EFFECTORS AND REGULATORS OF CELLULAR IMMUNE RESPONSE IN AUTOIMMUNE LIVER DISEASE

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EFFECTORS AND REGULATORS OF CELLULAR IMMUNE RESPONSE IN AUTOIMMUNE LIVER DISEASE

The role of TIM-3/Galectin-9 pathway

Rodrigo Gouveia Vasconcelos Rodrigues Liberal

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A Thesis submitted for the degree of Doctor of Philosophy at the

Faculty of Medicine of the University of Porto

Supervisor: Professor Diego Vergani

King's College London

Co-supervisor: Professor Maria de Fátima Machado Henriques Carneiro

Faculty of Medicine of the University of Porto

EFECTORES E REGULADORES DA RESPOSTA IMUNE CELULAR NA DOENÇA HEPÁTICA AUTO-IMUNE

O papel da via *TIM-3/Galectin-9*

Rodrigo Gouveia Vasconcelos Rodrigues Liberal

2012

Dissertação de candidatura ao grau de Doutor apresentada à

Faculdade de Medicina da Universidade do Porto

Orientador: Professor Diego Vergani

King's College London

Co-orientador: Professora Maria de Fátima Machado Henriques Carneiro

Faculdade de Medicina da Universidade do Porto

A presente **Dissertação de candidatura ao grau de Doutor**, apresentada à **Faculdade de Medicina da Universidade do Porto**, está redigida na língua inglesa, em virtude do trabalho que lhe serve de suporte ter sido realizado em grande parte no Reino Unido, no ***Institute of Liver Studies, Department of Transplantation Immunology and Mucosal Biology, King´s College London School of Medicine at King´s College Hospital***, sob a orientação do *Professor Diego Vergani*.

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TABLE OF CONTENTS

INDEX OF FIGURES

CHAPTER I General introduction

CHAPTER II Introduction to the present study

CHAPTER III Methods

CHAPTER IV Frequency, phenotype and function of regulatory T cells in autoimmune liver disease

CHAPTER V Role of galectin-9 regulatory T cells in autoimmune liver disease

CHAPTER VI Contribution of TIM-3 to the determination of effector T cell fate in autoimmune liver disease

INDEX OF TABLES

CHAPTER I General introduction

CHAPTER II Introduction to the present study

CHAPTER III Methods

CHAPTER IV Frequency, phenotype and function of regulatory T cells in autoimmune liver disease

CHAPTER V Role of galectin-9 regulatory T cells in autoimmune liver disease

CHAPTER VI Contribution of TIM-3 to the determination of effector T cell fate in autoimmune liver disease

Appendix I

LIST OF ABBREVIATIONS

[A]	Active
[R]	Remission
A	Adenine
AASLD	American Association for the Study of the Liver
ABC	Avidin-biotin-complex
ADCC	Antibody-dependent cell-mediated cytotoxicity
ADH	Alcohol dehydrogenase
ADP	Adenosine diphosphate
AIH	Autoimmune hepatitis
AIH-1	Autoimmune hepatitis type-1
AIH-2	Autoimmune hepatitis type-2
AILD	Autoimmune liver disease
AIRE	Autoimmune regulator
AISC	Autoimmune sclerosing cholangitis
ALT	Alanine aminotransferase
AMA	Anti-mitochondrial antibodies
AMP	Adenosine monophosphate
ANA	Antinuclear antibodies
AP	Alkaline phosphatase
APC	Allophycocyanin
APCs	Antigen-presenting cells
APECED	Autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy
ASGPR	Asialoglycoprotein recepto
AST	Aspartate aminotransferase
ATP	Adenosine triphosphate
BD	Becton-Dickinson
BSG	British Society for Gastroenterology
BTLA	B and T lymphocyte attenuator

cAMP	Cyclic AMP
cANCA	Cytoplasmic anti-neutrophil cytoplasmic antibody
Cbfβ	Core binding factor β
CD	Crohn´s disease
CD39	Ectonucleoside triphosphate diphosphohydrolase-1
CIA	Collagen-induced arthritis
CIE	Counter-immune-electrophoresis
CLP	Common lymphoid progenitor
CMV	Cytomegalovirus
CNS	Central nervous system
cpm	Counts per minut
CRD	Carbohydrate recognition domain
CSF	Cerebrospinal fluid
cTECs	Cortical thymic epithelial cells
CTL	Cytotoxic T lymphocyte
CTLA-4	Cytotoxic T lymphocyte antigen 4
cTregs	Conventional Tregs
Cy	Cychrome
CyA	Cyclosporine A
CYP2D6	Cytochrome P4502D6
DAB	3,3´-diaminobenzidine
DC	Dendritic cell
d-AIH	*de novo* autoimmune hepatitis
DID	Double-dimension immune-diffusion
DMSO	Dymethyl sulphoxide
DN	Double negative
DNA	Deoxyribonucleic acid
DP	Double positive
DR5	Death receptor 5

dsRNA	Double-stranded RNA
DST	Donor-specific transfusion
EAE	Experimental autoimmune encephalomyelitis
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
ERCP	Endoscopic retrograde cholangiopancreatography
FACS	Fluorescence-activated cell sorting
FasL	Fas ligand
FCS	Foetal calf serum
FITC	Flourescein isothiocyanate
FOXP3	Forkhead winged helix transcription factor P3
FSC	Forward scatter
G	Guanine
GAL	Galectin
GALT	Gut-associated lymphoid tissue
GATA-3	GATA binding protein-3
GGT	Gamma glutamyl transpeptidase
GITR	Glucocorticoid-induced tumour necrosis factor receptor family-related protein
HAVCR	Hepatitis A virus cellular receptor
HBV	Hepatitis B virus
HCV	Hepatitis C virus
HEp2	Human epithelial type 2
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
HS	Healthy subject
HSC	Haemopoietic stem cell
HSC	Hepatic stellate cells
HSV	Herpes Simplex Virus

IAIHG	International Autoimmune Hepatitis Group
IB	Immunoblot
IBD	Inflammatory bowel disease
ICCS	Intracellular cytokine staining
IDO	Indoleamine 2,3-deoxigenase
IFN-γ	Interferon-gamma
Ig	Immunoglobulin
IIF	Indirect immunofluorescence
IL	Interleukin
INR	International normalised ratio
IPEX	Immunodysregulation, polyendocrinopathy and enteropathy, X-linked syndrome
Irf-4	Interferon regulatory factor-4
ITAM	Immunoreceptor tyrosine-based activation motif
iTregs	Peripherally-induced adaptive Tregs
KIM-1	Kidney injury molecule-1
LAG-3	Lymphocyte activation gene 3
LAP	Latency associated peptide
LC-1	Liver cytosol type
LD	Linkage desiquilibrium
LKM-1	Liver kidney microsomal type 1
LP	Liver pancreas
LSECS	Liver sinusoidal endothelial cells
LSP	Liver specific protein
LT	Liver transplantation
MAb	Monoclonal antibody
MBP	Myelin basic protein
MF	Microfilament
MHC	Major histocompatibility complex

MMF	Mycophenolate mofetil
MOG	Myelin oligodendrocyte glycoprotein
MS	Multiple sclerosis
mTECs	Medullary thymic epithelial cells
N/A	Not applicable
NAFLD	Non-alcoholic fatty liver disease
neg	Negative
NFAT	Nuclear factor of activated T cells
NF-κB	Nuclear factor-κB
NK	Natural killer
NKT	Natural killer T
NOD	Non-obese diabetic
Nrp-1	Neuropilin
NS	Non significant
nTregs	Natural T-regs
nv	Normal value
OVA	Ovalbumin
pANCA	Perinuclear anti-neutrophil cytoplasmic antibody
pANNA	Peripheral anti-nuclear neutrophil antibody
PBC	Primary biliary cirrhosis
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PD-1	Programmed death 1
PDH	Pyruvate dehydrogenase
PE	Phycoerythrin
PerCP	Peridinin-chlorophyll-protein complex
PI3K	Phosphotidylinositol 3-kinase
PMA	Phorbol 12-mystrate 13-acetate

Pos	Positive
PSC	Primary sclerosing cholangitis
PtdSer	Phosphatidylserine
RA	Rheumatoid arthritis
RIA	Radio-immune-precipitation assay
RISC	RNA-induced silencing complex
RNAi	RNA interference
RORα	Retinoic acid-related orphan receptor alpha
RORγt	Retinoic acid-related orphan receptor gamma t
RPMI	Roswell Park Memorial Institute
Runx1	Runt-related transcription factor 1
SEM	Standard error of the mean
SEM	Standard error of the mean
siRNA	Small-interfering RNA
SLA	Soluble liver antigen
SLE	Systemic lupus erythematosus
SMA	Smooth muscle antibody
sn-RNPs	Small nuclear ribonucleoproteins
SP	Single positive
SSC	Side scatter
STAT	Signal transducer and activator of transcription
T	Tubules
T1D	Type 1 diabetes
TAPR	T cell and airway phenotype regulator
Tbet	T box expressed in T cells
TCR	T cell receptor
Teff	Effector T cell
TGF-β	Transforming growth factor beta
Th	T helper

TIM	T-cell Ig and mucin domain
TLR-4	Toll-like receptor 4
TNBS	2,4,6-trinitrobenzene sulfonic acid
TNF	Tumour necrosis factor
TNFR	Tumour necrosis factor receptor
TPMT	Thiopurine methyltranferase
TRAIL	Tumour-necrosis-factor-related apoptosis-inducing ligand
Tregs	Regulatory T cells
Tris-EDTA	Trishydroxymethylaminomethane/ethylene diamine tetraacetic acid
tRNP$^{(ser)sec}$	tRNA suppressor-associated antigenic protein
TSDR	Treg-cell specific demethylated region
UC	Ulcerative colitis
UDCA	Ursodeoxycholic acid
UGT	Uridine glucuronosyltransferase
V	Vessels

ABSTRACT

Autoimmune hepatitis (AIH), an immune-mediated liver disorder with a strong female preponderance, is characterised by hypergammaglobulinaemia, seropositivity for autoantibodies and interface hepatitis on histology. The aetiology of AIH is unknown, though both genetic and environmental factors are involved. CD4 effector lymphocytes are the main orchestrators of liver damage, their proliferation and pro-inflammatory cytokine secretion being correlated with the activity and severity of liver disease. A wealth of data indicates that in AIH the extent of self-reactive CD4 T cell effector immune responses is associated with defective $CD4^{pos}CD25^{pos}$ regulatory T cells (Tregs), a lymphocyte subset central to immune-tolerance maintenance. Whether impaired immune-regulation in AIH is due to a reduced number of Tregs, intrinsic defects in their function; or effector cell resistance to their suppression or even a combination of all them is unknown. Recent studies have shown that functional Tregs express very low levels of CD127, whereas activated T cells were characterised by the expression of this marker, indicating CD127 as a good phenotypic marker for the quantification of these cell population. On the other hand, murine studies have shown that galectin-9 (GAL-9), a member of the galectin family expressed by Tregs, inhibits Th1 effector immune responses after binding to the T-cell-imunoglobulin-and-mucin-domain-3 (TIM-3), its receptor on CD4 effector cell. The aims of this Thesis were thus to investigate possible causes of functional Treg impairment by exploiting the role of CD127 and that of TIM-3/GAL-9 pathway in patients with autoimmune liver disease.

Seventy-seven patients with anti-nuclear (ANA) and/or smooth-muscle antibody (SMA) positive AIH, including thirty nine patients diagnosed with AIH/sclerosing cholangitis (AISC) overlap syndrome, were studied. When considered together they are

referred to as autoimmune liver disease (AILD). Circulating $CD127^{neg}$ and $GAL\text{-}9^{pos}CD4^{pos}CD25^{pos}$ and $TIM\text{-}3^{pos}CD4^{pos}CD25^{neg}$ T cell phenotype was assessed by flow cytometry. $CD127^{neg}$ Treg ability to suppress was evaluated in a proliferation assay following co-culture with $CD25^{neg}$ responder cells. To determine whether GAL-9 expression is essential to Treg function, Tregs were treated with small-interfering-RNA to repress GAL-9 translation; Treg suppressor function was assessed by a reduction in target cell proliferation. To evaluate whether TIM-3 expression renders $CD4^{pos}CD25^{neg}$ T cells amenable to Treg control, purified $CD4^{pos}CD25^{neg}TIM\text{-}3^{pos}$ ($TIM\text{-}3^{pos}$) and $CD4^{pos}CD25^{neg}TIM\text{-}3^{neg}$ ($TIM\text{-}3^{neg}$) cells were co-cultured with Tregs.

The frequency of $CD4^{pos}CD25^{pos}CD127^{neg}$ cells was reduced in AILD patients compared to HS. Compared to conventional $CD4^{pos}CD25^{pos}$ (cTregs), $CD127^{neg}$ Tregs from both AILD and HS had a) higher numbers of $FOXP3^{pos}$, $CTLA\text{-}4^{pos}$ and $IL\text{-}10^{pos}$ cells; b) lower numbers of $T\text{-}bet^{pos}$, $RORC^{pos}$, $IFN\gamma^{pos}$ and $IL\text{-}17^{pos}$ cells; and c) similar numbers of $TGF\text{-}\beta^{pos}$ cells. $CD127^{neg}$ Tregs inhibited $CD25^{neg}$ cell proliferation more effectively than cTregs, though less markedly in AILD than in HS. In AILD, treatment with anti-IFNγ and anti-IL-17 neutralising antibodies ameliorated the suppressive ability of cT-regs, while leaving unchanged that of $CD127^{neg}$ Tregs; exposure to anti-IL-10 neutralising antibodies reduced Treg suppression in HS, but not in AILD.

In AILD the frequency of GAL-9 expressing Tregs was reduced, and inversely correlated with IgG levels and autoantibodies titres. Within $GAL\text{-}9^{pos}$ cells the frequency of: a) $FOXP3^{pos}$ cells was lower in AILD than in HS; b) $Tbet^{pos}$, $GATA\text{-}3^{pos}$ and $RORC^{pos}$ cells was similar in AILD and HS; c) IL-10-producing cells was lower in AILD than in HS but higher than in the $GAL\text{-}9^{neg}$ Treg fraction for both; d) TGF-β-producing cells was lower in AILD than in HS; e) IFN-γ- and IL-17-producing cells was

higher in AILD than in HS. siRNA treatment of GAL-9^{pos} Tregs reduced drastically Treg ability to suppress responder cell proliferation. Treatment with anti-IL-10 neutralising antibodies reduced Treg ability to suppress $CD25^{neg}$TIM-3^{pos} cell proliferation, while did not affect TIM-3^{neg} cell proliferation.

In AILD TIM-3^{pos} cells within $CD4^{pos}CD25^{neg}$ cells and their $Tbet^{pos}$ and $RORC^{pos}$ subsets were fewer and contained higher numbers of IFN-γ^{pos} and IL-17^{pos} cells than in HS. In patients and HS TIM-3^{pos} cells proliferated less vigorously and were more susceptible to Treg control than TIM-3^{neg} cells. The frequency of TIM-3^{pos} cells inversely correlated with the levels of AST.

In conclusion reduced numbers of $CD127^{neg}$ Tregs, as well as of reduced expression of GAL-9 on Tregs and of TIM-3 on $CD4^{pos}CD25^{neg}$ effector cells contribute to impaired immune-regulation in AILD by rendering Tregs less capable of suppressing, and effector cells less prone to Treg control

RESUMO

A hepatite autoimune (HAI) é um processo inflamatório hepatocelular auto perpetuado de etiologia desconhecida e que se caracteriza por hipergamaglobulinémia, positividade para auto-anticorpos e hepatite de interface na histologia. A sua etiologia é desconhecida, embora fatores genéticos e ambientais estejam envolvidos. Os linfócitos T CD4 efetores são os principais orquestradores da lesão hepática, sendo que a sua proliferação e secreção de citocinas pró-inflamatórias se correlacionam com a atividade e gravidade da doença. Vários estudos mostraram que na HAI a magnitude da resposta imune dos linfócitos CD4 auto-reativos está associada a um defeito das células T $CD4^{pos}CD25^{pos}$, designados por linfócitos T reguladores (Tregs), uma população celular essencial para a manutenção da tolerância imune. Desconhece-se, no entanto, se este distúrbio imunorregulatório na HAI é devido a uma redução do número de Tregs, a um defeito intrínseco da sua função, ou a resistência das células efetoras à inibição exercida pelas Tregs, ou ainda a uma combinação de todos estes mecanismos. Estudos recentes mostraram que as Tregs funcionais se caracterizam pela expressão muito baixa ou mesmo ausente de CD127, enquanto as células T ativadas se distinguem pela expressão desta molécula, indicando que o CD127 poderá ser um bom marcador fenotípico para a distinção destas duas populações celulares. Por outro lado, estudos em ratinhos demonstraram que a galectina-9 (GAL-9), um dos membros da família da galectinas e que é expressa pelas Tregs, inibe as respostas Th1 após se ligar ao *T cell immunoglobulin and mucin domain 3* (TIM-3), o seu recetor nas células efetoras. Posto isto, esta Tese teve como objetivo investigar possíveis causas responsáveis pelo comprometimento funcional das Tregs, explorando o papel desempenhando pelo CD127 e pela via TIM-3/GAL-9 na doença hepática auto-imune.

Foram estudados setenta e sete doentes, incluindo 39 doentes com o diagnóstico de síndrome de sobreposição HAI-colangite esclerosante. Quando considerados em conjunto, HAI e síndrome de sobreposição serão referidos como doença hepática autoimune (DHAI). A frequência e fenótipo das células $CD4^{pos}CD25^{pos}CD127^{neg}$ e $GAL\text{-}9^{pos}$ bem como das células $CD4^{pos}CD25^{neg}TIM\text{-}3^{pos}$ foram avaliados por citometria de fluxo. A função supressora das Treg foi avaliada, após cultura com células $CD25^{neg}$. Para determinar se a expressão da GAL-9 é essencial para a sua função, as Tregs foram tratadas com *small-interfering-RNA* para reprimir a tradução da GAL-9. Para avaliar se a expressão de TIM-3 confere susceptibilidade às células $CD4^{pos}CD25^{neg}$ T efetoras à ação supressora das Tregs, foram purificadas células $CD4^{pos}CD25^{neg}TIM\text{-}3^{pos}$ ($TIM\text{-}3^{pos}$) e $CD4^{pos}CD25^{neg}TIM\text{-}3^{neg}$ ($TIM\text{-}3^{neg}$).

A frequência das células reguladoras $CD4^{pos}CD25^{pos}CD127^{neg}$ está reduzida nos doentes com DHAI. No entanto, esta população quando comparada com as Tregs convencionais (ie, aquelas $CD4^{pos}CD25^{pos}$, cTregs) caracteriza-se por uma expressão mais elevada de FOXP-3, CTLA-4, IL-10, e uma expressão reduzida de Tbet, RORC, IFN-γ e IL-17, e expressão semelhante de TGF-β. As Tregs $CD127^{neg}$ inibiram a proliferação das células T $CD25^{neg}$ com maior eficácia que as cTregs. Nos doentes com DHAI, o tratamento com anticorpos monoclonais anti-IFN-γ e anti-IL-17 melhorou a capacidade supressora das cTregs, mantendo inalterada aquela das Tregs $CD127^{neg}$. O tratamento com anti-IL-10 resultou numa redução da função supressora das Tregs em indivíduos saudáveis, mas não nos doentes com DHAI.

Na DHAI, a frequência das Tregs que expressam GAL-9 está reduzida e correlaciona-se com os níveis de IgG e com os títulos de auto-anticorpos. No seio das células $GAL\text{-}9^{pos}$, a(s) frequência(s) de: a) células $FOXP3^{pos}$ é inferior na DHAI quando

comparado com os controlos saudáveis; b) células Tbet^{pos}, GATA-3^{pos} e RORC^{pos} são semelhantes nos dois grupos de indivíduos; c) células produtoras de IL-10 é menor na DHAI do que nos controlos, e mais elevada na fração GAL-9^{neg} em ambos os grupos; d) células produtoras de TGF-β é menor na DHAI do que nos controlos; e) células produtoras de IFN-γ bem como de IL-17 são mais elevadas na DHAI do que os controlos saudáveis. O tratamento das Tregs GAL-9^{pos} com siRNA reduziu drasticamente a sua capacidade de suprimir a proliferação das células-alvo.

Na DHAI, a frequência das células TIM-3^{pos} no seio das células CD4^{pos}CD25^{neg} e das suas populações Tbet^{pos} e RORC^{pos} foi inferior que nos controlos saudáveis. As células TIM-3^{pos} proliferaram menos vigorosamente e demonstraram ser mais suscetíveis ao controlo exercido pelas Tregs do que as células TIM-3^{neg}.

Em conclusão, redução do número das Tregs CD127^{neg}, bem como diminuição da expressão de GAL-9 pelas Tregs e de TIM-3 pelas células efetoras contribuem para o defeito na imunorregulação na DHAI, tornando as Tregs menos capazes de suprimir, e as células efetoras menos suscetíveis a serem reguladas.

CHAPTER I

General introduction

1. Definition of autoimmune hepatitis

Autoimmune hepatitis (AIH) is a severe hepatopathy of unknown cause progressing to cirrhosis and liver failure if not treated. AIH was first described in 1950 by Jan Waldenström, who reported a series of young women affected by a severe form of fluctuating persistent hepatitis associated with acneiform rashes, spider angiomas, anovulatory amenorrhea and marked elevations of serum immunoglobulins (Waldenström 1950). Subsequent observations reporting the presence of lupus erythematosus cells and antinuclear antibodies (ANA) in a proportion of patients, led to postulate that a loss of immunological tolerance was the basis of this condition, that Mackay termed "lupoid hepatitis" in 1956 (Mackay et al. 1956, Joske and King 1955). Studies performed in the early 1960s reported a good response to steroid treatment (Mackay and Wood 1963); three clinical controlled trials carried out in the following decade established the life-saving value of corticosteroids in the treatment of patients with "HBsAg-negative hepatitis" (Cook et al. 1971, Soloway et al. 1972, Murray-Lyon et al. 1973, Kirk et al. 1980). In the following years AIH was recognised as a separate clinical entity which was named "chronic active autoimmune hepatitis" (Gurian et al. 1985, Mackay 1994, McFarlane 1996). That term continued to be used until the end of the 1980s; after two international working meetings held in 1992 (Brighton, UK) and 1994 (Los Angeles, USA), it was substituted by the term of "autoimmune hepatitis" on the basis that the disease appears to have a genetic predisposition and is therefore *a priori* chronic and is often characterised by a fluctuating course with periods of treatment-induced or spontaneous remission and consequently is not always active (Mackay 1993, Ludwig 1995). The panels of the two working meetings composed the International Autoimmune Hepatitis Group (IAIHG). The IAIHG was responsible for

the introduction of an established (Johnson and McFarlane 1993) and meanwhile revised (Alvarez et al. 1999) scoring system for the diagnosis of AIH. More recently, a simplified score intended to be used in the clinical practice has been proposed by the group (Hennes et al. 2008).

Based on the type of serum autoantibodies, two types of AIH are recognised: type 1 (AIH-1), positive for ANA and/or anti-smooth muscle antibody (SMA), and type 2 (AIH-2), defined by the positivity for anti-liver kidney microsomal type 1 antibody (anti-LKM-1) or for anti-liver cytosol type 1 antibody (anti-LC-1).

2. Epidemiology

The actual prevalence of AIH is unknown. Few descriptive epidemiological studies are available and the majority of them do not rely on standard criteria for patients´ inclusion, since they were performed prior to the introduction of the IAIHG scoring system. Moreover, early studies did not exclude patients with chronic hepatitis C. In a study conducted in a Norwegian population, Boberg et al. found a mean annual incidence of 1.9 cases per 100,000 people per year with a prevalence of 16.9 cases per 100,000 people (Boberg et al. 1998). Another study, conducted in a Spanish population, reported an annual incidence of 0.83 cases per 100,000 inhabitants in the population aged > 14 years with a prevalence of 11.6 cases per 100,000 (Primo et al. 2004); it should be pointed out, however, that this was a hospital-based study, consequently limited by tertiary referral bias, which can result in an underestimation of incidence and prevalence and in an overestimation of disease severity. A study that examined the prevalence of AIH in Alaskan natives reported a prevalence of definite AIH of 34.5 cases per 100,000 (Hurlburt et al. 2002); this was the first study to use the IAIHG

scoring system and the rate observed was over twice as high as that of the other previous population based study, the Norwegian one referred to earlier. A study from the United Kingdom, conducted in a secondary care referral centre, showed that the annual incidence of AIH is 3.5 per 100,000 inhabitants (Whalley et al. 2007). More recently, an epidemiological study, carried out in New Zealand and in which standardised inclusion criteria were deployed, reported an annual incidence of 2.0 cases of AIH per 100,000 and a point prevalence of 24.5 cases per 100,000 (Ngu et al. 2010). In Asia, most reports come from Japan, where AIH is considerably less frequent than in Western countries with an incidence estimated between 0.08 and 0.15 cases per 100,000 people per year (Toda et al. 1997, Nishioka 1998). Similarly, AIH was long thought to be uncommon in China (Lam et al. 1980); however, with a more refined diagnostic work-up of patients, AIH has increasingly been reported in that country (Qiu et al. 2011).

The prevalence of AIH-2, which affects mainly children and young adults, is unknown, also owing to the fact that the diagnosis is often overlooked. At King´s College Hospital tertiary paediatric hepatology referral centre there has been a seven-fold increase in the incidence of both types of AIH over the last decade (Vergani 2007). AIH represents approximately 10% of some 400 new paediatric referrals per year, two thirds of the cases being AIH-1 and one-third AIH-2 (Vergani 2007).

AIH, though characterised by a strong female preponderance (ratio female/male = 3.6/1; Manns et al. 2010a), occurs in children and adults of both sexes. Although AIH was believed to be a disease of the young and middle aged population, it is now clear that it affects adults of all ages, including individuals older than 60 (Al-Chalabi et al. 2006, Czaja and Carpenter 2006a).

3. Clinical Presentation

AIH is characterised by highly variable modes of presentation and clinical manifestations (Krawitt 2008). In 30 to 40% of patients the onset of AIH is ill defined, mimicking that of acute hepatitis due to other causes (Crapper et al. 1986). Rarely, AIH presents as fulminant hepatic failure (Kessler et al. 2004). The majority of adult patients, however, have an insidious onset with a clinical course characterised by progressing fatigue, relapsing jaundice, weight loss and sometimes arthralgia (Vergani and Mieli-Vergani 2011a). A presentation with complications of portal hypertension, such as gastrointestinal bleeding or hypersplenism, can occasionally occur (Gregorio et al. 1997). Finally, some patients are completely asymptomatic and are diagnosed after incidental discovery of abnormal liver function tests. At least a third of patients, regardless of the mode of presentation, have histological evidence of cirrhosis at the time of diagnosis, indicating that they probably have had subclinical disease for a long time (Krawitt 2006). Even in the group of patients presenting with acute disease, liver biopsy often shows signs of advanced fibrosis or cirrhosis (Lohse and Mieli-Vergani 2011).

AIH may occasionally develop during pregnancy or post-partum in a previously asymptomatic patient (Schramm et al. 2006, Samuel et al. 2004). Furthermore, post-partum exacerbations may occur in patients whose condition improved during pregnancy (Heneghan et al. 2001), possibly due to the natural pregnancy-associated immunosuppressive state (Aluvihare et al. 2004).

A family history of autoimmune diseases is present in 40% of patients. Also, at least 20% of AIH patients have concomitant autoimmune diseases or will develop them during follow-up (Gregorio et al. 1997; Table 1.1.).

Table 1.1. Extra-hepatic diseases associated with autoimmune hepatitis.

Disease	Frequency	Reference
Common		
Thyroiditis	10 – 23%	Werner et al., 2008, Al-Chalabit et al., 2008, Muratori et al. 2009
Diabetes	7 – 9%	Werner et al., 2008
Inflammatory bowel disease	2 – 8%	Perdigoto et al., 1992, Anagnostopoulos et al., 2006,
Rheumatoid arthritis	2 – 5%	Nobili et al., 2005, Muratori et al., 2009
Psoriasis	3%	Werner et al., 2008
Sjögren´s syndrome	1 – 4%	Matsumoto et al., 2005, Werner et al., 2008, Muratori et al., 2009
Mixed connective tissue disease	2.5%	Muratori et al., 2009
Systemic lupus erythematosus	1 – 2%	Irving et al., 2007, Werner et al., 2008, Muratori et al. ,2009
Coeliac disease	1 – 2%	Kaukinen et al., 2002, Villalta et al., 2005, Caprai et al., 2008
Glomerulonephritis	1%	Werner et al., 2008,
Multiple sclerosis	1%	Nunez et al., 2004, Muratori et al., 2009
Case reports		
Anti-phospholipid syndrome		Uthman and Khamashta, 2007
Autoimmune pancreatitis		Quinet de Andrade et al., 2000
Felty syndrome		Inaba et al., 1995, Sema et al., 2005
Fibrosing alveolitis		Paul et al., 1991
Haemolytic anemia		Gurundu et al., 2001
Idiopatic thrombocytopenia purpura		Yamaike et al., 2002
Mononeuritis multiplex		Luth et al., 2006
Myastenia gravis		Han et al., 2000
Panniculitis		Banerjee et al., 1989, Fujiwara et al., 2000,
Polyglandular autoimmune syndrome type 1		Michele et al., 1994
Polymiositis		Ko et al., 1995
Uveitis		Romanelli et al., 2006, Romanelli and Almerigogna, 2009
Vitiligo		Sacher et al., 1990

Complications in AIH are those seen in any other progressive liver disease: in some patients, despite immunosuppressive treatment, chronic hepatitis progresses to cirrhosis and ultimately to hepatocellular carcinoma (HCC; Krawitt 2006). HCC, however, occurs rarely in AIH, where it is less frequent than in patients with chronic viral hepatitis (Yeoman et al. 2008).

4. Diagnosis

4.1. Scoring systems

The diagnosis of AIH is based on the presence of elevated transaminase and immunoglobulin (Ig) G levels, positivity for circulating autoantibodies, and a picture of interface hepatitis on histology (Table 1.2.).

Table 1.2. Descriptive criteria for the diagnosis of autoimmune hepatitis.

Feature	Definite AIH	Probable AIH
Serum biochemistry	Any abnormality in serum aminotransferases, especially if the serum alkaline phosphatase is not elevated. Normal levels of α1-anti-trypsin and caeruloplasmin	Same as for definite AIH but patients with abnormal serum concentrations of copper or caeruloplasmin may be included contigent on the exclusion of Wilson´s disease by appropriate investigations
Serum immunoglobulins	Total serum globulins or gamma-globulin or IgG concentrations > 1.5 times upper limit of normal	Any elevation of serum globulin, gamma-globulin or IgG over the upper limit of normal
Serum autoantibodies	ANA, SMA or anti-LKM-1 titres ≥ 1:80. Lower titres (particularly anti-LKM-1) may be significant in children. Negative AMA	Same as for definite but at titres ≥ 1:40 or presence of other specified autoantibodies
Liver histology	Interface hepatitis of moderate or severe activity with or without lobular hepatitis or bridging necrosis. No biliary lesions, granulomas or other preeminent changes suggestive of a different etiology	Same as for definite AIH
Viral markers	Negativity for markers of current infection with hepatitis A, B and C viruses	Same as for definite AIH
Other etiological markers	Average alcohol consumption < 25 g/day. No recent use of hepatotoxic drugs	Average alcohol consumption < 50 g/day and no recent use of hepatotoxic drugs. Patients who have consumed large amounts of alcohol or who have recently taken potentially hepatotoxic drugs may be included, if there is clear evidence of continuing liver damage after abstinence from alcohol or withdrawl of the drug

AIH, autoimmune hepatitis; *AMA*, anti-mitochondrial antibodies; *ANA*, anti-nuclear antibodies; *SMA*, anti-smooth muscle antibodies; *anti-LKM-1*, anti-liver kidney microsomal type 1 antibodies
Adapted from Alvarez F, Berg PA et al. J Hepatol 1999; 31: 929-9

The criteria for the diagnosis of AIH have been established and revised by the IAIHG (Johnson and McFarlane 1993, Alvarez et al. 1999). This diagnostic system, which includes positive and negative scores, grades clinical, laboratory and histological

features of AIH, including response to treatment. The system was originally designed for research purposes with the intent of standardising the diagnosis of AIH throughout the literature, but it later proved to be also clinically useful in assessing patients with few or atypical features of the disease (Czaja and Carpenter 1996, Czaja and Manns 2010).

In the IAIHG scoring system, differences between a definite and probable diagnosis of AIH relate mainly to the degree of serum gamma-globulin or IgG elevation, levels of ANA, SMA or anti-LKM-1 and exposure to alcohol, medications or infections that can cause liver injury. Cholestatic laboratory and histological changes carry a negative score. In rare cases, the presence of autoantibodies to asialoglycoprotein receptor (anti-ASGPR), LC-1, soluble liver antigen/liver pancreas (anti-SLA/LP) and atypical perinuclear anti-neutrophil cytoplasmic antibodies (pANCA) supports a probable diagnosis of AIH in the absence of conventional autoantibodies. Response to steroids strengthens the diagnosis of AIH and has been incorporated into the scoring system. A definite diagnosis before steroid treatment requires a score higher than 15, while a definite diagnosis after steroid treatment requires a score higher than 17 (Table 1.3.).

The diagnositic criteria for children are slightly different from those of adults. In view of the fact that healthy children are very rarely positive for autoantibodies, titres as low as 1:20 for ANA and SMA and 1:10 for anti-LKM-1 are compatible with the diagnoses of type 1 and type 2 AIH respectively (Mieli-Vergani et al. 2009).

Table 1.3. International autoimmune hepatitis group revised diagnostic scoring system.

Parameter	Feature	Score
Sex	Female	+2
ALP:AST (or ALT) ratio	> 3	–2
	1.5 – 3	0
	< 1.5	+2
Serum globulins or IgG (times above normal)	> 2.0	+3
	1.5 – 2.0	+2
	1.0 – 1.5	+1
	< 1.0	0
ANA, SMA or anti-LKM-1 titres	> 1:80	+3
	1:80	+2
	1:40	+1
	< 1:40	0
AMA	Positive	–4
Viral markers of active infection	Positive	–3
	Negative	+3
Hepatotoxic drug history	Yes	–4
	No	+1
Average alcohol	< 25 g/day	+2
	> 60 g/day	–2
Histological features	Interface hepatitis	+3
	Plasma cells	+1
	Rosettes	+1
	None of the above	–5
	Biliary changes [a]	–3
	Atypical changes [b]	–3
Immune diseases	Thyroiditis, colitis, other	+2
HLA	DR3 or DR4	+1
Seropositivity for other autoantibodies	Anti-SLA/LP, actin, ASGPR, p-ANNA	+2
Response to therapy	Remission	+2
	Relapse	+3

Pre-treatment score >15: definite AIH; 10-15: probable AIH; Post-treatment score >17: definite AIH; 12-17: probable AIH

ALP, alkaline phosphatase; *AST*, aspartate aminotransferase; *ALT*, alanine aminotransferase; *IgG*, immunoglobulin G; *ANA*, anti-nuclear antibody; *SMA*, anti-smooth muscle antibody; *anti-LKM-1*, anti-liver kidney microsomal type 1 antibodies; *AMA*, anti-mitochondrial antibodies; *SLA/LP*, soluble liver antigen/liver pancreas; *ASGPR*, asialoglycoprotein receptor; *p-ANNA*, peripheral anti-nuclear neutrophil antibody*; HLA*, human leukocyte antigen

[a] Including granulomatous cholangitis, concentric periductal fibrosis, ductopenia, marginal bile duct proliferation and cholangiolitis

[b] Any other prominent feature suggesting a different aetiology

Adapted from Alvarez F, Berg PA et al. J Hepatol 1999; 31: 929:938

Recently, a simplified scoring system has been proposed by the IAIHG (Hennes et al. 2008). This system, intended to be used in the clinical practice, assesses only four criteria: autoantibodies, IgG, histology and exclusion of viral hepatitis (Table 1.4.).

Table 1.4. Simplified criteria for the diagnosis of autoimmune hepatitis.

Variable	Cut-off	Points
ANA or SMA	≥ 1:40	1
ANA or SMA	≥ 1:80	2 [a]
or anti-LKM-1	≥ 1:40	
or SLA	Positive	
IgG	> upper limite of normal	1
	> 1.10 times upper limit of normal	2
Liver histology	Compatible with AIH	1
	Typical of AIH	2
Absence of viral hepatitis	Yes	2

Score ≥ 6: probable AIH; ≥7: definite AIH
ANA, anti-nuclear antibody; *SMA*, anti-smooth muscle antibody; *anti-LKM-1*, anti-liver kidney microsomal antibody type 1; *SLA*, soluble liver antigen; *IgG*, immunoglobulin G; *AIH*, autoimmune hepatitis
[a] Addition of points achieved for all autoantibodies cannot exceed a maximum of 2 points
Adapted from Hennes EM, Zeniya M et al. Hepatology 2008; 48: 169-176

4.2. Laboratory abnormalities

AIH is characterised by elevated levels of transaminases. In general the increase in aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels is much more striking than that observed for the levels of bilirubin and alkaline phosphatase (AP). Nonetheless, in some patients cholestasis is present; in these cases, extra-hepatic obstruction and cholestatic forms of viral hepatitis, drug-induced disease, primary biliary cirrhosis (PBC), primary sclerosing cholangitis (PSC), and overlap syndromes (see section 5.) must be taken into consideration (Krawitt 2008).

Another laboratory feature typical of AIH, albeit not always present, is a generalised elevation of serum globulins and particularly gamma globulins mainly due to an increase in the IgG fraction (McFarlane 2002). The serum autoantibodies typically present in AIH include: ANA, SMA, anti-LKM-1, and anti-LC-1 (see section 4.3.). Anti-mitochondrial antibodies (AMA), typically present in PBC, are occasionally present in patients with AIH (Bhat et al. 2009) . Since autoantibodies may also be found in other liver diseases their presence as such is not diagnostic of AIH.

4.3. Diagnostic autoantibodies

A key diagnostic criteria shared by all the scoring systems referred above is the presence of autoantibodies to nuclear components, smooth muscle and liver kidney microsome type 1 (Table 1.5.). Testing for these autoantibodies should be performed in all cases of cryptogenic liver disease (Vergani et al. 2004). Autoantibody detection not only assists in the diagnosis but also allows the differentiation of AIH into type 1 and type 2. Positivity for ANA and/or SMA – characterising AIH-1 –, and for anti-LKM-1 – which defines AIH-2 –, tend to be mutually exclusive (Vergani et al. 2004); in those rare cases in which they are present simultaneously, the clinical course is similar to that of AIH-2. Antibodies to LC-1, SLA/LP, and to neutrophil cytoplasmic antigens may be helpful to assist in the diagnosis of those patients negative for the defining AIH autoantibodies; for this reason, they were also included in the original and revised IAIHG diagnostic scoring systems (Johnson and McFarlane 1993, Alvarez et al. 1999).

Figure 1.1. provides an algorithm for the use of autoantibodies in the diagnosis of AIH.

Table 1.5. Autoantibodies and their targets in autoimmune liver diseases.

Autoantibody	Target antigen (s)	Liver disease	Value in AIH	Conventional method of detection	Molecular based assays
ANA	Multiple targets including: chromatin ribonuclueoproteins ribonucleoprotein complexes	AIH PBC PSC Drug-induced Chronic hepatitis C Chronic hepatitis B NAFLD	Diagnostic of AIH-1	IIF	N/A
SMA	Microfilaments (filamentous actin) Intermediate filaments (vimentin, desmin)	Same as ANA	Diagnostic of AIH-1	IIF	N/A
Anti-LKM-1	Cytochrome P450 2D6	AIH-2 Chronic hepatitis C	Diagnostic AIH-2	IIF	ELISA, IB, RIA
Anti-LC-1	Forminino-transferase cyclodeaminase	AIH-2 Chronic hepatitis C	Diagnostic of AIH-2 Prognostic of severe disease	IIF, DID, CIE	ELISA, RIA
SLA/LP	tRNP(Ser)Sec	AIH Chronic hepatitis C	Diagnostic of AIH Prognostic of severe disease, relapse and treatment dependence	Inhibition ELISA	ELISA, IB, RIA
Atypical p-ANCA (p-ANNA)	Nuclear lamina proteins	AIH PSC/AISC	Point towards diagnosis of AIH	IIF	N/A
AMA	E2 subunits of 2-oxo-acid dehydrogenase complexes, particularly PDC-E2	PBC	Against diagnosis of AIH	IIF	ELISA, IB, RIA

ANA, anti-nuclear antibodies; *SMA*, anti-smooth muscle antibodies; *anti-LKM-1*, anti-liver kidney microsomal antibody type 1; *anti-LC-1*, anti-liver cytosol antibody type 1; *SLA/LP*, soluble liver antigen/liver pancreas; *pANCA*, perinuclear anti-neutrophil cytoplasmic antibodies; *pANNA*, peripheral anti-nuclear neutrophil antibodies; *AMA*, anti-mitochondrial antibodies; *AIH*, autoimmune hepatitis; *PBC*, primary biliary cirrhosis; *PSC*, primary sclerosing cholangitis; *NAFLD*, non-alcoholic fatty liver disease; *IIF*, indirect immunofluorescence; *DID*, double-dimension immune-diffusion; *CIE*, counter-immune-electrophoresis; *ELISA*, enzyme-linked immunosorbent assay; *IB*, immunoblot; *RIA*, radio-immune-precipitation assay; *N/A*, not applicable

Recognition and interpretation of immunofluorescence patterns is not always straightforward; such an operator-dependent technique allied to the relative rarity of AIH leads, not infrequently, to reporting errors. In addition, clinical interpretation errors

may occur due to the relative lack of standardisation of the tests and to a degree of unfamiliarity of some clinicians with the AIH disease spectrum. In this regard, the IAIHG has established a serology committee in order to define guidelines and develop procedures and reference standards for more reliable testing (Vergani et al. 2004).

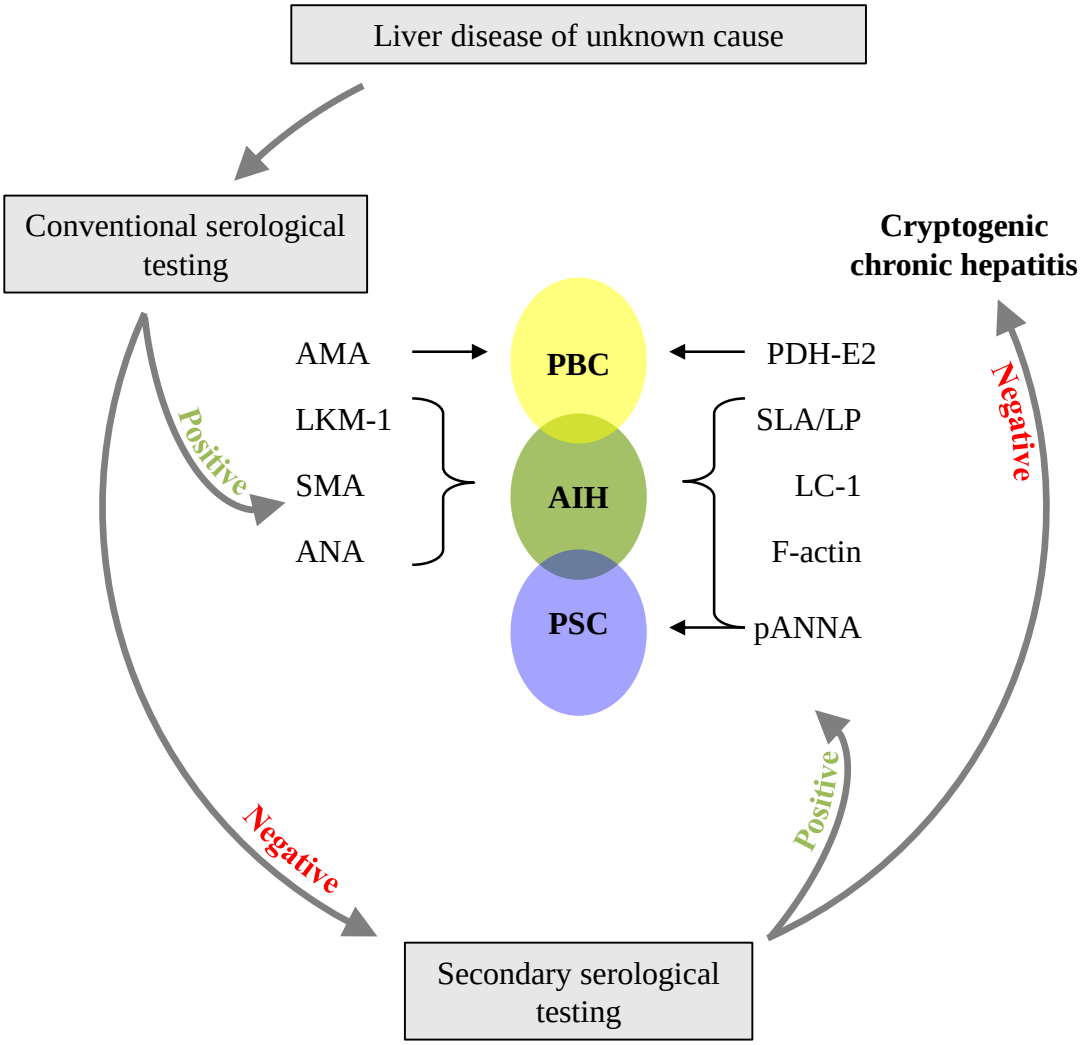


Figure 1.1. Serological tests in the evaluation of acute or chronic hepatitis of unknown cause. The initial serological assessments for antinuclear (*ANA*), anti-smooth muscle (*SMA*), anti-liver kidney microsome type 1 (*LKM-1*), and anti-mitochondrial (*AMA*) antibodies. The results of these conventional tests direct the diagnostic effort. If one or more tests are positive, the diagnosis of autoimmune hepatitis (*AIH*) or primary biliary cirrhosis (*PBC*) should be pursued. If these tests are negative, other serological assessments are appropriate, including tests for antibodies to actin (*F-actin*), soluble liver antigen/liver pancreas (*SLA/LP*), liver cytosol type 1 (*LC-1*), the E2 subunits of the pyruvate dehydrogenase complex (*PDH-E2*), peripheral anti-nuclear neutrophil antibodies (*pANNA*). These results may suggest other diagnoses, including primary sclerosing cholangitis (*PSC*), overlap syndromes or cryptogenic chronic hepatitis.
Adapted from Manns MP, Czaja AJ et al. Hepatology 2010; 51: 2193-2213.

4.3.1. Anti-nuclear antibodies

ANA, the first AIH-associated autoantibody (Mackay et al. 1956), is readily detectable and gives a nuclear staining in rodent kidney, stomach and liver sections (Figure 1.2.). In most cases, its pattern, especially in the liver, is homogeneous, with a coarsely or finely speckled pattern observed less frequently (Czaja et al. 1997).

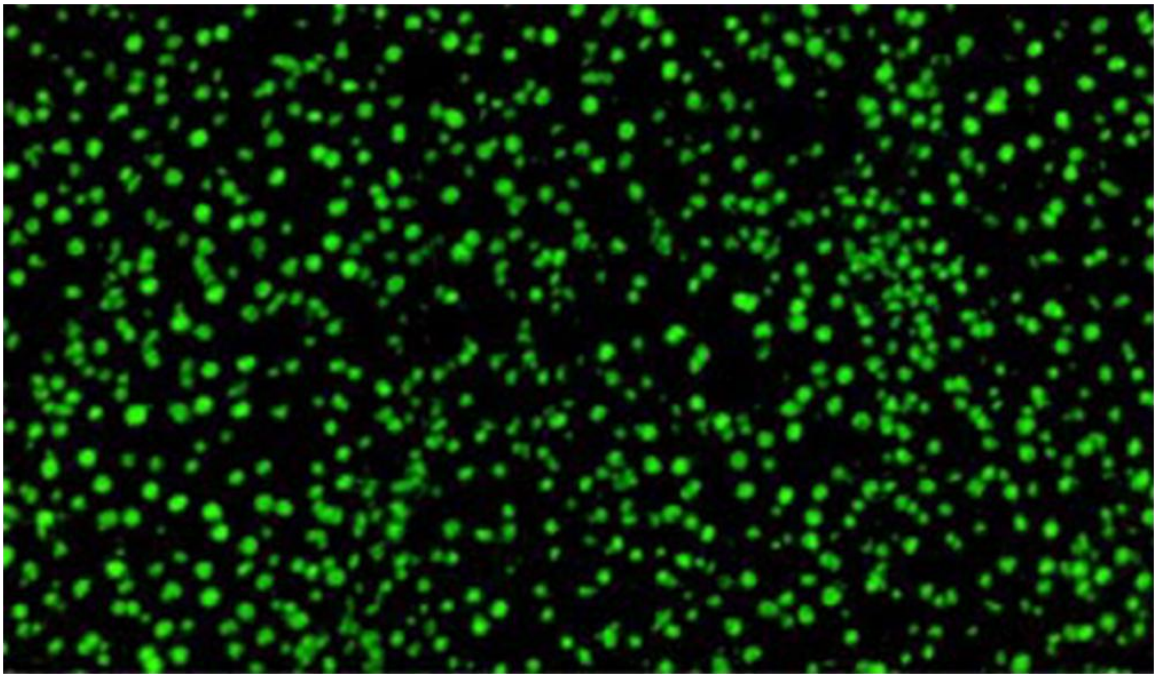


Figure 1.2. Antinuclear antibodies in autoimmune hepatitis. Immunofluorescence pattern of antinuclear autoantibodies on rodent liver section.

Human epithelial type 2 (HEp2) cells, characterised by prominent nuclei, should be used for a clearer definition of the nuclear pattern (Figure 1.3.); however, they should not be used for screening purposes due to a high positivity rate in adult and paediatric healthy populations (Tan et al. 1997, Hilario et al. 2004). In AIH, ANA target antigens are heterogeneous and not completely defined. ANA have been found to be reactive with single- and double-stranded deoxyribonucleic acid (DNA), small nuclear ribonucleoproteins (sn-RNPs), centromeres, histones, chromatin and cyclin A (Burlingame et al. 1993, Czaja et al. 1994, Strassburg et al. 1996a). The mechanism

leading to ANA production in AIH is not clear, although it has been related to the release of nuclear components following hepatocyte injury and/or a loss of B cell tolerance to several nuclear components (Nishioka 1998). In AIH, ANA titre is clinically relevant if higher than 1/40 in adults and than 1/20 in children (Vergani et al. 2004), in whom it correlates with disease activity (Gregorio et al. 2002).

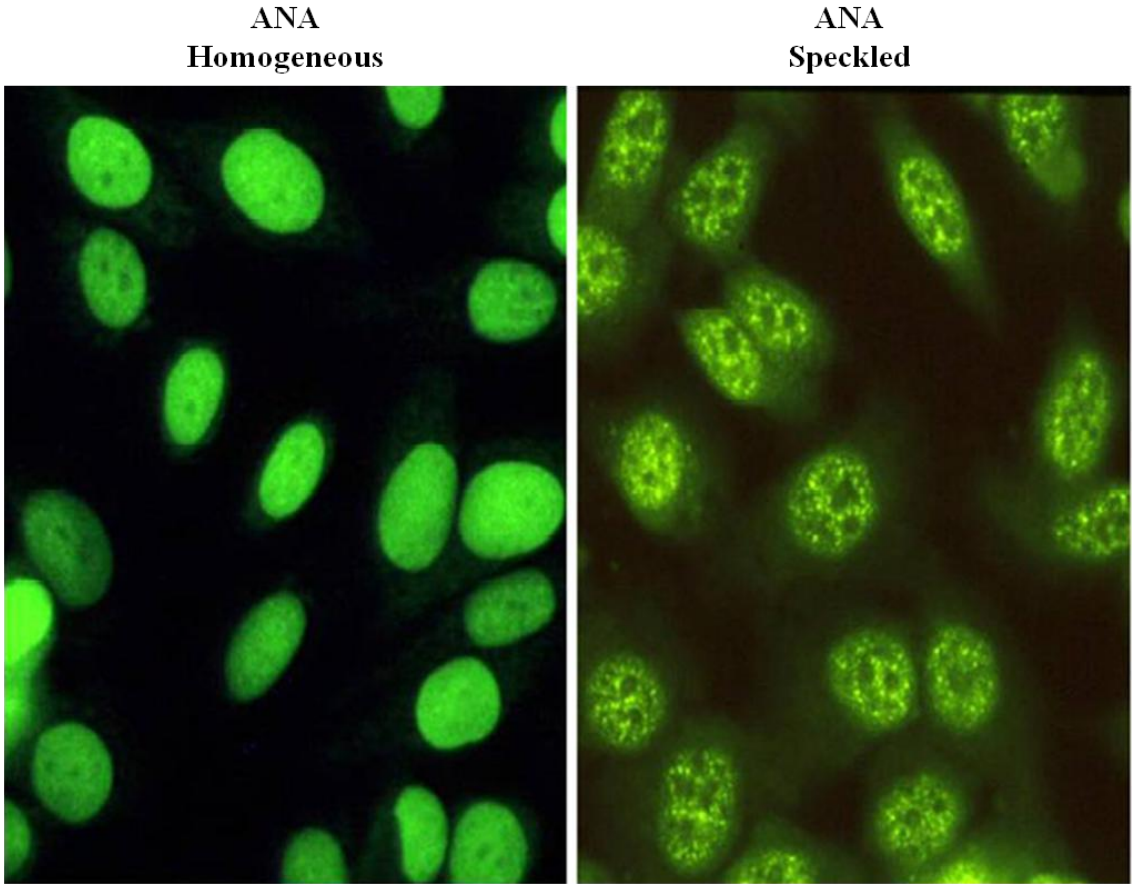


Figure 1.3. Anti-nuclear antibody immunofluorescence pattern on human epithelial type 2 cells. The homogeneous pattern (*left*) is the most common in autoimmune hepatitis (AIH). The speckled pattern (*right*) is less common in AIH, but more frequent in other conditions (e.g. chronic hepatitis C infection).

ANA have also been identified in up to 52% of patients with PBC (Strassburg and Manns 2002), a disease condition associated with the presence of AMA. However, while no AIH-specific ANA has been reported so far, PBC-specific ANA have been demonstrated and are recognisable by immunofluorescence giving a multiple nuclear

dot or a rim-like membranous pattern on HEp-2 or HeLa cells used as substrate (Szostecki et al. 1987, Bandin et al. 1996, Wesierska-Gadek et al. 1996). ANA are present not only in other autoimmune disorders such as systemic lupus erythematosus (SLE), Sjögren syndrome and scleroderma (Tan 1989) but also in patients with viral hepatitis, drug-induced hepatitis, and alcoholic and non-alcoholic fatty liver disease (Strassburg and Manns 2002, Adams et al. 2004); thus, though considered useful as diagnostic markers, ANA are not specific for AIH

4.3.2. Anti-smooth muscle antibodies

SMA antibodies are detected on rodent kidney, stomach and liver sections, where they stain the walls of the arteries. In the stomach they also stain the muscularis mucosa and the lamina propria. On the renal substrate, it is possible to visualise the V (vessels), G (glomeruli) and T (tubules) patterns (Bottazzo et al. 1976; Figure 1.4.). The VG and VGT patterns are more specific for AIH than the V pattern. The VGT pattern corresponds to the "F-actin" or microfilament (MF) pattern observed when cultured fibroblasts are used as substrate (Muratori et al. 2002). Neither the VGT nor the anti-MF patterns are, however, entirely specific for AIH. SMA are directed against components of the cytoskeleton such as actin and non-actin components including tubulin, vimentin, desmin, and skeletin (Strassburg and Manns 2002).

In AIH, they usually occur at titres higher than 1/80, though in very young patients with AIH-1 the titres may be as low as 1/20 (Gregorio et al. 1997). Like ANA, SMA are not specific for AIH, since their presence – particularly its V pattern – has also been reported in advanced liver disease of other aetiologies, infectious diseases and rheumatic disorders (Manns and Vogel 2006).

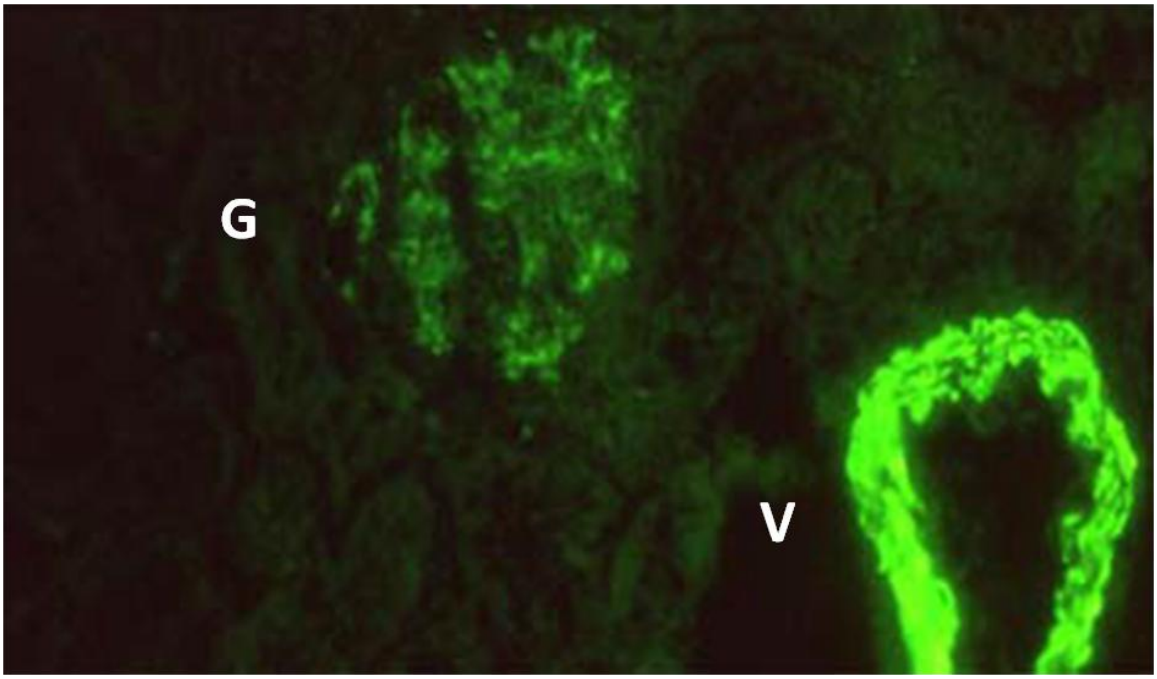


Figure 1.4. Anti-smooth-muscle antibodies in autoimmune hepatitis. Immunofluorescent pattern of anti-smooth-muscle antibodies (SMA) on rodent renal section. SMA stains the glomeruli (*G*) and arterial vessels (*V*).

It has been reported that antibodies to actin are found in a subset of patients that also produce SMA and are characterised by reactivity to F-actin in laboratory tests (Lidman et al. 1976, Toh 1979). Depending on the assay, antibodies to actin can be more specific than SMA for the diagnosis of AIH and for prognostic purposes (Fusconi et al. 1990). Indeed, patients that test positive for anti-actin antibodies in at least two different assays present at an earlier age and are less responsive to corticosteroids than patients without these antibodies (Czaja et al. 1996).

4.3.3. Anti-liver kidney microsomal type 1 antibodies

Anti-LKM-1 antibodies, the AIH-2 serological marker, stain the hepatocellular cytoplasm and the P3 portion of the renal tubules on rodent substrates. Since both anti-LKM-1 and AMA stain liver and kidney, they can be misdiagnosed (Bogdanos et al.

2008). Compared to anti-LKM-1, AMA stains the liver more faintly and the renal tubules more diffusely with an accentuation of the distal ones (Figure 1.5.). Moreover, while AMA stains gastric parietal cells, anti-LKM-1 does not (Rizzetto et al. 1973).

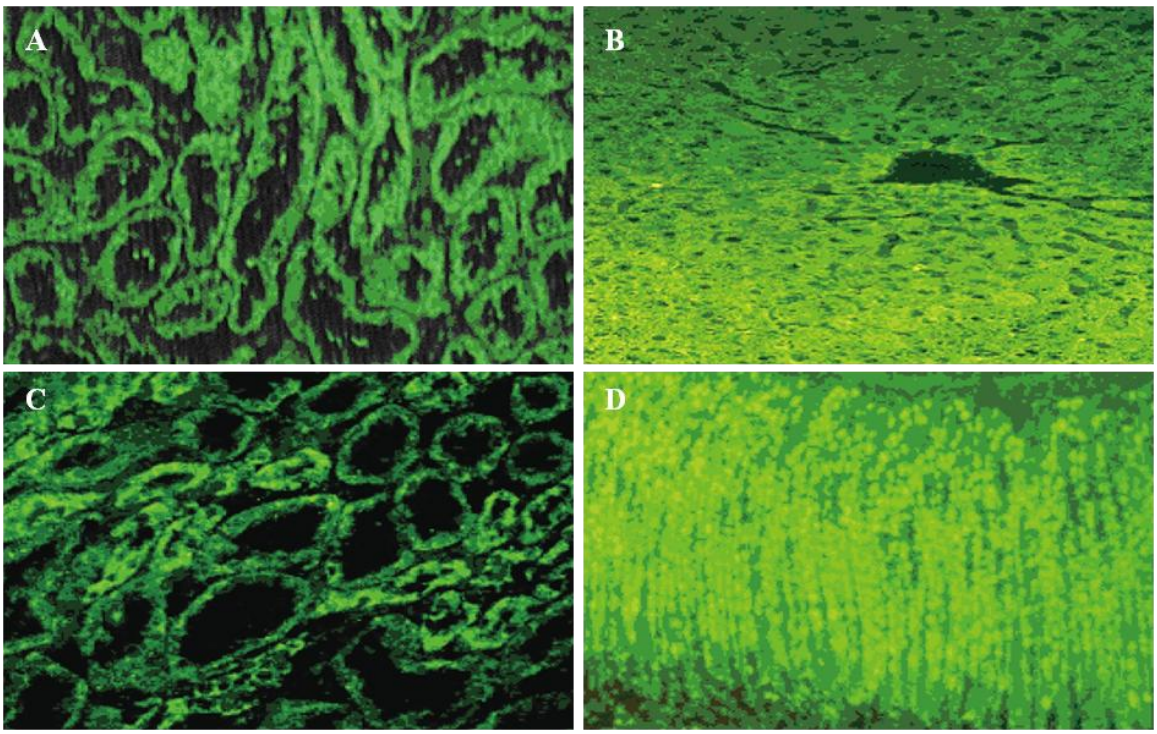


Figure 1.5. Immunofluorescence pattern of anti-liver kidney microsomal type 1 and anti-mitochondrial antibodies. Anti-liver kidney microsomal type 1 (anti-LKM-1) antibodies (*A&B*) stain stronger the proximal tubules (*A*), while anti-mitochondrial (AMA) antibodies (*C&D*) stain the smaller, distal tubules (*C*) of rodent kidney. Since these specificities are sometimes confused, the use of rodent liver (*B*) and stomach (*D*) sections is recommended to discriminate between the two reactivities; AMA typically stain the gastric parietal cells while anti-LKM-1 stain the liver but not the stomach.

The identification of the molecular targets of anti-LKM-1 – cytochrome P4502D6 (CYP2D6) –, and AMA – enzymes of the 2-oxo-acid dehydrogenase complexes –, have led to the establishment of immune-assays based on the use of recombinant or purified antigens. Commercially available enzyme-linked immunosorbent assays (ELISAs) are accurate for detecting anti-LKM-1, at least in the context of AIH-2, and reasonable for the detection of AMA. Thus, if

immunofluorescence remains doubtful, the use of molecularly based immune-assays may be of help (Vergani et al. 2004, Mieli-Vergani et al. 2009).

Anti-LKM-1 titre is clinically relevant if equal or higher than 1/40 in adult patients and 1/10 in subjects up to the age of 18 years (Vergani et al. 2004). Intriguingly, anti-LKM-1 are also present in up to 10% of patients with chronic hepatitis C virus (HCV) infection, and their titre has been found to be associated with disease activity (Lenzi et al. 1990).

A second type of anti-LKM antibodies (anti-LKM-2), nowadays of historical interest, was associated with hepatitis induced by ticrynafen, an uricosuric diuretic, withdrawn from clinical use in the United States in 1980 because of its severe hepatotoxicity. Anti-LKM-2 antibodies were found to react most intensely with the cells in the initial third of the proximal tubule and their target antigen was defined as cytochrome P4502C9 (CYP2C9; Lecoeur et al. 1996). Anti-LKM-3, which target members of the uridine glucuronosyltransferases (UGTs) family 1, give an immunofluorescent pattern similar to anti-LKM-1, though they occur mainly in hepatitis delta (D) patients; their presence, however, has also been reported in up to 19% of patients with AIH-2 (Philipp et al. 1994, Strassburg et al. 1996b, Csepregi et al. 2001).

4.3.4. Anti-liver cytosol type 1 antibodies

Anti-LC-1 antibodies stain the cytoplasm of liver cells with relative sparing of centrilobular area (Martini et al. 1988), but is usually obscured by concurrent presence of anti-LKM-1 (Figure 1.6.). In the presence of anti-LKM-1, anti-LC-1 can be detected by the use of liver cytosol in double-dimension immunodiffusion, or counter-immunoelectrophoresis, and a positive reference serum (Muratori et al. 1995).

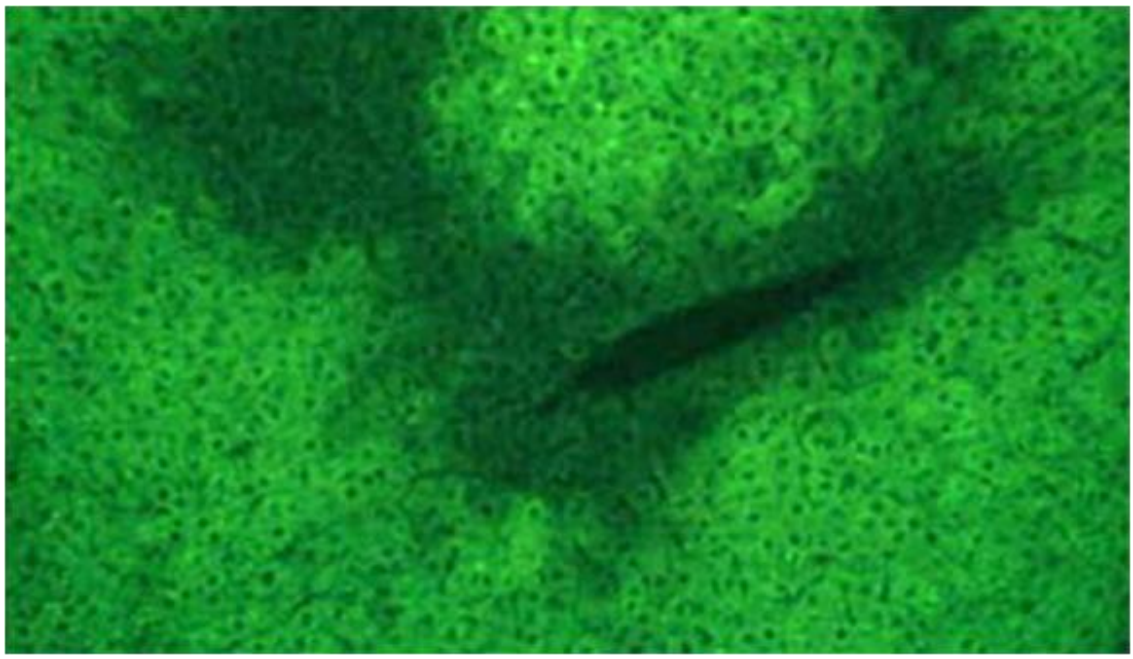


Figure 1.6. Anti-liver cytosol type 1 antibodies. Immunofluorescence pattern of anti-liver cytosol type 1 (anti-LC-1) antibodies on a rodent liver section: they stain the cytoplasm of hepatocytes with a weakening of the stain around the central vein.

Anti-LC-1 antibodies have been found either alone or in association with anti-LKM-1 in patients with AIH-2 (Abuaf et al. 1992). Their presence scores positively towards the diagnosis of AIH-2, allowing prompt initiation of treatment. Anti-LC-1 antibodies have also been reported in patients with HCV (Lenzi et al. 1995) and in AIH-1 (Han et al. 1995). The molecular target has been identified as the formimino-transferase cyclodeaminase, an enzyme involved in folate metabolism and most highly expressed in the liver (Lapierre et al. 1999). The presence and titre of anti-LC-1 antibodies has been found to correlate with disease activity and represents a potentially useful marker of residual hepatocellular inflammation in AIH (Muratori et al. 1998).

4.3.5. Anti-soluble liver antigen/liver-pancreas antigen antibodies

Anti-SLA and anti-LP, originally described as individual antibodies, share the same antigenic target and have subsequently been identified as the same autoantibody

(Wies et al. 2000). Anti-SLA/LP are detected by radioimmunoassay and ELISA but cannot be detected by immunofluorescence (Vergani et al. 2009).

The presence of anti-SLA/LP in the absence of conventional antibodies, has led to the proposed definition of a third group of AIH (AIH-3; Manns et al. 1987). However, early reports used a cut-off point for conventional autoantibody levels higher than those currently used for the diagnosis of AIH; therefore, this entity has not been accepted by the IAIHG. Nevertheless, anti-SLA/LP appears to be highly specific for the diagnosis of AIH, and its detection at the time of diagnosis identifies patients with a more severe disease and a worse outcome (Ma et al. 2002a).

Screening of cDNA expression libraries using high titre anti-SLA serum has allowed to identify UGA tRNA suppressor-associated antigenic protein ($tRNP^{(ser)sec}$) as the anti-SLA target (Costa et al. 2000). More recently, this target antigen has been renamed as Sep (O-phosphoserine) tRNA:Sec (selenocysteine) tRNA synthase (Palioura et al. 2009). Molecularly based diagnostic assays have become available, but their full evaluation is still underway (Vergani et al. 2009).

4.3.6. Anti-neutrophil cytoplasmic antibodies

ANCAs react to cytoplasmic components of neutrophils and give a perinuclear (pANCA) or a cytoplasmic (cANCA) pattern. In AIH-1, akin to PSC and inflammatory bowel disease (IBD), pANCAs are frequently detected, but they are atypical, since they react with peripheral nuclear membrane components (hence the name of peripheral anti-nuclear neutrophil antibody [pANNA]; Bogdanos et al. 2009). In contrast to AIH-1, pANNA is absent in AIH-2. Detection of pANNA aids the diagnosis of AIH, particularly in the absence of other autoantibodies (Vergani et al. 2004).

4.3.7. Anti-asialoglycoprotein receptor antibodies

Attempts to identify putative auto-antigens specifically expressed on the hepatocyte surface in AIH have led to the description of a crude liver extract preparation known as the liver specific protein (LSP) and its major component, the asialoglycoprotein receptor (ASGPR; McFarlane et al. 1984). ASGPR, also designated hepatic lectin, is a type II transmembrane glycoprotein. It is the only known liver-specific auto-antigen, and is constitutively expressed on the hepatocellular membrane. Antibodies to ASGPR are observed in 88% of all patients with AIH and can co-exist with ANA, SMA and anti-LKM-1 (Strassburg and Manns 2002). However, they are not disease specific, as they can also be found in viral hepatitis, drug-induced hepatitis and PBC (Treichel et al. 1993). In AIH the levels of anti-ASGPR correlate with inflammatory disease activity and may be used as an additional marker to monitor treatment efficacy (Treichel et al. 1994).

4.4. Histology

Since transaminases and IgG levels do not reflect the extent of histological inflammatory activity, or the presence or absence of cirrhosis, liver biopsy is mandatory not only to confirm the diagnosis but also to evaluate the severity of liver damage.

Hepatitis at the portal-parenchymal interface, known as interface hepatitis, is typical, though not exclusively observed in AIH (Czaja and Carpenter 1997). This picture is characterised by a lymphoplasmacytic infiltrate that crosses the limiting plate and invading the liver parenchyma (Figure 1.7.). Lymphocytes, plasma cells and histiocytes surround individual dying hepatocytes at the portal-parenchymal interface and in the lobule. Though plasma cells are usually abundant at the interface and

throughout the lobule, their presence in low number does not exclude the diagnosis of AIH (Vergani et al. 2009).

Other lesions that may be present include hepatocyte swelling and pycnotic necrosis. Fibrosis is present in all but the mildest forms of the disease. In contrast to patients with an insidious course, those presenting with acute liver failure show histologic features that predominate in the centrilobular area (Stravitz et al. 2011). Many patients presenting with fulminant hepatic failure tend to have massive necrosis and multilobular collapse; importantly, they have less fibrosis than those presenting with a more insidious course (Krawitt 2006).

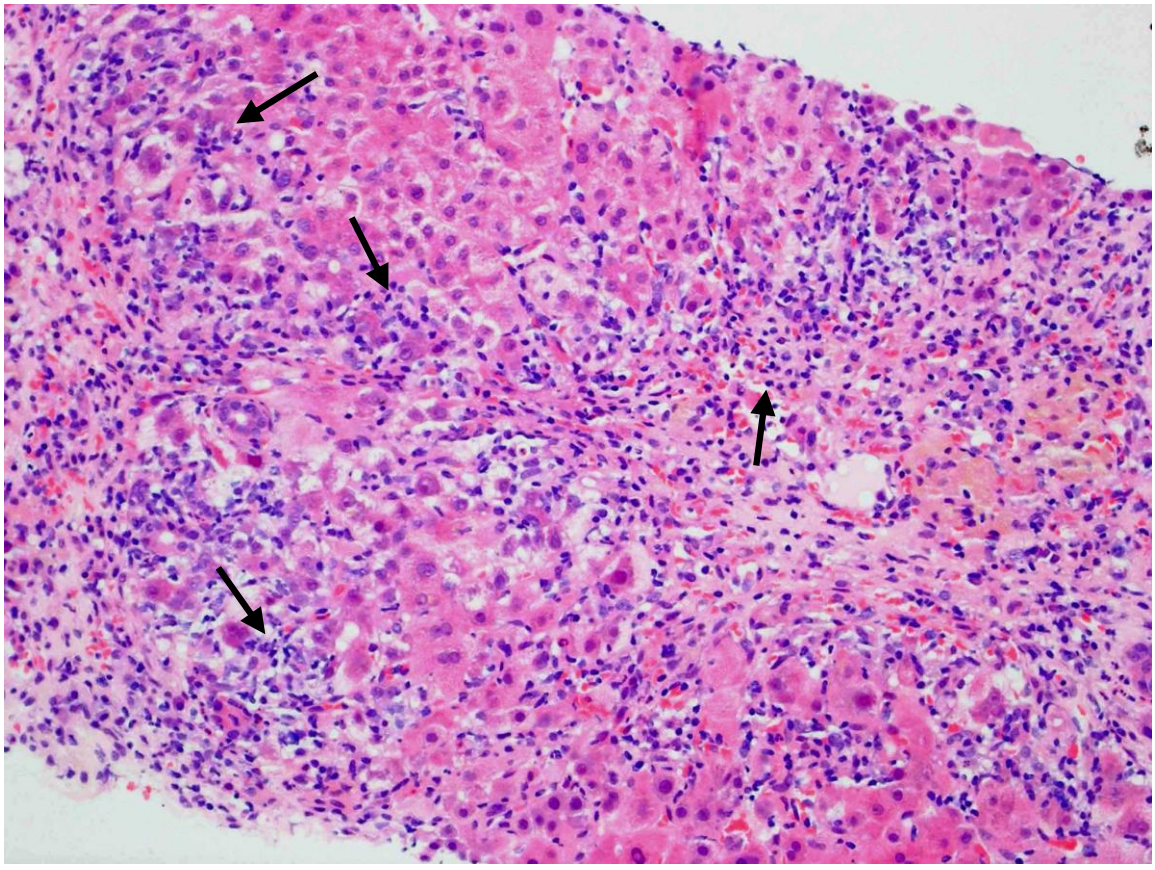


Figure 1.7. Histology of autoimmune hepatitis. The portal and periportal inflammatory infiltrate characteristic of autoimmune hepatitis is composed of lymphocytes, monocytes/macrophages and plasma cells (arrows showing plasma cell infiltratation), and is known as interface hepatitis. Haematoxilin & eosin staining (Picture kindly provided by Dr Alberto Quaglia, Institute of Liver Studies, King´s College Hospital).

Despite the fact that sampling variation may occur in needle biopsy specimens, especially in the presence of cirrhosis, the severity of the histological appearance is usually of prognostic value. However, even patients with cirrhosis at presentation respond well to immunosuppressive treatment (Mieli-Vergani and Vergani 2002).

Although the characteristic portal-parenchymal lesion usually spares the biliary tree, a small proportion of patients may show inflammatory changes surrounding the bile ducts, suggesting the presence of an overlap with sclerosing cholangitis, which occurs more frequently in the pediatric setting (Gregorio et al. 2001).

5. Overlap syndromes

The term “overlap syndrome” has been introduced to the field of hepatology to describe variant forms of AIH presenting with concomitant features of AIH and PBC or PSC (Czaja 1996). Standardisation of diagnostic criteria for overlap syndromes has not been achieved so far. There is still controversy as to whether these overlap syndromes represent distinct entities or are variants of the major autoimmune liver diseases (Beuers 2005). In this regard, a recent position statement published by the IAIHG advocates that patients with overlapping features should not be categorised as separate diagnostic entities, but instead considered to be part of the ‘‘classical’’ diseases (Boberg et al. 2011).

The common forms that have been described are: 1) PBC with features of AIH and 2) AIH with biliary features suggestive of PSC. Although they usually occur at the same time (termed as “true” overlap syndromes), they can also occur sequentially, i.e. patients present with features of one disease and subsequently develop characteristics of the other (Abdo et al. 2002).

5.1. Primary biliary cirrhosis-autoimmune hepatitis overlap syndrome

AIH and PBC are the most prevalent autoimmune liver diseases. Liver function tests typically show a hepatitic pattern in AIH, and a predominantly cholestatic pattern in PBC; moreover, while an elevation in IgG level is typical of AIH, an increase in IgM is present in the majority of PBC patients (Vierling 2004). Patients presenting with features of both diseases have been described since the 1970s (Kloppel et al. 1977, Okuno et al. 1987). In 1996, Czaja reported that PBC-AIH overlap was present in 8% of patients with AIH or PBC (Czaja 1996). This information was then confirmed by Chazouillères et al. who found that PBC-AIH overlap accounted for 9% of PBC patients (Chazouilleres et al. 1998). Subsequently, the same authors reported that AIH can develop in patients with long standing PBC (Poupon et al. 2006). Accordingly, it is speculated that in PBC, the overlap syndrome represents one end of a spectrum of immune-mediated injury in which damage to hepatocytes is more prominent and may assume the form of interface hepatitis (Lohse et al. 1999).

5.2. Autoimmune hepatitis-primary sclerosing cholangitis overlap syndrome

AIH-PSC overlap syndrome has been described in various reports over the last few decades, and is now assumed to be present in a considerable proportion of patients with autoimmune liver disease (el-Shabrawi et al. 1987, Rabinovitz et al. 1992, Gohlke et al. 1996, McNair et al. 1998). AIH-PSC overlap is characterised by ANA and/or SMA seropositivity, hypergammaglobulinaemia and interface hepatitis – all features typical of "classical" AIH – in conjunction with cholestatic biochemical alterations, frequent concurrence of IBD, and histological features of fibrous obliterative cholangitis, ductopenia, portal tract oedema and/or bile stasis (Floreani et al. 2005). The

diagnosis is supported by cholangiographic changes of intra-hepatic and/or extra-hepatic PSC (Kaya et al. 2000). In children, overlapping features of AIH and PSC are much more common than in adults and the term autoimmune sclerosing cholangitis has been coined (Gregorio et al. 2001; see section 7.2.).

6. Treatment

The goal of AIH treatment is to obtain early complete remission, to prevent disease progression, and to maintain this in the long term on the lowest possible dose of medication (Vergani and Mieli-Vergani 2011b). With the exception of a fulminant presentation with encephalopathy, AIH responds satisfactorily to immunosuppressive treatment whatever the degree of liver impairment, with a reported remission rate around 80% (Krawitt 2006).

6.1. Standard treatment

In the early 70s, three randomised clinical trials in adults with AIH provided the basis for the current immunosuppressive regimens, collectively suggesting that treatment with prednisolone improves liver function tests, ameliorates symptoms and prolongs survival (Cook et al. 1971, Soloway et al. 1972, Murray-Lyon et al. 1973). Although azathioprine was not able to induce remission when used on its own, it did allow the maintenance of remission in association with a significantly reduced dose of steroids (Johnson et al. 1995). Initial treatment with prednisone (or prednisolone) with or without azathioprine should be instituted as soon as the diagnosis is made, and not delayed for 6 months as suggested in the early studies (Lohse and Mieli-Vergani 2011). Standard treatment regimens are summarised in Table 1.6.

The initial approach to therapy partly depends upon histologic findings (Krawitt 2006). Standard treatment is indicated in any patient with evidence of interface hepatitis, with or without fibrosis or cirrhosis (Manns et al. 2010a). Transaminase and IgG levels do not correlate with histological damage and consequently provide limited help with respect to treatment initiation. In patients with portal inflammation only, institution of therapy is determined by AST and IgG levels, and/or by the presence of symptoms (Krawitt 2008). The therapeutic approach in patients with a milder form of the disease – who may be asymptomatic or pauci-symptomatic, and are detected incidentally after discovery of abnormal liver function tests – is less clear (Vergani and Mieli-Vergani 2011b). The benefit of therapy is undefined and may be so low that the risk of corticosteroid side effects might outweigh its possible benefits; this is particularly relevant when starting therapy in post-menopausal women and elderly patients (Al-Chalabi et al. 2006).

Table 1.6. Immunosuppressive treatment regimens for adults and children with autoimmune hepatitis.

Population	Initial regimen	Maintenance
Adults	Prednis(ol)one 60 mg/day OR Prednis(ol)one 30-60mg/day in combination with azathioprine 1-2 mg/kg/day	Prednis(ol)one 10 mg/week reduction until 20 mg/day, followed by of 5 mg/week reduction until 10 mg/day and by 2.5 mg/week reduction to reach maintenance (5 mg/day) Azathioprine 1-2 mg/kg/day if in combination with prednis(ol)one or 2 mg/kg/day if alone
Children	Prednis(ol)one 1-2 mg/kg/daily (up to 60 mg/daily) for two weeks either alone or in combination with azathioprine 1-2 mg/kg/day	Prednis(ol)one taper over 6-8 weeks to 0.1-0.2 mg/kg/day or 5mg/day Azathioprine 1-2 mg/kg/day if added initially

Adapted from Manns MP, Czaja AJ et al. Hepatology 2010; 51: 2193-2213.

Although some patients may remain in remission after drug treatment is withdrawn, most require long-term maintenance therapy. Despite the absence of firm guidelines, it is cautious not to attempt withdrawal of immunosuppression within 2

years of diagnosis (Manns et al. 2010a). During withdrawal attempts, it is essential to closely monitor the liver function tests, as relapse may be severe and even fatal. Patients who have successfully stopped immunosuppression should undergo long-term follow-up, as relapse can occur even 10 years later (Vergani and Mieli-Vergani 2011b).

6.1.1. Adverse side-effects

Corticosteroid-related side-effects must be considered and may influence the decision of treatment and the choice of medications (Table 1.7.). The most common side effect of steroid treatment is cushingoid changes, which affect the majority of patients after prolonged treatment (Summerskill et al. 1975). Less common, but severe, side-effects include osteoporosis, vertebral collapse, diabetes, cataract, hypertension and psychosis. These conditions are not necessarily contra-indications for the use of corticosteroids, but their presence may require special precautions and monitoring. In 13% of patients the development of such complications leads to dose reduction or even premature drug withdrawal; the most common reasons for treatment withdrawal are cosmetic changes or obesity, osteopenia with vertebral collapse, and brittle diabetes (Manns et al. 2010a).

Complications of azathioprine in AIH affect 10-20% of patients and include cholestatic hepatitis, veno-occlusive disease, pancreatitis, nausea and vomiting, rash and bone marrow suppression; these complications usually subside upon drug withdrawal (Manns et al. 2010a; Table 1.7.). The major side-effect of azathioprine is cytopenia; the risk to develop it relates to low erythrocyte concentration of thiopurine methyltranferase (TPMT) activity (Lennard et al. 1989, Ben Ari et al. 1995). The genes encoding TPMT are highly polymorphic, and deficiency of TPMP is present in 0.3-0.5% of the

population (Gisbert et al. 2007), though not all patients with deficiency experience bone marrow failure (Kaskas et al. 2003). While azathioprine is contra-indicated in homozygotes for TMTP deficiency, heterozygotes tolerate azathioprine at low doses and the level of enzyme activity may actually increase with continued administration of the drug (Cuffari et al. 2004; Czaja and Carpenter 2006b). Determination of enzyme activity is for this reason warranted only in the presence of pretreatment cytopenia, cytopenia developing during therapy or administration of higher-than-conventional doses of azathioprine (Czaja 2008).

Table 1.7. Side effects associated with standard treatment of autoimmune hepatitis.

Prednisolone-related	Azathioprine-related
Common	
Facial rounding	Nausea
Dorsal hump striae	Emesis
Weight gain	Rash
Acne	Fever
Facial hirsutism	Cytopenia
Alopecia	
Uncommon	
Osteopenia	Pancreatitis
Vertebral compression	Opportunistic infections
Cataracts	Arthralgia
Diabetes (brittle)	Cholestatic liver injury
Emotional instability	
Hypertension (labile)	
Rare	
Pancreatitis	Malabsorption
Opportunistic infections	Malignancy
	Bone marrow failure
	Teratogenicity

A well described complication of continuous immunosuppressive therapy is the development of malignancies (Wang et al. 1989, Werner et al. 2009). The incidence of extra-hepatic neoplasms in treated AIH patients is 1 in 194 patients-year, and the probability of tumor occurrence is 3% after 10 years (Wang et al. 1989, Manns et al. 2010a). Tumours do not have a predominant cell type, and they are not related to age, sex, treatment regimen or cumulative duration of treatment (Wang et al. 1989, Johnson et al. 1995). Nevertheless, since the risk of malignancy associated with chronic low dose azathioprine therapy has been reported to be 1.4-fold higher than that of age- and sex-matched normal populations, the beneficial effect of this drug as a corticosteroid-sparing agent must be must be counterbalanced with its risks (Wang et al. 1989).

6.2. Alternative treatments

In the group of patients that show failure to standard therapy, intolerance or low compliance to standard immunosuppression, alternative immunosuppressive treatments have been proposed (Yeoman et al. 2010). Decisions regarding the use of such regimens have to be based, however, on the scarce data available, mainly on the basis of studies conducted on small numbers of patients or case reports.

These alternative treatments include mycophenolate mofetil (MMF; Richardson et al. 2000), tacrolimus (Van Thiel et al. 1995), cyclosporine (Fernandes et al. 1999), methotrexate (Burak et al. 1998, Venkataramani et al. 2001), cyclophosphamide (Kanzler et al. 1997), ursodeoxycholic acid (UDCA; Czaja et al. 1999a), and rituximab (Barth and Clawson 2010; Table 1.8.). Although some encouraging results have been described, the progress with these non-standard treatments has evolved slowly and none has yet been incorporated into a standard management algorithm.

MMF is a purine antagonist that selectively inhibits proliferation of activated lymphocytes but is not dependent on TPMP activity (Heneghan and McFarlane 2002). It improves various symptoms of AIH, but many patients may also experience drug intolerance – headache, diarrhoea, nausea, dizziness, hair loss, and neutropenia (Vergani and Mieli-Vergani 2011b). In patients for whom standard immunosuppression fails to induce stable remission, or who are intolerant to azathioprine, MMF, together with prednisolone, is currently the treatment of choice (Manns et al. 2010a).

Calcineurin inhibitors, cyclosporine and tacrolimus, have been used as a rescue treatment for difficult-to-treat cases of AIH. As large studies in this subgroup of patients are lacking, they should be used with caution (Vergani and Mieli-Vergani 2011b).

Table 1.8. Alternative treatments for autoimmune hepatitis.

Drug	Advantages	Disadvantages
Mycophenolate mofetil	Favourable toxicity profile Experience as a transplant immunosuppressant	Contradictory reports regarding its efficacy
Cyclosporine	Potent immunosuppressant Experience in the transplant setting	Renal toxicity
Tacrolimus	Potent immunosuppressant Experience in the transplant setting	Renal toxicity
Budesonide	High first pass metabolism in the liver Immunosuppressive action	Ineffective in the cirrhotic liver
Cyclophosphamide	Efficacy	Dependency on continuous therapy Hematological side effects
Methotrexate	Favourable toxicity profile	Efficacy yet to be shwon
Ursodeoxycholic acid	Putative immunomodulatory capacities	Efficacy yet to be shown
Rituximab	Relatively favourable toxicity profile	Efficacy yet to be shown

Budesonide, a corticosteroid with the highest affinity for the glucorticoid receptor and with a high first pass metabolism, is presently receiving considerable attention as an alternative to prednisone or prednisolone in AIH treatment. First reports were somewhat contradictory, but in a recently published large European study a

combination of budesonide and azathioprine was reported to induce remission in a higher proportion of noncirrhotic patients with fewer adverse effects than medium-dose standard steroids and azathioprine. In this study, remission was achieved in 60% of the budesonide group but in only 39% of the prednisone group. (Manns et al. 2010b). It should be noted, however, that the latter remission rate is much worse than that reported in both adults and children (~80%) when a higher starting dose of prednisone is used. Moreover, budesonide cannot be used in cirrhotic patients, who represent at least a third of the AIH population (Hempfling et al. 2003).

Figure 1.8. provides an algorithm of the treatment strategy for adult patients with AIH as suggested by the British Society for Gastroenterology (BSG) and the American Association for the Study of the Liver (AASLD) published guidelines.

6.3. Liver transplantation

Liver transplantation (LT) is the ultimate treatment for AIH patients presenting with fulminant liver failure, or who develop end-stage chronic liver disease and for those with hepatocellular carcinoma that meet the transplant criteria (Reich et al. 2000, Mottershead and Neuberger 2008).

A combination of prednisolone and a calcineurin inhibitor is the most common immunosuppressive regimen used after LT, leading to a very successful outcome with reported 5-year and 10-year patient survival of 80-90% and 75% respectively (Manns et al. 2010a).

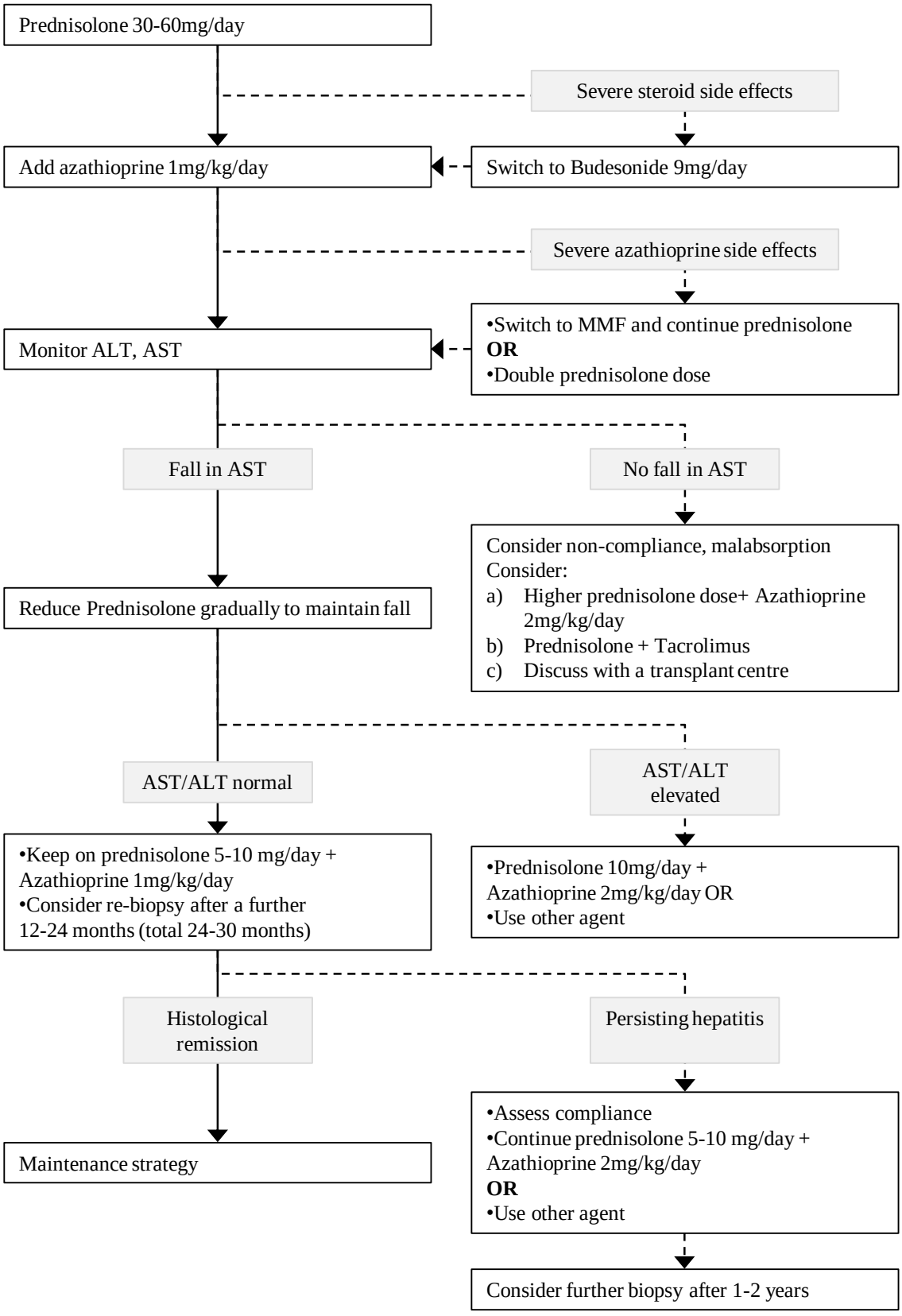


Figure 1.8. Treatment approach for adult patients with autoimmune hepatitis.
ALT, alanine aminotransferase; *AST*, aspartate aminotransferase; *MMF*, mycophenolate mofetil
Adapted from Gleeson D, Heneghan MA. Gut 2011; 60: 1611-1629

6.3.1. Recurrence of autoimmune hepatitis after liver transplantation

Although LT is a highly successful mode of treatment for AIH, primary disease recurs in ~30% of the patients (Neuberger et al. 1984, Gautam et al. 2006). Diagnosis of recurrent AIH is based on biochemical abnormalities, the presence of autoantibodies, interface hepatitis on liver histology and/or steroid dependence (Demetris et al. 2006, Manns et al. 2010a). Awareness of its existence and subsequent appropriate management has led, during the last few years, to a decrease in its frequency, and more importantly to a better outcome in patients diagnosed with this condition (Rowe et al. 2008). In this regard, it is of particular importance to be cautious when weaning immunosuppression off in patients who underwent LT for AIH since corticosteroid discontinuation may increase the risk for recurrent disease.

Additionally, it has been reported that AIH can also arise *de novo* following LT for non-autoimmune liver diseases. This form of graft dysfunction, called *de novo* autoimmune hepatitis, is characterised by features identical to those of classical AIH, namely hypergammaglobulinaemia, positivity for autoantibodies and interface hepatitis (Kerkar et al. 1998, Liberal et al. 2012). This condition, though reported in adults, seems to be much more prevalent in children (see section 7.3.)

7. Autoimmune liver disease in children

In children, there are two liver disorders in which liver damage is likely to arise from an autoimmune attack: “classical” AIH and AIH-sclerosing cholangitis overlap syndrome (also termed autoimmune sclerosing cholangitis). A possible autoimmune pathogenesis has also been postulated in the case of *de novo* autoimmune hepatitis following LT.

7.1. Comparison between AIH-1 and AIH-2

The distinction between type 1 and type 2 AIH is particularly relevant in paediatrics, since anti-LKM-1-positive disease is rare, though not absent, in adults (Gregorio et al. 1997, Oettinger et al. 2005). Anti-LKM-1-positive patients present with higher levels of bilirubin and transaminases than those who are ANA/SMA positive; in addition, AIH-2 presents significantly more frequently with fulminant hepatic failure compared to AIH-1. Apart from children with fulminant presentation, a severely impaired hepatic synthetic function, as assessed by the presence of both prolonged prothrombin time and hypoalbuminemia, is more common in ANA/SMA-positive than in anti-LKM-1-positive patients. The vast majority of patients have increased levels of IgG, but some 20% do not, thus normal IgG levels do not exclude the diagnosis of AIH. Partial IgA deficiency is more common in AIH-2 than in AIH-1 (Gregorio et al. 1997). The severity of interface hepatitis at diagnosis is similar in both types, but cirrhosis on initial biopsy is more frequent in AIH-1 than AIH-2, suggesting a more chronic course of the disease in the former (Gregorio et al. 1997).

7.2. Autoimmune sclerosing cholangitis

In recent years, it has become increasingly evident that in children and young adults, sclerosing cholangitis is often associated with florid autoimmune features, including elevated titres of autoantibodies, especially ANA and SMA, hypergammaglobulinaemia, and interface hepatitis on liver biopsy (Table 1.9.).

Table 1.9. Clinical presentation of childhood autoimmune liver disease.

Parameter	AIH-1	AIH-2	AISC
Median age in years	11	7	12
Mode of presentation (%)			
Acute hepatitis	47	40	37
Acute liver failure	3	25	0
Insidious onset	38	25	37
Complication of chronic liver disease	12	10	26
Associated immune-diseases (%)	22	20	48
Inflammatory bowel disease (%)	20	12	44
Abnormal cholangiogram (%)	0	0	100
ANA/SMA (%)	100	25	96
Anti-LKM-1 (%)	0	100	4
pANNA (%)	45	11	74
Anti-SLA (%)	58	58	41
Interface hepatitis (%)	66	72	35
Biliary features (%)	28	6	31
Cirrhosis (%)	69	38	15

ANA, anti-nuclear antibodies; *SMA*, anti-smooth muscle antibodies; *anti-LKM-1*, anti-liver kidney microsomal type 1 antibody; *pANNA*, peripheral anti-nuclear neutrophil antibodies; *SLA*, soluble liver antigen; *IgG*, immunoglobulin G.
From Gregorio GV, Portmann B et al. Hepatology.2001; 33: 544-553

Since these features are shared with AIH and are often not accompanied by elevated alkaline phosphatase or gamma glutamyl transpeptidase (GGT) levels at disease onset, the diagnosis of sclerosing cholangitis relies on cholangiographic studies. In childhood, this condition is called autoimmune sclerosing cholangitis (AISC) and is as prevalent as AIH type 1, as shown in a prospective study conducted over a period of 16 years (Gregorio et al. 2001). In this study, all children with serological – positive autoantibodies and high IgG levels – and histological – interface hepatitis – features of autoimmune liver disease underwent a cholangiogram at time of presentation. Approximately half of them had bile ducts changes characteristic of sclerosing cholangitis (Figure 1.9. and 1.10.), though these were generally less advanced than

those observed in adult PSC, and were therefore diagnosed as having AISC (Gregorio et al. 2001). Importantly, a quarter of the children with AISC, despite abnormal cholangiograms, had no histological features pointing to a bile duct involvement, and the diagnosis of AISC was only possible because of the cholangiographic studies (Figure 1.11.). Virtually all AISC patients were seropositive for ANA and/or SMA. In contrast to AIH, which is a predominantly a female disease, AISC affects equally boys and girls (Gregorio et al. 2001).

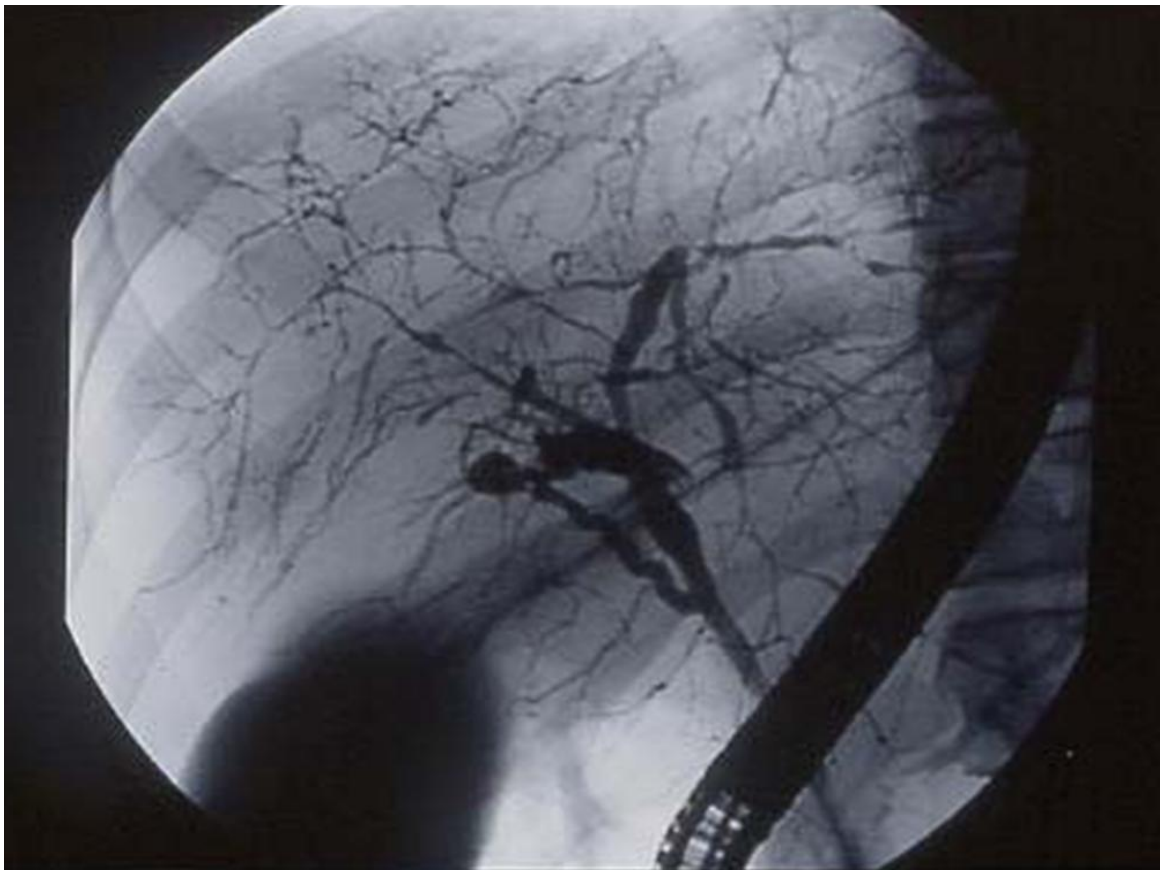


Figure 1.9. Endoscopic retrograde cholangiopancreatography (ERCP) in autoimmune sclerosing cholangitis. ERCP showing widespread bile duct strictures and dilatations in a child with autoimmune sclerosing cholangitis.

In the 16-year follow-up study it was shown that the mode of presentation of AISC was similar to that of AIH-1. Concomitant presence of IBD was, however, more frequent in AISC (45%), than in AIH-1 (20%). At the time of presentation, liver

function tests did not help in discriminating between AIH and AISC, though AP/AST ratio was significantly higher in AISC (Table 1.10.). pANNA was present in 74% of children with AISC vs 45% in AIH-1 and 11% in AIH-2 (Gregorio et al. 2001). Clinical, laboratory, and histological features of AIH-1, AIH-2 and AISC are compared in Table 1.9.

Table 1.10. Biochemical presentation of childhood autoimmune liver disease.

	AIH	AISC
Bilirubin (nv < 20 μmol/L)	35 (4-306)	20 (4-179)
Albumin (nv > 35 g/L)	35 (25-47)	39 (27-54)
AST (nv < 50 IU/L)	333 (24-4830)	102 (18-1215)
INR (nv < 1.2)	1.2 (0.96-2.5)	1.1 (0.9-1.6)
GGT (nv < 50 IU/L)	76 (29-383)	129 (13-948)
AP (nv < 350 IU/L)	356 (131-878)	303 (104-1710)
AP/AST ratio	1.14 (0.05-14.75)	3.96 (0.20-14.20)

AST, aspartate aminotransferase; *INR*, international normalised ratio; *GGT*, gamma glutamyltranspeptidase; *AP*, alkaline phosphatase; *nv*, normal values
From Gregorio GV, Portmann B et al. Hepatology.2001; 33: 544-553

The same study also showed that AISC is more common than sclerosing cholangitis without autoimmune features, autoantibody-negative sclerosing cholangitis having been observed in only 9 children referred over the 16-year study period.

In the absence of a cholangiography, AISC patients are diagnosed and treated as AIH, though evidence of bile duct damage can become apparent during follow-up. AISC responds to immunosuppression in terms of reduction of parenchymal inflammation, but bile duct disease progresses despite treatment in 50% of patients. Following favourable reports in adult PSC (Mitchell et al. 2001), UDCA is usually added to the regimen, though there are no data regarding its efficacy in arresting disease progression. The medium-term prognosis is good, with a reported 7-year survival of 100%, though 15% of patients require LT during follow-up (Gregorio et al. 2001).

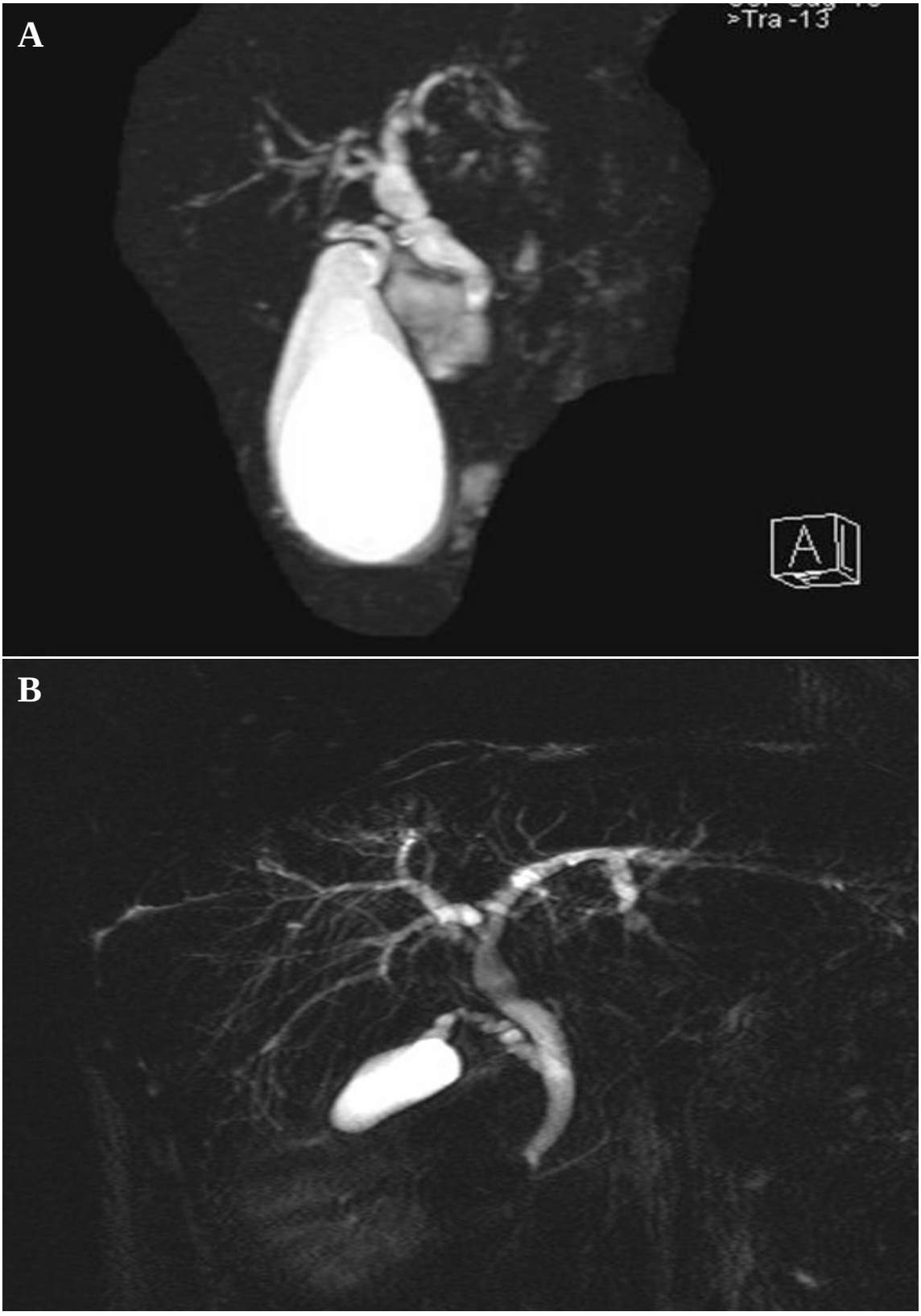


Figure 1.10. Magnetic resonance cholangiopancreatography (MRCP) in autoimmune sclerosing cholangitis. MRCP showing only gross (*A*), and both gross and subtle biliary changes (*B*) in children with autoimmune sclerosing cholangitis.

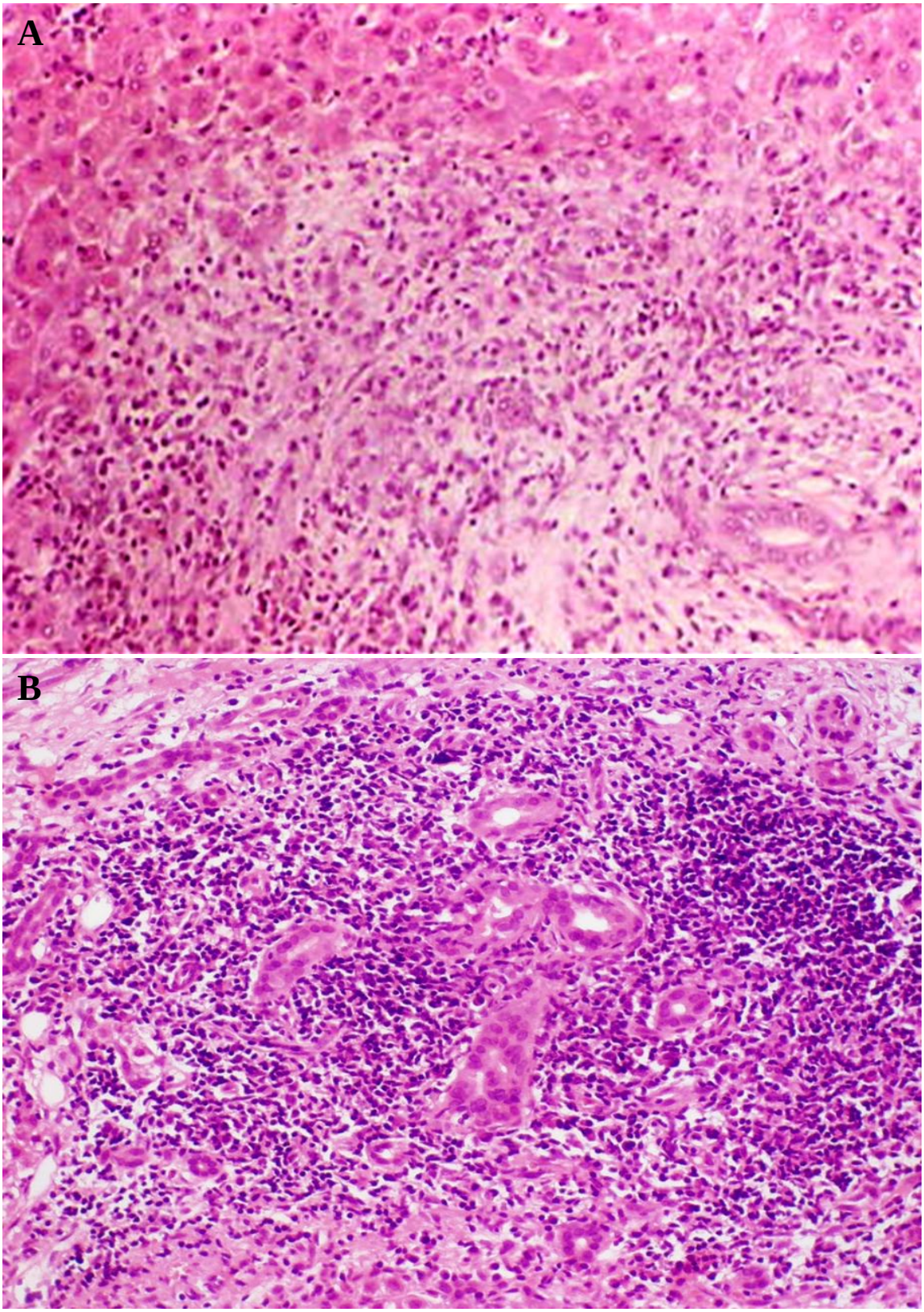


Figure 1.11. Histology of autoimmune sclerosing cholangitis. Interface hepatitis without obvious biliary changes in a patient with autoimmune sclerosing cholangitis (*upper panel*). Interface hepatitis with abundant plasma cell infiltration and clear bile duct damage in another child with autoimmune sclerosing cholangitis (*bottom panel*).

Evolution from AIH to AISC has been documented suggesting that AIH and AISC are part of the same nosological spectrum (Gregorio et al. 2001).

Whether childhood AISC and adult PSC belong to the same disease spectrum remains undefined, since no prospective study in a large cohort of patients has investigated the presence of bile duct damage at disease onset in adults with features of autoimmune liver disease. Interestingly, in a retrospective study, a high proportion of adults initially diagnosed as having AIH-1 were found to have sclerosing cholangitis on magnetic resonance cholangiography (Abdalian et al. 2008).

Criteria for the diagnosis of childhood autoimmune liver disease are depicted in Table 1.11. An algorithm for the treatment decision of children with autoimmune liver disease is provided in Figure 1.12.

Table 1.11. Criteria for the diagnosis of autoimmune liver disease in childhood.

Criteria
Elevated transaminases
Positivity for circulating autoantibodies • ANA and/or SMA (titre ≥ 1:20) = **AIH-1** or **AISC** • Anti-LKM-1 (titre ≥ 1:10) = **AIH-2** • Anti-LC-1 = **AIH-2**
Elevated immunoglobulin G (in 80% of cases)
Liver biopsy: • Interface hepatitis • Multilobular collapse
Exclusion of viral hepatitis
Exlusion of Wilson´s disease
Exclusion of non-alcholic steatohepatitis
Cholangiogram: • Normal = **AIH** • Abnormal = **AISC**

AIH, autoimmune hepatitis; *AISC*, autoimmune sclerosing cholangitis; *ANA*, anti-nuclear antibodies; *SMA*, anti-smooth muscle antibodies; *anti-LKM-1*, anti-liver kidney microsomal type 1 antibody; *anti-LC-1*, anti-liver cytosol type antibody

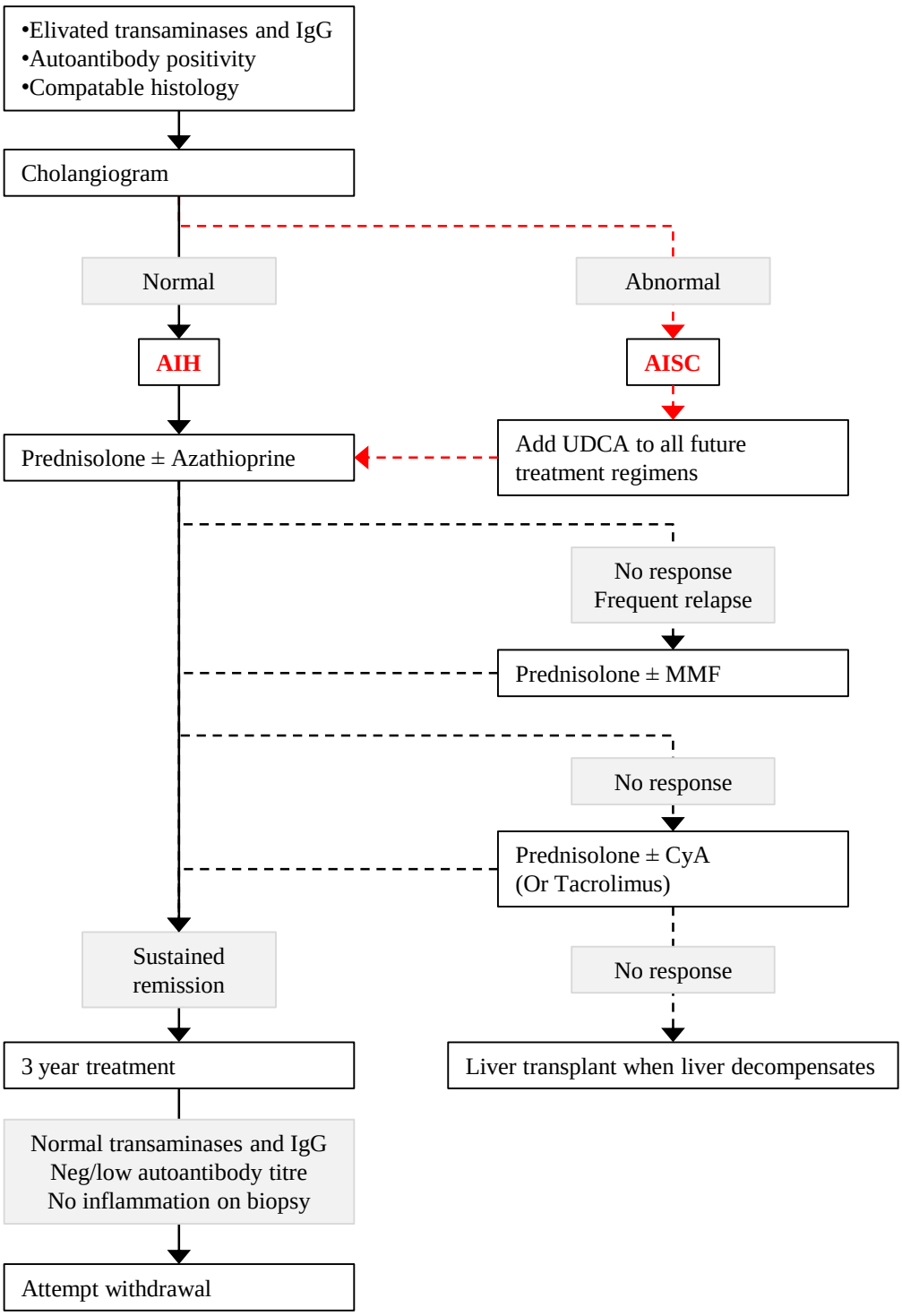


Figure 1.12. Algorithm for treatment decision in children with autoimmune liver disease. *IgG*, immunoglobulin G; *AIH*, autoimmune hepatitis; *AISC*, autoimmune sclerosing cholangitis; *UDCA*, ursodeoxycholic acid; *MMF*, mycophenolate mofetil; *CyA*, cyclosporine A; *neg*, negative
Adapted from Mieli-Vergani G, Vergani D. Best Pract Res Clin Gastroenterol. 2011; 25: 783-795

7.3. *De novo* autoimmune hepatitis after liver transplantation

Post-transplant *de novo* AIH (d-AIH) was initially described in 1998, and in contrast to the recurrence of the disease in patients transplanted for AIH, this condition affects patients transplanted for disorders other than AIH. In the first report of d-AIH, over a 5-year period, seven children (4% of 180 LT recipients) developed a form of graft dysfunction with features identical to those of classical AIH, namely hypergammaglobulinaemia, positivity for circulating autoantibodies (one ANA, two ANA and SMA, one gastric-parietal-cell antibody, and three atypical anti-LKM-1), and histological features of chronic hepatitis with portal and periportal inflammation (Figure 1.13.).

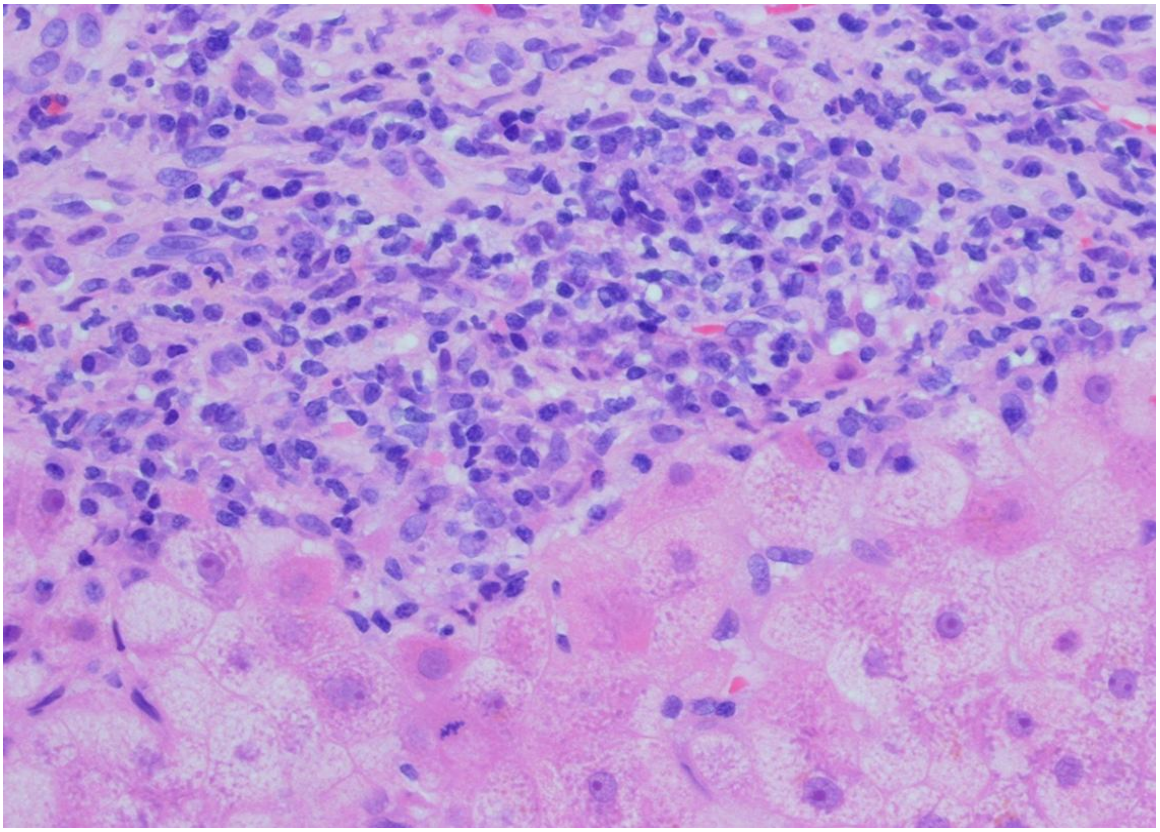


Figure 1.13. Histology of *de-novo* autoimmune hepatitis. The portal tract is densely infiltrated by mononuclear cells with a conspicuous presence of plasma cells, that extend and disrupt the limiting plate.

The index case did not respond to a short course of high dose steroids for rejection, but only to the classical treatment of AIH. None of the children were transplanted for autoimmune liver disease, none were hepatitis C virus positive, and all had serum concentrations of cyclosporine A or tacrolimus within therapeutic anti-rejection levels at the time of diagnosis of d-AIH (Kerkar et al. 1998). Since that report, several other groups have reported the occurrence of d-AIH after LT. Its prevalence in children ranges from 2.35% to 6.2% ; the indications for LT so far reported include: extra-hepatic biliary atresia, Alagille syndrome, acute liver failure, α-1-antitrypsin deficiency, primary familial intra-hepatic cholestasis, PSC, and Budd-Chiari syndrome (Hernandez et al. 2001, Gupta et al. 2001, Andries et al. 2001, Spada et al. 2001, Miyagawa-Hayashino et al. 2004, Venick et al. 2007)

Awareness that treatment with prednis(ol)one alone or in combination with azathioprine or MMF is successful in d-AIH has led to excellent graft and patient survival (Salcedo et al. 2002). It is of interest that these patients do not respond satisfactorily to short courses of high dose steroids for cellular rejection, making it essential to reach an early diagnosis to avoid graft loss.

CHAPTER II

Introduction to the present study

1. The immune system

The word immunity, which derives from the Latin *immunis* – meaning exempt or free from burden – refers to the mechanisms used by the body as protection against foreign agents. The immune system is therefore an organized network of cells and molecules with specialised roles in defending against infection.

Immunity requires the recognition and elimination or containment of infectious organisms. This is achieved by two different types of response. Innate (natural) immune responses depend on invariant receptors recognising conserved constituents of pathogens, and are activated immediately upon encounter of them. Though crucially important, innate responses can be overcome by pathogens and they do not lead to immunological memory, occurring to the same extent after each subsequent exposure. In contrast, the acquired (adaptive) immune system can recognise a specific molecular feature of a pathogen and provides enhanced protection against reinfection (Delves and Roitt 2000a).

The hallmark of adaptive immunity in advanced vertebrates is the existence of lymphocytes, which execute and regulate immune responses. Adaptive immunity can be classified into humoral – the antibody response produced by B cells – and cellular or T cell-mediated immune responses (Delves and Roitt 2000b).

1.1. Humoral immune response

B lymphocytes, which are responsible for antibody production, are the key mediators of the humoral immune response (Delves and Roitt 2000a). These cells obtained their name from early experiments on antibody production in birds, in which surgical removal of a lymphoid organ known as bursa de Fabricius resulted in a

complete inability to produce antibody. These antibody-producing cells then became known as bursa-derived, or B cells (Warner et al. 1962, Cooper et al. 1966, Moore and Owen 1966). In contrast to birds, mammals do not have a bursa; rather, B cells originate and mature within the bone-marrow before being released into the circulation. During foetal life, the liver is also an important site for B lymphocyte development (Delves and Roitt 2000b). B cells bear on their surface the B cell antigen receptor (BCR), which is a membrane bound form of the antibody that the B lymphocyte will secrete upon activation and differentiation into plasma cells (Ollila and Vihinen 2005). Antibodies can contribute to immunity in three main ways: firstly, antibodies can bind pathogens or their toxic products, thereby preventing their access to cells that they might infect or destroy, in a process known as "neutralisation"; secondly, antibodies bound to and facilitate the uptake of the pathogen by phagocytic cells that are specialised to destroy ingested bacteria ("opsonisation"); lastly, antibodies binding to a surface of a pathogen can activate the proteins of the complement cascade, which can ultimately lead to the formation of pores in the targeted pathogen and to its direct destruction. Altogether, neutralisation, opsonisation, and complement activation will eventually result in the scavenging and degradation of the pathogen or its toxic products by macrophages (MacLennan et al. 2003). Other than producing antibodies, another major role of B cells is that to present antigens and provide activation signals to T cells (see section 2.).

1.2. Cellular immune response

Seminal studies performed during the 1950s demonstrated that lymphocytes were responsible for two measurable immune responses: antibody production and graft rejection (Gowans 1957, Simonsen 1957). Later, it was shown that mice thymectomised

immediately after birth were defective at rejecting a tissue graft (Martinez et al. 1962); thus the lymphocytes that were thought to derive from the thymus were called thymus-dependent (T) lymphocytes or T cells.

T cells develop from progenitors derived from pluripotent hematopoietic stem cells in the bone marrow. These migrate through the blood to the thymus, where a stepwise rearrangement of genes encoding their unique receptor for antigen takes place. Viable T cells at an immature stage then leave the thymus to populate the lymph nodes. These naive T cells may become activated in the lymph node by antigens presented to them by antigen-presenting cells (APCs). The activated lymphocytes then proliferate, forming a clonally derived battalion of cells with specificity for the invader, and differentiate into functionally polarised lymphocyte subsets. Depending on its function and the type of cytokines produced, the cell is referred to as effector or regulator.

2. Generation of T cell responses

2.1. T cell activation

The initiation of an adaptive immune response requires the recognition of specific peptides, presented by APCs, by the T cell receptor (signal 1), as well as additional co-stimulatory signals, also provided by the APC (signal 2). A third signal, comprising cytokines produced by the APC, or otherwise present in the environment, also contributes to T cell differentiation.

2.1.1. Signal 1 – antigen recognition by T cells

T cells are defined by the surface expression of a receptor for antigen – the T cell receptor (TCR), composed of two different polypeptide chains, termed the TCRα

and TCRβ chains, which are linked by a disulfide bond. A minority of T cells (about 5% in humans) bear an alternative receptor composed of polypeptide chains designated γ and δ (Davis and Bjorkman 1988). The two subgroups are typically known as αβ and γδ T cells respectively. The function of the γδ TCR in immune responses is not entirely clear. For the purpose of this thesis the term TCR and T cell will be adopted to mean the αβ receptor and αβ T cell respectively.

Unlike immunoglobulins, which act as antigen receptors for B cells, TCRs cannot bind an antigen in its native (natural) state. In contrast, TCRs recognise short sequences of amino acids derived by intracellular proteolysis of the antigen. These peptides are then transported to the cell surface and presented to the TCR by a major histocompatibility complex (MHC) molecule (Konig 2002). MHC molecules are highly polymorphic glycoproteins encoded by genes within the major histocompatibility complex (*MHC*). There are two classes of MHC molecules – MHC class I and MHC class II. Under physiological conditions, peptides derived from proteins synthesised endogenously are presented *via* MHC class I molecules, whereas exogenous antigens, which are first endocytosed and then processed by proteolytic enzymes, are presented in association with MHC class II molecules (Delves and Roitt 2000b).

Two main T cell subsets can be defined by the expression of the cell-surface accessory molecules, CD4 and CD8. A third of T cells express CD8. As these cells are typically associated with target cell killing, the term cytotoxic T lymphocyte (CTL) is often used. Two thirds of T cells express the related glycoprotein CD4 on their surface. Since the function of mature CD4 T cells is primarily to induce activation of other immune cells, they have been termed T helper (Th) cells. The CD4 and CD8 accessory molecules have specific functions. While CD8 binds to an invariant part of MHC class I

molecules, recognising antigens presented by them, CD4 binds to and recognises peptides presented by MHC class II molecules. MHC class I expression by all nucleated cells enables infected cells to signal their condition to CD8 T cells and to establish cellular contacts that ultimately results in the death of the infected cell and its pathogenic content. In contrast, MHC class II molecules are expressed only by few specialised APCs (macrophages, dendritic cells and B cells), since recognition of peptides embraced by MHC class II molecules leads CD4 T cells to produce cytokines and govern the type and the extent of an adaptive immune response (Delves and Roitt 2000b).

In addition to the TCR, which interacts with the peptide-MHC complex, without itself possessing any intrinsic signalling properties, there are other cell surface molecules critically involved in T cell activation. Among these are the CD3γ, CD3δ, and CD3ε protein chains that in association with a disulfide linked ζ chain homodimer constitute the CD3 complex (Werlen and Palmer 2002). Each CD3 complex molecule contains an immunoreceptor tyrosine-based activation motif (ITAM). ITAMs comprise tyrosine residues, which upon phosphorylation by associated kinases, lead ultimately to the transcriptional activation of various gene sequences (Guy and Vignali 2009). This interaction between peptide-MHC and TCR, which is transduced by the CD3 complex, is generally designated ‘signal 1’ in the process of T cell activation.

2.1.2. Signal 2 – role of co-stimulation

Signalling through the TCR complex is not by itself sufficient to lead to the activation of a naive T cell. A second signal, known as ‘signal 2’ or co-stimulation, is also required and is provided by APCs (Lafferty et al. 1980). Signalling in the absence

of co-stimulation results in T cell anergy, a phenomenon that renders T cells unable to respond to subsequent antigen exposure (Schwartz et al. 1989, Jenkins et al. 1990). Thus, co-stimulation is pivotal in determining whether the encounter of a T cell with the antigen results in activation or anergy.

The best characterised and most effective T cell co-stimulatory pathway involves the CD28 receptor, and its two co-stimulatory ligands CD80 and CD86 (also known as B7-1 and B7-2 respectively; Greenwald et al. 2005; Figure 2.1.). While CD28 is constitutively expressed on all T cells in mice and on 95% of CD4 and 50% of CD8 T cells in humans, CD80 and CD86 are expressed mainly on APCs. Engagement of CD28 by CD80 or CD86 leads to the phosphorylation of a tyrosine in the cytoplasmic tail of CD28, and to the activation of the phosphotidylinositol 3-kinase (PI3K) pathway (Rudd et al. 2009). Together with the TCR signal, this pathway ultimately leads to the enhancement of interleukin (IL)-2 gene expression, up-regulation of cell-survival genes, promotion of energy metabolism (glucose uptake and glycolysis rate), and facilitation of cell-cycle progression, therefore resulting in the vigorous expansion and differentiation of antigen-stimulated T cells (Sharpe and Abbas 2006).

Conversely, TCR signalling can be attenuated by inhibitory molecules. These include cytotoxic T lymphocyte antigen 4 (CTLA-4), programmed death 1 (PD-1) and B and T lymphocyte attenuator (BTLA). Of these, CTLA-4 has been the most extensively studied. It shares 30% sequence homology with CD28, binding to the same ligands (Alegre et al. 2001). However, CTLA-4 binds CD80/CD86 at a much higher affinity than CD28 (Linsley et al. 1991), and delivers an inhibitory signal to the activated T cell, by preventing IL-2 production and arresting cell-cycle progression (Salomon and Bluestone 2001). Thus, CTLA-4 limits the expansion of activated T cells.

The pivotal role of CTLA-4 as a negative immune regulator is highlighted by the observation that mice genetically deficient in CTLA-4 expression develop a fatal lymphoproliferative disease characterised by progressive accumulation of T cells in peripheral lymphoid organs as well as in solid organs (Waterhouse et al. 1995, Tivol et al. 1995).

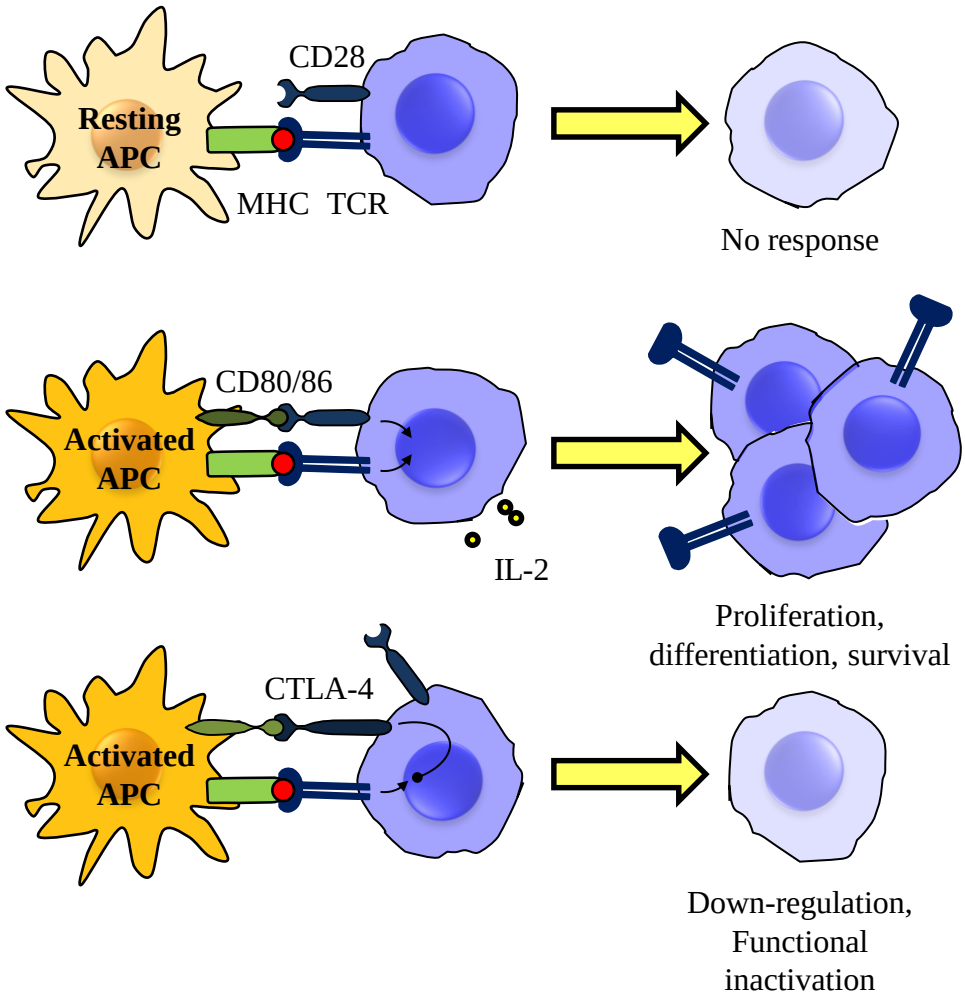


Figure 2.1. Role of CD80/CD86 and CD28/CTLA-4 pathway in T cell activation. Resting antigen-presenting cells (APCs) express few or no CD80/CD86 co-stimulatory molecules and thus fail to activate naïve T cells (*upper panel*). Cytokines produced in response to microbes activate APCs and lead to up-regulation of co-stimulatory molecules. CD80/86 – CD28 interaction stimulates the expansion and differentiation of naïve T cells (*middle panel*). Cytotoxic T lymphocyte antigen 4 (CTLA-4), the high affinity receptor for CD80 and CD86, is up-regulated on activated T cells. CD80/86 – CTLA-4 interaction inhibits T cell proliferation (*bottom panel*).

Although the interaction between CD80/CD86 and CD28/CTLA-4 molecules remains the best characterised (Figure 2.1.), other co-stimulatory and inhibitory molecules and their interaction have also been described. These pathways can be subdivided into two major categories according to the type of receptors they use: 1) members of the immunoglobulin (Ig) superfamily, which comprises the B7/CD28, CD2, and T-cell Ig and mucin domain (TIM) families (see section 7.3.1.), and 2) members of the tumour necrosis factor (TNF)/TNF receptor (TNFR) family (Sharpe 2009).

2.1.3. Signal 3 – cytokine environment

Upon activation, naïve T cells differentiate into subsets with specific effector functions. This decision is largely determined by the cytokine milieu present at the time of T cell activation in the peripheral immune compartment. These cytokines, produced either by APCs or T cells, are generally referred to as 'signal 3'(Gutcher and Becher 2007).

2.2. Differentiation of effector CD4 T cells

CD4 T cells play a central role in immune protection. They help B cells to make antibodies, enhance CD8 T cell responses, regulate macrophage functions, and recruit neutrophils, eosinophils and basophils to sites of infection and inflammation. Through their production of cytokines and chemokines, CD4 T cells orchestrate immune responses against a vast array of pathogenic microorganisms, and regulate the extent and persistence of these immune responses.

Based on their cytokine profile, two distinct effector CD4 T cell subsets were initially proposed: Th1 and Th2 cells (Mosmann et al. 1986). Almost twenty years later,

the Th17 cell subset emerged as a third major effector population of cells that could derive from their naïve CD4 precursors (Murphy et al. 2003, Aggarwal et al. 2003, Cua et al. 2003; Figure 2.2.). Almost simultaneously, an immune-regulatory T cell population was generated *in vitro* from naïve CD4 T cells (Chen et al. 2003, Fantini et al. 2004). These cells were labelled peripherally-induced adaptive regulatory T cells to distinguish them from the previously described thymus-derived natural regulatory cells (Zheng et al. 2004; the role of each regulatory T cell population will be discussed in section 5.). More recently, the existence of another two CD4 T cell subsets, namely "Th9" (Dardalhon et al. 2008, Veldhoen et al. 2008) and "Th22" T cell subsets (Duhen et al. 2009, Trifari et al. 2009) has been postulated, although lineage-specific transcription factors are as yet unidentified.

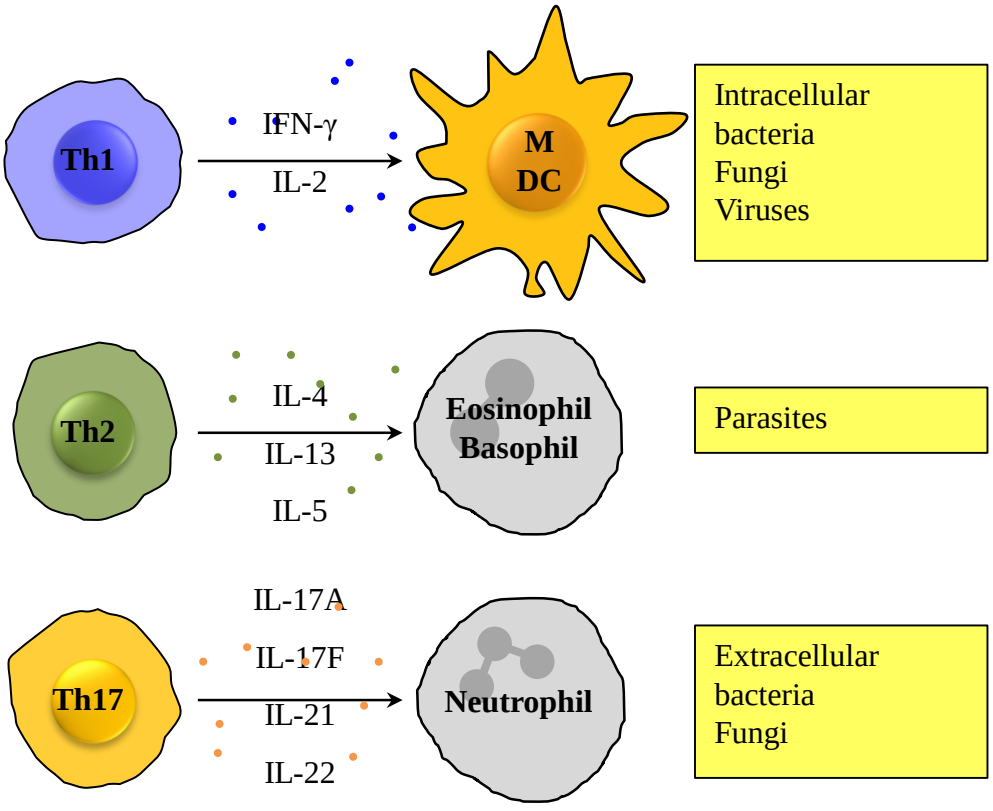


Figure 2.2. T helper cell subsets. Cytokine profile, target cell and corresponding types of infection are shown for each CD4 T helper (Th) cell subset.

2.2.1. Th1 cells

Th1 cells produce high levels of interferon-gamma (IFN-γ), promoting both phagocyte activation and the production of opsonizing and complement-fixing antibodies, thus playing an important role in protection against intracellular pathogens (Suzuki et al. 1988, Mosmann and Coffman 1989). In humans, Th1 cells play a particularly important role against mycobacterial infections (Zhu and Paul 2008). In addition to IFN-γ, their signature cytokine, Th1 cells produce lymphotoxin, TNF-α, and IL-2 (Mosmann and Coffman 1989, Paul and Seder 1994).

The full differentiation of Th1 cells requires the cooperation of type 1 interferons and IL-12 (Parronchi et al. 1992, Manetti et al. 1993). The presence of dendritic cell (DC)-derived IFN-α and/or natural killer (NK) cell-derived IFN-γ may lead to the activation of the signal transducer and activator of transcription (STAT)-1 in naïve CD4 T cells. Activated STAT-1 up-regulates the expression of T box expressed in T cells (Tbet; Afkarian et al. 2002), the master regulator for Th1 cell differentiation and IFN-γ production (Szabo et al. 2000). Ectopic expression of Tbet results in the induction of IFN-γ-polarised cells, whereas Tbet-deficient cells produce diminished amounts of IFN-γ (Szabo et al. 2002). Although Tbet induces the production of IFN-γ, it has been shown that IFN-γ itself, through STAT-1 activation, can induce the expression of Tbet, highlighting an important autocrine loop that serves as a powerful amplification mechanism for IFN-γ production and Th1 differentiation (Lighvani et al. 2001).

STAT-4 activation by IL-12 is also critical for Th1 responses. STAT-4-deficient mice display impaired Th1 cell differentiation along with an increase in Th2 responses, suggesting that STAT-4 acts to suppress Th2-mediated immunity (M. H. Kaplan et al. 1996b, Thierfelder et al. 1996). Interestingly, it was shown that GATA binding protein

3 (GATA-3), the Th2 lineage-specific transcription factor, antagonises Th1 responses by down-regulating STAT-4, rather than interacting directly with Tbet (Usui et al. 2003). The IL-12/STAT-4 pathway also favours Tbet up-regulation, in a similar way to the IFN-γ/STAT-1 pathway (Y. Yang et al. 2007, Usui et al. 2006), although STAT-4 may also induce IFN-γ production independently of Tbet (Zhu and Paul 2010).

At later stages of Th1 differentiation, expression of the IL-18 receptor alpha chain (IL-18Rα) is increased. IL-18Rα up-regulation requires the IL-12/STAT-4 pathway and is further enhanced by IFNγ (Micallef et al. 1996, Robinson et al. 1997, Stoll et al. 1998). IL-12 together with IL-18 can induce IFNγ production by Th1 cells in the absence of TCR stimulation (Yang et al. 2001). Such antigen-independent cytokine production is probably important for amplifying Th1 responses by recruiting other pre-existing Th1 cells (Smeltz et al. 2002, Okazawa et al. 2002).

2.2.2. Th2 cells

Th2 are key participants in the host response against extracellular pathogens, such as helminths (Mosmann and Coffman 1989, Romagnani 1991). They produce a vast array of cytokines, including IL-4, IL-5, IL-10, and IL-13. IL-4 is the cytokine responsible for IgE class switching in B cells (Kopf et al. 1993). Like IFN-γ for Th1 cells, IL-4 is the positive feedback cytokine for Th2 cell differentiation (Le Gros et al. 1990, Swain et al. 1990). IL-5 plays a critical role in recruiting eosinophils (Coffman et al. 1989) and IL-10 suppresses Th1 cell proliferation and inhibits DC function (Moore et al. 2001). IL-13 is similar in structure and activity to IL-4, enhancing B cell responses and augmenting Th2 cell development (Wynn 2003). In addition, IL-13 is considered critical for helminth expulsion (Urban et al. 1998).

The interaction of IL-4 with its receptor results in the phosphorylation of STAT-6, which in turn induces the expression of the Th2 master regulator GATA-3, and consequently promotes the activation of the Th2 characteristic cytokines IL-4, IL-5 and IL-13 (M. H. Kaplan et al. 1996a, Zheng and Flavell 1997, Kurata et al. 1999, Zhu et al. 2001). GATA-3 may also induce its own expression when its levels reach a certain threshold (Ouyang et al. 2000). Th2 differentiation is completely abolished when GATA-3 is conditionally deleted in peripheral CD4 T cells (Zhu et al. 2004, Pai et al. 2004). However, GATA-3 alone is not sufficient to drive IL-4 production. IL-2-mediated activation of STAT-5 is also necessary (Zhu et al. 2003). Thus, cooperation of STAT-5 with GATA-3 accounts for full Th2 differentiation (Zhu et al. 2006, Ho et al. 2009; Figure 2.3.). Importantly, the mutual regulation of Th1 and Th2 cell polarisation is induced not only by IL-4 and IFN-γ respectively, but also by the Th1 and Th2 lineage specific transcription factors. Accordingly, GATA-3 can down-regulate STAT-4 (Usui et al. 2003), and strong STAT-5 activation inhibits Tbet expression (Zhu et al. 2003). Conversely, Tbet can directly suppress GATA-3 expression (Usui et al. 2006) (Figure 2.3.)

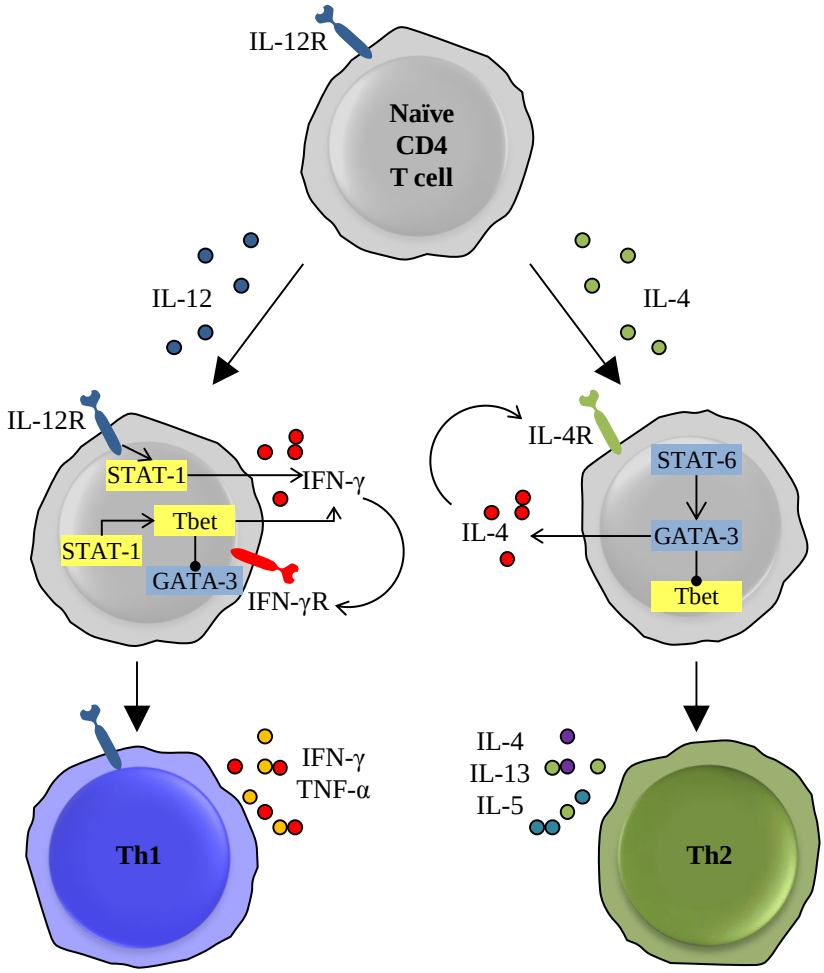


Figure 2.3. Cross regulation of T helper 1 and 2 cell differentiation. At the time of T cell activation, the cytokines present in the milieu are responsible for the differentiation pathway chosen. Cross regulation ensures the efficient expansion of one lineage. In T helper (Th) 1 differentiation IL-12 induces the activation of signal transducer and activator of transcription 4 (STAT-4) that induces interferon-γ (IFN-γ) transcription. IFN-γ feeds back positively, activating the transcription factor T box expressed in T cells (Tbet) *via* STAT-1. Tbet promotes IFN-γ production and inhibits Th2 cytokine expression *via* GATA-3. Th1 differentiation is promoted in an IL-4 rich milieu. IL-4 activates STAT-6 to increase the expression of GATA-3. GATA-3 drives the expression of Th2 cytokines while suppressing Tbet and consequently Th1 development.

2.2.3. Th17 cells

In recent years, the Th1/Th2 binary model of CD4 effector T cell differentiation has been challenged following the discovery of Th cells that produce IL-17 (consequently named Th17 cells), and exhibit effector functions distinct from Th1 and Th2 cells (Langrish et al. 2005).

Th17 cells are important mediators of protection against extracellular pathogens not effectively handled by either Th1 or Th2 cells (Korn et al. 2009, Weaver et al. 2006). Produced in large quantities by Th17 cells, IL-17A is responsible for most of the Th17-mediated effects, and is therefore considered the prototypic cytokine of the IL-17 family, which includes five additional members (IL-17 B, C, D, E and F; Kolls and Linden 2004). In addition to IL-17A (commonly referred to as IL-17 also in this thesis), Th17 cells co-produce IL-17F (Liang et al. 2006). These two cytokines exert overlapping effector functions: upon cross-linking of their receptor they both induce production of pro-inflammatory cytokines (IL-6, IL-1 and TNF), pro-inflammatory chemokines (CXCL-1, CXCL-6 and IL-8) and metalloproteinases, ultimately leading to the recruitment of neutrophils to the site of inflammation (Bettelli et al. 2008). Since their receptors (IL-17AR and IL-17CR) are expressed on both hematopoietic and non-hematopoietic cells, IL-17A and IL-17F are able to induce inflammation at several levels. Although the majority of Th17 cells express both IL-17A and IL-17F, it is clear now that there are Th cells expressing only IL-17A, only IL-17F or both (Liang et al. 2007, Chang and Dong 2007).

Besides IL-17A and IL-17F, Th17 cells also produce IL-21 and IL-22, neither of which are Th17-exclusive cytokines, although preferentially produced and released by Th17 cells (Liang et al. 2006, Korn et al. 2007a, Y. Zheng et al. 2007a). Whereas IL-17 has broad effects on many cell types, IL-21 acts on other cells such as B lymphocytes and further amplifies Th17 responses (Nurieva et al. 2007). IL-22, a member of the IL-10 family, is produced by activated T cells and NK cells (Wolk and Sabat 2006). It mediates its effects through a receptor complex expressed primarily within tissues, particularly by epithelial cells of the gastrointestinal tract and skin, but absent on

immune cells (Kotenko et al. 2001, Moore et al. 2001). IL-22 is a bifunctional cytokine with both pro- and anti-inflammatory effects: while promoting tissue inflammation in response to extracellular pathogens (Aujla et al. 2008), it also increases acute-phase reactant production by hepatocytes, thus protecting them from acute liver inflammation (Zenewicz et al. 2007).

In contrast to Th1 and Th2 cell differentiation, which is dependent upon their respective effector cytokines, Th17 cell differentiation does not require IL-17. Instead, a combination of the anti-inflammatory cytokine transforming growth factor beta (TGF-β) and the pro-inflammatory and pleiotropic cytokine IL-6 is required to induce the differentiation of a naïve T cell into a Th17 lymphocyte (Mangan et al. 2006, Veldhoen et al. 2006, Bettelli et al. 2006). In the absence of IL-6, Th17 cells can also be generated by a combination of TGF-β and IL-21, but IL-6 is a comparatively stronger inducer of Th17 immunity (Korn et al. 2007a, Zhou et al. 2007). IL-21, a member of the IL-2 family of cytokines, is also produced in large amounts by Th17 cells, therefore acting in an autocrine fashion to amplify Th17 differentiation (Nurieva et al. 2007). In addition to the factors mentioned above, IL-1β can synergize with IL-6 to induce Th17 cells (Acosta-Rodriguez et al. 2007). Lastly, although the differentiation of naïve T cells into Th17 cells does not require IL-23, its presence is essential for the expansion and maintenance of the Th17 population. Indeed, recent data have shown that T cells cultured in the presence of TFG-β and IL-6 do not cause tissue inflammation unless also cultured in the presence of IL-23 (Stumhofer et al. 2007, McGeachy et al. 2007). On the basis of these data, Bettelli et al. proposed a three-step model essential for the full differentiation of Th17 cells: induction of Th17 differentiation by a combination of TGF-β and IL-6, followed by amplification of Th17 cell frequency by IL-21, and lastly

maintenance/stabilisation of the phenotype of previously differentiated Th17 cells by IL-23 (Bettelli et al. 2008; Figure 2.4.).

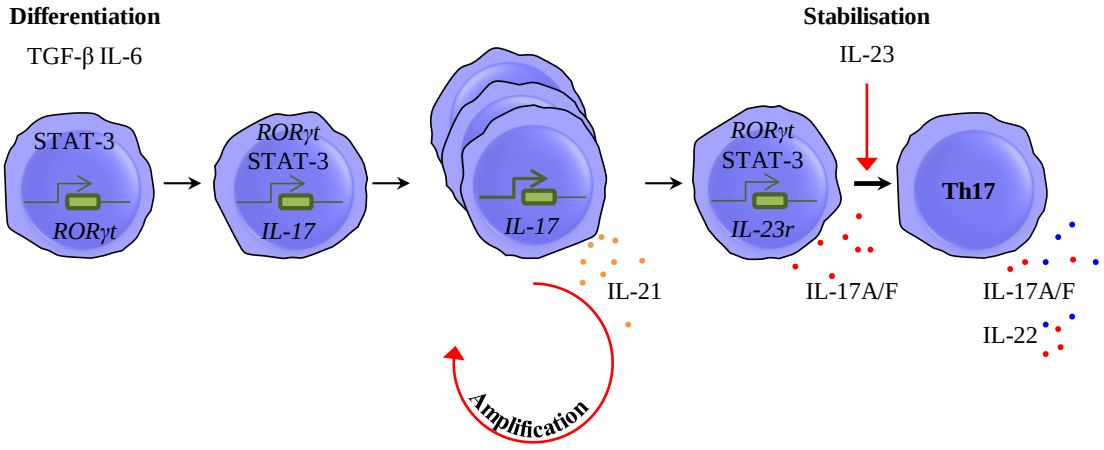


Figure 2.4. Steps in Th17 cell differentiation. Different factors control the initial differentiation, the amplification and the stabilisation of Th17 cells. Whereas transforming growth factor-β (TGF-β), together with IL-6, are the differentiation factors for Th17 cells, IL-21, which is produced by Th17 cells themselves, acts in a positive feedback loop to amplify the frequency of Th17 cells. Lastly, IL-23 expands and stabilises Th17 cells to produce their effector cytokines.

Analogous to Tbet in Th1 and GATA-3 in Th2 cells, Th17 development relies on the presence of a lineage-specific transcription factor, identified as the retinoic acid-related orphan receptor gamma t (RORγt) (Park et al. 2005, Harrington et al. 2005; Figure 2.3.). RORγt is the master regulator that drives Th17 cell lineage differentiation (Ivanov et al. 2006). Its expression is induced in naïve CD4 T cells within 8 hours of TCR engagement along with TGF-β and IL-6. Over-expression of RORγt promotes Th17 differentiation, whilst RORγt-deficient T cells produce very low levels of IL-17 in response to TGF-β, IL-6 or IL-21 (Ivanov et al. 2007). The residual IL-17 production by RORγt-deficient cells is dependent on the activity of a related nuclear receptor, RORα, also up-regulated in Th17 cells. Although RORα deficiency results in minimally reduced IL-17 production, loss of both RORγt and RORα completely abrogates Th17 differentiation (X. O. Yang et al. 2008). The induction of RORγt is dependent on

STAT-3, a transcription factor activated by IL-6, IL-21 and IL-23 that has been shown to play an important role in the expression of IL-17. Conditional deletion of STAT-3 in T cells prevents the development of Th17 cells, and over-expression of an active form of STAT-3 increases IL-17 production (X. O. Yang et al. 2007, Harris et al. 2007). STAT-3 affects Th17 differentiation by up-regulating RORγt, which is upstream of IL-17; thus both transcription factors seem to cooperate, and the presence of both is required for IL-17 production (X. O. Yang et al. 2007, X. O. Yang et al. 2008).

Cytokines and transcription factors for the differentiation of the three effector Th cell subsets are summarised in Figure 2.5.

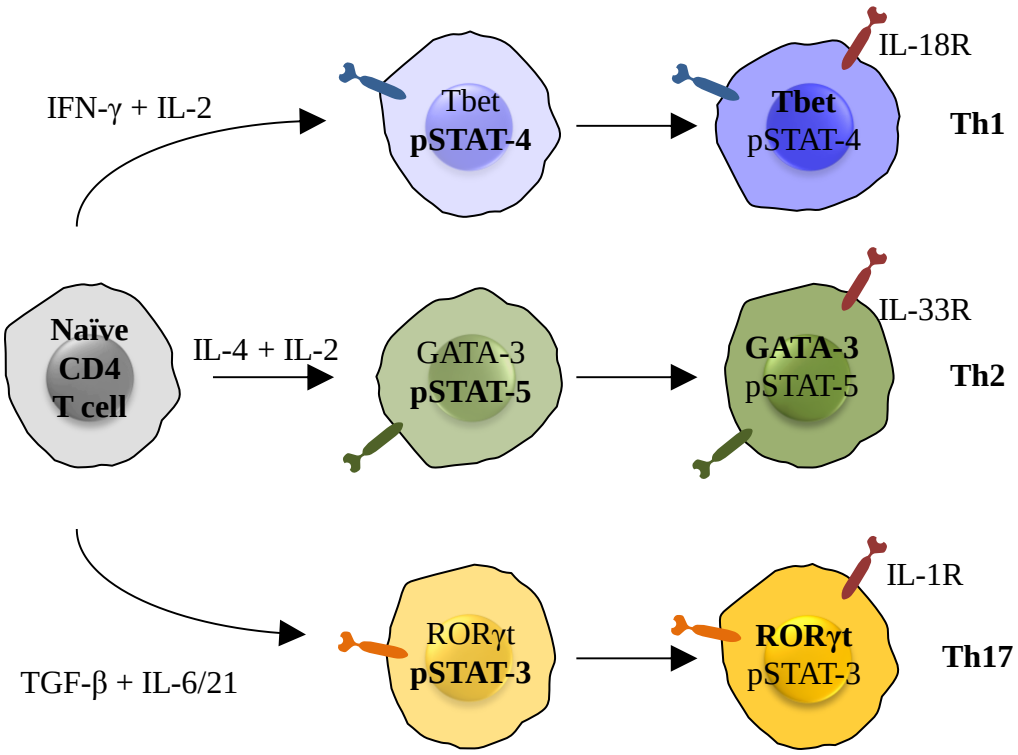


Figure 2.5. Cytokines and transcription factors critical for effector T helper (Th) cell differentiation. Upon T cell receptor (TCR) activation triggered by antigen-presenting cells (APCs), naïve CD4 T cells differentiate into distinct Th lineages depending on the types of cytokines present in the environment. The differentiation process involves up-regulation of master transcriptional factors and activation of signal transducers and activators of transcription (STAT) proteins. At later stages of Th cell differentiation, different Th cells preferentially express an interleukin-1 (IL-1) family receptor. Together with a STAT protein, the IL-1 family cytokine induces effector cytokine production from Th cells in a TCR-independent manner.

3. Tolerance

As discussed in the previous section, the immune system is equipped with very powerful effector mechanisms that can eliminate a wide variety of pathogens. However, during early days of the study of Immunity, Paul Ehrlich realised that these effector mechanisms could, if directed against the host, cause severe tissue damage – a phenomena that he termed *horror autotoxicus*. Thus, if unrestrained immune reactions directed towards self-tissues do occur, they would result in a wide spectrum of chronic syndromes generally coined autoimmune diseases.

Since the T cell arm of the immune system includes an incredibly large repertoire of T cell clones, each containing a unique receptor for antigen, it allows the immune system to respond to a almost infinite variety of antigens (Jiang and Chess 2006). As a consequence, this diverse repertoire contains T cells recognising the body´s own antigens – self-reactive T cells – that drive unwanted autoimmune responses. For this reason, the immune system has evolved several mechanisms to restrain such T cells, and therefore prevent damage to self tissues. This process that leads to the elimination or neutralisation of self-reactive cells is known as tolerance, and a breakdown in the working of this system can lead to autoimmunity (Kamradt and Mitchison 2001). Immunological tolerance is multi-layered, encompassing a network of mechanisms, that allows maintenance of protective immunity while averting autoimmunity (Mathis and Benoist 2010). These layers are generally classified into two broad categories: central tolerance that shapes the T cell repertoire in the thymus and peripheral tolerance, which relates to the regulation of T cell responses in the periphery (Figure 2.6.).

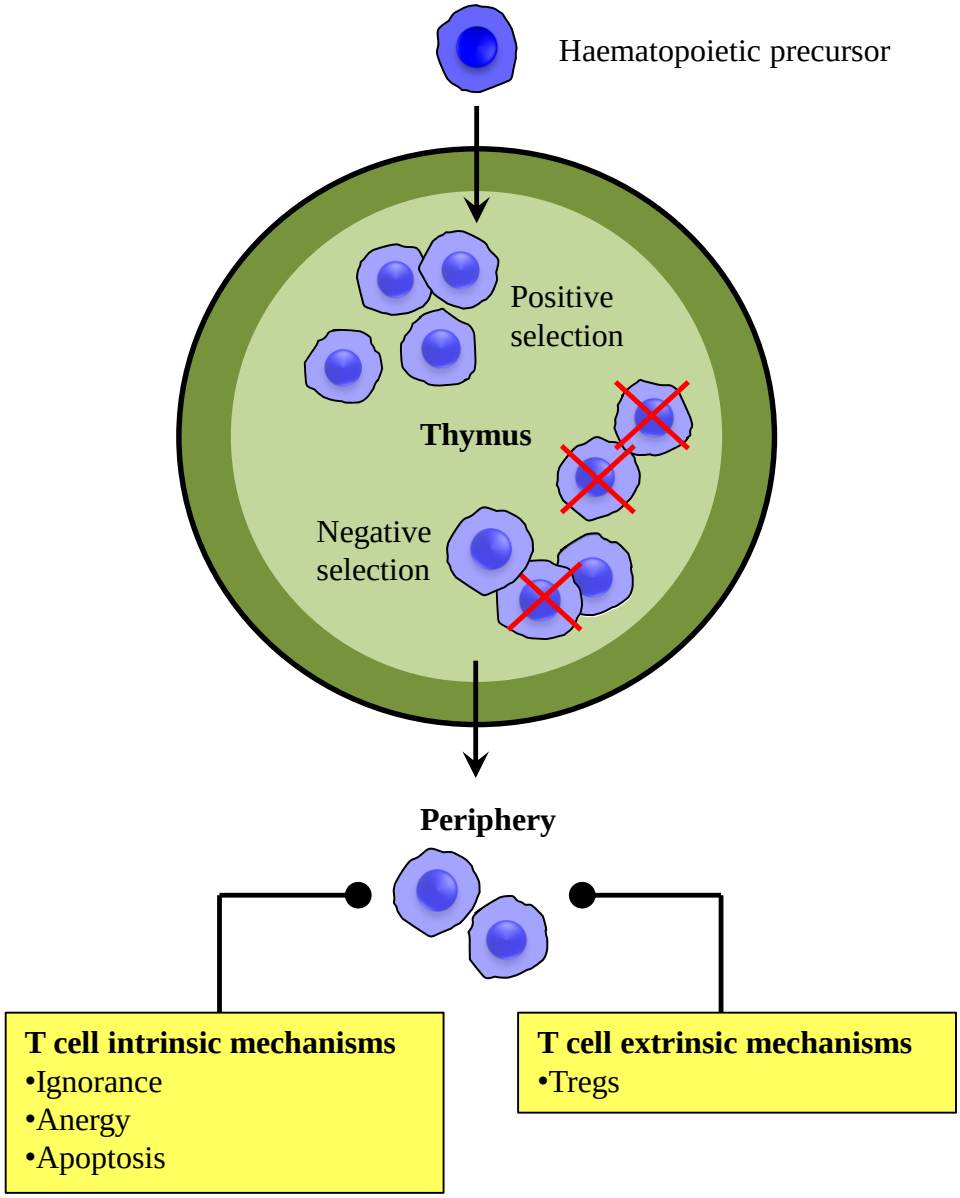


Figure 2.6. Mechanisms of immunological tolerance. Haematopoietic precursors migrate to the thymus where they undergo positive and negative selection based on the nature of their T cell receptor interactions with peptide/major histocompatibility complex. Although the vast majority of self-reactive T cells are effectively deleted at this stage, few escape to the periphery where they are kept under control by peripheral tolerance mechanisms acting either directly on the self-reactive T cell (T cell intrinsic) or indirectly *via* additional cells (T cell extrinsic).

3.1. Central tolerance

Central tolerance refers to those events that, occurring during the early life of a lymphocyte, focus the adaptive immune system´s attention on pathogens while steering it away from healthy tissue. It is induced at the primary sites of lymphocyte

development - bone marrow and thymus – and it includes the mechanisms by which antigen-receptor recognition of self-antigen at these sites results in self-tolerance (Hogquist et al. 2005).

This process of lymphocyte development begins in the bone marrow, where multipotent haemopoietic stem cells (HSCs) commit to one of two lineages, myeloid or lymphoid. The first stage of commitment to the lymphoid lineage is represented by the common lymphoid progenitor (CLP), which can give rise to T, B and natural killer (NK) cells (Kondo et al. 1997). While B cell development continues in the bone marrow, further T cell development is preceded by the migration and entry of CLPs into the thymus (Graf 2008), an organ that supports the development and repertoire generation of T cells (Miller 1961). After commitment to the T cell lineage, which depends on Notch receptor signalling, developing lymphocytes pass through a series of different phases characterised by changes in the status of their TCR genes (Deftos and Bevan 2000), culminating in the expression of a functional TCR. TCR generation encompasses a process of somatic recombination, in which non-contiguous gene sequences are randomly recombined in order to produce high levels of "recombinatorial" diversity (Petrie et al. 1995). The imprecise nature of recombination leads to 'junctional' diversity, further increasing the TCR repertoire and leading to the production of non-functional TCRs and TCRs with specificity for self molecules (Goldrath and Bevan 1999).

After arriving as precursors from the bone marrow, thymocytes lack expression of the surface molecules characteristic of mature T cells. At this point these cells lack the TCR as well as the CD4 and CD8 co-receptors, thus they are called CD4/CD8 double negative (DN) thymocytes (Spits 2002, Gill et al. 2003, Ciofani and Zuniga-

Pflucker 2010). Proliferation and differentiation of DN thymocytes into CD4/CD8 double positive (DP) cells is driven by a distinct surface receptor, composed of a TCRβ chain and an invariant pre-TCRα chain (Rodewald and Fehling 1998). In the thymic cortex, cortical thymic epithelial cells (cTECs) and other APCs display a wide variety of self-peptide: MHC complexes. About 97% of DP cells fail to recognise any of these and since they do not receive a survival signal, they die by neglect (Werlen et al. 2003). The remaining DP thymocytes, binding self-peptide: MHC complexes with sufficient avidity, are positively selected (Santori et al. 2002), surviving and undergoing the programme of differentiation into CD4 or CD8 single positive (SP) thymocytes. Thus, the process of positive selection favours the selection of self-reactive T cells (Hengartner et al. 1988, von Boehmer and Kisielow 1990, Jiang and Chess 2006).

However, some of these cells, after entering the thymic medulla, die by clonal deletion when they interact with overly high affinity with self-peptide: MHC complexes (Palmer 2003), thereby preventing the majority of self-reactive T cells from entering the periphery (Takahama 2006). This process of negative selection is made possible by the ectopic presentation of tissue-specific antigens by medullary thymic epithelial cells (mTECs), dependent at least in part on the transcriptional factor autoimmune regulator (AIRE; Zuklys et al. 2000, Derbinski et al. 2005).

Although clonal deletion is the predominant mechanism by which tolerance is maintained, a small proportion of T cells can undergo receptor editing (Nemazee and Hogquist 2003) or enter into a state of anergy (Hammerling et al. 1991).

Despite the numerous mechanisms of central tolerance described above, it is clear that self-reactive T cells still escape to the periphery (Williams et al. 1998, Bouneaud et al. 2000). A number of possibilities can be invoked to explain this

phenomenon : a) some thymocytes simply do not encounter the limited number of APCs expressing their cognate ligand as they mature; b) the avidity between the self-reactive thymocyte and the self-peptide : MHC complex may be too low to allow deletion (Mueller 2010); c) antigens that are developmentally expressed may not be present in the thymus during negative selection, resulting in a failure to induce central tolerance (Goverman 2011); d) mutations in key signal-transducing molecules can lead to disturbances in the balance of positive and negative selection, therefore allowing T cells to escape negative selection (von Boehmer and Melchers 2010); and lastly e) the presence of TCRs with unusual binding properties can render their interaction with MHC molecules asymmetrical or incomplete, therefore permitting self-reactive T cells to escape deletion (Hahn et al. 2005).

3.2. Peripheral tolerance

Because central tolerance is not always effective, various mechanisms of peripheral T cell tolerance fine tune the self-reactive T cell repertoire, being therefore essential to prevent autoimmunity. These mechanisms can be divided into those intrinsic to the self-reactive T cell, and those implying the intervention of additional subsets of cells (Walker and Abbas 2002; Figure 2.7.).

One way to prevent autoimmune destruction by a given T cell clone is to delete that specificity from the repertoire: autoreactive cells with high avidity for self molecules die by apoptosis (Mallone et al. 2005), following engagement of death receptors such as Fas (a process known as activation induced cell death) or the Bcl-2 related protein Bim (activated T cell autonomous death; Hildeman et al. 2002, Marrack and Kappler 2004).

Alternatively, T cell encounter with a self-antigen may lead to functional inactivation – a phenomenon termed T cell anergy (Jenkins and Schwartz 1987). This may happen due to the intrinsic properties of self-reactive T cells able to escape to the periphery that render them less able to initiate an immune response. They are characterised by the high surface expression of inhibitory receptors such as CD5 (Hawiger et al. 2004), CTLA-4 (Eggena et al. 2004), and PD-1 (Nishimura et al. 1999, Keir et al. 2006), as well as higher intracellular levels of members of the ubiquitin ligase family (Bonnevier et al. 2005, Fathman and Lineberry 2007). These molecules target signalling machinery, such as the TCR and the CD28 co-receptor, for destruction (Schartner et al. 2007). In some cases this high threshold for activation is coupled with a low avidity for self molecules and/or with a lack of co-stimulation provided by APCs.

A last intrinsic mechanism of peripheral tolerance implicates that self-reactive T cells may never encounter their specific self-antigen, therefore remaining in a status of ignorance. This may be due to the restricted trafficking patterns of both naïve and antigen-experienced T cells. In the latter case, lymphocyte trafficking ability is conferred by DCs during antigen presentation and is characterised by the up-regulation of tissue-homing molecules (Mora et al. 2005). In the absence of inflammatory signals, DCs have tolerogenic properties and are able to prevent T cell migration from the lymphoid tissues to peripheral sites (Bianchi et al. 2009).

In addition to these intrinsic peripheral mechanisms, self-reactive lymphocytes are ultimately controlled by a separate subset of CD4 T cells – the regulatory T cells.

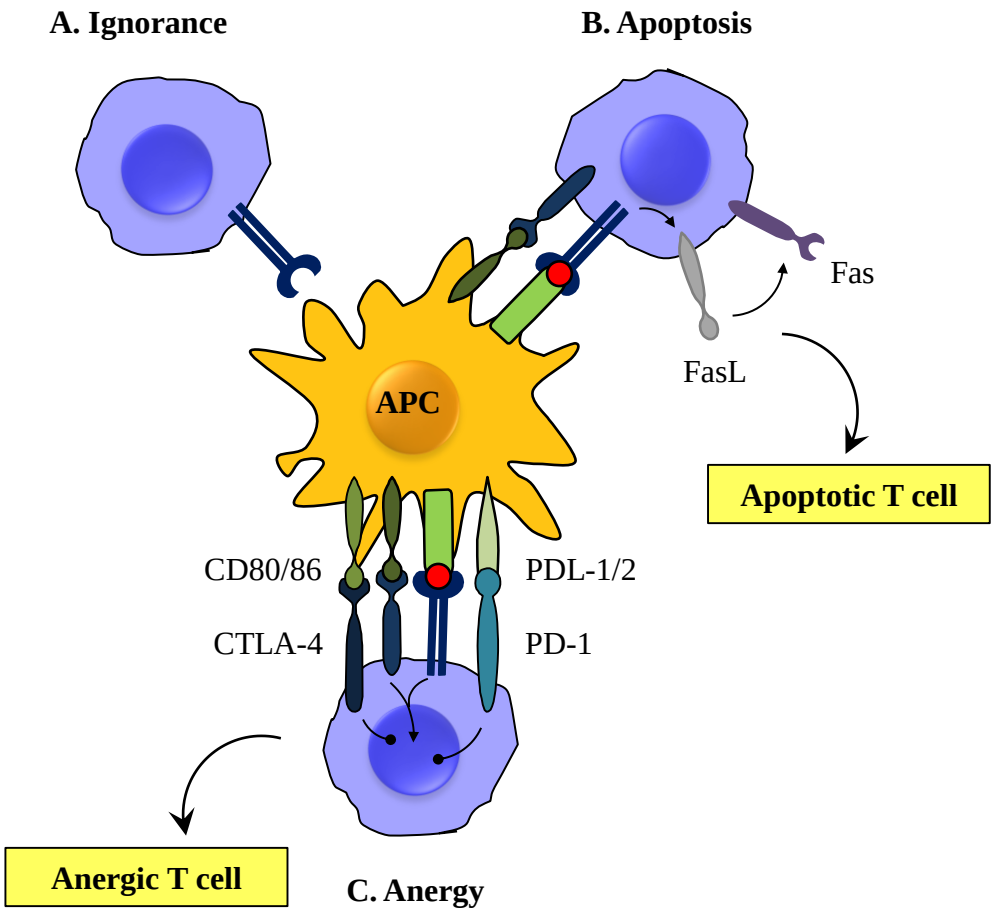


Figure 2.7. T cell intrinsic mechanisms of peripheral tolerance. Self-reactive T cells may never encounter the self-antigen they recognise therefore existing in a state of ignorance (*A*). Encounter of self-reactive T cell with its antigen may result in anergy – a process involving the interaction of the T cell molecules cytotoxic T lymphocyte antigen 4 (CTLA-4) or programmed death 1 (PD-1) with their ligands, CD80/86 and programmed death ligand (PDL) 1 and 2 respectively (*B*). Lastly, self-reactive T cells may be deleted following contact with self-antigen by activation-induced cell death involving up-regulation of T cell Fas ligand (FasL) and subsequent signalling through the death receptor Fas (*C*).

3.3. Regulatory T cells

The concept that T cells could regulate immune responses dates back to the 1970s, when the existence of cells with suppressor properties was reported. This cell population was defined as being able to dominantly avert unwanted autoimmune responses (Gershon and Kondo 1970, Benacerraf et al. 1975). During the following two decades, the idea of suppressor cells fell out of favour, partly due to some irreproducible

and odd functional properties, and mainly to the lack of reliable distinguishing molecular markers.

Fortunately, the field of T "suppressor" cells was reborn by the observations of Sakaguchi et al. that a subset of CD4 T lymphocytes constitutively expressing the IL-2 receptor α-chain (known as CD25) possesses potent immunosuppressive functions both *in vitro* and *in vivo*. These lymphocytes have been re-named "regulatory T cells" (Tregs; Sakaguchi et al. 1995). Tregs actively suppress pathological and physiological immune responses, thereby contributing to the maintenance of immunological self-tolerance and immune homeostasis.

3.3.1. Phenotypic characteristics of regulatory T cells

CD25

As mentioned in the previous section, the historical and prototypical Treg is characterised by the expression of CD25. Although CD25 is not a faithful marker due to its up-regulation on cells undergoing activation, it should be emphasised that Tregs express the highest levels of CD25 and do so constitutively, whereas expression by conventional CD4 T cells is transient and lower. Moreover, $CD4^{pos}CD25^{high}$ T cells have been shown to suppress immune responses (Takahashi et al. 1998, Thornton and Shevach 1998). Importantly, transfer of T cell suspensions depleted of $CD25^{pos}$ T cells produces autoimmune disease in athymic nude mice, whereas co-transfer of a small number of $CD4^{pos}CD25^{pos}$ T cells inhibits the development of autoimmunity (Sakaguchi et al. 1995). The high level of expression of CD25 on Treg cells suggests the importance of IL-2 for these cells. Indeed, presence of IL-2 is required for homeostasis and metabolic fitness of Tregs (Fontenot et al. 2005), and therefore for maintenance of

self-tolerance, as shown by the evidence that IL-2 neutralisation (Setoguchi et al. 2005) or deficiency (Kramer et al. 1995) determines a reduction in the number of Tregs, leading to a wide spectrum of autoimmune manifestations (O'Shea et al. 2002). Thus, CD25 expression is a functional characteristic of Tregs, being commonly used as a marker in Treg research.

FOXP3

The $CD25^{high}$ population is highly enriched in what is still considered the most reliable marker for the identification of Tregs, the forkhead winged helix transcription factor FOXP3 (Fontenot et al. 2003, Hori et al. 2003). Its importance for the development and function of Tregs is highlighted by the fact that mutations in the *Foxp3* gene in mice result in fatal autoimmune lymphoproliferative disease. In mice the spontaneous scurfy mutation, in which a loss of function in the *Foxp3* gene is observed, results in a complete abrogation of Tregs and death of the mice at 3-4 weeks of age (Brunkow et al. 2001). Similarly, humans with immunodysregulation, polyendocrinopathy and enteropathy, X-linked syndrome (IPEX), who also show mutations in the *FOXP3* gene, have a range of symptoms that are consistent with an altered suppressive function of Tregs (Wildin et al. 2001).

Although the mechanisms of action of *FOXP3* have not yet been fully characterised, recent evidence suggests that it may act as both a transcriptional repressor and activator (Marson et al. 2007, Y. Zheng et al. 2007b). The genes regulated by *FOXP3* are not necessarily targeted by *FOXP3* itself; instead its effects is exerted *via* other transcription factors such as nuclear factor of activated T cells (NFAT), nuclear factor-κB (NF-κB; Bettelli et al. 2005) and the runt-related transcription factor 1-core

binding factor β (Runx1-Cbfβ) heterodimer (Kitoh et al. 2009), leading to the inhibition of their target genes expression (among others, the genes coding for IL-4 and IFN-γ). *Foxp3* also interacts with *Foxo3a* and *Foxo1*, which induce transcription by binding to the *Foxp3* promoter (Harada et al. 2010).

Interestingly, several investigators have suggested that the transcription factors Tbet (Koch et al. 2009) and interferon regulatory factor-4 (Irf-4; Zheng et al. 2009) are differentially expressed in FOXP3^{pos} Tregs following exposure to specific inflammatory environments, which may allow for the differential regulation of target genes in an adaptive manner.

CTLA-4 and GITR

Tregs also express high levels of CTLA-4 and glucocorticoid-induced tumour necrosis factor receptor family-related protein (GITR; Sakaguchi 2004, McHugh et al. 2002). In normal mice exposure of Tregs to an anti-CTLA-4 monoclonal antibody leads to their functional inactivation (Takahashi et al. 2000), and CTLA-4-deficient mice succumb to fatal autoimmune disease (Bachmann et al. 1999).

GITR, a member of TNFR superfamily (Nocentini et al. 1997), appears to act as a negative regulator of Treg function: its stimulation abrogating Treg-mediated suppression (Shimizu et al. 2002); in contrast, it provides positive co-stimulation to other effector T cells (Kanamaru et al. 2004). However, and similar to CD25 expression, CTLA-4 and GITR are also expressed by activated T cells, therefore limiting their use as markers of Tregs (Linsley et al. 1992). Nevertheless, several molecules have recently been identified that can help to distinguish Tregs from activated T cells.

Latency associated peptide

Latency associated peptide (LAP) forms a latent complex with TGF-β1, an important cytokine involved in Treg cell development and function (Nakamura et al. 2004). LAP is not only useful for distinguishing between Tregs and activated $FOXP3^{neg}$ or $FOXP3^{pos}$ effector T cells (Tran et al. 2009), as its expression characterises a Treg subset with high levels of FOXP3, CTLA-4, and GITR (Chen et al. 2008) but LAP can also be used as a marker for TGF-β expressing Tregs, which exhibit higher ability to suppress compared to the $CD4^{pos}CD25^{pos}LAP^{neg}$ population.

While the LAP^{pos} subpopulations suppressive function is TGF-β dependent and is mediated by both cell-to-cell contact and soluble factors, the function of LAP^{neg} cells is mainly cell contact dependent (Chen et al. 2008).

CD39

CD39 (ectonucleoside triphosphate diphosphohydrolase-1, ENTPD1), an ectoenzyme that degrades adenosine triphosphate (ATP) to adenosine monophosphate (AMP), represents another surface marker of Tregs (Borsellino et al. 2007, Deaglio et al. 2007). In mice, it is present on virtually all $CD4^{pos}CD25^{pos}FOXP3^{pos}$ Tregs. In humans, CD39 expression is limited to a subset of $FOXP3^{pos}$ regulatory effector/memory-like T cells (Dwyer et al. 2007). In addition to CD39, murine and a subset of human Tregs also express CD73, an ecto-5'-nucleotidase, which acting in tandem with CD39, converts AMP into adenosine (Zimmermann 1992); hence, these suppressor cells are eventually able to catalyse ATP/adenosine diphosphate (ADP) into the immunosuppressive nucleoside adenosine (Kobie et al. 2006, Borsellino et al. 2007, Deaglio et al. 2007). The expression of both CD39 and CD73 therefore serves as a

phenotypic signature of Tregs, also having important mechanistic implications for their functional suppressive activity by catalyzing the generation of pericellular adenosine (Dwyer et al. 2007).

CD127

Recent studies have shown that functional Tregs express very low levels of the IL-7 receptor alpha (CD127), whereas activated $CD25^{pos}$ T were characterised by the expression of this marker (Banham 2006). In the same year, another study reported that CD127 was down-regulated on all human T cells after activation. While the majority of effector and memory T cells were found to re-express it, $FOXP^{pos}$ T cells remained $CD127^{low}$ (Liu et al. 2006). A detailed information about CD127 is provided in section 7.1.1.

3.3.2. Regulatory T cell subsets

Tregs are normally classified according to their developmental pathway into thymus-derived natural Tregs (nTregs) and peripherally-induced adaptive Tregs (iTregs; Figure 2.8.). Both nTregs and iTregs share a similar phenotype and have a contact-dependent mechanism of action that is poorly understood (Horwitz et al. 2008).

nTregs represent the most common Treg type accounting for 5-10% of and 1-2% of peripheral $CD4^{pos}$ T cells in mice (Belkaid et al. 2002) and 1-2% in humans (Baecher-Allan et al. 2001). The pivotal role of the thymus in their generation was elucidated in early studies showing that mice undergoing thymectomy on day 3 of life suffered from T cell-mediated autoimmune disease, which could be reversed by $CD4^{pos}$ T cell infusion (Nishizuka and Sakakura 1969). A two-step instructive model of nTreg

development in the thymus has recently been proposed (Lio and Hsieh 2008). Firstly, autoreactive Treg precursors are exposed to strong TCR signals following high affinity binding to self-peptide: MHC molecules expressed on the surface of thymic epithelial cells (Jordan et al. 2001). This results in the up-regulation of CD25 and other components of the proximal IL-2 signalling pathway (Burchill et al. 2008). The second step involves co-stimulation mediated by the binding of CD28 present on developing T cells with its ligands expressed by thymic APCs (Salomon et al. 2000). Following co-stimulation, Treg precursors begin to secrete IL-2 and up-regulate their expression of CTLA-4, GITR and, most importantly, FOXP3 (Tai et al. 2005).

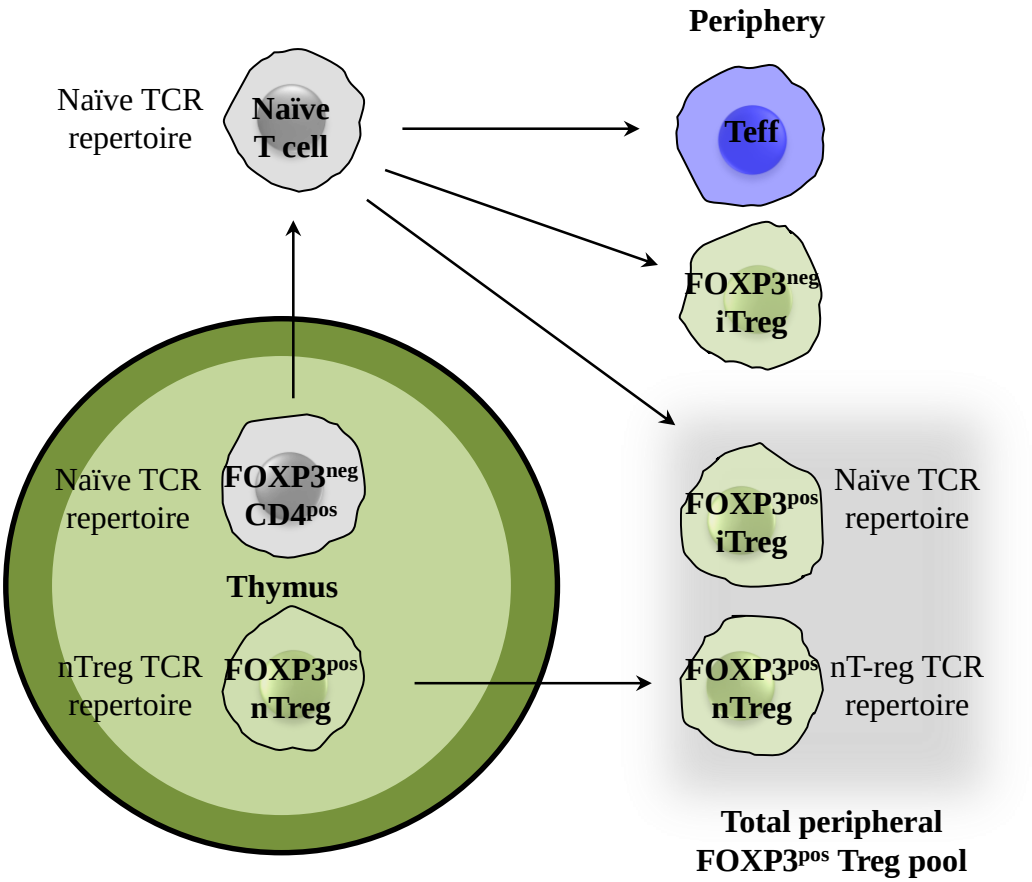


Figure 2.8. Thymic and peripheral generation of regulatory T cells. Thymus-derived natural regulatory T cell (nTregs) differentiate in the thymus and then migrate to peripheral tissues. Peripherally-induced adaptive regulatory T cells (iTregs) differentiate in secondary lymphoid organs and tissues. The peripheral population of FOXP3^{pos} regulatory T cells (Tregs) comprises both nTregs and iTregs.

As mentioned earlier, the avidity of TCRs for self-peptide: MHC complexes within the thymus determines the fate of the developing thymocytes – death by neglect, positive selection, Treg generation and negative selection (Klein et al. 2009). These alternative fates can be encompassed by an affinity threshold model (Palmer and Naeher 2009). It should be noted that the boundaries between these are likely to be flexible *in vivo* and influenced by the number of engaged TCRs and the availability of co-stimulatory signals (Figure 2.9.). The term "functional avidity" has been coined to account for these additional influences (Scherer et al. 2004).

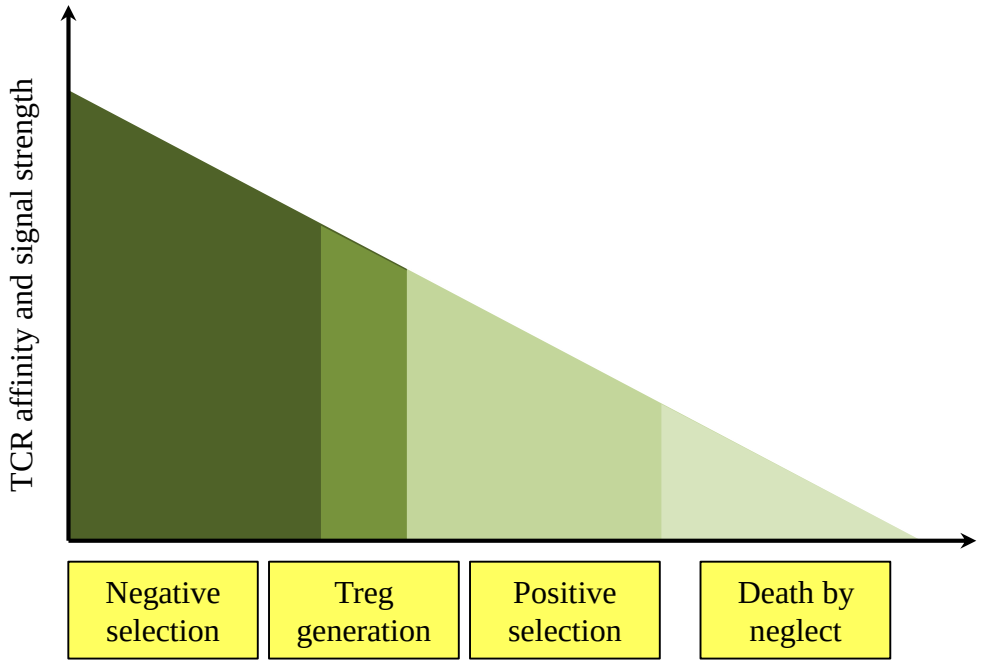


Figure 2.9. The affinity threshold model of central tolerance and regulatory T cell generation. Positive selection of T cells occurs upon receipt of "appropriate" levels of T cell receptor (TCR) stimulation. Aberrantly high levels of stimulation result in clonal deletion, while low levels lead to death by neglect. Regulatory T cell (Treg) generation occurs at levels between that of positive and negative selection.

In recent years it became evident that $FOXP3^{pos}$ Tregs can also be generated outside the thymus under a variety of conditions (Curotto de Lafaille and Lafaille 2009). $FOXP3^{pos}$ iTreg development requires TCR stimulation and the presence of TGF-β, for

both *in vitro* and *in vivo* generation (Chen et al. 2003). Addition of TGF-β to TCR-stimulated naive $CD4^{pos}$ T cells induces transcription of FOXP3, acquisition of anergic and suppressive activity. The mechanism responsible for TGF-β-induced transcription of FOXP3 involves cooperation of the transcription factors STAT-3 and NFAT at a *Foxp3* gene enhancer element (Fantini et al. 2004, Josefowicz and Rudensky 2009). Using IL-2-deficient mice and IL-2 neutralisation, it has been demonstrated that TGF-β-dependent generation of iTregs also requires the presence of this cytokine (S. G. Zheng et al. 2007). iTregs are known to play an essential role in the gut-associated lymphoid tissue (GALT), due to continuous exposure to dietary antigens and commensal gut bacteria (Curotto de Lafaille and Lafaille 2009). Within this environment, a subset of DCs characterised by the expression the integrin CD103, can convert naïve T cells into Tregs *via* the production of retinoic acid (Coombes et al. 2007).

A number of studies have provided evidence that shed light on the differences between nTregs and iTregs. Both populations express FOXP3 and suppress immune responses through contact-dependent mechanisms and by the production of soluble factors (Vignali et al. 2008, Sakaguchi et al. 2010). nTregs are stable with respect to retaining regulatory function and FOXP3 expression in the periphery. Studies on the methylation of CpG motifs in the *Foxp3* locus of nTregs identified complete demethylation within an evolutionary conserved region upstream of exon 1, named Treg-cell specific demethylated region (TSDR; Curotto de Lafaille and Lafaille 2009). nTregs are also unique in that they express the transcription factor Helios (Thornton et al. 2010). Although iTregs express the same surface markers, they can be distinguished from nTregs based on FOXP3 DNA methylation patterns and their lack of Helios expression.

Besides FOXP3^{pos} Tregs, other adaptive regulatory T cell subsets develop as a consequence of antigen exposure in the periphery. Tr1 cells can be generated from naïve CD4 T cells by antigenic stimulation in the presence of IL-10, both *in vitro* and *in vivo* (Roncarolo et al. 2006). These cells produce IL-10 and TGF-β, which have been demonstrated to be critical for their suppressive effect (Groux et al. 1996). Tr1 cells do not express FOXP3, yet some of their properties *in vitro* are very similar to those of FoxP3^{pos} Tregs. Antigen-specific TGF-β-secreting cells, termed Th3 cells (Weiner 2001), were originally propagated from animals that became tolerant to orally administered protein antigen (Faria and Weiner 2005). Their relationship with other regulatory T cell subsets has not been fully characterised, but it has been reported that oral tolerance induction generates both CD25^{pos}FOXP3^{pos} as well as CD25^{neg}FOXP3^{neg} T cells with regulatory properties (Sun et al. 2006).

A variety of other T cell subpopulations including CD8^{pos}CD28^{neg}, CD4^{neg}CD8^{neg}, and γδ T cells have also been reported to exert immunosuppressive activity (Shevach 2006). There is so far little evidence that they play crucial roles in natural self-tolerance.

3.3.3. Mechanisms of suppression

A key issue of current Treg research is to understand their mechanisms of suppression (Sakaguchi et al. 2010). Tregs can exert their suppressive function either through a contact-dependent mechanism – by directly targeting the function of effector T cells as well as by modulating the maturation and/or function of dendritic cells, which in turn are required for the activation of the former – and/or through contact-independent mechanisms (Shevach 2009). From a functional and practical perspective,

the suppression mechanisms deployed by Treg cells can be grouped into four "modes of action": suppression by inhibitory cytokines, suppression by cytolysis, suppression by metabolic disruption and suppression by modulation of DC maturation or function (Vignali et al. 2008; Figure 2.10.).

Suppression by inhibitory cytokines

The role of TGF-β as a suppressor molecule still remains a matter of debate. The majority of studies failed to demonstrate that Treg function *in vitro* could be reversed by an anti-TGF-β neutralising antibody (Piccirillo et al. 2002). However, since preventing its production leads to the development of an autoimmune inflammatory disease in mice , TGF-β should contribute, at least in part, to *in vivo* suppression in this mouse model (Sakaguchi et al. 2009a). Though controversial, TGF-β may act as a mediator of suppression in membrane-bound form (Nakamura et al. 2001). It may condition effector T cells to be amenable to suppression, and may contribute to the differentiation of other T cells into Treg-like cells (infectious tolerance; Fahlen et al. 2005, Andersson et al. 2008). In addition to TGF-β, IL-10 production may be utilised by Tregs under certain conditions. IL-10 has been shown to be essential for the prevention of colitis in mouse models of IBD (Asseman et al. 1999), and allograft rejection (Hara et al. 2001, Kingsley et al. 2002).

More recently, IL-35, a new member of the IL-12 family of cytokines, has been shown to be expressed by Tregs and to be required for their maximal suppressive activity (Collison et al. 2007). Ectopic expression of IL-35 conferred regulatory activity to naïve T cells and recombinant IL-35 suppressed T cell proliferation *in vitro* (Collison et al. 2007, Gavin et al. 2007).

Suppression by cytolysis

Another potential mechanism of Treg-mediated suppression is cell-contact dependent cytolysis of target cells mediated by granzymes A and B (Grossman et al. 2004, X. Cao et al. 2007) in perforin dependent and independent manners (Gondek et al. 2005). Recently it has been shown that activated Tregs induce apoptosis of effector T cells through a tumour-necrosis-factor-related apoptosis-inducing ligand–death receptor 5 (TRAIL–DR5) pathway. Blockade of TRAIL abrogates cytolysis and suppression (Ren et al. 2007). In addition, Galectin-1, which can induce T cell apoptosis, has been shown to be highly expressed by mouse and human Tregs. Knock-out and blockade of Galectin-1 abrogates Treg suppressive function (Garin et al. 2007).

Suppression by metabolic disruption

Several mechanisms of Treg suppression have been recently described that can be collectively referred to as "metabolic disruption" of effector T cells (Vignali et al. 2008). Since Tregs are characterised by high expression levels of the high affinity IL-2R (CD25), they compete with other T cells for local IL-2 consumption (Thornton and Shevach 1998, de la Rosa et al. 2004). Consequently, actively dividing effector T cells are deprived of IL-2 and undergo Bim-mediated apoptosis (Pandiyan et al. 2007). Another suppressive mechanism employed by Tregs relates to the dual expression of CD39 and CD73. These surface ectoenzymes catalyse the degradation of extracellular nucleotides (e.g. ATP), leading to generation of pericellular adenosine (Kobie et al. 2006, Deaglio et al. 2007). Adenosine suppresses effector T cell function through activation of the adenosine receptor 2A, leading to subsequent intracellular cyclic AMP

accumulation and the depletion of pro-inflammatory ATP (Sitkovsky and Ohta 2005, Borsellino et al. 2007).

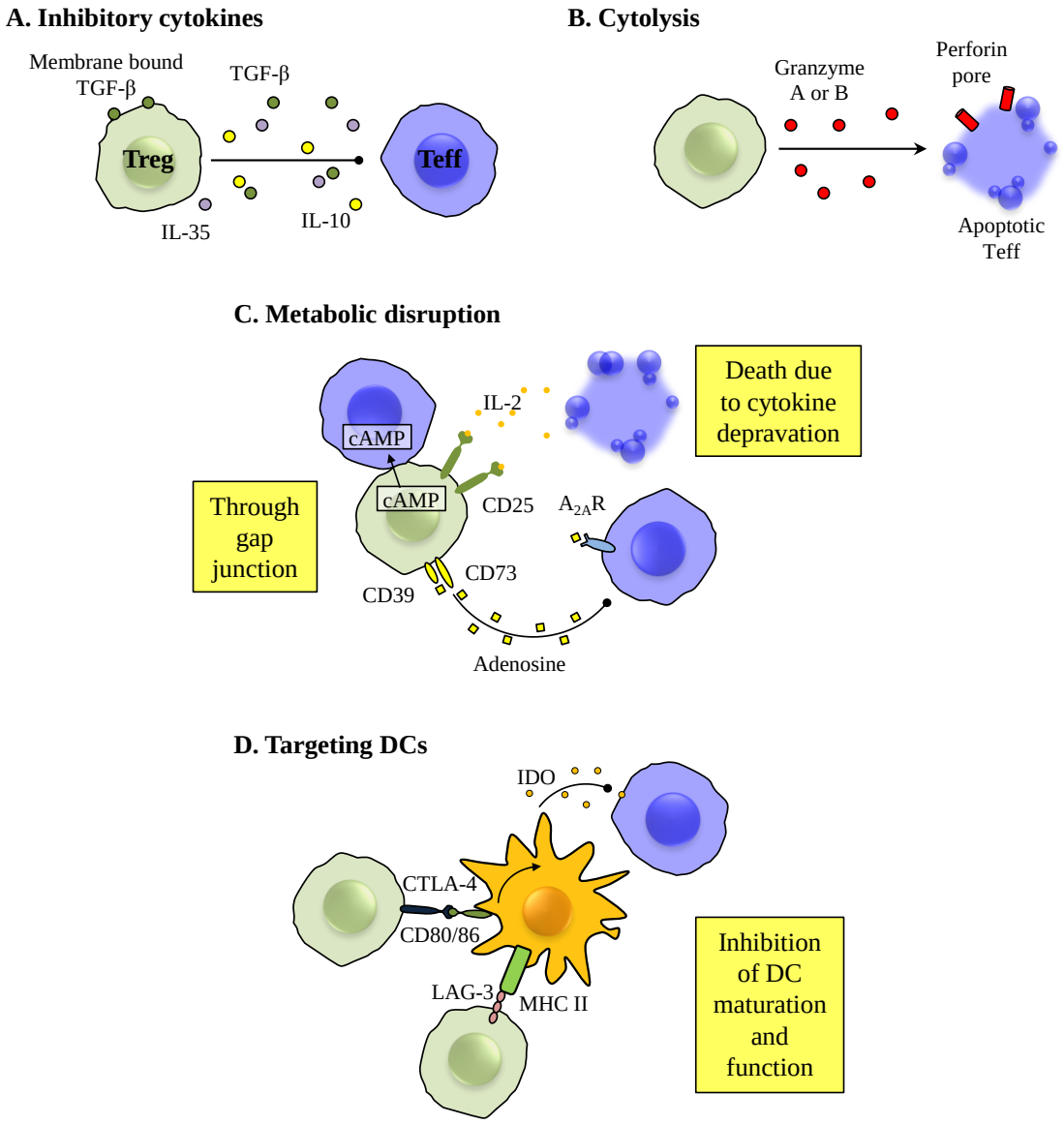


Figure 2.10. Mechanisms of suppression employed by regulatory T cells. The various potential mechanisms of suppression employed by regulatory T cells (Tregs) can be grouped into 4 modes of action: (*A*) inhibitory cytokines including interleukin (IL)-10, transforming growth factor β (TGF-β), and IL-35; (*B*) cytolysis including granzyme A- and B-dependent and perforin-dependent killing mechanisms; (*C*) metabolic disruption including CD25-dependent cytokine deprivation-mediated apoptosis, cyclic AMP (cAMP)-mediated inhibition, and CD39 and/or CD73-generated adenosine-mediated suppression; (*D*) targeting dendritic cells (DCs) including mechanisms that modulate DC maturation and or function, such as lymphocyte activation gene 3 (LAG-3) – MHC class II-mediated suppression of DC maturation and cytotoxic T lymphocyte antigen 4 (CTLA-4) – CD80/86-mediated induction of indoleamine 2,3-deoxigenase (IDO) which is an immunosuppressive molecule made by DCs.
Adapted from Vignali DAA, Collinson LW et al. Nat Rev Immunol 2008; 8: 523-532.

Suppression by targeting DCs

In addition to the effect on T cell function, Tregs also modulate the maturation and function of DCs.

A core Treg suppressive mechanism is delivered *via* the surface expression of CTLA-4. The importance of CTLA-4 for Treg function has been shown by the use of CTLA-4 specific blocking antibodies (Read et al. 2000) or Treg-specific *Ctla-4* knock-out mice (Wing et al. 2008) – in the absence of functional CTLA-4, Treg-mediated suppression of effector T cells was reduced, with consequent ensuing of autoimmune disease. Mechanistically, CTLA-4 binding leads to the down-regulation and sequestration of the co-stimulatory molecules CD80 and CD86 on DCs (Cederbom et al. 2000, Sakaguchi et al. 2009b). In addition, CTLA-4 binding to CD80/CD86 also leads to the up-regulation of the enzyme indoleamine 2,3-dioxygenase (IDO) (Grohmann et al. 2002). IDO catabolises the essential amino acid tryptophan, therefore limiting the access to this pro-inflammatory resource (Munn et al. 1999, Grohmann et al. 2002). The products of tryptophan catabolism are the immunosuppressive kynurenines (Belladonna et al. 2007).

In addition to inducing the production of immunosuppressive molecules by DCs, Tregs may also down-modulate the capacity of DCs to initiate immune responses. Recent studies have suggested that cell surface lymphocyte activation gene 3 (LAG-3, also known as CD233) binds to MHC class II molecules with very high affinity, preventing the maturation and the ability of DCs to activate effector T cells (Liang et al. 2008). The role of LAG-3 for Treg function was highlighted by studies showing that LAG-3 deficiency or specific blockade led to a reduced immune-suppressive activity, and that its forced expression can confer suppressive capacity on $CD4^{pos}$ T cells (Huang

et al. 2004). Lastly, neuropilin (Nrp-1) has been proposed to play a role in Tregs/DC cross-talk, by promoting long interactions between Tregs and immature DCs, therefore slowing the initiation of an immune response (Mizui and Kikutani 2008). Given that Nrp-1 is differentially expressed by Tregs (Bruder et al. 2004), this may confer to them an advantage over naïve T cells in modulating the function of DCs.

In conclusion, this section has described various cellular and molecular events that contribute to overall Treg-mediated suppression. However, it should be pointed out that none of these accounts entirely for Treg-mediated suppression. In fact, it is possible that various combinations of several mechanisms are operating, depending on the milieu and the type of immune response. In view of their role in the maintenance of immune-tolerance, it is not surprising that enhanced or reduced Treg number and/or suppressive function is implicated in several diseases.

3.3.4. Tregs and disease

Tregs play an important role in a range of diseases. They are implicated in the pathogenesis of allergy (Ling et al. 2004), chronic infection (Belkaid and Rouse 2005), and cancer (Beyer and Schultze 2006). They are also required for the maintenance of tolerance to self, thus preventing autoimmune disease.

The role of Tregs in protecting from autoimmunity was first identified in mice in which the absence or depletion of Tregs resulted in the development of pan-autoimmunity, with manifestations of gastritis, thyroiditis, diabetes and IBD (Sakaguchi et al. 1995, Brunkow et al. 2001). Subsequent studies in animal models of autoimmune disease showed that Treg defects may account for the development of autoimmunity and that the disease could be reversed by the adoptive transfer of Tregs (Sakaguchi and

Sakaguchi 2005). Later, it was confirmed the presence of Tregs in human peripheral blood and their ability to suppress T cell proliferation *in vitro* (Baecher-Allan et al. 2001, Stephens et al. 2001).

The importance of T cell regulation in human disease is highlighted by the severe inflammation and autoimmune manifestations that occurs in individuals who suffer from IPEX, the immunodysregulation determined by mutation in the *FOXP3* gene. These individuals develop a broad range of autoantibodies, insulin-dependent diabetes, thyroiditis, eczema, haemolytic anaemia and IBD, and in the absence of a bone marrow transplant, they die at an early age (Wildin et al. 2001). Defective regulation by Tregs has been documented in other, more common autoimmune diseases.

Multiple sclerosis

Multiple sclerosis (MS) is an inflammatory disease of the central nervous system (CNS), characterised by focal demyelination, loss of oligodendrocytes, axonal damage and astrogliosis (Hafler 2004). In MS, self-reactive $CD4^{pos}$ T cells target myelin-based antigens, have the ability to migrate to the CNS and display unique effector functions (McFarland and Martin 2007). Evidence implicating Tregs in the pathogenesis of MS derives from studies on both experimental autoimmune encephalomyelitis (EAE), a murine model of MS, and human MS.

First, it has been shown that animals expressing a transgenic TCR specific for myelin basic protein (MBP) and lacking recombination-activating genes are deprived of Tregs and spontaneously develop EAE, implicating these cells in EAE prevention (Hori et al. 2003). Other studies have conversely shown that Treg-depletion resulted in an increased susceptibility to EAE, whereas the adoptive transfer of Tregs led to a reduced

EAE incidence (Kohm et al. 2002, Hori et al. 2003). The ability of Tregs to suppress CNS inflammation once the disease is established is, however, controversial: in spite of Treg accumulation in the CNS, effector T cells have been found to be resistant to Treg-mediated suppression during active disease due to a highly pro-inflammatory micro-environment (Kohm et al. 2002, Korn et al. 2007b).

In the human setting, most of the studies have shown no difference in the percentage of Treg cells in the peripheral blood between MS patients and healthy subjects, irrespective of the disease activity (Viglietta et al. 2004, Haas et al. 2007, Michel et al. 2008). In the cerebrospinal fluid (CSF), on the other hand, Treg numbers have been found to be increased compared to those observed in the periphery of MS patients (Feger et al. 2007, Venken et al. 2008a). To date, only one study has reported a decreased number of $FOXP3^{pos}$ Tregs in this condition. This defect was, however, limited to those patients with relapsing-remitting MS, and was corrected after treatment with IFN-β (Venken et al. 2008b). Recent studies, analysing different Treg subsets in MS, have identified a decrease in the naive or recent thymic emigrant Tregs, a population characterised by the expression of CD31 and CD45RA, and which has been associated with a higher ability to suppress (Haas et al. 2007, Venken et al. 2008a). Similarly, Fletcher et al. reported a decrease in the population of Tregs expressing CD39 (Fletcher et al. 2009), a molecule that has also been associated with Treg suppressive function (see section 5.3.3.). Lastly, Tregs from MS have been reported to have an increased percentage of $CD127^{pos}$ cells (Michel et al. 2008), which defines an activated cell subset deprived of suppressive function. The studies analysing Treg function consistently reported an impaired ability of Tregs to suppress the proliferation and IFN-γ secretion by effector T cells in patients with MS (Viglietta et al. 2004, Haas

et al. 2005, Michel et al. 2008). Collectively these data suggest that while the studies on the frequency and phenotype of Tregs in MS are inconsistent, those on the function of these cells consistently show an impaired ability to suppress.

Type 1 diabetes

Type 1 diabetes (T1D) is a T cell mediated disease that results in inflammation and destruction of the pancreatic islet beta cells with consequent loss of insulin production, and a lifelong dependency on exogenous insulin (Bluestone et al. 2010).

Experiments performed in non-obese diabetic (NOD) mice, a spontaneous model of T1D, have shown that the development of diabetes is tightly controlled by Tregs (You et al. 2008). Indeed, diabetes transfer, which is observed following the infusion of T cells from diabetic mice into immune-incompetent syngeneic recipients is prevented by co-injection of Tregs from the spleen or the thymus of young prediabetic mice (Herbelin et al. 1998, You et al. 2005, You et al. 2007). In addition, treatment of young NOD mice with CD25-depleting antibodies accelerated the onset of the disease (Billiard et al. 2006). Furthermore, adoptive transfer of Tregs into NOD mice was shown to ameliorate the disease (Tang et al. 2004b).

Many studies have investigated the role of Treg in patients with T1D. Similarly to what observed in MS, whether the frequency of Tregs is reduced or normal in subjects with T1D remains controversial. Although an initial study has reported a significant decrease in terms of Treg numbers in patients with newly diagnosed or established T1D (Kukreja et al. 2002), subsequent studies found no difference in the number of Tregs in the peripheral blood of individuals with T1D compared with healthy subjects (Putnam et al. 2005, Lindley et al. 2005, Brusko et al. 2005, Brusko et al.

2007). However, a recent study examining different Treg subsets reported an increase in the $CD45RA^{neg}FOXP3^{low}$ T cell fraction in subjects with new-onset diabetes compared with healthy subjects; this cell subset was characterised by production of high levels of IL-17 and absence of suppressive function (Marwaha et al. 2010), suggesting that early disease is associated with a reduced number of functional Tregs. Akin to Treg numbers, several groups have shown that Tregs isolated from patients with T1D are functionally impaired (Putnam et al. 2005, Lindley et al. 2005, Brusko et al. 2005, Tree et al. 2006). Recent studies have demonstrated that in addition to intrinsic Treg defects, impaired immunosuppression in T1D is, in part, due to the resistance of effector T cells to Treg-mediated suppression (Schneider et al. 2008, Lawson et al. 2008).

Rheumatoid arthritis

Rheumatoid arthritis (RA) is a systemic autoimmune disease characterised by progressively destructive joint inflammation, destruction of articular cartilage and bone and synovial hyperplasia (McInnes and Schett 2011).

As observed in other autoimmune condition, collagen-induced arthritis (CIA), an experimental model of RA, is exacerbated by Treg depletion (Morgan et al. 2003). However, at variance with MS and T1D, the diseased tissue in RA patients, i.e. the synovium, can be obtained, this enabling analysis of Treg number and function in the target tissue and comparison with the peripheral blood.

Analyses of Treg frequency in the peripheral blood of subjects with RA resulted in contradictory observations. Several studies have reported decreased (Cao et al. 2004, Lawson et al. 2006, Sempere-Ortells et al. 2009) or normal (Ehrenstein et al. 2004, Liu et al. 2005, Lin et al. 2007) Treg frequencies, whereas other groups have shown an

increase in the number of Tregs in the periphery of RA patients (van Amelsfort et al. 2004, Kao et al. 2007, Han et al. 2008). Despite these differences, there is general consensus that the frequency of Tregs cells is higher in the synovial fluid of patients with RA than in controls (Cao et al. 2004, Mottonen et al. 2005, Lawson et al. 2006).

The evaluation of the suppressive function of Tregs in RA has also resulted in heterogeneous findings. While initial studies showed that Tregs isolated from either the blood or the synovium of RA patients maintained their ability to suppress the proliferation of effector cells (Cao et al. 2003, Mottonen et al. 2005, Lawson et al. 2006), Ehrenstein et al. reported that Tregs in RA were unable to suppress the responder cells´ production of IFN-γ and TNF-α (Ehrenstein et al. 2004). This functional defect could be, however, corrected after disease control with anti-TNF-α specific agents.

Systemic lupus erythematosus

SLE is a systemic autoimmune disease characterised by the presence of autoantibodies and immune complexes that target multiple organ systems, including the skin, joints, kidneys and CNS (Tsokos 2011). Although SLE is thought to be largely mediated by B cells (Tsokos 2011), Treg deficiency results in the development of lupus-like features, including nephritis and positivity for DNA-specific antibodies (Sakaguchi et al. 1995), thus suggesting that impairment of Treg-mediated suppression may have a role in the pathogenesis of SLE.

Numerous studies have evaluated the frequency and phenotype of circulating Tregs in SLE. Most of them reported a decreased frequency of Tregs in the peripheral blood of SLE patients (Crispin et al. 2003, Miyara et al. 2005, Mellor-Pita et al. 2006, Suen et al. 2009). However, other groups have reported normal (Alvarado-Sanchez et

al. 2006, Venigalla et al. 2008) or increased (Suarez et al. 2006) frequencies of circulating Tregs in patients with SLE. Phenotypic analysis showed that Tregs isolated from active SLE patients were characterised by a more activated phenotype and by significantly lower expression of FOXP3 compared to patients with inactive disease (Valencia et al. 2007, Bonelli et al. 2008).

From a functional point of view, several groups reported that Tregs isolated from active SLE patients were defective at suppressing the proliferation and cytokine production of effector T cells, while Tregs obtained from inactive SLE exhibited normal suppressive function (Valencia et al. 2007). However, Venigalla et al. showed that Tregs lacking CD127 expression were quantitatively and qualitatively normal (Venigalla et al. 2008). The authors of these studies have further shown that responder cells isolated from patients with active SLE were less sensitive to the suppressive function of autologous or normal donor Tregs than those from patients with inactive SLE (Venigalla et al. 2008), suggesting that the defect in T cell suppression observed in active SLE was due to the resistance of effector cells to Treg mediated suppression and not to abnormal regulatory T cell function. In summary, defects in the number and function of Treg cells, as well as resistance of effector T cells to suppression have been established in patients with SLE. The observations of numerous possible regulatory defects may reflect the systemic character of this disease (Buckner 2010).

Inflammatory bowel disease

The idiopathic IBD comprises two types of chronic intestinal disorders: Crohn´s disease (CD) and ulcerative colitis (UC). Despite pathological differences, both diseases are thought to be orchestrated by T cells and to result from a breakdown of

immune-tolerance in the gut (Abraham and Cho 2009). The key contribution of Tregs to immune-tolerance maintenance in the gut is best exemplified by the occurrence of wasting disease and gastritis observed in Treg-depleted mice, and by the amelioration of disease following adoptive transfer of Tregs (Sakaguchi et al. 1995). A role for Tregs in the regulation of human bowel inflammatory disease is supported by the finding that individuals with IPEX syndrome develop severe bowel inflammation as a component of their illness (Wildin et al. 2002).

Similar to RA, studies focusing on the role of Tregs in IBD have benefited from the possibility to sample and examine the frequency and function of these cells not only in the peripheral blood but also in the target organ. As for the conditions described above, the studies evaluating the frequency of Tregs in the peripheral blood of patients with IBD have given contradictory results. While two studies reported an increase in the number of circulating Tregs (Takahashi et al. 2006, Saruta et al. 2007), other studies demonstrated that Tregs were decreased in number in patients with IBD compared to controls (Maul et al. 2005, Li et al. 2010, Eastaff-Leung et al. 2010). Notwithstanding the differences found in the periphery, analysis of the target organ consistently showed an increase in the percentage of $FOXP3^{pos}$ cells in the *lamina propria* and in the mesenteric lymph nodes, particularly in and near the inflamed tissue (Maul et al. 2005, Li et al. 2010, Eastaff-Leung et al. 2010, Rahman et al. 2010). However, two groups have reported that this increase in the frequency of infiltrating Tregs is also observed in other non-related inflammatory conditions, such as in diverticulitis and infectious enteritis (Maul et al. 2005, Uhlig et al. 2006), indicating that the increased frequency of Tregs in IBD is similar to that accompanying all types of gut inflammation.

Functional studies performed with Tregs cells obtained from peripheral blood, mesenteric lymph nodes or *lamina propria* of IBD patients pointed to a similar suppressive activity compared to that exerted by Tregs obtained from healthy individuals. Interestingly, however, treatment of IBD patients with anti-TNF-α antibodies resulted in an increase in the number and function of Tregs, suggesting that control of disease activity is associated with the presence of functional Tregs.

In summary, though there is still controversy whether the number and/or phenotype of Tregs are altered in autoimmune diseases, defects in Treg suppressive function has been consistently reported. Similarly, loss of tolerance to hepatic self-antigens leading to liver autoimmunity has been shown to be accompanied by impairment in Treg immune-regulation.

4. Breaking tolerance – pathogenesis of autoimmune liver disease

4.1. Liver as an immunological organ

The liver is regarded as a unique anatomical and immunological organ, continuously exposed to antigen-rich blood (containing pathogens, toxins, tumor cells and self-antigens) from the gastro-intestinal tract (Racanelli and Rehermann 2006). The liver is highly enriched in phagocytic cells, APCs and lymphocytes and is an active site for the production of cytokines, complement components and acute phase proteins (Mackay 2002). Within the liver, many cell populations can act as APCs, namely endothelial cells, subendothelial stellate cells, hepatocytes, and dendritic cells (Crispe 2009; Figure 2.11.). Infiltrating liver lymphocytes are present throughout the parenchyma, as well as in the portal tracts; these include cells of the innate (NKT and NK cells) and adaptive immune systems (B and T cells; Racanelli and Rehermann

2006). Immune-regulatory mechanisms are required to determine whether an antigen encounter will result in immunological tolerance or reactivity. Liver autoimmunity ensues when self-tolerance is lost (Vergani and Mieli-Vergani 2008).

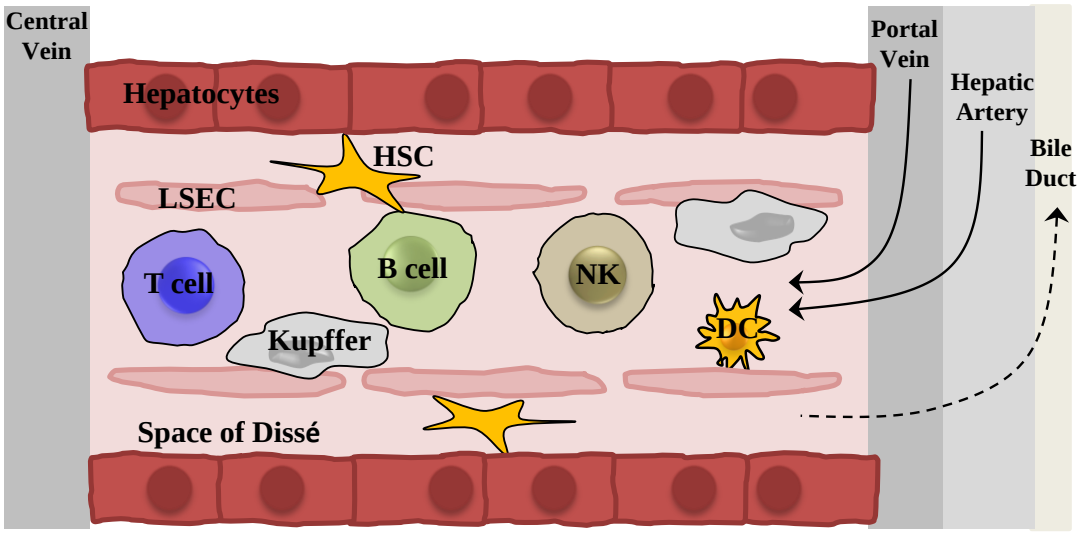


Figure 2.11. Liver as an immunological organ. Immune cells in the liver comprise those belonging to the innate arm of the immune system, namely hepatocytes, liver sinusoidal endothelial cells (LSEC), hepatic stellate cells (HSC), Kuppfer cells, dendritic cells (DCs), natural killer (NK) cells; and those belonging to the adaptive immune system, such as B and T cells. LSECs separate the blood stream from hepatocytes forming the space of Dissé.

4.2. Genetics of AIH

Genetic studies have identified a number of genes conferring susceptibility to AIH.

AIH is a "complex trait" disease, therefore not following any Mendelian pattern of inheritance. Its mode of inheritance is unknown, though like other human complex trait disorders, it involves one or more genes that, acting alone or in concert, and interacting with environmental factors, increase or reduce the risk of the trait (Donaldson 2004b).

In AIH, the strongest genetic associations relate to genes located within the *MHC* – the human leukocyte antigen (*HLA*) region – on the short arm of chromosome 6

(Table 2.1.), particularly those encoding *DRB1* alleles (Donaldson 2002, Donaldson 2004a, Donaldson 2004b). The role of class II MHC molecules is to present peptide antigens to CD4 T cells, suggesting that HLA class II antigen presentation and T cell activation are involved in the pathogenesis of AIH (Vergani et al. 2002). In Europe and North America, the alleles conferring susceptibility to AIH-1 are *DRB1*0301* and *DRB1*0401* which encode the HLA DR3 and DR4 antigens respectively (Donaldson et al. 1991, Strettell et al. 1997). Both heterodimers contain a K (lysine) residue at position 71 of the *DRB1* polypeptide and the hexameric amino acid sequence L (leucin) L (leucin) E (glutamic acid) Q (glutamine) K (lysine) R (arginine) at position 67-72 (Donaldson 2002, Donaldson 2004a). Amongst many associations reported to date, the presence of either HLA DR3 or DR4 are the only associations considered strong enough to positively point towards the diagnosis of AIH according to the revised diagnostic scoring system elaborated by the IAIHG (Alvarez et al. 1999). In Japan, Argentina and Mexico, susceptibility is linked to *DRB1*0405* and *DRB1*0404*, alleles encoding R (arginine) rather than K (lysine) at position 71, but sharing the motif LLEQ-R with *DRB1*0401* and *DRB1*0301* (Czaja and Donaldson 2000). Thus, K or R at position 71 in the context of LLEQ-R may be critical for susceptibility to AIH, favouring the binding of autoantigenic peptides, complementary to this hexameric sequence (Czaja et al. 2002). However, an alternative model based on valine/glycine dimorphism at position 86 of the DR-β polypeptide has been proposed, better representing the key HLA associations in patients from Argentina and Brazil (Donaldson 2002, Donaldson 2004a). In a study from Japan, AIH-1 patients were found to have *DRB1* alleles which encode histidine at position 13 (Donaldson 2004b).

Taken together, these data indicate that different populations show distinct genetic associations and that the peptides presented by MHC class II molecules to the T cell receptors are distinct and may derive from different antigens. Thus, these HLA associations are potentially indicative of the prevailing environmental insults triggering AIH-1 in different environments. In this regard, it is interesting to note that in South America possession of HLA *DRB1*1301* allele, which predisposes to paediatric AIH-1 in this population, is also associated with persistent infection with the endemic hepatitis A virus (Vergani et al. 2009).

Recently, a meta-analysis study, aiming to identify common HLA class II alleles conferring susceptibility to AIH-1 in Latin America, reported that the serological group DQ2 constitutes a risk factor, whilst the possession of DR5 or DQ3 is associated with protection against AIH occurrence (Duarte-Rey et al. 2009).

Susceptibility to AIH-2 is conferred by the possession of HLA DR7 (*DRB1*0701*) and DR3 (*DRB1*0301*), patients positive for *DRB1*0701* having a more aggressive disease and more severe prognosis (Ma et al. 2006). Another study has suggested that the possession of *HLA-DQB1*0201* is the major genetic determinant for children with AIH-2, although *HLA-DQB1*0201* is in linkage disequilibrium with *DRB1*0701* and *DRB1*0301*, both associated with AIH-2 (Djilali-Saiah et al. 2004). In AIH-2, HLA alleles have also been found to influence the autoantigenic humoral response, with *DRB1*0301* being associated with seropositivity for both anti-LKM-1 and anti-LC-1 antibodies and *DRB1*0701* predominant amongst patients seropositive for anti-LKM-1 only. Additionally, children positive for *DRB1*0701* develop anti-LKM-1 antibodies characterised by a more restricted specificity compare to children positive for *DRB1*0301* (Djilali-Saiah et al. 2006).

Table 2.1. HLA associations in autoimmune hepatitis.

HLA locus	Allele association	AIH-1	AIH-2
HLA-B	B8	LD with DRB1*0301 Severe disease course Relapse after drug withdrawl More frequent requirement for LT	
HLA-C	Cw7	Susceptibility in United Kingdom (LD with DRB1*0301	
HLA-DRB1	DRB1*0301	Susceptibility in Europe and North America Younger age at onset, higher rate of treatment failure, relapse after drug withdrawl, and requirement for LT than DR4 More expression of SLA/LP	Susceptibility in children Associated with seropsitivity for both anti-LKM-1 and anti-LC-1
	DRB1*0401	Susceptibility in Europe and North America Later age at onset than DR3 Higher frequency in women Associated with concurrent immunological diseases Low frequency of progression to hepatic failure and death Higher frequency of ANA positivity	
	DRB1*0404	Susceptibility in Mexico	
	DRB1*0405	Susceptibility in Japan and Argentina	
	DRB1*0701		Susceptibility in Europe and in Brazil Predominant amongst patients positive for only anti-LKM-1 Aggressive disease course and worse prognosis
	DRB1*1301	Susceptibility in South America and in DR3/DR4-negative North American patients	Early age at onset in Brazil
	DRB1*1401	Susceptibility in India	
	DRB1*1501	Protection in United Kingdom	
HLA-DQ	DQB1*0201	Susceptibility in United Kingdom and South America	Susceptibility in Europe and in North America (LD with DRB1*0301 and DRB1*0701)
	DQB1*0301	Protection in South America	
	DQB1*0601	Susceptibility in Brazil (LD with DRB1*1301)	Susceptibility in Germany

HLA, human leukocyte antigen; *AIH*, autoimmune hepatitis; *LD*, linkage desiquilibrium; *LT*, liver transplantation; *SLA/LP*, soluble liver antigen/liver pancreas; *anti-LKM-1*, anti-liver microsomal antibody type 1 antibodies; *anti-LC-1*, anti-liver cytosol type 1 antibodies; ANA, anti-nuclear antibodies

Other genes within the *HLA* region and shown to be associated with AIH susceptibility include the *IgA* and complement factor 4A (*C4A*) genes (Gregorio et al. 1997). IgA deficiency, frequently found in patients with AIH, is genetically linked to

the MHC locus, particularly with HLA-DR1 and DR7 (Vorechovsky et al. 1999, De la Concha et al. 2002). Moreover, low levels of C4a are present in 69% of children with AIH, this deficiency being genetically determined (Vergani et al. 1985).

A number of genes outside the MHC may also be involved in susceptibility to AIH. As mentioned earlier, CTLA-4 (also known as CD152) is a molecule present on the surface of T cells, which interacts, in competition with CD28, with CD80 and CD86 ligands on APCs, transmitting an inhibitory signal to T cells. A transition from A (adenine) to G (guanine) in exon 1 of the *CTLA-4* gene has been shown to confer susceptibility to several autoimmune diseases, including AIH-1 in caucasians from North America (Agarwal et al. 2000); however, this finding was not confirmed in Brazilian patients with AIH-1 or AIH-2 (Bittencourt et al. 2003). A polymorphism at position 308 in the tumor necrosis factor α gene promoter (*TNFA*) occurs more frequently in patients with AIH-1 from Europe and North America than in healthy subjects and is associated with a poorer response to steroids (Czaja et al. 1999b), a finding that was not observed in Japanese patients (Yoshizawa et al. 2005). A polymorphism at position 670 in the *FAS* gene promoter was found to influence AIH susceptibility and progression, leading to a more aggressive disease with an early development of cirrhosis (Agarwal et al. 2007). Polymorphisms in the vitamin D receptor – capable of activating macrophages and monocytes, preventing dendritic cells differentiation, and inhibiting Th1 cell function – were shown to contribute to the development of autoimmune liver disease (Vogel et al. 2002).

A form of AIH resembling AIH-2 has been described in some 20% of patients with autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED). This condition, also known as autoimmune polyendocrine syndrome 1, is a monogenic

autosomal recessive disorder, and is characterised by a variety of organ-specific autoimmune diseases, the most common of which are hypoparathyroidism and primary adrenocortical failure, accompanied by chronic mucocutaneous candidiasis (Liston et al. 2005, Mathis and Benoist 2009). At variance with many other autoimmune diseases, APECED is associated with mutations of a single gene – designated autoimmune regulator 1 (*AIRE1*). The *AIRE1* gene sequence consists of 14 exons containing 45 different mutations, with a 13-bp deletion at nucleotide 964 in exon 8 accounting for more than 70% of APECED alleles in the United Kingdom (Simmonds and Gough 2004). *AIRE1*, which codifies a transcription factor, is highly expressed in medullary epithelial cells and other thymic stromal cells involved in clonal deletion of self-reactive T cells. Interestingly, APECED has a high level of variability of symptoms, especially between populations. Since various gene mutations have the same effect on thymic transcription of ectopic genes in animal models, it is likely that the clinical variability across human populations relates to environmental or genetic modifiers. Of the latter, the most likely to synergise with AIRE mutations are polymorphisms in the HLA region. HLA molecules are not only highly variable and strongly associated with multiple autoimmune diseases but they are also able to affect thymic repertoire selection of self-reactive T cell clones (Longhi et al. 2010). Carriers of a single *AIRE1* mutation do not develop APECED. However, although the inheritance pattern of APECED indicates a strictly recessive disorder, there are anecdotal reports of mutations in a single copy of *AIRE* being associated with human autoimmunity of a less severe form than classically defined APECED (Simmonds and Gough 2004, Liston et al. 2005). The role of *AIRE1* heterozygote status in the development of AIH remains to be defined. *AIRE1* mutations have been reported in three children with severe AIH-2 and extra-

hepatic autoimmune manifestations (Lankisch et al. 2005), and in four children with AIH-1 and a family history of autoimmune disease (Lankisch et al. 2009).

4.3. Molecular mimicry

In patients with increased genetic susceptibility to AIH, immune responses to liver autoantigens may be triggered by molecular mimicry, a process where immune responses to external pathogens become directed toward structurally similar self-components (Vento et al. 1984, Bogdanos et al. 2001).

Autoimmunity is a common feature during chronic HBV and HCV infections, where 50% of patients eventually develop autoantibodies such as ANA and SMA (Gregorio et al. 1999, Gregorio et al. 2003). In addition, 10% of chronic HCV patients are positive for LKM-1, this positivity being correlated with disease severity and adverse reactions to interferon treatment (Lenzi et al. 1999). Thus, HBV and HCV infection may play a role in the initiation of the autoimmune process; however, a strong link between autoimmunity and viral hepatitis has been shown only for HCV. Interestingly, reactivity against $CYP2D6_{193-212}$, identified as a major B-cell epitope in AIH-2, is also found in 50% of HCV infected patients with seropositivity for anti-LKM-1 antibodies (Bogdanos et al. 2001). In HCV-positive/anti-LKM-1-positive patients the presence of cross-reactivity between antibodies directed against homologous regions of HCV (NS5B $HCV_{2985-2990}$) and cytomegalovirus (exon $CMV_{130-135}$; Kerkar et al. 2003) has been demonstrated. Moreover, Manns et al. reported a sequence homology between $CYP2D6_{254-271}$ and the amino acid (aa) sequence 310-324 of E1 HCV and the aa sequence 156-170 of IE1 75 Herpes Simplex Virus 1 (HSV-1). As anti-LKM-1 antibodies cross-react with homologous regions of CYP2D6, HCV, CMV and HSV, a

"multi-hit" mechanism for the generation of these antibodies and possibly of AIH-2 may be envisaged (Manns et al. 1991, Bogdanos et al. 2001). According to this model, sequential exposure to common viral pathogens like CMV or HSV may establish permissive immunologic conditions by priming a cross-reactive subset of T cells in a genetically predisposed host. Depending on the level of exposure and the degree of genetic susceptibility, a minority of recurrently infected individuals may progress to autoimmune disease. It is therefore conceivable that an as yet unidentified virus infection may be part of the origin of the autoimmune attack in AIH (Vergani et al. 2009).

Further support of this model has been provided by an interesting case-report describing a 10-year-old girl who acquired HCV infection following LT for end-stage liver disease caused by α1-anti-trypsin deficiency. Two weeks after HCV infection IgM anti-LKM-1 appeared, followed by IgG anti-LKM-1, a finding suggesting HCV as a trigger of a primary anti-LKM-1/anti-CYP2D6 autoimmune response (Mackie et al. 1994).

A link between HCV infection and breakdown of immune tolerance has also been postulated by a recent study by Agmon-Levin et al., who reported that serum anti-HCV antibodies were found in up to 8.7% of patients with autoimmune diseases, including cryoglobulinaemia, Hashimoto's thyroiditis and IBD (Agmon-Levin et al. 2009).

In addition to these environmental and genetic factors, there is increasing evidence that a failure of mechanisms maintaining tolerance to liver autoantigens is central to the development of AIH.

4.4. Mechanisms of autoimmune liver damage

The mechanisms leading to autoimmune liver damage have been a focus of intense investigation over the past three decades. However, the initial trigger of centred autoimmunity remains elusive.

The histological picture of interface hepatitis (see Chapter I, section 4.4.), the hallmark of the disease and composed of lymphocytes, plasma cells, and macrophages was the first to suggest that an autoaggressive cellular immune attack was the basis of this condition. Whatever the initial trigger, this massive recruitment of activated inflammatory cells is likely to cause damage. Immunohistochemical studies performed in the early 1990s have identified a predominance of T lymphocytes expressing the α/β T cell receptor (Senaldi et al. 1992). Amongst T cells, the majority are positive for the CD4 helper/inducer phenotype, and a sizeable minority for the CD8 cytotoxic phenotype. Lymphocytes of a non T cell lineage are fewer and include NK cells, macrophages, B cells and plasma cells (Senaldi et al. 1992).

Regardless of the factors triggering the autoimmune process, the pathogenic mechanism leading to liver damage is part of a complex scenario, which requires the intervention of both innate and adaptive arms of the immune system.

Liver damage is probably orchestrated by $CD4^{pos}$ lymphocytes that recognise a self-antigenic peptide. The autoimmune response is initiated after the peptide is embraced by a HLA class II molecule and presented to a naïve $CD4^{pos}$ T helper cell (Th0) by an APC, in the presence of the appropriate co-stimulatory signals, induced by the interaction of CD28 on Th0 and CD80/CD86 on APC. Once activated, and depending on the type of cytokines present in the microenvironment and on the nature of the antigen, Th0 cells can differentiate into Th1, Th2 and Th17 cells. These effector

cells initiate a cascade of immune reactions largely determined by the cytokines they produce:

a) Th1 cells secrete mainly IL-2 and IFN-γ; IFN-γ is considered the main orchestrator of tissue damage since it not only stimulates CD8 cells, enhances the expression of HLA class I, and induces expression of HLA class II molecules on hepatocytes (Lobo-Yeo et al. 1990, Senaldi et al. 1991), but also activates monocytes/macrophages, which in turn release IL-1 and TNF-α;

b) Th2 cells produce IL-4, IL-10 and IL-13, cytokines that induce the maturation of B cells into plasma cells, with consequent production of autoantibodies;

c) Th17 cells, which arise in the presence of TGF-β and IL-6, produce IL-17, IL-22, TNF-α, and CCL-20 (Bettelli et al. 2006). In both mice and humans Th17 immunity has been recently associated with autoimmune disease (Miossec et al. 2009). Th17 cells have also been implicated in the pathogenesis of another autoimmune liver disease, PBC (Harada et al. 2009). Although their contribution to liver damage in AIH is still under investigation, a recent paper showed an increased number of Th17 cells in both the peripheral blood and liver of patients with AIH compared to healthy controls (Zhao et al. 2011).

If Tregs are numerically and functionally impaired in AIH (see section 4.5.), effector responses are perpetuated with consequent liver cell destruction by the direct action of CTL, cytokines released by Th1, and possibly Th17 cells and monocytes/macrophages, complement activation or engagement of natural killer cells by autoantibodies bound to the hepatocyte surface (Vergani and Mieli-Vergani 2008). Mechanisms leading to and/or perpetuating the autoimmune liver attack in AIH are depicted in Figure 2.12.

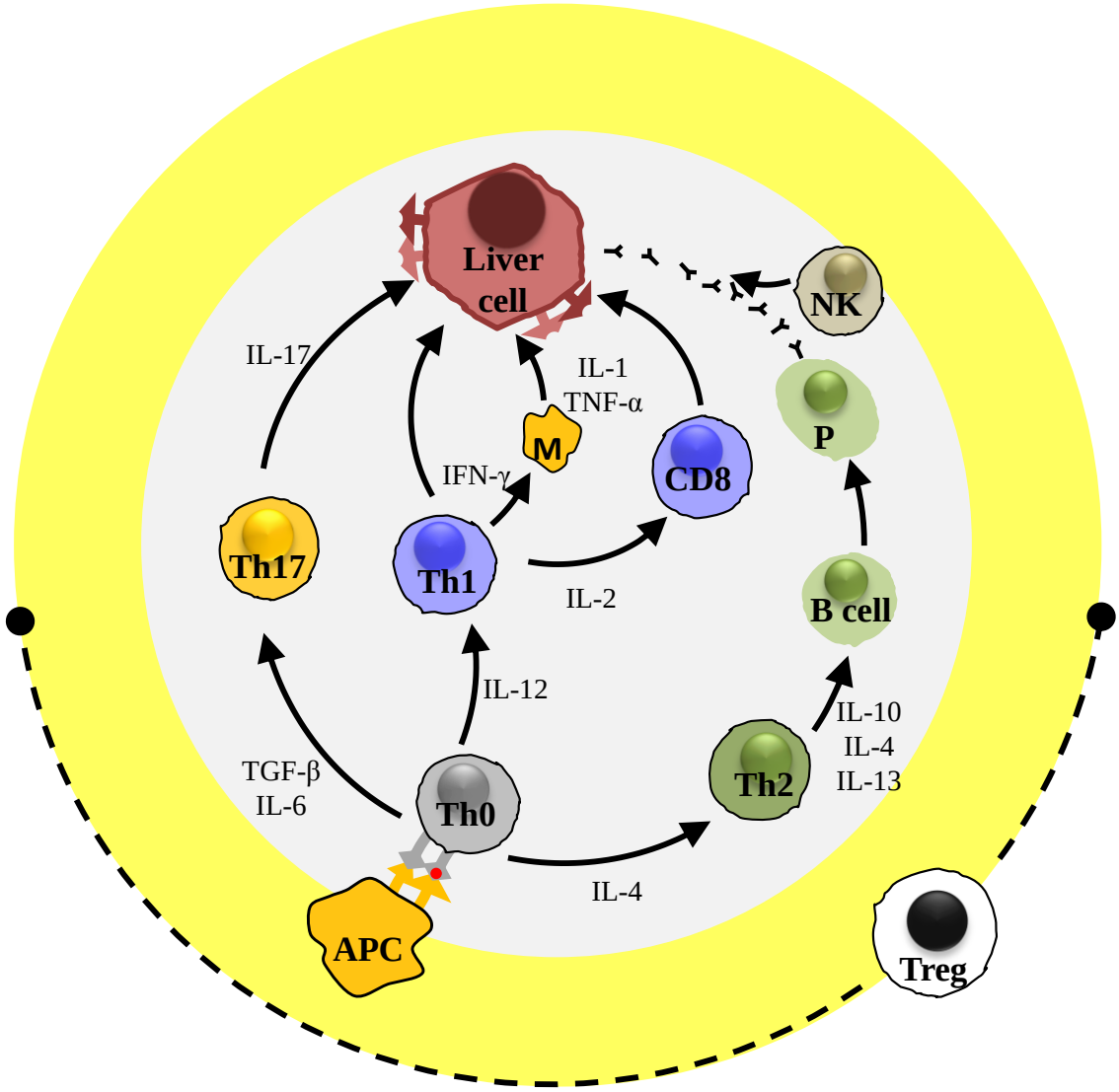


Figure 2.12. Autoimmune attack to the liver cell. An autoantigenic peptide is presented to an uncommitted T helper (Th0) lymphocyte within the HLA class II molecule of an antigen-presenting cell (APC). Th0 cells become activated and, according to the cytokines present in the microenvironment and the nature of the antigen, differentiate into Th1, Th2, or Th17 cells, initiating a series of immune reactions determined by the cytokines they produce: Th2 secrete mainly IL-4, IL-10 and IL-13, and direct autoantibody production by B lymphocytes; Th1 secrete IL-2 and IFN-γ, which stimulate T cytotoxic lymphocytes (CTL), enhance expression of class I and induce expression of class II HLA molecules on hepatocytes and activate macrophages; activated macrophages release IL-1 and tumour necrosis factor alpha (TNF-α). If regulatory T cells do not oppose, a variety of effector mechanisms are triggered: liver cell destruction could derive from the action of CTL; cytokines released by Th1 and recruited macrophages; complement activation or engagement of Fc receptor-bearing cells such as natural killer (NK) lymphocytes by the autoantibody bound to the hepatocyte surface. The role of the recently described Th17 cells, which arise in the presence of transforming growth factor beta (TGF-β) and IL-6, is under investigation.

4.4.1. Humoral immune response

Several autoantibodies have been reported to contribute to liver damage in AIH. The titres of anti-liver-specific membrane lipoprotein (LSP) antibody, as well as antibodies to the LSP components ASGPR and alcohol dehydrogenase (ADH) correlate with biochemical and histological indices of disease severity (Jensen et al. 1978, McFarlane et al. 1986). The role of these autoantibodies in the autoimmune liver attack has been suggested by the finding that hepatocytes, isolated from patients with AIH, are coated with immunoglobulins and are susceptible to cytotoxicity when exposed to autologous Fc receptor bearing mononuclear cells (Vergani et al. 1979, Vergani et al. 1987). More recently, anti-SLA antibodies, targeting the UGA suppressor tRNP-associated antigenic protein ($tRNP^{(ser)sec}$; Wies et al. 2000, Palioura et al. 2009), were found in 50% of AIH-1 and AIH-2 patients, defining a disease course more severe compared to seronegative patients (Ma et al. 2002a).

In AIH-2, the evidence that anti-LKM-1 antibodies are involved in liver damage stems from studies showing that CYP2D6 is also expressed on the surface of hepatocytes, and therefore may be susceptible to recognition by anti-LKM-1 antibodies (Muratori et al. 2000). Following the identification of CYP2D6 as the target antigen of LKM-1, the search for B cell epitopes within CYP2D6 has been the focus of intensive research. In AIH-2 anti-LKM-1 antibodies recognise linear regions (autoepitopes) of CYP2D6 in a hierarchical manner. Thus, the main linear B cell epitope, $CYP2D6_{193-212}$ is recognized by 93% of the patients, $CYP2D6_{257-269}$ by 85%, $CYP2D6_{321-351}$ by 53%, and two additional minor epitopes $CYP2D6_{363-389}$ and $CYP2D6_{410-429}$ are recognized by 7% and 13% respectively (Kerkar et al. 2003). Using the expression of both full-length and a series of truncated CYP2D6 proteins in an eukaryotic system, Ma et al. have

provided a conformational epitope mapping of CYP2D6, showing that the antigenicity is confined to the C terminal portion of the molecule and that it increases stepwise towards the C terminal (Ma et al. 2002b).

4.4.2. Cellular immune response

Early investigations of the cellular immune mechanisms involved in the pathogenesis of autoimmune liver damage concluded that AIH patients had circulating lymphocytes "sensitised" to liver antigens and able to kill target cells *in vitro*. Studies performed using T and non-T cell subsets obtained from the peripheral blood of AIH patients and xenogenic target cells demonstrated that cytotoxic cells were present in the non-T cell subpopulation (Vergani et al. 1979). Involvement of an antibody-dependent cell-mediated cytotoxicity (ADCC) in the autoimmune liver attack has been suggested by experiments showing that cytotoxic lymphocytes were located within the non-T cell compartment and that aggregated IgG were able to block cytotoxic activity. Such involvement was later confirmed by the observation that hepatocytes carrying IgG on their surface were susceptible to damage by lymphocytes from healthy individuals (Vergani et al. 1987). In the early 90s, clonal analysis studies showed that cytotoxicity against liver-specific antigens could also be detected within the T cell compartment (Wen et al. 1990).

Subsequent studies conducted by Wen et al. demonstrated that children with AIH display a 10-fold higher frequency of liver antigen-specific precursors in their circulation when compared to healthy subjects (Wen et al. 2001). Experiments of clonal analysis revealed a high frequency of CD4 T cells expressing the HLA-DR molecule amongst IL-2R positive cells. Neutralization of HLA-DR, CD4 and IL-2R led to the

conclusion that these clones follow the classical rules of immune recognition, being able to recognize antigens in the context of class II HLA molecules (Wen et al. 1990). Subsequent investigations showed that in AIH the largest numbers of clones generated from the peripheral blood are $CD4^{pos}$ T cells bearing the αβ T cell receptor. In contrast, the highest proportion of clones obtained from liver biopsies of the same patients are $CD4^{neg}CD8^{neg}$ T cells bearing the γδ T cell receptor or $CD8^{pos}$ αβ T cells. Both types of liver-derived clones are able to proliferate in response to liver-specific antigens, such as ASGPR and ADH; clones that proliferate in the presence of ASGPR are also able to induce the production of ASGPR-specific autoantibodies from B lymphocytes *in vitro* (Wen et al. 2001).

CD4 T cell immune responses

CD4 T cell immune responses have mainly been studied in AIH-2, where the target autoantigen is known. The evidence that autoantibodies to CYP2D6 belong to the IgG isotype – thus implicating a CD4-dependent class switch – and, that the majority of T cells infiltrating the liver in AIH are CD4 lymphocytes, suggested a role for these cells in AIH pathogenesis. $CYP2D6_{262\text{-}285}$ specific T cell clones generated from liver tissue and peripheral blood express a Th1 CD4 phenotype (Lohr et al. 1991, Lohr et al. 1992). To define the specificity of *ex vivo* CYP2D6-reactive T cells in AIH-2, overlapping peptides covering the whole CYP2D6 molecule were constructed; this study showed that T cells from HLA *DRB1*0701* positive patients recognise seven CYP2D6 regions, four of which are also partially recognized by T cells of *DRB1*0701* negative patients (Ma et al. 2006). Whilst distinct peptides induce production of IFN-γ, IL-4, or IL-10, peptides inducing IFN-γ and proliferation were found to overlap. An

overlap between sequences inducing T and B cell responses has also been observed. The number of epitopes and the quantity of cytokine produced by T cells have been reported to correlate with biochemical and histological markers of disease activity. These results indicate that the T cell response to CYP2D6 in AIH-2 is polyclonal, involves multiple effector types targeting different epitopes, and is associated with hepatocyte damage (Ma et al. 2006).

CD8 T cell immune responses

In the early 1990s CD8 T cell clones specific for ASGPR were described in AIH patients (Wen et al. 1990). More recently, CYP2D6-specific CD8 T cells, capable of producing IFN-γ and of exerting cytotoxicity upon recognition of CYP2D6 epitopic sequences in an HLA class I restricted fashion, have been identified. Involvement of CYP2D6-specific CD8 T cell immune responses in the liver damage is suggested by the correlation between the extent of CYP2D6-specific CD8 T cell immune responses and indices of disease activity and by the presence of self-reactive CD8 T lymphocytes within the portal tracts (Longhi et al. 2007).

γδ T cells

γδ T cells bear a TCR composed of rearranged γ and δ chains instead of the conventional αβ heterodimeric TCR (Kreslavsky et al. 2010). Although representing a small proportion of circulating lymphocytes, γδ T cells are abundant in the liver (Girardi 2006). According to the rearranged Vδ chain, two γδ T cell subsets are generally recognised: Vδ1 and Vδ2 cells. While the former are highly abundant among intraepithelial lymphocytes but scarcely represented in the peripheral blood, the latter

constitute up to 80% of the whole circulating γδ T cell population. In terms of function, Vδ1 cells are known to possess both regulatory and effector function, whereas Vδ2 cells are mainly effectors and involved in the defence against pathogens and tumours (Girardi 2006).

In the early 1990s, Wen et al. reported a higher frequency of γδ T cells in children with AIH compared to controls. Later, the same authors showed that a higher number of γδ T cell clones could be obtained from liver biopsy specimens of AIH patients than from their peripheral blood. Moreover, they showed that these γδ T cells, in comparison to those bearing the αβ TCR, are more cytotoxic against liver-derived cell-lines, therefore suggesting a role for these cells in the autoimmune liver attack.

A paper by Ferri et al. has recently renewed interest in γδ T cells and in understanding their contribution to liver damage in AIH, showing that the number of these cells is elevated during the active phases of disease. Moreover, γδ T cells not only display an inverted Vδ1/Vδ2 ratio, but are also more frequently positive for IFN-γ and granzyme B than normal controls. The frequency γδ T cells positive for granzyme B correlated with transaminase and bilirubin levels, a finding that suggests a role for these cells in the initiation and/or perpetuation of liver injury (Ferri et al. 2010).

Monocytes

Monocytes/macrophages represent a major component of the portal/periportal cellular infiltrate in AIH. Compared to health, monocytes isolated from the peripheral blood of children with AIH, displayed more vigorous spontaneous migration, and produced more TNF-α over IL-10, and expressed higher levels of Toll-like receptor 4 (TLR-4) – a molecule that, like other members of the Toll-like receptor family,

recognises pathogen-associated molecular patterns, and whose engagement is key to the initiation of adaptive immune responses (Hoshino et al. 1999). Marked monocytes´ activation during active disease suggests active participation of these cells in liver damage, possibly promoted by autoreactive cells belonging to the adaptive arm of the immune system (Longhi et al. 2009).

Collectively the studies described above indicate an involvement of both humoral and cellular immune responses in the pathogenesis of AIH, involvement which is likely to be permitted by a failure of immune homeostatic processes.

4.5. Impairment of regulatory T cells

Occurrence of autoimmune diseases is determined by the breakdown of immune-regulatory mechanisms that in health are responsible for maintaining immunological tolerance against self-antigens (see section 3.3.). In the context of AIH, seminal studies conducted during the 1980s were able to a) demonstrate an impairment of cells with "suppressor" function, which would eventually revert following *in vitro* exposure of therapeutic doses of steroids (Hodgson et al. 1978, Nouri-Aria et al. 1982, Vento et al. 1984), and to b) show that this defect involved a subpopulation of T lymphocytes controlling immune responses against a liver-specific membrane autoantigen (Vento et al. 1984). Those were the seeds for a series of subsequent studies, that almost 20 years later, clearly indicated that impairment in regulatory T cells is essential to the loss of immune tolerance in AIH and to the emergence of uncontrolled effector immune responses (Longhi et al. 2004, Longhi et al. 2005, Longhi et al. 2006, Longhi et al. 2008, Ferri et al. 2010, Longhi et al. 2011).

Among the multiple T cell subsets with suppressive function, the regulatory T cells (Tregs), defined by the expression of CD4, the IL-2 receptor α chain (CD25), and the transcription factor FOXP3, have emerged as having a central role in maintaining immune-tolerance to autoantigens (Sakaguchi et al. 1995, Sakaguchi et al. 2010; see section 5.3.).

Both children and adults with AIH display a reduced frequency of $CD4^{pos}CD25^{high}$ Tregs that, compared to healthy subjects, express lower levels of FOXP3 (Longhi et al. 2004, Longhi et al. 2005, Ferri et al. 2010). This defect partly relates to the liver disease stage, being more evident at presentation and at relapse than during drug-induced remission. The observation that the frequency of Tregs is inversely correlated with anti-SLA and anti-LKM-1 antibody titres, suggest that Treg reduction favours the serologic manifestations of AIH (Longhi et al. 2004).

Tregs isolated from patients with AIH are also functionally impaired as they fail to restrain the proliferation and IFN-γ production of CD4 and CD8 effector T cells (Longhi et al. 2004, Longhi et al. 2005), and are also defective at inducing a regulatory milieu that would support and enhance their own function (Longhi et al. 2006). When considering their interaction with innate immune cells, Tregs from AIH patients have been found to aberrantly enhance the activation of monocytes (Longhi et al. 2009).

The observation that corticosteroid therapy can partially restore Treg indicates that in AIH these cells, though impaired, have the potential to expand and regain their function (Longhi et al. 2004, Longhi et al. 2005).

Using a polyclonal T cell stimulation strategy (that engages the T cell receptor *via* CD3 and the co-stimulatory molecule CD28, while providing exogenous IL-2; a key cytokine for Treg survival and growth), Tregs can be expanded from circulating

$CD4^{pos}CD25^{pos}$ Tregs, and generated *de novo* from non-regulatory $CD4^{pos}CD25^{neg}$ T cells in healthy subjects and, though to a lesser extent, also in patients with AIH (Longhi et al. 2008). Interestingly, expanded Tregs express higher levels of FOXP3, and therefore suppress more efficiently when compared to freshly isolated Tregs (Longhi et al. 2008).

Since the expansion strategy mentioned above provides strong TCR signals, it may tend to preferentially expand effector rather than regulatory T cells; thus, it will be essential in view of future clinical application to either expand highly purified and consequently highly effective Tregs; or, alternatively, to create the right environment in which Tregs can perform at their best, namely through the inhibition of the aforementioned IL-17 expression (Longhi et al. 2012).

Studies conducted in mice have shown that Tregs with autoantigen specificity suppress more efficiently than their non-antigen-specific counterparts. In contrast to AIH-1, were reactivity to a univocal autoantigenic target is lacking, in AIH-2 not only is the key autoantigen known (CYP2D6; Gueguen et al. 1988), but also the antigenic regions ($CYP2D6_{217-260}$ and $CYP2D6_{305-348}$), targeted by B, CD4 and CD8 T cells is well characterised (Kerkar et al. 2003, Longhi et al. 2007). Thus, AIH-2 seems to be the ideal model for attempting reconstitution of self-tolerance by specific immunological intervention. In this regard, antigen-specific Tregs from patients with AIH-2 have been found to suppress CD4 and CD8 T cell responses more potently than polyclonally expanded Tregs. The most efficient suppression of autoreactive T cells was achieved when Tregs exposed to CYP2D6 peptides were co-cultured with semi-mature dendritic cells loaded with the same peptide (Longhi et al. 2011). For this reason autoantigen-

specific Tregs would represent an optimal tool for achieving a tailored immne-tolerance reconstitution in AIH.

Among other cells with suppressive potential, NKT cells, a subset of T cells that co-express NK cell markers and that are highly represented within the liver (see section 4.1.), have been suggested to play an important role in regulating immune responses in the context of autoimmune liver diseases (Santodomingo-Garzon and Swain 2011). NKT cells are numerically reduced in the peripheral blood of AIH patients, particularly during the active phases of disease and this number is partially restored during drug-induced remission; their behaviour, therefore, mirrors that of $CD4^{pos}CD25^{high}$ regulatory T cells (Ferri et al. 2010). In addition, NKT cells from AIH patients produce lower quantities of the regulatory cytokine IL-4 compared to healthy controls (Ferri et al. 2010). Evidence of impaired immune-regulation in AIH is summarised in Table 2.2.

Table 2.2. Evidence of impaired immune-regulation in AIH.

Evidence	Reference
AIH-1	
Impairment of "suppressor" cell function, which recovers after in vitro exposure of steroids	Nouri-Aria et al., 1982
Defect in a T cell population that controls immune reponses against a liver-specific antigen	Vento et al., 1984
Tregs are defective in number and function	Longhi et al., 2004, Longhi et al., 2005
Tregs fail to restrain the proliferation and IFN-γ production by CD4 and CD8 T cells	Longhi et al., 2004, Longhi et al., 2005
Tregs are defective at promoting secretion of regulatory cytokines by their targets	Longhi et al., 2006
Tregs aberrantly enchance activation anf function of monocytes	Longhi et al., 2009
NKT cells are reduced in number	Ferri et al., 2010
NKT cells contain low frequencies of IL-4-producing cells	Ferri et al., 2010
AIH-2	
Tregs are defective in number and function	Longhi et al., 2004, Longhi et al., 2009
Tregs fail to restrain the proliferation and IFN-γ production by CD4 and CD8 T cells	Longhi et al., 2004, Longhi et al., 2005

AIH, autoimmune hepatitis, *Tregs*, regulatory T cells; *IFN-γ*, interferon gamma; *NKT cells*, natural killer T cells; *IL-4*, interleukin-4

4.6. Animal models of AIH

Animal experiments have contributed much to the understanding of pathogenic mechanisms of several conditions. In AIH, the ideal model should be characterised by a well-defined initiating event followed by chronic inflammation leading to fibrosis. Although several murine models of AIH have been reported so far, none of them faithfully reproduces the human condition.

Since in AIH-2 the autoantigen is well defined, researchers have focused their attention in this disease subtype. One model is based on the immunisation of C57BL/6 female mice with a plasmid containing the cDNA for the antigenic region of human CYP2D6 and formimino-transferase cyclodeaminase, targets of anti-LKM-1 and anti-LC-1 respectively, together with the end of the terminal region of murine CTLA-4 (Lapierre et al. 2004). Only after three immunisations and when a plasmid containing the cDNA encoding IL-12 was also used, antigen specific autoantibodies were produced, and a modest liver damage accompanied by portal and periportal infiltration of CD4, CD8 T and, B cells was observed. When the same immunisation protocol was used in different mouse strains, no inflammatory changes were observed highlighting the importance of a specific genetic background in the development of the disease (Lapierre et al. 2004). More recently the same group demonstrated that in the same model the severity of the disease depends on the age at the time of the xeno-immunisation as 7-week-old females of C57BL/6 background developed more severe disease with higher levels of serum alanine aminotransferase and autoantibody titre than younger and older females (Lapierre et al. 2010). Another model of AIH-2 was generated after infecting CYP2D6 transgenic mice with an Adenovirus-CYP2D6 vector (Holdener et al. 2008). While focal hepatocyte necrosis was seen both in mice treated

with the Adenovirus-CYP2D6 vector and control mice treated with Adenovirus alone, only the former developed chronic histological changes, including fibrosis, reminiscent of AIH. The hepatic lesion was associated to a specific immune response to an immunodominant region of CYP2D6 and a cytotoxic T cell response to Adenovirus-CYP2D6 vector infected target cells. Though these two experimental approaches provide useful information on the possible pathogenic mechanisms leading to AIH-2, a model faithfully reproducing AIH in humans is still missing.

The role of antigen-specific T cells in the induction and perpetuation of experimental hepatitis has recently been provided by a transgenic mouse model, characterised by the expression of chicken ovalbumin (OVA) on the surface of hepatocytes (Buxbaum et al. 2008). Repeated injections of OVA-specific T cells resulted in chronic hepatitis characterised by lobular and portal inflammation. Hepatic damage was determined by CD8 OVA-specific T cells and was enhanced by OVA-specific $CD4^{pos}$ T cell help (Buxbaum et al. 2008). In contrast, Robinson et al. reported that in *Tgf-b*$^{-/-}$ mice, a model characterised by the development of fulminant hepatitis, $CD4^{pos}$ T cells are the most important source of IFN-γ relevant to pathology, with no significant contribution from $CD8^{pos}$ T cells (Robinson et al. 2009). Taken together, it is possible that involvement of different immune-mediated mechanisms may determine different types of disease presentation and outcome.

To elucidate the roles of immune-regulatory mechanisms involved in the development of AIH, Kido et al. recently developed a mouse model of spontaneous AIH by inducing a concurrent loss of two controlling mechanisms, namely $FOXP3^{pos}$ regulatory T cells and the PD-1-mediated signalling. Following neonatal thymectomy, which is associated with a dramatic reduction in the number of nTregs in the periphery,

PD-1 deficient mice developed fatal AIH characterised by severe $CD4^{pos}$ and $CD8^{pos}$ T cell infiltration and massive lobular necrosis, and elevated titres of ANA. Importantly, adoptive transfer of Tregs was able to suppress the progression of fatal hepatitis after AIH initiation, confirming the role of self-reactive T lymphocytes in the development of AIH liver damage as well as that of Tregs in maintaining tolerance (Kido et al. 2008). Further evidence implicating Tregs in AIH pathogenesis, was provided by the work of Lapierre et al. mentioned above, which showed that xeno-immunised C57BL/6 male mice developed minimal liver inflammation which was associated with a higher percentages of $FOXP3^{pos}$ Tregs both in peripheral blood and liver compared to immunised female mice.

In summary, both human and animal studies have provided wide evidence that in AIH the effector functions of CD4 and CD8 T cells are pathogenically important and associated with defective Tregs. However, what causes Treg defects in AIH remains largely unknown.

5. Causes of regulatory T cell impairment in autoimmune liver disease

As shown in section 3.3.4., autoimmunity can result from a loss of Treg immune-regulation due to: 1) reduced numbers of Tregs; 2) intrinsic defects in Treg function; and/or 3) effector cell resistance to Treg suppression.

5.1. Inadequate numbers of Tregs

An obvious first step in determining whether Treg defects contribute to human autoimmunity is to assess whether there is an inadequate number of functional Tregs, thereby leading to impaired immune-regulation.

Although it has been shown that the frequency of $CD4^{pos}CD25^{pos}$ cells are significantly reduced in AIH patients (see section 4.5.), studies conducted in other human autoimmune diseases have demonstrated that the pool of Tregs defined by the expression of CD25 are frequently contaminated by effector T cells, rendering difficult the precise quantification of functional Tregs. In the past few years, the identification of new markers has allowed a more refined Treg quantification and has enabled the distinction of *bona fide* Tregs from effector cells. One of these molecules is the IL-7 receptor (IL-7R) or CD127.

5.1.1. CD127

CD127, the IL-7R, has recently been reported to be useful in discriminating between $CD25^{pos}$ Tregs and $CD25^{pos}$ activated T cells (Seddiki et al. 2006), because many non-regulatory T cell subsets are IL-2-independent and IL-7-dependent (von Freeden-Jeffry et al. 1995). However, Tregs, which require IL-2 for survival, do not require neither IL-7 nor the IL-7R. Down-regulation of CD127 on Treg cells was confirmed by using microarray gene-expression profiling studies of $CD4^{pos}CD25^{high}$ and $CD4^{pos}CD25^{neg}$ T cells, and by using quantitative polymerase chain reaction to confirm CD127 mRNA reduced expression (Liu et al. 2006).

A link between CD127 and FOXP3 has also been suggested based on the observation that *Foxp3* physically interacts with the promoter region of CD127, which could explain the down-regulation of CD127 characteristic of Tregs (Liu et al. 2006). In addition, it has been shown that the $CD127^{neg}$ population is characterised by high suppressive activity, in contrast to the non-suppressive $CD127^{pos}$ subset (Seddiki et al. 2006).

Codarri et al. have recently explored the role of CD127 expressing cells in the context of solid organ transplantation (Codarri et al. 2007), where it is known that activated allospecic CD4 T cells and Tregs play a key role by either participating in allograft rejection or promoting tolerance (Graca et al. 2002, Benghiat et al. 2005). The authors reported that the $CD4^{pos}CD25^{pos}$ T cell population in kidney and liver transplant recipients were frequently $CD127^{pos}$, whereas in healthy subjects the majority of these cells were $CD127^{neg}$. They have further shown that this cell population contained allospecic CD4 T cells and secreted effector cytokines such as TNF-α and IFN-γ, being substantially more expanded in patients with documented chronic rejection than in the peripheral blood of stable transplant recipients. Moreover, in patients with chronic rejection $CD127^{pos}$ cells accounted for 50% of the total CD4 T cells infiltrating the allograft, clearly indicating an association between CD127 expression by $CD4^{pos}CD25^{pos}$ cells and T cell effector functions (Codarri et al. 2007).

Analysis of the functional properties of $CD4^{pos}CD25^{high}CD127^{neg}$ Tregs have been provided by a study in relapse-remitiing MS patients (Michel et al. 2008), a condition associated with defective Treg function (see section 3.3.4). The authors showed that, at variance with $CD4^{pos}CD25^{high}$, the regulatory potency of $CD4^{pos}CD25^{high}CD127^{low}$ cells was similar in patients and in age-matched control subjects. Conversely, the $CD4^{pos}CD25^{high}CD127^{pos}$ T cell population in MS patients proliferated more vigorously and produced higher quantities of effector cytokines, explaining, at least in part, the previous observation of an impaired suppressive capacity of $CD4^{pos}CD25^{high}$ cells in MS patients (Michel et al. 2008).

Taken together these studies highlight the importance of reduced CD127 expression as a marker of *bona fide* Treg cells. I have thus hypothesised that

unrestrained effector mechanisms in autoimmune liver disease are permitted by a reduced number of functional Tregs, i.e. those characterised by absent expression of CD127.

5.2. Intrinsic defect in Treg function

Akin to cell numbers, another possible cause for impaired immune-regulation in autoimmunity setting is represented by defects in the Treg cell function that are intrinsic to these cells. In this regard, the galectin family of molecules has recently received attention, given its role in immune-regulation and because of the association of some of its members with Treg´ mechanisms of suppression.

5.2.1. Galectins

Lectins are non-enzymatic and non-immunoglobulin proteins that bind carbohydrates. Galectins, previously termed S-type lectins, are a family of evolutionarily conserved animal lectins that bind N-acetyllactosamin-containing glycans (Cooper and Barondes 1999, Rabinovich and Toscano 2009). They have pleiotropic roles in immune responses, being involved in pathogen recognition, in shaping the course of adaptive immune responses and in fine-tuning inflammatory responses (Rabinovich et al. 2007b, R. Y. Yang et al. 2008).

Fifteen galectins have been identified in mammals, 11 of which are also present in humans – galectins 1-4, 7-10, and 12-14 (Liu and Rabinovich 2005). Structurally, galectins share a common basic fold and at least one conserved carbohydrate recognition domain (CRD) of approximately 130 amino acids responsible for

carbohydrate binding (Leffler et al. 2004). Galectins are traditionally classified in three different groups on the basis of structural similarities:

a) prototype galectins, which have one CRD and exist as monomers or dimers (galectin-1, galectin-2, galectin-5, galectin-7, galectin-10, galectin-11, galectin-13, galectin-14 and galectin-15);

b) tandem-repeat type galectins, which contain two different CRDs separated by a linker of up to 70 amino acids (galectin-4, galectin-6, galectin-8, galectin-9 and galectin-12);

c) the chimera-type galectin-3, which contains a CRD connected to a non-lectin amino-terminal region (Figure 2.13.).

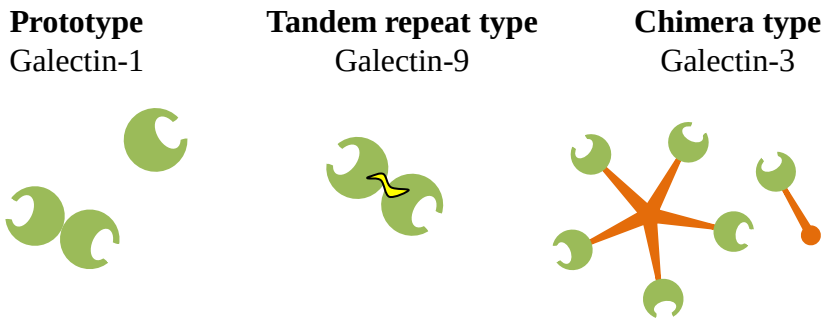


Figure 2.13. Representation of the structure of different members of the galectin family. Galectins can be subdivided into 3 groups: prototype galectins, which contain one carbohydrate recognition domain (CRD) and can form homodimers; tandem-repeat type galectins, which contain two distinct CRDs in tandem connected by a linker of up to 70 amino acids and are inherently bivalent; and the unique chimera-type galectin-3, which consists of unusual tandem repeats of proline- and glycine-rich short stretches fused onto the CRD.

Although galectins have features of cytosolic proteins, they are found not only intracellularly but also in the extracellular space. The mechanism of secretion is not clear since galectins lack the signal sequences required for classical secretory pathway *via* the endoplasmic reticulum (ER) and vesicles of the Golgi apparatus, and are therefore released through an unusual route that requires intact carbohydrate-binding activity of the secreted protein (Hughes 1999).

Once outside the cells, galectins bind to, and cross-link multiple glucoconjugates found on the cell surface or in the extracellular matrix, translating glycan-coded information into immune cell activation, differentiation and homeostatic programmes (Cooper and Barondes 1999).

Like many other receptor-ligand systems, galectins can trigger a cascade of transmembrane signalling events; through this mechanism, they can modulate processes that include apoptosis, cytokine secretion, and cell adhesion and migration (Rabinovich et al. 2007a). However, such interactions are limited to galectins that are secreted; some of the family members remain associated with cell membranes or function within the intracellular compartment, participating in processes that are essential for basic cellular functions such as pre-mRNA splicing, regulation of cell cycle progression and cell growth, and modulating apoptosis *via* interaction with Bcl-2 family members in the cytoplasm (Liu et al. 2002, Wang et al. 2004). These functions are typically independent of their sugar-binding activities and rather rely on protein-protein interactions (Rabinovich et al. 2002). Interestingly, the same member – e.g. galectin-3 – may exert paradoxical effects depending on whether it acts from the outside or inside the cell. If in the former it triggers cell death, in the latter it protects the cell from apoptosis (Nangia-Makker et al. 2007, Liu and Rabinovich 2005).

Although the majority of galectins have a wide tissue distribution, some of them are expressed with restricted tissue specificity (Rabinovich et al. 2002). Within the immune system, galectins are expressed by virtually all immune cells, either constitutively or in an inducible fashion, and are significantly up-regulated by activated B and T cells, activated macrophages, and DCs (Blaser et al. 1998, Rabinovich et al. 1998, Zuniga et al. 2001). Recently, studies using gene expression arrays have indicated

high expression of galectins in Tregs (Sugimoto et al. 2006, Ocklenburg et al. 2006, Garin et al. 2007).

The expression of galectins is regulated during the activation and differentiation of immune cells, and may be significantly altered under several pathological conditions (Rabinovich et al. 2007a). Moreover, the expression of galectins may be modulated by cytokines, chemokines and hormones (Camby et al. 2006).

5.2.2. Galectins and tolerance

Galectins play a vital role in central and peripheral tolerance (Dhirapong et al. 2009; Figure 2.14.). Since a more detailed description of the biological functions of galectin-9 (GAL-9) will be provided later, this section will primarily focus on other members, particularly galectin-1 (GAL-1) and galectin-3 (GAL-3) that along with GAL-9 are each representative of the different subfamilies mentioned above.

A key feature of the galectin family members is their influence on T cell development, function and homeostasis. The best studied family member, GAL-1, has been shown to control T cell growth and apoptosis during thymic development and after stimulation in the periphery (Perillo et al. 1995). GAL-1 is expressed on thymic epithelial cells (Baum et al. 1995), where it promotes clonal deletion of thymocytes (Perillo et al. 1997), and therefore contributes to central tolerance by eliminating self-reactive lymphocytes. In the periphery, GAL-1 induces apoptosis of activated, but not of naïve T cells (Blaser et al. 1998).

In addition to its pro-apoptotic properties, GAL-1 is known to elicit IL-10 production (van der Leij et al. 2004) and to boost the suppressive activity of Tregs. Indeed, exposure to GAL-1 promoted the differentiation of Tregs *in vitro* (Juszczynski

et al. 2007). Moreover, in a mouse model where colitis is induced by 2,4,6-trinitrobenzene sulfonic acid (TNBS), administration of GAL-1 resulted in expansion of IL-10-producing cells, which successfully suppressed autoimmune inflammation (Santucci et al. 2003). In this regard, proteomic analysis of human Tregs have identified galectin-10 (GAL-10) as a putative marker of Tregs, since it was expressed only by $CD4^{pos}CD25^{pos}$ cells and treatment with GAL-10-specific small-interfering RNA (siRNA) resulted in complete abrogation of Treg suppressive activity (Kubach et al. 2007).

As mentioned earlier, GAL-3 has the unique ability of acting in a dual manner – it can either prevent or induce apoptosis of T cells depending on its intra or extracellular localisation respectively (Hsu et al. 2009). While intracellular GAL-3 protects from apoptosis induced by Fas ligation or a broad spectrum protein kinase inhibitor staurosporine (Yang et al. 1996), the same protein located exogenously may promote cell death, as exemplified by its apoptotic effect on T cells (Fukumori et al. 2003). It should be noted, however, that most studies to date point to a pro-inflammatory role of GAL-3. Indeed, elevated levels of serum GAL-3 have been reported in various inflammatory conditions, such as rheumatoid arthritis (Ohshima et al. 2003) and Behcet´s disease (Lee et al. 2007). Also, a role for GAL-3 in the development of airway inflammation in a murine model of asthma has been demonstrated (Zuberi et al. 2004, Lopez et al. 2006). Furthermore, GAL-3-deficient mice consistently developed fewer inflammatory cell infiltrations following thioglycollate injection in the peritoneal cavity than the wild-type mice, thus supporting GAL-3 as a positive regulator of inflammatory responses (Hsu et al. 2000).

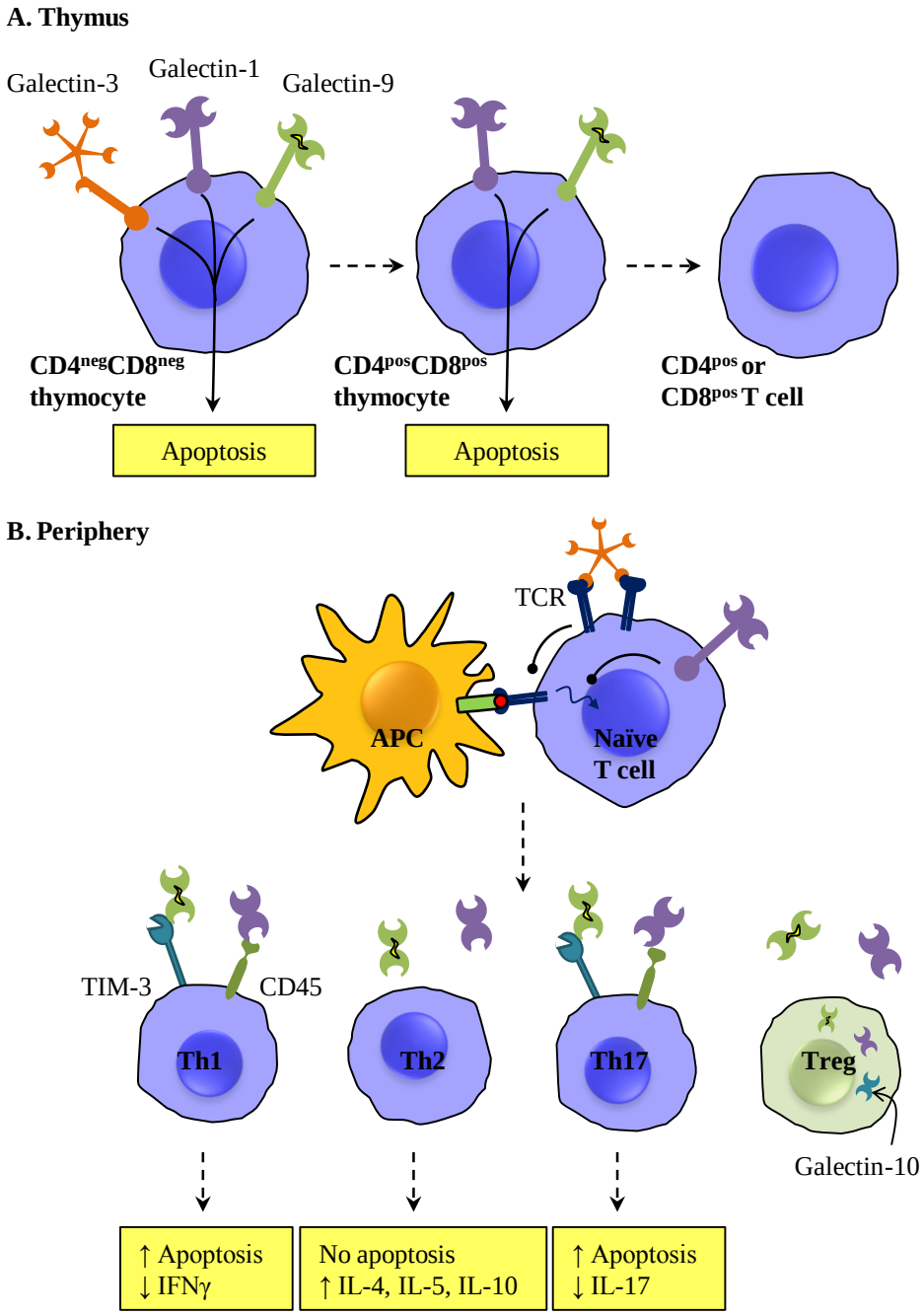


Figure 2.14. Contributions of galectins to central and peripheral tolerance. In the thymus galectin-1, galectin-3 and galectin-9 induce apoptosis in double negative ($CD4^{neg}CD8^{neg}$) or double positive ($CD4^{pos}CD8^{pos}$) thymocytes, suggesting a role for the these galectins in regulating central tolerance (*A*). Once in the periphery, galectin-9 blocks early T cell receptor (TCR)-mediated activation signals and prolongs the survival of naïve T cells. Galectin-3 forms lattices with complex N-glycans to limit TCR clustering, thus increasing agonist threshold for TCR signalling. Following T cell activation, galectin-1, galectin-3 and galectin-9 bind to particular glycosylated receptors and trigger distinct intracellular events to induce T cell death. In addition, galectin-1, galectin-9 and galectin-10 may contribute to the suppressive activity of regulatory T cells (Tregs).

5.2.3. Galectin-9

GAL-9, also known as ecalectin, belongs to the tandem-repeat class of galectins, consisting of two homologous but distinct CRDs (N-terminal, 148 amino acids; C-terminal, 149 amino acids) connected by a linker peptide (Hirabayashi et al. 2002, Wada and Kanwar 1997). Frame-shift mutations leading to a truncated product with only one functional CRD were found in the coding sequence of GAL-9 in some cancer cells (Lahm et al. 2000). Three isoforms arising from alternative splicing of the original transcript and differig in the length of the linker region, have been identified (Chabot et al. 2002). The full-length protein may also be post-transcriptionally cleaved by enzymes such as thrombin (Nishi et al. 2006). To date, there is little or no information as to whether these GAL-9 isoforms exhibit different biological functions.

GAL-9 was first identified as a selective eosinophil chemoattractant, inducing superoxide production and prolonging cell survival (Matsumoto et al. 1998). Subsequent studies revealed that, similar to other galectins, GAL-9 modulates a variety of biological functions such as cell aggregation and adhesion and apoptosis of eosinophils, cancer cells and T cells (Wada et al. 1997, Saita et al. 2002, Kageshita et al. 2002). It can be localised both intracellularly and, albeit unusual for a galectin, on the cell surface. Release from the cell surface to a soluble form requires the action of certain matrix metalloproteinases (Hirashima et al. 2004). Almost simultaneously, GAL-9 was also described as a urate transporter in the kidney, where it functions as a voltage-sensor and mediates transport of urate (Lipkowitz et al. 2004).

GAL-9 has also been shown to possess a broad array of immunological activities. Firstly, it was demonstrated that GAL-9 was involved in the regulation of thymic epithelial cell–thymocyte interactions, promoting apoptosis of the latter (Wada

et al. 1997). Subsequent studies using several different cell lines have shown that GAL-9 preferentially induced cell death of activated $CD4^{pos}$ T cells through the calcium-influx-calpain-capsase-1 pathway (Kashio et al. 2003).

In 2005, Zhu and colleagues identified GAL-9 as the ligand of T-cell-immunoglobulin-and-mucin-domain-3 (TIM-3), a protein selectively expressed on terminally differentiated Th1 but not Th2 cells (Zhu et al. 2005).

Functional studies conducted within the same investigation suggested that GAL-9 induces cell death in Th1 but not in Th2 cells and that Gal-9-induced cell death is dependent on TIM-3. In the context of EAE, *in vivo* administration of GAL-9 in mice immunised with myelin oligodendrocyte glycoprotein (MOG) immunodominant peptide specifically reduced the numbers of MOG-specific IFN-γ-producing Th1 cells. Moreover, treatment with GAL-9-specific siRNA during the induction of EAE resulted in blunting of disease (Zhu et al. 2005). Thus, GAL-9 engagement of TIM-3 serves to negatively regulate Th1 immunity.

Interestingly, GAL-9 expression is itself up-regulated by IFN-γ (Imaizumi et al. 2002, Asakura et al. 2002). This fact introduces a paradigm whereby IFN-γ, responsible for tissue inflammation, also induces an inhibitory ligand – GAL-9 – which then selectively deletes Th1 cells and thereby preventing protracted inflammation in the target organ. Since loss of IFN-γ would disrupt the feedback loop, this could be the underlying mechanism that explains why IFN-γ deficient mice develop a more severe form of EAE (Ferber et al. 1996).

Seki et al. showed that exogenous administration of GAL-9 significantly suppressed CIA in a dose-dependent manner (Seki et al. 2008). Interestingly, GAL-9 treatment resulted in a dramatic reduction of IL-17 levels in the joint, which was

mirrored by an increase in the percentage of Tregs. On the other hand, GAL-9-deficient mice became susceptible to CIA, presumably due to a decreased number of Tregs. Furthermore, GAL-9 treatment prevented the differentiation of Th17 cells from naïve T cells, when these were cultured in the presence of TGF-β and IL-6 – cytokines that drive the differentiation of Th17 cells. The same *in vitro* study showed that GAL-9 up-regulated both FOXP3 mRNA expression and Treg differentiation in the presence of TGF-β (Seki et al. 2008). In line with this, further evidence supporting a role for GAL-9 in regulating immune responses has been provided by Sehrawat et al. in the context of experimental allergic conjunctivitis (Sehrawat et al. 2009), and Lv et al. using a mouse model of viral myocarditis (Lv et al. 2011). In both studies, administration of exogenous GAL-9 resulted in disease recovery, not only by promoting apoptosis of $CD4^{pos}$ effector cells, but also by inducing $FOXP3^{pos}$ Treg conversion and limiting Th17 generation *in vitro*.

In the human setting, a role for GAL-9 in the differentiation of human $FOXP3^{pos}$ Tregs was provided later in patients with HCV infection. In this study, GAL-9 treatment consistently resulted in the expansion of Tregs, contraction of effector CD4 T cells, and apoptosis of HCV-specific CTLs (Mengshol et al. 2010).

Human GAL-9 was first cloned from tissue of a patient with Hodgkin´s disease (Tureci et al. 1997) and was found to be widely distributed (Wada and Kanwar 1997). Indeed, GAL-9 can be potentially expressed on almost all cells.

Within the immune system, GAL-9 is predominantly expressed on naïve $CD4^{pos}$ effector T cells and Tregs. Upon T cell activation, GAL-9 is down-regulated on the effector compartment, but importantly maintained on Tregs (Sabatos et al. 2003, Sanchez-Fueyo et al. 2003).

In this thesis I aim to explore whether GAL-9 expression by Tregs relates to their suppressive function and whether Treg impairment in AIH results from a defective GAL-9 expression.

5.3. Effector cell resistance to Treg suppression

A third mechanism that may contribute to an impaired immune-regulation relates to the susceptibility of effector T cells to Treg control (Walker 2009). Resistance of effector T cells to Treg action has been observed in several animal models of autoimmunity, in which inflammation and tissue destruction progress despite the presence of functional Tregs at the site of inflammation (Gregori et al. 2003, Monk et al. 2005, Korn et al. 2007b, D'Alise et al. 2008). Importantly, the same trend is now emerging from human studies, especially from those in the context of SLE (Venigalla et al. 2008, Vargas-Rojas et al. 2008). Amongst the possible ways by which effector T cells may become resistant to suppression one may depend on TIM-3. TIM-3 is a member of the TIM family of proteins and has been recently linked with effector cell susceptibility to Treg control.

5.3.1. TIM family of proteins

In 2001, the *Tim* family of genes was cloned from within the *Tapr* (the T cell and airway phenotype regulator) locus as a novel allergy and asthma susceptibility gene (McIntire et al. 2001). Since then, there has been increasing evidence indicating that this family play a crucial role in the regulation of immune responses, not only in allergy and asthma, but also in the response to viral infections, transplant tolerance, and autoimmunity (Su et al. 2008, Rodriguez-Manzanet et al. 2009). The family consists of

eight members (*Tim-1* to *Tim-8*) on mouse chromosome 11B1.1, and three ortholog members (*TIM-1*, *TIM-3*, and *TIM-4*) on human chromosome 5q33.2, a region that has been repeatedly associated with asthma, allergy and autoimmunity (Marsh et al. 1994, Encinas and Kuchroo 2000, McIntire et al. 2004). Each of these genes encode a type 1 membrane protein with similar structure, consisting of an N-terminal Ig-like domain, a mucin-like domain, a single transmembrane domain, and an intracellular tail (Meyers et al. 2005b). While the intracellular tails of TIM-1 and TIM-3 contain tyrosine phosphorylation motifs that are involved in transmembrane signalling, TIM-4 has a short intracellular tail devoid of tyrosines (Kuchroo et al. 2003; Figure 2.15.).

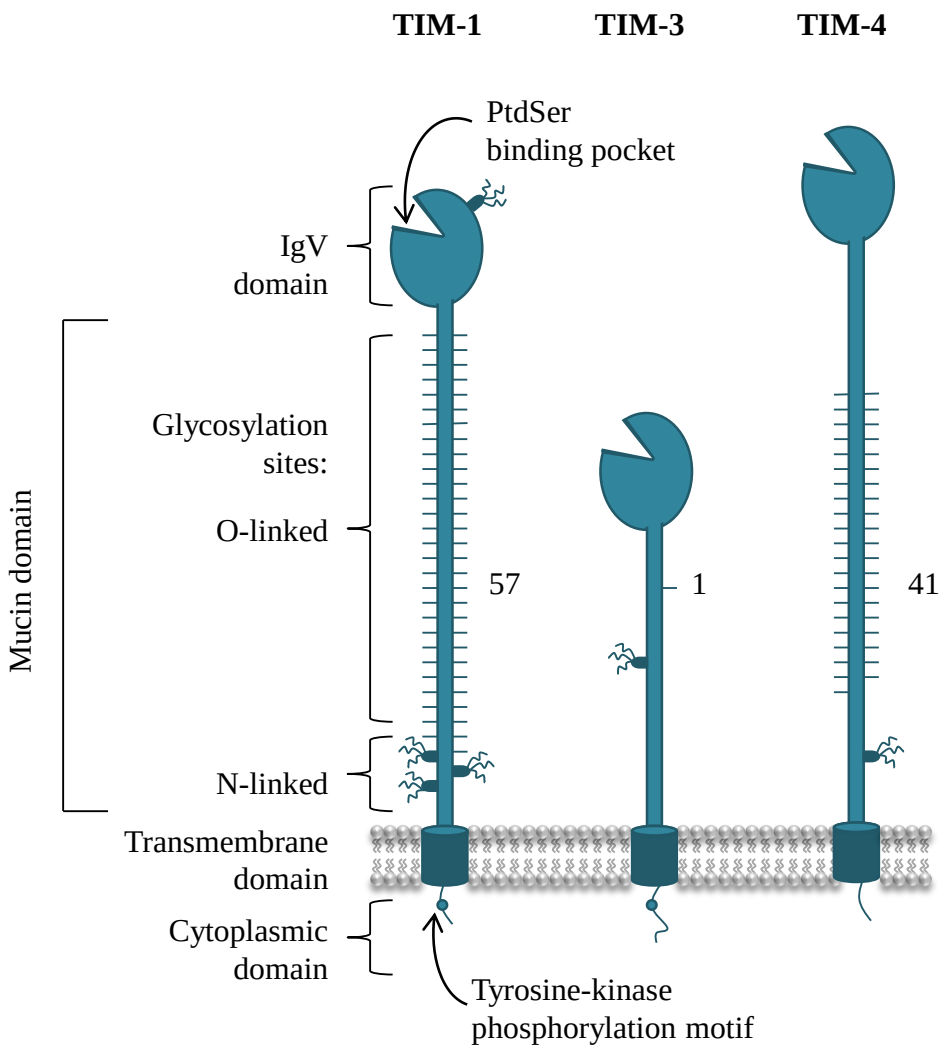


Figure 2.15. Schematic representation of human TIM domain protein structures.

These molecules are involved in several important immunological functions, including T cell activation, induction of T cell apoptosis and T cell tolerance, and the clearance of apoptotic cells (Freeman et al. 2010). Although this thesis focuses on TIM-3, a brief overview of the other TIM family members expressed in humans is given in the next section.

5.3.2. TIM-1

TIM-1 was first identified in monkeys (G. Kaplan et al. 1996) and then in humans (Feigelstock et al. 1998) as the hepatitis A virus cellular receptor (HAVCR), the receptor exploited by hepatitis A for viral entry. Later, TIM-1 was identified as a cell surface protein highly expressed on proximal tubular epithelial cells after acute kidney injury – and therefore termed kidney injury molecule-1 (KIM-1; Ichimura et al. 1998). TIM-1 is also up-regulated in urine and tissue samples of patients with renal cell carcinoma (Vila et al. 2004).

Within the immune system, TIM-1 is expressed on activated but not naïve $CD4^{pos}$ T cells (Umetsu et al. 2005). Upon CD4 T cell differentiation, it is preferentially expressed on Th2 cells, since Th1 and Th17 cells express little or no TIM-1 (Meyers et al. 2005a, Nakae et al. 2007b). It is now clear that TIM-1 is also expressed by other immune cells, namely mast cells (Nakae et al. 2007a), invariant NKT cells (Khademi et al. 2004), and a subpopulation of B cells (Sizing et al. 2007).

Several ligands for TIM-1 have been identified including TIM-4 (Meyers et al. 2005a), TIM-1 itself (Santiago et al. 2007), IgAλ (Tami et al. 2007), and phosphatidylserine (PtdSer; Kobayashi et al. 2007).

Given the association of *TIM-1* polymorphisms with allergic disease both in humans and in mouse models (McIntire et al. 2003), a number of studies have sought to elucidate the role of TIM-1 in these processes. *In vitro* treatment with anti-TIM-1 monoclonal antibodies (mAb) provided a potent co-stimulatory signal that increased T cell proliferation and IL-4 cytokine production (Umetsu et al. 2005). *In vivo*, the administration of the mAb along with antigen also resulted in the increase of antigen-specific T cell proliferation and production of IL-4 and IFN-γ (Umetsu et al. 2005). Moreover, agonistic TIM-1 mAb prevented respiratory tolerance, providing further support that TIM-1 co-stimulation activates T cells.

Recently, it has been shown that TIM-1 co-stimulation abrogates allogeneic transplant tolerance by reducing the expression of FOXP3, and thereby preventing Treg development (Degauque et al. 2008). Since the induction of respiratory tolerance to antigen is also associated with generation of antigen-specific Tregs, TIM-1 co-stimulation may prevent tolerance induction not only by enhancing Th cell development, but also by hindering Treg development (Rodriguez-Manzanet et al. 2009).

Interestingly, different TIM-1 mAbs, which recognise distinct epitopes of TIM-1, result in different outcomes. While TIM-1 mAbs recognising exon 4 of the mucin domain exacerbated inflammation and increased production of Th2 cytokines, a mAb reactive with the Ig domain had an anti-inflammatory effect in a mouse model of asthma (Sizing et al. 2007). In addition, alternative outcomes may also derive from different levels of signal strength provided by TIM-1 engagement. In this regard, administration of the agonistic high-affinity TIM-1 mAb during the induction of autoimmunity enhanced pathogenic Th1 and Th17 responses and increased the severity

of EAE, whereas a lower affinity mAb, increased Th2 responses and inhibited the development of EAE. Thus, TIM-1 has been proposed to have both activating and inhibitory effects in immune responses (Xiao et al. 2007). However, the precise mechanisms that regulate the different outcomes of TIM-1 signalling by different mAbs are not yet known (Freeman et al. 2010).

5.3.3. TIM-4

The role of TIM-4, another TIM molecule present in mice and humans, is less clearly defined. In contrast to TIM-1 and TIM-3, TIM-4 is not expressed on T cells but is primarily found on APCs, including DCs, macrophages, and peritoneal B cells (Meyers et al. 2005a, Kobayashi et al. 2007).

Although initially identified as the ligand for TIM-1 (Meyers et al. 2005b), it is now unclear whether direct interaction occurs (Sizing et al. 2007). Both mouse and human TIM-4 are receptors for PtdSer, being capable of binding and engulfing apoptotic bodies (Kobayashi et al. 2007). However, TIM-4 may also have other unidentified ligands.

Current evidence shows that the interaction between TIM-4 and its putative ligand results in promotion of Th2 responses. Several studies suggest that TIM-4 may play a role in maintaining oral tolerance and in the prevention of food allergy. TIM-4 up-regulation on murine intestinal mucosa DCs, following exposure to *Staphylococcus* enterotoxin B, promoted Th2 polarisation and intestinal allergic responses – which were inhibited in mice pre-treated with TIM-4 mAb (P. C. Yang et al. 2007). However, the results of *in vitro* studies, in which TIM-4 Ig fusion proteins have been used, are contradictory. High concentrations of TIM-4 Ig led to an increased T cell proliferation;

on the other hand, lower doses mediated an inhibitory signal (Meyers et al. 2005b). TIM-4 Ig has also been shown to both activate and inhibit naïve T cells, while treatment of pre-activated T cells enhances activation (Mizui et al. 2008). Since in these studies, T cells were stimulated with anti-CD3/anti-CD28 in the absence of APCs, it is difficult to translate such conclusion to an *in vivo* model. This is particularly relevant considering that TIM-4 can act as PtdSer receptor, simply because the absence of APCs eliminates the process of apoptotic body engulfment (Freeman et al. 2010).

Importantly, TIM-4, in contrast to other members of the family, does not contain any tyrosine phosphorylation motifs in its intracellular tail, hence raising the question of whether it can directly mediate transmembrane signalling (Rodriguez-Manzanet et al. 2009).

5.3.4. TIM-3

TIM-3 was originally discovered as a result of generation and screening of a panel of 20.000 mAbs in an attempt to distinguish Th1 and Th2 cells based on cell surface expression profiles (Monney et al. 2002). Expression of TIM-3, a 281 amino acid cell surface protein, was detectable on CD4 and CD8 T cells only after several rounds of antigenic stimulation in the presence of Th1 polarising conditions (ie. IL-12 + anti-IL-4). Up-regulation of TIM-3 *in vivo* also required several rounds of cell division, being closely associated with IFN-γ production. In contrast, TIM-3 was neither expressed by naïve T cells nor Th2 cells (Monney et al. 2002, Sanchez-Fueyo et al. 2003). Later, TIM-3 was also shown to be expressed on murine Th17 cells (Nakae et al. 2007b).

In humans, TIM-3 is expressed on terminally differentiated Th1 cells, on Th17 cells, and on some $CD8^{pos}$ T cells (Hastings et al. 2009). However, TIM-3 expression is not limited to cells of the adaptive immune system. Indeed, subsets of cells belonging to the innate arm also express TIM-3, including human NK cells, monocytes (Khademi et al. 2004), and DCs (Anderson et al. 2007).

In addition to the full-length, cell-surface expressed protein, a splice variant of TIM-3 was found in mice that lacks the mucin and transmembrane domains and is thought to be a soluble form of TIM-3 (Sabatos et al. 2003, Geng et al. 2006). Although it seems to possess the binding specificity of the full-length protein, little is known about how this alternate TIM-3 splice form influences immune responses (Rodriguez-Manzanet et al. 2009).

TIM-3, a negative regulator of T cell responses

Initial studies of the functional role of TIM-3 in the regulation of T cell responses showed that it acts as an inhibitory molecule. Administration of an anti-TIM-3 mAb during induction of EAE worsened disease progression, leading to increased mortality and to an atypical and acute form of the disease, characterised by high numbers of inflammatory foci in the central nervous system (CNS; Monney et al. 2002). Histological examination of the CNS of anti-TIM-3 mAb treated mice showed high numbers of activated macrophages in the demyelinating lesions, concurrent with the expansion of the monocytes population in the peripheral blood (Monney et al. 2002). In addition, polymorphisms in *TIM-3* gene have been associated with the development of RA in humans (Chae et al. 2004), and airway hyper-reactivity in mice (McIntire et al. 2001).

To better understand the mechanisms by which TIM-3 operates *in vivo*, fusion proteins (TIM-3.Ig) containing either the full length or soluble extracellular forms of TIM-3 fused to a human Fc tail were generated (Sabatos et al. 2003, Sanchez-Fueyo et al. 2003). The administration of TIM-3.Ig to ovalbumin-immunised mice resulted in a Th1 cell hyperproliferation similar to that observed following treatment with anti-TIM-3 mAb. Interestingly, administration of TIM-3.Ig also led to the production of massive quantities of Th1 cytokines (IFN-γ and IL-2; Sabatos et al. 2003). Moreover, in NOD mice, administration of either anti-TIM-3 mAb or TIM-3.Ig fusion protein similarly resulted in the acceleration of diabetes onset and progression (Sanchez-Fueyo et al. 2003).

As mentioned above, GAL-9 was later identified as a ligand of TIM-3. GAL-9 triggering of TIM-3 on Th1 cells was shown to induce calcium flux, cell aggregation and cell death *in vitro*.

In contrast to TIM-3.Ig treatment, *in vivo* administration of GAL-9 caused selective loss of IFN-γ-producing cells and led to the amelioration of EAE, providing further support for TIM-3 as a negative regulator of Th1 responses (Zhu et al. 2005).

Collectively, these data demonstrate that the interaction between TIM-3 and GAL-9 serves as a mechanism to dampen Th1 immunity by selective deletion of TIM-3^{pos} Th1 cells. Thus, the TIM-3/GAL-9 pathway may have evolved to control population expansion of Th1 cells in the immune compartment and to prevent prolonged inflammation in target tissues (Zhu et al. 2005, Anderson and Anderson 2006; Figure 2.16.).

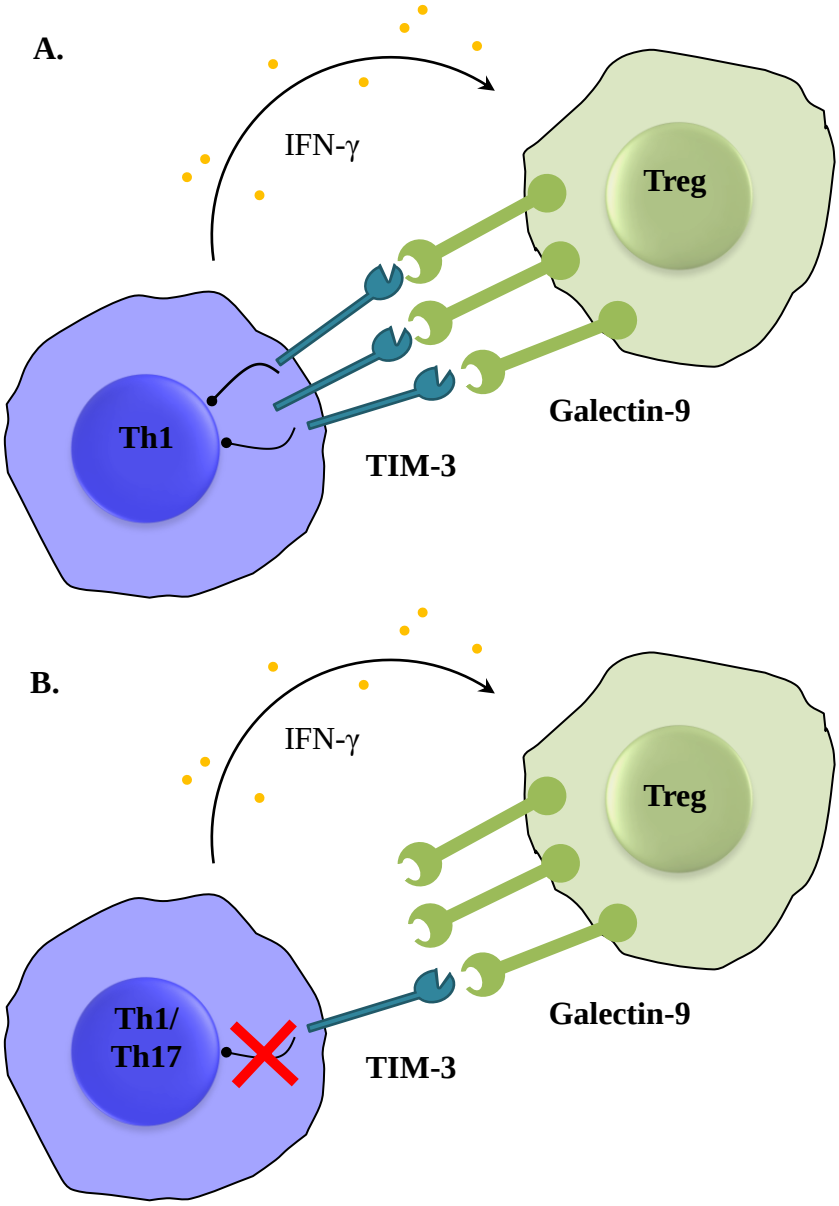


Figure 2.16. TIM-3-mediated immune-regulation. Th1 cells produce IFN-γ, which up-regulates galectin-9 and consequently triggers cell death in T cell immunoglobulin and mucin domain 3 (TIM-3)-positive cells, leading to termination of Th1 responses at tissues sites (*A*). In disease states, low levels of TIM-3 may allow Th1 cells to escape galectin-9 induced cell death. Similarly, Th17 cells, reported to express lower levels of TIM-3, may be less susceptible to galectin-9-mediated suppression (*B*).

TIM-3 and T cell exhaustion

Virus-specific T cells in human immunodeficiency virus (HIV), hepatitis B virus (HBV) and HCV chronic infections progressively develop a range of functional impairments in cytokine production, cytotoxic activity, and proliferative capacity – a

phenomenon termed “exhaustion” (Klenerman and Hill 2005, Freeman et al. 2006). Exhaustion of $CD4^{pos}$ and $CD8^{pos}$ T cells had initially been associated with sustained expression of the inhibitory molecule PD-1, which results in the inhibition of T cell proliferation and cytokine production. Blockade of the PD-1/PDL-1 pathway resulted in a partial restoration of T cell function *in vitro* (Day et al. 2006, Urbani et al. 2006, Wherry et al. 2007). However, since not all exhausted cells expressed PD-1, and hence were not rescued by blockade of the PD-1/PDL-1 pathway (D'Souza et al. 2007), it was likely that other molecules contributed to the exhaustion associated with chronic infections (Hafler and Kuchroo 2008). In this regard, relatively recent studies have implicated TIM-3 in mediating T cell exhaustion during the course of chronic viral infections.

Jones et al. reported the up-regulation of TIM-3 on both $CD4^{pos}$ and $CD8^{pos}$ T cells from individuals with acute and progressive chronic HIV infection, but not in chronically infected individuals who had controlled the infection or in healthy subjects. Interestingly, TIM-3-expressing cells did not overlap with the $PD\text{-}1^{pos}$ population, suggesting that TIM-3 and PD-1 expression mark distinct subsets of exhausted T cells. The frequency of $TIM\text{-}3^{pos}$ cells correlated positively with viral load and inversely with $CD4^{pos}$ T cell counts. HIV-specific T cells expressing high levels of TIM-3 proliferated less and produced reduced levels of cytokines compared to their $TIM\text{-}3^{neg}$ counterparts. Lastly, the demonstration that the blockade of TIM-3 pathway with a mAb or TIM-3.Ig enhances virus-specific T cell responses *ex vivo* clearly shows that TIM-3 plays a critical role in suppressing the overall T cell response to HIV (Jones et al. 2008).

In the context of HCV chronic infection, it has been shown that TIM-3 expression is also increased on $CD4^{pos}$ and $CD8^{pos}$ T cells. However, in contrast to HIV,

expression of TIM-3 by T cells highly overlapped with that of PD-1. The proportion of dually PD-1/TIM-3 expressing cells was greatest in liver-resident T cells, and significantly more in HCV-specific than in cytomegalovirus-specific T cells used as controls. TIM-3-expressing cells were characterised by a senescent phenotype, a central rather than an effector memory profile, and lower production of IFN-γ and TNF-α compared to the TIM-3^{neg} population. Treatment with anti-TIM-3 mAb strongly enhanced T cell proliferation and IFN-γ production and decreased IL-10 production in response to HCV peptide antigen (Golden-Mason et al. 2009).

In an attempt to identify patients at risk of developing viral persistence during and after acute HCV infection, McMahan et al. have analysed the impact of TIM-3 and PD-1 co-expression on T cells in the outcome of patients with acute HCV infection. They have shown that the level of dual TIM-3 and PD-1 expression on HCV-specific $CD8^{pos}$ cells predated the development of viral persistence, providing greater prognostic information than single expression and viral load. Higher expression of these inhibitory molecules correlated with impaired IFN-γ production and reduced cytotoxic activity. Importantly, while blockade of either TIM-3 or PD-1 pathways enhanced HCV-specific $CD8^{pos}$ T cell proliferation to a similar extent, hepatocyte-directed cytotoxicity was increased predominantly after TIM-3 blockade, leading the authors to speculate that TIM-3 and PD-1 may be associated with distinct steps that mediate functional T cell exhaustion (McMahan et al. 2010).

TIM-3 and tolerance

The TIM-3/GAL-9 pathway is also suggested to play an important role in establishing tolerance. Long-lasting tolerance to allogeneic grafts can be achieved by

donor-specific transfusion (DST) and CD154 (CD40L) mAb treatment, an effect mediated by enhanced donor-specific Treg suppression. However, blocking TIM-3 mediated immune-regulation by the concurrent administration of TIM-3.Ig compromised the function of donor-specific Tregs, and prevented the induction of tolerance (Sanchez-Fueyo et al. 2003). Similarly, TIM-3 deficient mice were also refractory to tolerance induction with DST and anti-CD154 mAb. Indeed, both TIM-3.Ig treated and Tim-3 deficient mice exhibited increased T cell proliferation, and production of IL-2 after administration of high-dose aqueous antigen compared to controls (Sabatos et al. 2003). The exact mechanisms by which TIM-3 mediates tolerance induction remain to be elucidated.

TIM-3 and autoimmunity

The role of TIM-3 in the modulation of human autoimmune disease has been most thoroughly studied in MS. $CD4^{pos}$ T cell clones derived from the CSF of MS patients expressed lower levels of TIM-3, despite producing higher quantities of IFN-γ than those from the CSF of control subjects. *In vitro* Th1 polarisation significantly enhanced IFN-γ secretion but not TIM-3 expression among clones from patients relative to those from controls. Treatment of normal $CD4^{pos}$ T cells or Th1 cells with TIM-3 specific siRNA reduced TIM-3 expression and resulted in increased proliferation and IFN-γ secretion (Koguchi et al. 2006). These data are in agreement with the observations mentioned in the above section that TIM-3 up-regulation on exhausted T cells in chronic viral infections leads to less T cell activation (Sakuishi et al. 2011). Collectively, these observations suggest that dysregulated expression of TIM-3 in MS may render effector cells refractory to inhibition, thereby causing immunopathology.

Another study performed in patients with MS aimed at assessing TIM-3 expression and function on *ex vivo* $CD4^{pos}$ T cells isolated from the periphery. CD4 cells from control subjects expressed higher levels of TIM-3 mRNA compared to untreated MS patients. The authors also demonstrated that during T cell stimulation, the presence of anti-TIM-3 mAb enhanced IFN-γ production in healthy subjects, but not in untreated patients, demonstrating a defect in TIM-3 immune-regulation. Interestingly, treatment with glatiramer acetate or IFN-β – effective treatments in reducing the frequency of relapses in MS patients – increased TIM-3 mRNA and reversed the functional defect, providing a potential mechanistic explanation for the lack of immune-regulation associated with self-reactive T cells in MS (Li Yang et al. 2008).

To date, it remains unclear whether Th1 cells in MS are somehow selected for lower TIM-3 expression or whether there is a genetic deficiency in up-regulation of TIM-3 (Anderson and Anderson 2006, Li Yang et al. 2008).

However, regardless of the mechanism, lower TIM-3 expression on Th1 cells prevent the resolution of tissue inflammation, possibly contributing to chronic disease states. Moreover, IL-17-producing Th17 cells have been demonstrated in murine models to be highly pathogenic, and recently these cells have been shown to express TIM-3 (Nakae et al. 2007b), raising the possibility that TIM-3 may play a role in the regulation of this subset (Figure 2.13.).

In this thesis I will explore whether altered TIM-3 expression enables pathogenic T cells to avoid Treg control in patients with AIH.

6. Hypotheses and aims

The main aim of this thesis is to investigate possible causes of functional Treg impairment in patients with autoimmune liver disease. I will explore whether Treg reduced ability to suppress results from a primary Treg defect or by a low susceptibility of responder cells to Treg control.

Given the role played by Tregs in the maintenance of immune-tolerance , I plan to determine the frequency of $CD4^{pos}CD25^{high}$ Tregs expressing low levels of the activation molecule CD127 ($CD127^{low}$ Tregs), and to investigate to what extent absence or low levels of CD127 impact on Treg ability to suppress.

Because of the pivotal role played by the interaction between GAL-9 and TIM-3 in controlling effector immune responses while maintaining tolerance, I plan to evaluate the GAL-9 expression in Tregs and to assess whether this expression is associated to Treg ability to suppress. I will then determine the expression of TIM-3 by responder cells and evaluate whether this expression relates to the responsiveness of effector cells to Treg control.

The following hypotheses will be tested (Figure 2.17):

Hypothesis 1: $CD4^{pos}CD25^{pos}CD127^{neg}$ Tregs are numerically reduced in patients with autoimmune liver disease.

Hypothesis 2: Reduced GAL-9 expression leads to defective Treg function and to suboptimal control of effector immune responses.

Hypothesis 3: impaired TIM-3 expression by effectors results in low susceptibility to Treg control.

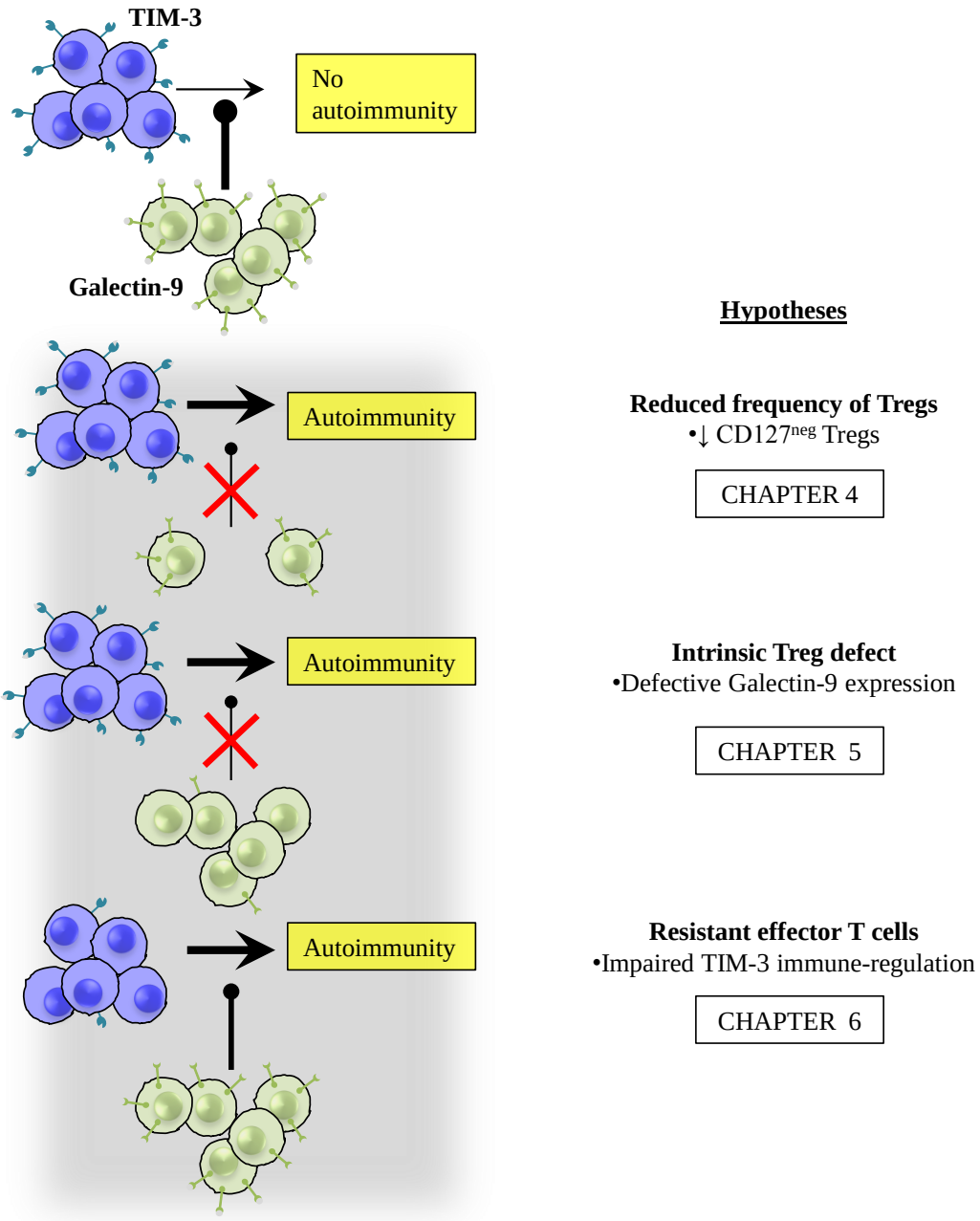


Figure 2.17. Hypotheses of the present study. Schematic representation of the three hypotheses that will be tested in this thesis. Impaired Treg suppression could derive from: 1) low numbers of regulatory T cells, particularly those characterised by low or absent expression of CD127 (see Chapter 4); 2) intrinsic defects in Treg cell function – i.e. defective Gal-9 expression (see Chapter 5); 3) low responsiveness of effector cells to Treg due to low TIM-3 expression (see Chapter 6).

CHAPTER III

Methods

1. Preparation of peripheral blood mononuclear cells (PBMCs)

PBMCs were isolated from heparinised blood by standard density gradient centrifugation. Ten to 20 mL of peripheral blood was venesected from each patient and healthy subject and mixed in a sterile syringe containing preservative free heparin at a final concentration of 10 U/mL. Aliquots of heparinised blood were subsequently layered on the top of an equal volume of Lymphoprep^{TM} (Ficoll) density gradient solution (Amersham Pharmacia Biotech, Ltd., Little Chalfont, United Kingdom) in a 50 mL sterile tube ensuring that a distinct interface between the two layers was maintained. The blood was then centrifuged at 500×*g* for 21 minutes at 20 ºC without brake, to ensure separation of the PBMCs from the denser Ficoll/erythrocyte layer below, and the less dense dilute plasma layer above. After centrifugation, the mononuclear cell layer was collected with a sterile pipette, transferred into a fresh tube and washed twice with Roswell Park Memorial Institute 1640 (RPMI 1640) medium (Invitrogen Life Technologies, Paisley, United Kingdom) at 300×*g* for 5 minutes with brake applied. After the second wash, PBMCs were resuspended in RPMI 1640 medium, supplemented with 2 mM L-glutamine, 25 mM HEPES, 100 U/mL benzyl penicillin, 0.1 mg/mL streptomycin, and 2.5 μg/mL amphotericin B with heat inactivated foetal calf serum (FCS, hereafter denoted "supplemented RPMI 1640").

Cells were counted using a haemocytometer and lymphocyte viability was determined by Trypan-blue exclusion. This method is based on the principle that live (viable) cells will not take up the dye, whereas dead (non-viable) cells will (Hoskins et al. 1956). Equal volumes of 0.4% Trypan-blue solution and lymphocyte suspension were mixed, then put on a Neubauer haemocytometer and examined under a light microscope. Cell viability was calculated as follows: % of viable cells = (number of

viable [non-stained] cells / number of total cells) x 100. The viability of freshly isolated cells always exceeded 98%.

2. PBMCs cryopreservation

After isolation from peripheral blood, PBMCs that were not used immediately were frozen in a solution containing 90% FCS and 10% dymethyl sulphoxide (DMSO, Sigma Aldrich Ltd., Gillingham, Dorset, United Kingdom) – a cryoprotectant that reduces the amount of ice present during freezing, thus reducing ionic stress (Pegg 2007).

Briefly, the cell suspension obtained after PBMCs isolation was centrifuged at 300×*g* for 5 minutes. The pellet obtained was then resuspended in 1 mL of cryoprotective solution per 5-10x10^6 cells prior to being transferred into sterile cryotubes. Immediately after the cryotubes were placed in pre-warmed Nalgene™ cryocontainer (Nalgene, Nalge Europe Ltd., Hereford, United Kingdom) that had been filled with 70% isopropanol according to the manufacturer's instructions to freeze to -70 ºC at a cooling rate of 1 ºC per minute. After 24 hours, vials were transferred into a -140 ºC freezer.

3. Thawing of cryopreserved PBMCs

If not properly thawed, lymphocytes´ viability and recovery may be affected and their function compromised. In general, cells should be thawed quickly but diluted slowly to remove DMSO. Cells with DMSO intercalated into their membranes are very fragile, and must be pelleted and handled gently (Pegg 2007).

Cryopreserved cells were thawed quickly (< 1 minute) by immersion and gentle shaking of the cryotubes in a 37 ºC water bath. Warm RPMI 1640 was then dropwise added into the cryotube until a final volume that was twice that of the cell suspension. The diluted cell suspension was transferred to 50 mL sterile tube containing 8 mL of warm RPMI 1640 for every cryotube of cells added. Cells were pelleted by centrifugation at 300×g for 7 minutes. After discarding the supernatant and gently flicking the tube to break up the pellet, 10 mL of RPMI 1640 were added for a second wash and centrifugation was carried out as above. After this second wash, cells were resupended in 1 mL of supplemented RPMI 1640 and cell number and viability was determined by Trypan-blue exclusion.

Although it has been suggested that cryopreservation of PBMCs may result in attenuation of their functionality (Tree et al. 2004, Axelsson et al. 2008), preliminary experiments where cell preparations from the same patients were tested before and after cryopreservation showed no significant difference in viability and behaviour in culture when assessed for proliferation and cytokine production in the case of effectors and ability to suppress in the case of regulatory T cells.

4. Flow cytometry

Flow cytometry is a technique that permits measurement and analyses of optical and fluorescence characteristics of particles of interest (e.g. cells), termed “events”, as they flow in a fluid stream through a beam of light (Brown and Wittwer 2000). These characteristics are determined using a flow cytometer – an optical-to-electronic coupling system – which records how the cell scatters an incident laser light and emits fluorescence (Virgo and Gibbs 2012). It is composed of three main systems: fluidics,

optics, and electronics. The fluidics system transports particles in a stream to the laser beam for interrogation. Then, the optics system, which consists of lasers and optical filters, illuminates the particles in the sample stream and directs the resulting light signals to the appropriate detectors (Watson 1999). Lastly, the electronics system converts the detected light signals into electronic signals that can be processed by the computer (Recktenwald 1993). Additionally, some instruments can be equipped with a cell-sorting device (Herzenberg et al. 2002); in these, the electronics system is also capable of performing sorting decisions by charging and deflecting particles (see section 6.2.2.).

When the cells pass through the laser beam, they scatter its light. Two scatter parameters are measured: forward scatter (FSC) and side scatter (SSC). FSC is proportional to the cell size; thus, lymphocytes have smaller FCS than monocytes and granulocytes. On the other hand, SSC, which is measured at an angle of 90º to the FSC, relates to the cells´ granularity and nuclear complexity/lobularity. For this reason, monocytes and granulocytes have a higher SSC than lymphocytes (Figure 3.2.). Additionally, antibodies conjugated to fluorescent dyes can bind specific proteins on cell membranes or inside cells. Once the cells pass through the light source, the fluorescent molecules are excited to reach a higher energy state. Upon returning to their resting states, the fluorochromes emit light energy at higher wavelengths. The use of multiple fluorochromes, each with similar excitation wavelengths but different emission wavelengths (or “colours”), allows several individual cell markers to be detected simultaneously (De Rosa et al. 2003, Perfetto et al. 2004).

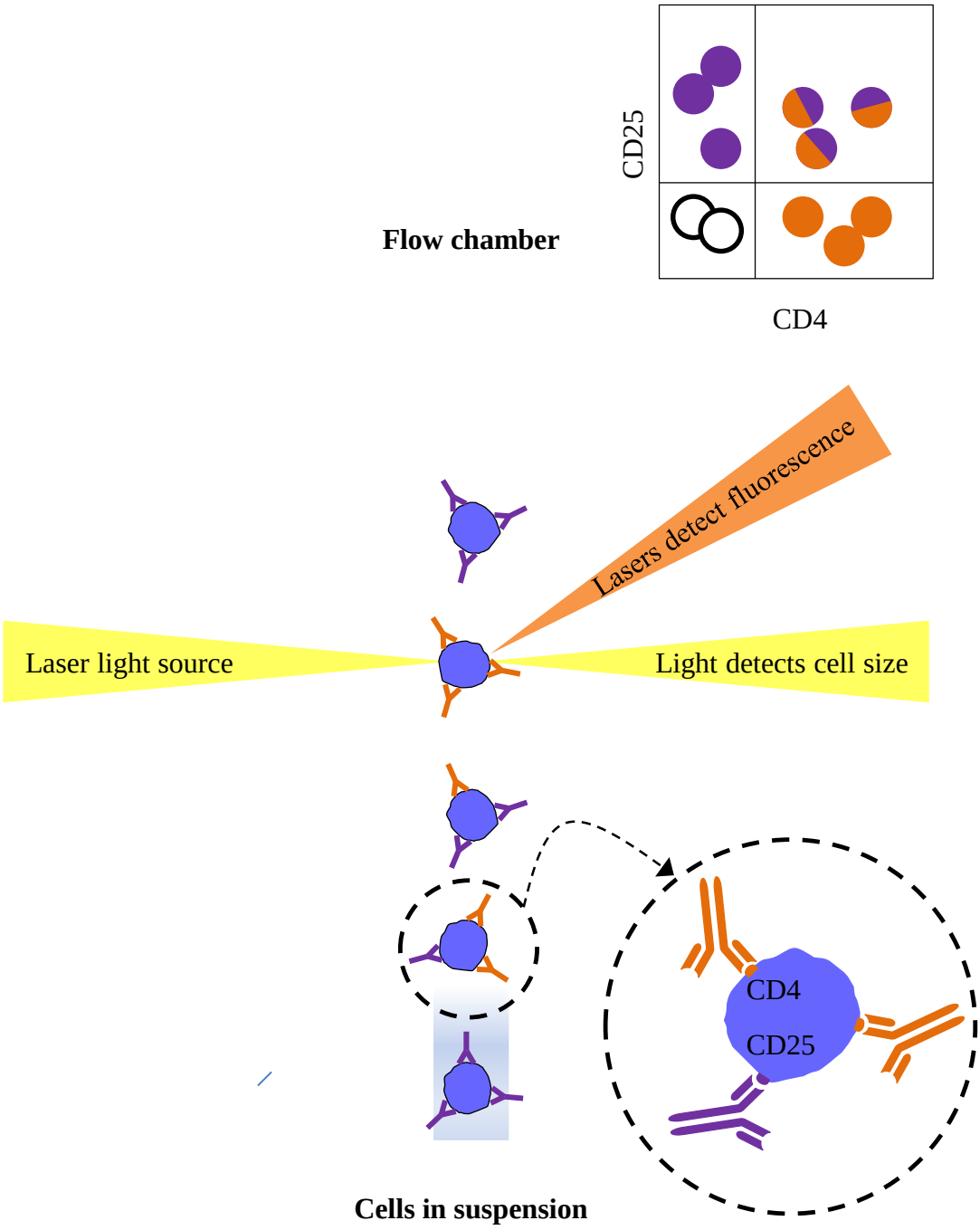


Figure 3.1. How the flow cytometer works. Fluorochrome-labelled monoclonal antibodies are added to a cell suspension. The labelled cell suspensions are passed through the flow cell of a flow cytometer. Each cell passes through the laser beam of the flow cytometer, a process termed single cell analysis. The fluorochrome of each labelled monoclonal antibody bound to the cell is excited by the laser light and emits fluorescence of a certain wavelength. The cells also scatter light at multiple angles. Photodetectors placed at forward angle and at right angles to the axis of the laser beam collect the emitted or scattered light. The signals from each photodiode are digitised and passed to a computer for storage, display, and analysis. A variety of histograms for visual display can be generated automatically or at the discretion of the operator.

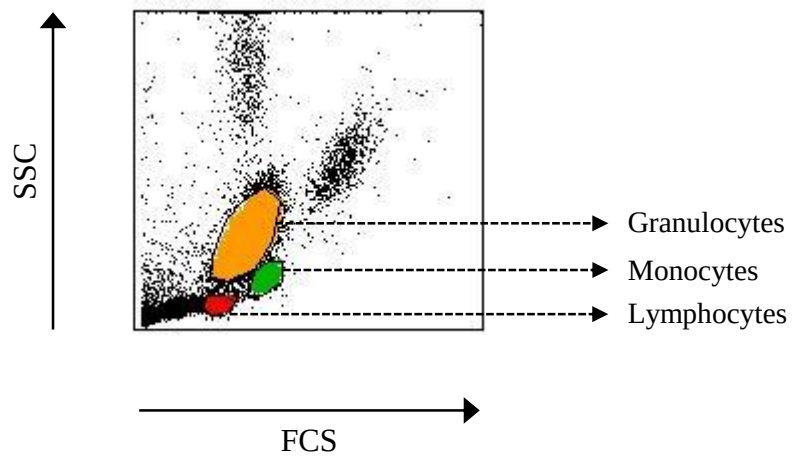


Figure 3.2. Analysis of lysed whole blood. Forward (FCS) and side scatter (SSC) characteristics allow the gating of three different populations: granulocytes (*yellow*), monocytes (*green*), and lymphocytes (*red*).

In the current study, flow cytometry was performed on a two lasers Becton-Dickinson (BD) FACSCanto™ II. BD FACSDiva™ software was used for analysis.

4.1. Surface antigens

Multi-colour flow cytometry was performed on fresh and/or frozen PBMCs, gated using unstained cells in order to exclude dead cells, granulocytes and monocytes. 3.5×10^5 unfractioned PBMCs were used for each experiment. Cells were resuspended in 200 μL of 1× phosphate buffered saline (PBS) containing 1% FCS (PBS/1% FCS). Cells were then stained with different combinations of fluorochrome-conjugated monoclonal antibodies (mAbs), including anti-CD4, anti-CD25, anti-CD127, anti-CD45RO, anti-CD45RA, anti-CCR7, and anti-TIM-3 (Table 3.2.). Cells were incubated at 4ºC in the dark for 30 minutes. Following incubation, PBMCs were washed once with 400 μL PBS/1% FCS at 300×*g* for 5 minutes, then resuspended in 500 μL, and stored at 4ºC until analysis.

Table 3.1. Antibodies used for flow cytometry.

Specificity	Fluorchrome	Clone	Volume (μL/test)	Company
CD4	APC-Cy7	RPA-T4	1.5	BD Biosciences[1]
CD25	PE-Cy7, APC, FITC, PE	M-A251	2	BD Biosciences[1]
CD127	PE, FITC	HIL-7R-M21	1	BD Biosciences[1]
TIM-3	PE, APC	344823	4	R&D Systems[2]
CD45RO	PE-Cy7, PE	UCHL1	2	BD Biosciences[1]
CD45RA	FITC	OX-33	2	BD Biosciences[1]
CCR7	PE-Cy7	3D12	2	BD Biosciences[1]
Galectin-9	N/A	ECA8	1.2	MBL[3]
IgG1	PE	A85-1	0.5	BD Biosciences[1]
CTLA-4	APC	BNI3	5	BD Biosciences[1]
FoxP3	APC, FITC, PE	PCH101	5	eBioscience[4]
T-bet	PerCP-Cy5.5, PE	4B10	5	eBioscience[4]
GATA-3	PE-Cy7	L50-823	5	BD Biosciences[1]
RORC	APC, PE	AFKJS-9	0.8	eBioscience[4]
IL-10	APC, PE	JES5-16E3	5	BD Biosciences[1]
TGF-β1	PerCP	27232	5	R&D Systems[2]
IFN-γ	APC, PE	45-15	3	IQ Products[5]
IL-2	APC	MQ1-17H12	0.8	BD Biosciences[1]
IL-17	FITC, PE	64DEC17	5	eBioscience[4]

3.5×10^5 cells were used per test
APC, allophycocyanin; *Cy7*, Cychrome 7; *PE*, phycoerythrin; *FITC*, flourescein isothiocyanate; *PerCP*, peridinin-chlorophyll-protein complex; *Cy5.5*, cychrome 5.5
[1] Oxford, UK; [2] Abingdon, UK; [3] Nagoya, Japan; [4] Hatfield, UK; [5] Groningen, The Netherlands

4.2. Intracellular staining

The percentage of cells positive for FOXP3, Tbet, GATA-3, RORC (transcription factors of Tregs, Th1, Th2 and Th17 cells respectively), and CTLA-4 was determined by intracellular staining. PBMCs were first suspended in PBS/1%FCS and stained with mAbs directed against surface markers, as described above. After 30 minutes incubation, cells were washed once, and then incubated at 4ºC for 20 minutes with 100 μL of BD Cytofix/Cytoperm^{TM} (BD Biosciences) – a formaldehyde and

saponin-containing solution that can be used for the simultaneous fixation and permeabilisation of cells, allowing the mAbs to penetrate into the cell and detect the intracellular protein (Tay et al. 1999). After one wash, cells were resuspended in 100 µl BD Perm/Wash^{TM} buffer solution containing a pre-determined optimal concentration of fluorochrome-conjugated anti-FOXP3, anti-Tbet, anti-GATA-3, anti-RORC, and anti-CTLA-4 mAbs. After a further 30 minutes incubation at 4ºC in the dark, cells were washed, resuspended in 500mL PBS/1%FCS, and analysed by flow cytometry.

Since a fluorochrome-conjugated mAb is not available, expression of GAL-9 was determined by an indirect method (Asakura et al. 2002, Saita et al. 2002). Briefly, following fixation/permeabilisation (see above), cells were washed and initially incubated with IgG1 anti-human GAL-9 mAb (MBL, Nagoya, Japan) at 4ºC in the dark. Following 30 minutes incubation, cells were counter-stained with PE-conjugated anti-IgG1 secondary antibody and incubated for further 30 minutes. Cells were finally washed and analysed by flow cytometry.

For intracellular cytokine staining (ICCS), PBMCs were seeded in 96-round bottom plates at 3.5×10^5 cells/well, and cultured in supplemented RPMI 1640. Phorbol 12-mystrate 13-acetate (PMA) (10 ng/mL) and ionomycin (500 ng/mL) were added to the culture to induce cytokine production by T cells (Pala et al. 2000). After incubation at 37 ºC and 5% CO_2 for 1 hour, Brefeldin A was added at a final concentration of 20 µg/mL, in order to prevent the transport of cytokines from the endoplasmic reticulum to the Golgi apparatus and hence to favour their intracellular accumulation (Dinter and Berger 1998). Following 5-hour incubation, PBMCs were transferred into FACS tubes, washed with 400 µL of PBS/1%FCS at 300×*g* for 5 minutes, then stained with antibodies for surface markers, fixed and permeabilised as described previously.

Finally, cells were counter-stained with fluorochrome-labelled anti-IFN-γ, anti-IL-17, anti-IL-2, anti-TGF-β and anti-IL-10 mAbs (Table 3.1.). Flow cytometry was performed as above.

5. Cell purification

5.1. Regulatory T cell subsets

5.1.1. Magnetic sorting

$CD4^{pos}CD25^{pos}$ (henceforth denoted as "Tregs") cells were isolated from PBMCs using immunomagnetic beads (Dynal Invitrogen, Oslo, Norway). This method includes two sequential steps: the negative selection of total $CD4^{pos}$ cells is followed by the positive selection of $CD25^{pos}$ cells.

In brief, PBMCs were resuspended in 500 µl supplemented RPMI 1640 and 200 µl Antibody Mix Human CD4 per 100×10^6 cells. This antibody mixture contains murine anti-human antibodies specific for CD14, CD16, CD56, CDw123, Glycophorin-A, CD36, CD8, and CD19 marking monocytes, DCs, granulocytes, NK cells, platelets, erythrocytes, $CD8^{pos}$ T cells, and B cells for depletion. Cells were incubated at 4^0C in the dark for 20 minutes, washed and loaded with 1 mL depletion beads coated with an Fc-specific human immunoglobulin G4 antibody (Depletion MyOne Beads) per 100×10^6 cells. Cells were incubated at room temperature for 15 minutes with rolling and tilting. The tube was placed in a magnetic stand for 6 minutes. All non-$CD4^{pos}$ T cells became attached to the wall of the tube as an effect of the magnetic field, thus allowing the transfer of the supernatant containing the bead-free $CD4^{pos}$ T cells to a new tube. The purity of $CD4^{pos}$ T cells, assessed by flow cytometry at this point, exceeded 95% (Figure 3.3.).

Following $CD4^{pos}$ isolation, the cells were resuspended in supplemented RPMI 1640 at 15×10^6 per mL. 200 μL of Dynabeads CD25 – immunomagnetic beads coated with anti-human CD25 antibodies – were added to the solution. After 25 minutes incubation at 4º C, the tube was placed in the magnet and the supernatant containing $CD4^{pos}CD25^{neg}$ T cell fraction (see section 6.2.) was collected. Finally, the residual beads harbouring the $CD25^{pos}$ population were resuspended in 500 μL supplemented RPMI 1640 with DETACHaBEAD (80 mL per 15 x 10^6 cells), which allows cell detachment from the beads. After 45 minutes incubation at room temperature, the tube was placed in the magnet, and the supernatant containing $CD4^{pos}CD25^{pos}$ cell fraction collected and transferred to a fresh tube. The purity of $CD4^{pos}CD25^{pos}$ Tregs was higher than 92% (Figure 3.3.).

$CD4^{pos}CD25^{pos}CD127^{neg}$ cells (hereafter denoted "$CD127^{neg}$ Tregs") were further purified from total $CD^{pos}CD25^{pos}$ Tregs. Because of the large number of cells required, this additional purification step was limited to those subjects from whom sufficient numbers of $CD4^{pos}CD25^{pos}$ T cells were obtained.

In brief, purified $CD4^{pos}CD25^{pos}$ cells were incubated with PE-conjugated anti-CD127 monoclonal antibodies at 4 ºC in the dark for 30 minutes. After washing, cells were incubated with microbeads conjugated to monoclonal anti-PE antibodies (Miltenyi Biotec, Bergisch-Gladbach, Germany) at 4°C for a further 15 minutes. Incubation was followed by magnetic separation using MS columns (Miltenyi Biotec). The flow-through cells were the $CD4^{pos}CD25^{pos}CD127^{neg}$ cells, while those eluted from the column corresponded to the activated $CD4^{pos}CD25^{pos}CD127^{pos}$ lymphocyte fraction. The purity of both subsets, assessed by flow cytometry, exceeded 90% (Figure 3.4.).

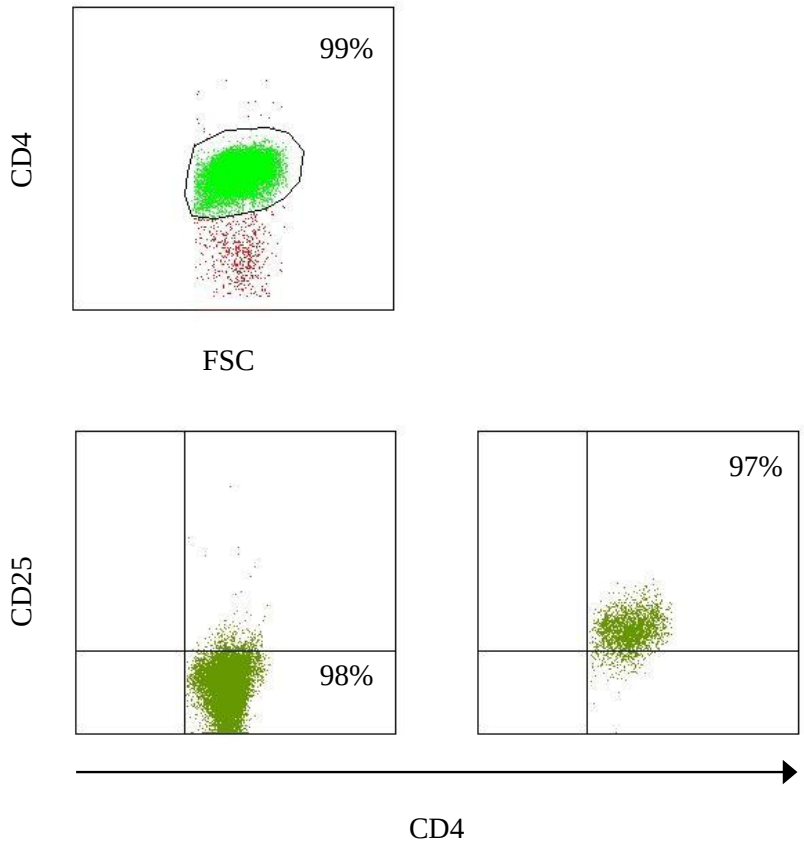


Figure 3.3. Flow cytometric assessment of $CD4^{pos}$, $CD4^{pos}CD25^{neg}$ and $CD4^{pos}CD25^{pos}$ T cell purity. Dot plot of anti-CD4 fluorescence (Y axis) versus FSC (X axis), showing high purity after negative selection of $CD4^{pos}$ cells (*upper panel*). Dot plots of anti-CD4 fluorescence (X axis) versus CD25 fluorescence (Y axis), after negative and positive selection of $CD25^{neg}$ (*bottom left*) and $CD25^{pos}$ (*bottom right*) populations respectively.

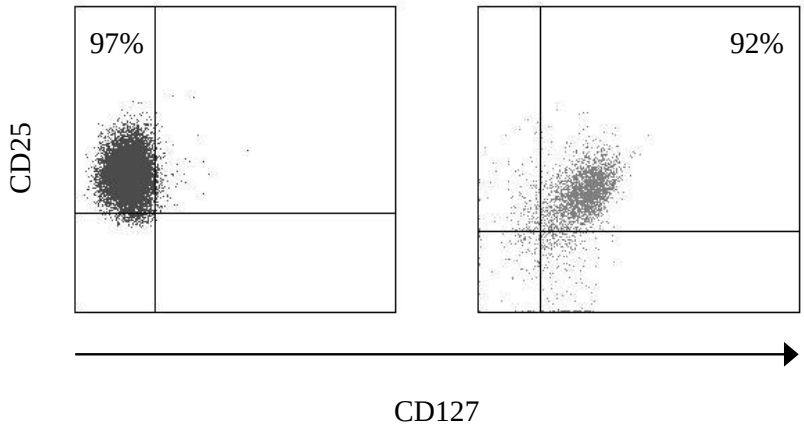


Figure 3.4. Flow cytometric assessment of $CD4^{pos}CD25^{pos}CD127^{neg}$ and $CD4^{pos}CD25^{pos}CD127^{pos}$ T cell purity. Dot plots of anti-CD127 fluorescence (X axis; arbitrary units, log scale) versus CD25 fluorescence (Y axis; arbitrary units, log scale) in a representative autoimmune hepatitis patient. Highly pure $CD25^{pos}CD127^{neg}$ (*left panel*) and $CD25^{pos}CD127^{pos}$ (*right panel*) population are seen in the respective quadrants. The purity of the two subpopulations is indicated as percentage of CD4 cells in the respective quadrant.

5.1.2. FACS sorting

$CD4^{pos}CD25^{pos}$ cells were also sorted into $CD4^{pos}CD25^{high}$ and $CD4^{pos}CD25^{low}$ populations using fluorescence-activated cell sorting (FACS™, Figure 3.5.).

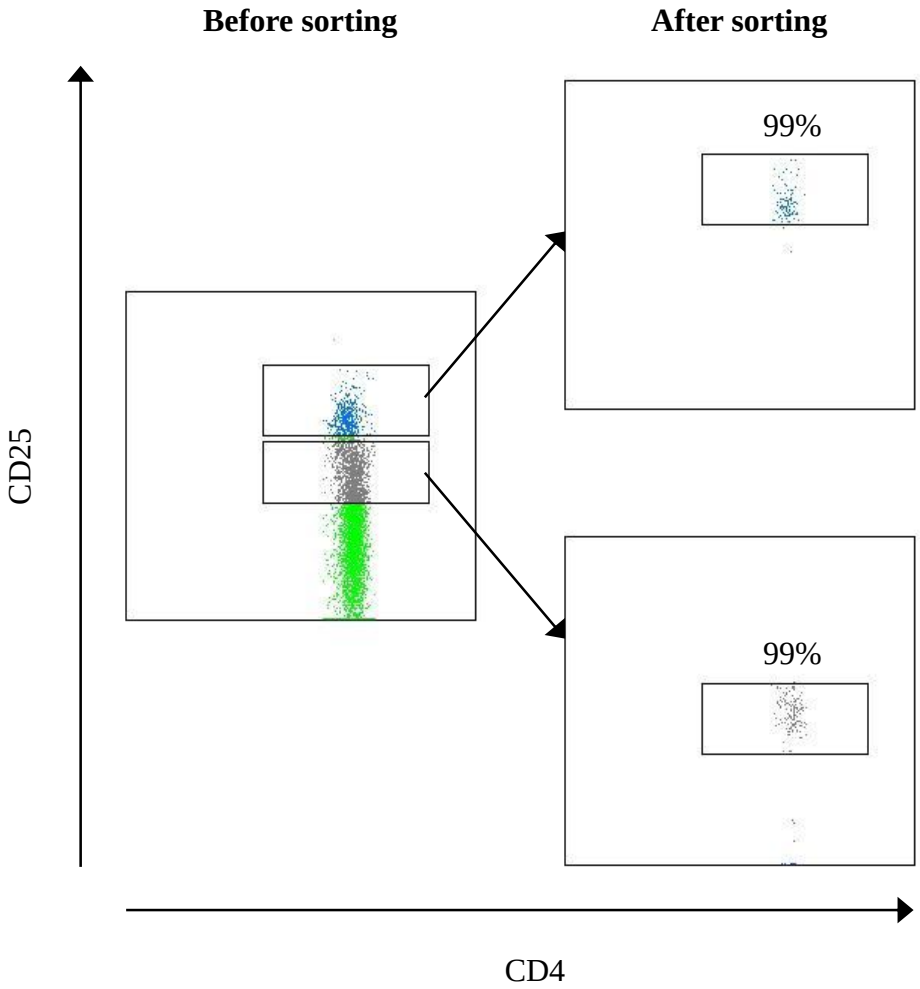


Figure 3.5. Purity of FACS-sorted regulatory T cell populations. Cells were sorted according to CD25 expression using BD FACSAria™. Dot plots showing high purity for both $CD4^{pos}CD25^{high}$ (*right top*) and $CD4^{pos}CD25^{low}$ (*right bottom*) populations after sorting in a representative healthy subject.

PBMCs were washed in PBS/1%FCS and resuspended at a density of 1×10^6 cells/ml prior to staining with 25 µl of anti-human CD25 PE and 15 µl of anti-human CD4 APC-Cy7 per $1x10^6$ cells. Unstained and single stained control populations were used as controls.

Cells were then washed twice with PBS/1%FCS, before filtering through 40 µm cell strainers (BD Biosciences) and resuspension at 8×10^6 cells/ ml in PBS. Ice-cold cells were sorted into $CD25^{high}$ (corresponding to the top 2% of $CD4^{pos}$ cells displaying the brightest CD25 fluorescence) and $CD25^{low}$ (corresponding to the remaining $CD4^{pos}$ cell population displaying low CD25 fluorescence) cells using a FACSAria™ (Becton Dickinson Immunocytochemistry Systems). Sorted lymphocytes were then washed in PBS and resuspended in supplemented RPMI 1640. The purity of both $CD25^{low}$ and $CD25^{high}$ cells exceeded 98% (Figure 3.6.).

5.2. Responder cells

$CD4^{pos}CD25^{neg}$ cells were isolated from PBMCs by two-step negative selection as described in 6.1.1. Their purity exceeded 95% (Figure 3.4.).

$CD4^{pos}CD25^{neg}$ cells were further purified according to the expression of TIM-3. In brief, $CD4^{pos}CD25^{neg}$ cells were incubated with PE-conjugated anti-TIM-3 mAbs for 30 minutes, then with microbeads conjugated to monoclonal anti-PE antibodies for further 15 minutes at 4˚C. $CD4^{pos}CD25^{neg}TIM\text{-}3^{neg}$ and $CD4^{pos}CD25^{neg}TIM\text{-}3^{pos}$ (henceforth "$TIM\text{-}3^{neg}$" and "$TIM\text{-}3^{pos}$") populations were purified by negative and positive selection respectively using MS columns (see section 6.1.2.). Their purity was greater than 90% (Figure 3.6.).

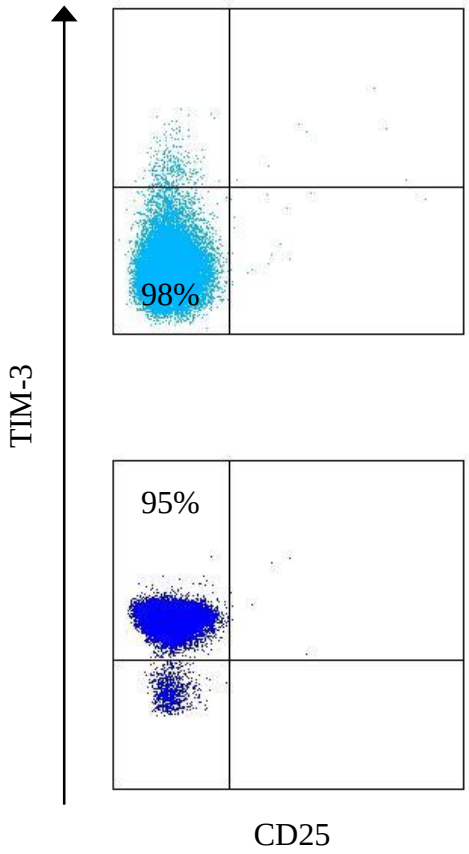


Figure 3.6. Purity of TIM-3^{pos} and TIM-3^{neg} cell subsets. CD4^{pos}CD25^{neg} cells were immunomagnetically-sorted according to the expression of TIM-3. Dot plots of anti-CD25 fluorescence (X axis; arbitrary units, log scale) versus TIM-3 fluorescence (Y axis; arbitrary units, log scale) in a representative autoimmune hepatitis patient. Purified CD25^{neg}TIM-3^{neg} (*top panel*) and CD25^{neg}TIM-3^{pos} (*bottom panel*) populations are seen in the respective quadrants where their percentage of purity is indicated.

6. Proliferation and suppression assays

6.1. Assessment of suppressive function of regulatory T cells

Immunomagnetically-purified CD4^{pos}CD25^{pos}, CD4^{pos}CD25^{pos}CD127^{neg}, CD4^{pos}CD25^{pos}CD127^{pos}, and FACS-sorted CD4^{pos}CD25^{low} and CD4^{pos}CD25^{high} cells were added to autologous CD4^{pos}CD25^{neg} effector cells at a ratio of 1/8. The Treg/effector cell ratio was selected as optimal on the basis of preliminary experiments in which ratios of 1/16, 1/8, and 1/4 were compared: 1/8 ratio was chosen as capable of exerting detectable regulatory function using the minimum number of cells. Cells were

seeded in a round-bottom 96-well plate and cultured in a humidified incubator at 37ºC and 5% CO_2 for 5 days in the presence of anti-CD3/anti-CD28 T cell expander capable of preserving the original T cell function (CD3/CD28 Dynabeads, Dynal Biotech), at a bead/cell ratio of 1/2. Recombinant IL-2 was added at 30 U/mL on day 1. Parallel cultures of $CD4^{pos}CD25^{neg}$ cells cultured on their own were performed under identical conditions and used as controls. All experiments were performed in duplicate. For the last 18 hours the cultures were pulsed with 0.25 μCi/well of ^{3}H-thymidine and harvested using a multi-channel harvester. The amount of incorporated ^{3}H-Thymidine was assessed by a β-counter (Canberra Packard Ltd., Pangbourne, United Kingdom), and expressed as counts per minute (cpm). The inhibition percentage was calculated using the formula: Inhibition (%) = (1 – cpm in the presence of Tregs/ cpm in the absence of Tregs) x 100.

6.2. Effector CD4 T cell proliferation and responsiveness to Treg control

To evaluate the responsiveness of effector cells to regulatory T cell-mediated suppression, $CD4^{pos}CD25^{neg}$, TIM-3^{neg}, and TIM-3^{pos} populations, stimulated with T cell expander and IL-2 (see section 6.1.), were co-cultured with $CD4^{pos}CD25^{pos}$, $CD4^{pos}CD25^{pos}CD127^{neg}$ and $CD4^{pos}CD25^{pos}CD127^{pos}$ cells under the same conditions described in 6.1. Control cultures using $CD4^{pos}CD25^{neg}$, TIM-3^{neg} and TIM-3^{pos} cells cultured on their own were performed under identical conditions. Following 5-day culture, cells were assessed for their proliferative response by ^{3}H-thymidine incorporation (see section 6.1.).

Percentage of target cell inhibition was determined using the formula shown in the previous section.

7. Neutralisation assays

To assess whether the suppressor function of regulatory T cells is related to the release of regulatory and/or effector cytokines, purified mouse monoclonal anti-human-IL-10 (clone 23278), anti-human-TGF-β (clone 9016), anti-human-IFN-γ (clone 25723), and anti-human-IL-17 (clone 41809) neutralising antibodies (all from R&D Systems, Abingdon, UK) were added at a final concentration of 10 μg/mL to purified $CD4^{pos}CD25^{pos}$, $CD4^{pos}CD25^{pos}CD127^{neg}$ and $CD4^{pos}CD25^{pos}CD127^{pos}$ T cell populations. After 12 hours, these T cell populations were added to autologous $CD4^{pos}CD25^{neg}$, TIM-3^{neg} and TIM-3^{pos} cells used as targets.

Similarly, to investigate whether the susceptibility of the responder cells to Treg control is related to the release of cytokines, $CD4^{pos}CD25^{neg}$, TIM-3^{neg} and TIM-3^{pos} cells were exposed to anti-IFN-γ, anti-IL-17, anti-IL-10, and anti-TGF-β neutralising antibodies (10 μg/mL) for 12 hours before $CD4^{pos}CD25^{pos}$, $CD4^{pos}CD25^{pos}CD127^{neg}$ cells were added. Cells were cultured under the same conditions described above. Following a 5-day culture, cells were tested for their proliferative response.

8. *Galectin-9* silencing

Gene silencing is the most effective strategy enabling evaluation of gene function, and it can be evaluated at both transcriptional and post-transcriptional levels. At the post-transcriptional level, small interfering RNAs (siRNAs) are one of the most recent tools that can be deployed to silence gene expression (Dorsett and Tuschl 2004). The RNA interference (RNAi) pathway, which was first described by Andrew Fire and Craig Mello in 1998 who observed that the injection of double-stranded RNA (dsRNA)

into the nematode *Caenorhabditis elegans*, led to a potent sequence-specific degradation of cytoplasmic mRNAs containing the same sequence as the dsRNA trigger (Fire et al. 1998). Following the demonstration that double-stranded siRNAs could trigger RNAi pathway in mammalian cells (Elbashir et al. 2001), siRNA has become a routine tool to study gene function (Corey 2007).

Gene expression can be blocked by either transfecting the siRNA into cells – a process in which the siRNA is complexed with lipids or other molecules to facilitate passage through cell membranes (Janowski et al. 2006) – or by introducing a vector that could express the siRNA within the cells (Tiscornia et al. 2006). Once inside the cell, the siRNA is bound by the proteins of the RNA-induced silencing complex (RISC). The RISC proteins facilitate the binding of the RNA sequences complementary to one of the two strands of the siRNA duplex (Filipowicz 2005). One strand of the siRNA (the sense or passenger strand) is lost from the complex, while the other strand (the antisense or guide strand) is matched with its complementary RNA target (Figure 3.7.). Recognition of mRNA by the antisense strand of the siRNA can cause destruction of the mRNA, therefore preventing its translation into a protein and thereby reducing the level of protein inside cell (Corey 2007).

siRNAs have been used to silence specific genes in different cell types with an impressive suppression ranging from 70 to 100%. In these cases RNAi-dependent gene silencing could be considered a true knock-out technology (Tuschl et al. 1999). However, in *in vivo* assays and cell cultures, it has been shown that siRNAs do not completely eliminate the encoded protein; therefore the effect produced by the siRNA should be considered a “knock-down” rather than a “knock-out” (Sifuentes-Romero et al. 2011).

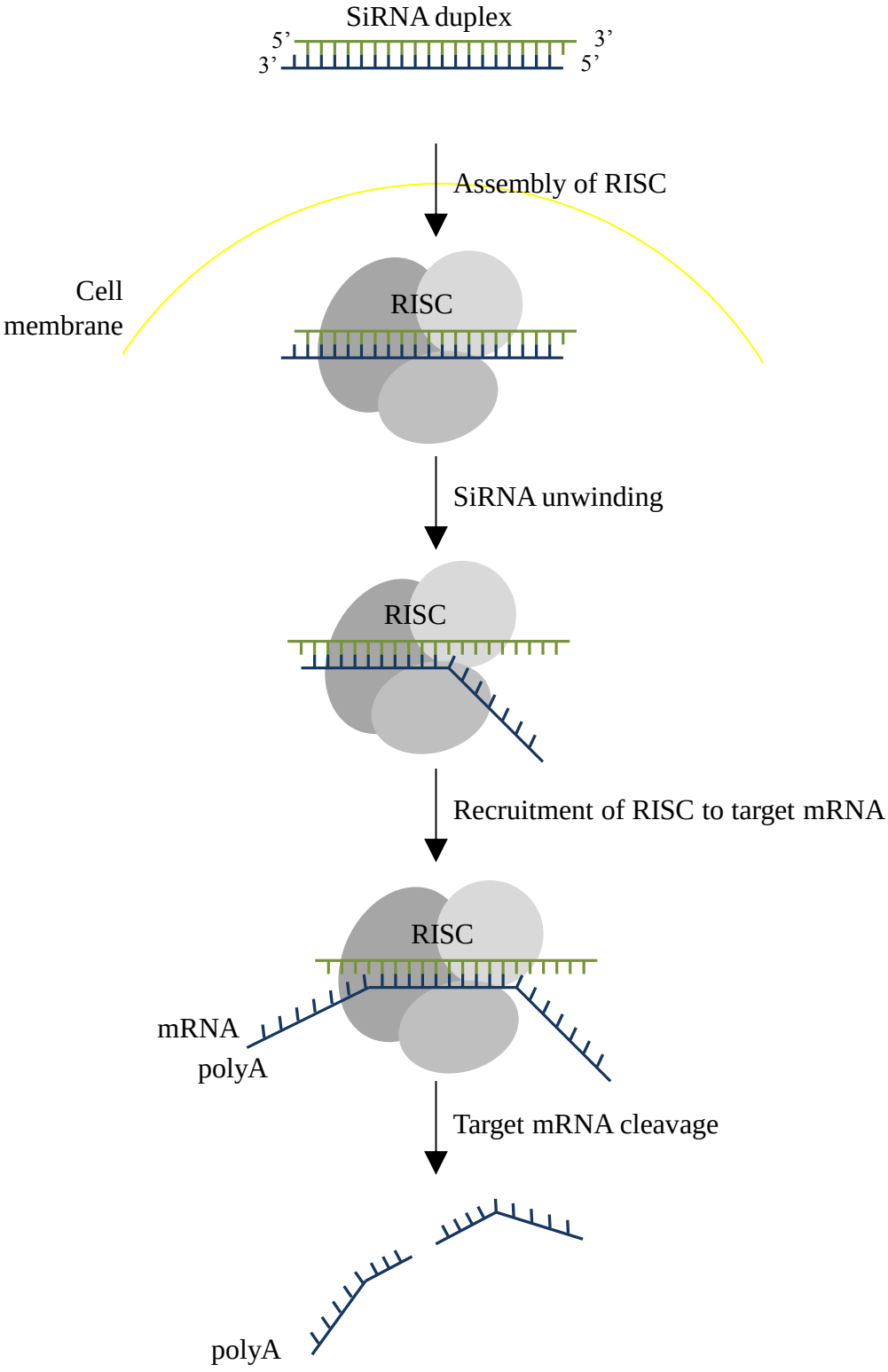


Figure 3.7. How small interfering RNAs work. Small interfering RNAs (siRNAs) enter cells and then bind to the RNA-induced silencing complex (RISC). The RISC proteins unravel the duplex and facilitate the search for mRNA sequences that are complementary to one of the RNA strands. Upon recognition of a complementary mRNA, RISC cleaves the mRNA and prevents translation.

8.1. Transfection procedure

GAL-9-specific siRNA (Invitrogen Life Technologies) used in this study contained three different sequences targeting all isoforms. A GAPDH Stealth RNAi and a "negative control Stealth RNAi" (Invitrogen Life Technologies) served as positive and negative controls for transfection.

Immunomagnetically purified $CD4^{pos}CD25^{pos}$, $CD25^{pos}CD25^{pos}CD127^{neg}$ and $CD25^{pos}CD25^{pos}CD127^{pos}$, and FACS-sorted $CD4^{pos}CD25^{high}$ and $CD4^{pos}CD25^{low}$ T cells were resuspended in Optimem medium (Invitrogen Life Technologies) at 2-3×10^6/ml, and then transfected using 8 μl Lipofectamine RNAiMax (Invitrogen Life Technologies). GAL-9-specific stealth siRNAs were used at a final concentration of 3 nM. GAL-9 stealth siRNA working concentration and Lipofectamine RNAiMax volume were determined after performing a series of preliminary experiments where stealth siRNA concentrations ranging from 0.75 to 24 nM and Lipofectamine RNAiMax volumes ranging from 1 to 16 μl per ml of total cell suspension were tested. A GAL-9 Stealth RNAi concentration of 3 nM and a Lipofectamine RNAiMax volume of 8 μl was the most effective at inducing gene knockdown with minimal toxicity to the cells.

8.2. Assessment of *Galectin-9* gene knock-down

Following overnight incubation at 37˚C and 5% CO_2, aliquots of 2.5×10^5 cells were collected to extract RNA and assess *GAL-9* expression by real-time PCR. Cells were lysed with TRizol reagent (Invitrogen Life Technologies) at a concentration of 0.1 ml/1 x 10^6 cells and total RNA was extracted. mRNA was reverse transcribed using Oligo-$(dT)_{12-18}$ primer (Invitrogen Life Technologies) and Omniscript Reverse Transcriptase (Qiagen Inc., Chatsworth, CA).

GAL-9 transcripts were quantified by real-time PCR using gene-specific probes and TaqMan Master Mix (Applied Biosystems, Warrington, UK). PCR amplification conditions were: 50°C for 2 minutes, 95°C for 10 minutes, 40 cycles of 95°C for 15 seconds, and 60°C for 1 minute. Samples were run in triplicate using a real-time PCR thermocycler (ABI Prism 7000 Sequence Detection System, Applied Biosystems, Foster City, CA), and results were analysed by matched software. Relative expression of *GAL-9* gene was determined by normalising to *GAPDH* expression according to the manufacturer's instructions.

8.3. Assessment of suppressive function of siRNA-treated cells

Following siRNA treatment, cells were washed and resuspended in supplemented RPMI 1640, and added to $CD4^{pos}CD25^{neg}$, $TIM\text{-}3^{pos}$ and $TIM\text{-}3^{neg}$ responder cells at a 1/8 ratio. Parallel cultures where siRNA-untreated $CD4^{pos}CD25^{pos}$, $CD25^{pos}CD25^{pos}CD127^{neg}$, $CD25^{pos}CD25^{pos}CD127^{pos}$, $CD4^{pos}CD25^{high}$, and $CD4^{pos}CD25^{low}$ T cells were added to responder cells served as controls. After a 5-day culture, cell proliferation was assessed by ^{3}H-thymidine incorporation and the percentage of cell proliferation inhibition calculated as detailed above.

9. Immunohistochemistry

The phenotype of lymphocytes infiltrating the liver tissue was investigated by immunohistochemistry in patients with a documented diagnosis of AILD. Liver biopsies were performed percutaneously according to the Menghini technique, with a 1.4 mm diameter needle. The biopsies were fixed in neutral formalin, embedded in paraffin, cut into 5 μm thick sections, and stained for CD4, FOXP3, TIM-3 and GAL-9 (Table 3.2.).

Tissue sections were then evaluated by a single pathologist, who graded the biopsies quantitatively for histological variables.

Table 3.2. Primary antibodies used for immunohistochemistry.

Specificity	Source	Clone	Dilution	Company
Galectin-9	Polyclonal goat	AF2405	1/300	R&D Systems[1]
TIM-3	Polyclonal goat	AF2365	1/50	R&D Systems[1]
CD4	Monoclonal mouse	4B12	Ready-to-use	Dako[2]
FoxP3	Monoclonal mouse	22510	1/100	Abcam[3]

[1] Abingdon, UK; [2] Glostrup, Denmark; [3] Cambridge, UK

Different immunohistochemistry methods were employed: 1) standard avidin-biotin-complex (ABC) method (Vector Laboratories, Burlingame, USA) – used for preliminary experiments aiming at determining the optimal dilution of anti-TIM-3, anti-GAL-9, and anti-FOXP3 primary antibodies; 2) EnVision+ system (Dako Cytomation, Glostrup, Denmark) – used for TIM-3 and GAL-9 single immunostaining, and for FOXP3/GAL-9 double immunostaining. Furthermore, CD4 and FOXP3 single immunostaining was performed using an autostainer (HX System Benchmark, Ventana Medical Systems, Tucson, AZ, USA), as per the manufacturer's instructions.

9.1. Single immunostaining

9.1.1. ABC method

ABC method is one of the most widely used staining techniques for immunohistochemistry. This technique relies on the high affinity that avidin – a large glycoprotein found in chicken eggs – has for the vitamin biotin. Avidin possesses four binding sites for biotin, but due to the molecular orientation of the binding sites, fewer than four molecules of biotin will actually bind. The biotin molecule is easily

conjugated to variety of biological molecules such as antibodies and enzymes (Hsu et al. 1981). In this method secondary antibodies are conjugated to biotin and function as links between tissue-bound primary antibodies and an avidin-biotin-peroxidase complex (Figure 3.8.).

For this purpose, liver tissue sample sections were first deparaffinised by placing them in xylene for 4 minutes, and then in ethanol for additional 4 minutes. After rehydration, epitope retrieval was achieved by boiling the sections either in a sodium citrate buffer (pH 6.0) or in a trishydroxymethylaminomethane/ethylene diamine tetraacetic acid (Tris-EDTA) buffer (pH 9) in a microwave (600 Watts twice for 5 minutes with a 2-minute interval). After cooling for 15 to 30 minutes, slides were placed in PBS for 5 minutes. Endogenous peroxidases were quenched by peroxidase-blocking solution (DakoCytomation, Denmark) for 30 minutes. Non-specific background staining was inhibited by a 10 min incubation of the sections with 5% horse serum (Vector Laboratories). Avidin-biotin blocking reagents were applied for 30 min to block endogenous biotin. Sections were incubated with polyclonal goat anti-human TIM-3, polyclonal goat anti-human GAL-9, and monoclonal mouse anti-human FOXP3 for one hour at room temperature. 1/10, 1/20, 1/50, 1/100, 1/200, 1/300, 1/500 and 1/1000 dilutions were tested for each primary antibody. Dilutions giving the strongest signal with minimal background were chosen. Thereafter, sections were incubated with biotin conjugated secondary antibody (anti-goat IgG, Vector Laboratories, diluted: 1:50) for 30 minutes. Immunoreactivity was visualized using avidin-biotin-peroxidase complex (ABC) kit reagents (Vector Laboratories), and 3,3´-diaminobenzidine (DAB, Dako Cytomation).. Sections were then weakly counterstained with haematoxylin and examined under light microscope.

9.1.2. Envision+ method

EnVision Systems are based on dextran polymer technology (Figure 3.9.). This detection system permits binding of a large number of enzyme molecules (horseradish peroxidase or alkaline phosphatase) to a secondary antibody *via* the dextran backbone. The benefits are many, including increased sensitivity, minimised non-specific background staining and a reduction in the total number of steps compared to the ABC method (Chilosi et al. 1994).

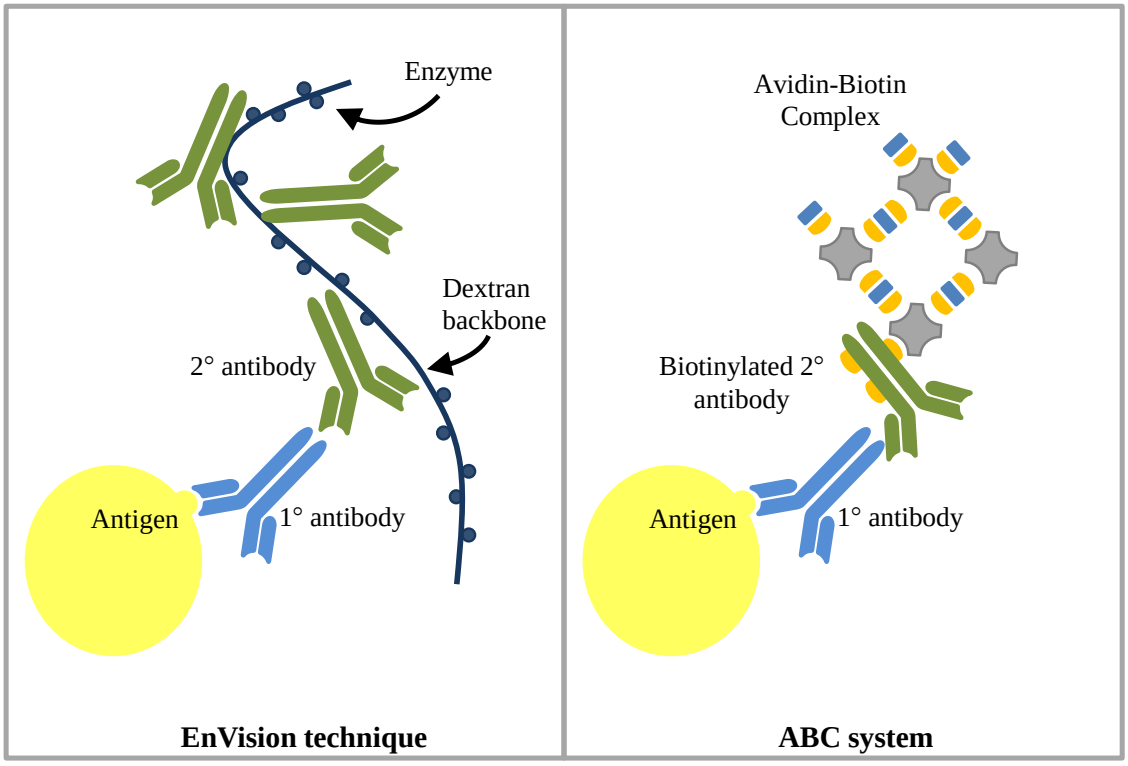


Figure 3.8. Immunohistochemistry techniques. In the EnVision technique, the primary antibody is attached to a dextran backbone which contains an average of 10 molecules of secondary antibody and 70 molecules of enzyme (peroxidase). In the ABC technology, the avidin-biotin enzyme reacts with the biotinylated secondary antibody.

Antigen unmasking was carried out by microwaving the slides twice in a Tris-EDTA buffer (pH 9.0) for 5 minutes with a 2-minute interval. After the endogenous peroxidase was blocked, deparaffinised sections were incubated with primary monoclonal antibodies for 1 hour at room temperature. The sections were then

incubated with goat anti-mouse immunoglobulins conjugated to a peroxidase-labelled dextran polymer (EnVision, DakoCytomation) at room temperature for 1 hour. The reaction products were developed through the immersion of the sections in DAB solution.

9.2. Double immunostaining

Double immunostaining for GAL-9 with FOXP3 was performed to assess whether these two proteins co-localise in the liver tissue. Deparaffinised sections intended for were microwaved twice in Tris-EDTA buffer (pH 9.0) for 5 minutes with a 2-minute interval.

Staining with the first primary antibody was performed in the same way used for single immunostaining, and signals were developed with DAB (brown product). After 3 washes with trishydroxymethylaminomethane-buffered saline (5 minutes each), the second primary antibody was added to the sections, and they were incubated for 1 hour at room temperature.

After they were washed in trishydroxymethylaminomethane-buffered saline, the second reaction was developed with an EnVision G\2 System/Alkaline Phosphatase kit (DakoCytomation; red product). Nuclei were lightly counterstained with haematoxylin.

10. Statistical analysis

Data were analysed using Microsoft Excel 2010 and Prism® 5 software (GraphPad; San Diego, CA).

The normality of variable distribution was assessed by the Kolmogorov-Smirnov goodness of fit test. When the hypothesis of normality was accepted ($p > 0.05$) results

were presented as mean and standard error of the mean (SEM), used instead of the standard deviation to describe the precision of the sample mean. Comparison between variables was performed using two-tailed paired or unpaired Student´s *t* test as appropriate. When the hypothesis of normality was rejected ($p < 0.05$), results were expressed as median and range and comparisons between variables were performed using two-tailed, non-parametric Wilcoxon or Mann-Whitney U tests for related and independent data sets respectively.

Correlation between variables was determined: by Pearson´s correlation coefficient in those cases the data conformed to a normal distribution; or by Spearman´s rank correlation coefficient if data were not normally distributed. A *p* value less than 0.05 was considered significant; *p* values ≥ 0.05 and ≤ 0.15 were considered to indicate a trend to significance.

11. Patients and controls

The experiments included in this thesis were performed in seventy-seven patients with autoimmune liver disease. Thirty healthy subjects, recruited from King´s College Hospital staff members, served as normal controls. Details of patients and controls included in individual parts of the study are given in the corresponding chapter. A table depicting all relevant clinical data from each individual patient is provided in Appendix I.

12. Ethics

In adherence to the Helsinki Principles, informed consent was obtained from all subjects whose biological material was used in the present study. Consent was obtained

from patients, if older than 16, and from parents/guardians, in cases of patients younger than 16. The study protocols were approved by the Ethics Committee of King´s College Hospital, London, United Kingdom.

CHAPTER IV

Frequency, phenotype and function of regulatory T cells in autoimmune liver disease

1. Background

A wealth of data indicates that $CD4^{pos}CD25^{pos}$ cells are key players in the maintenance of immune-tolerance as they prevent effector cells to react against self-antigens. Despite the advances made in understanding the mechanisms underlying Treg cell lineage commitment and function, purifying Treg cells – for basic and translational studies – is still largely reliant upon their CD4 and CD25 expression (Banham 2006). However, while in mice CD25 identifies a largely homogeneous regulatory population (Takahashi et al. 1998), human $CD4^{pos}$ T cells exhibit a continuous and primarily low expression of CD25, in which 2-4% express high levels and up to 30% express low levels of CD25 (Baecher-Allan et al. 2001). Further studies have shown that at variance with mice where the whole $CD4^{pos}CD25^{pos}$ T cell population exhibit regulatory function, in humans only the $CD4^{pos}$ cell subset expressing the highest CD25 fluorescence (the so-called $CD25^{high}$ population) displays a similarly strong regulatory function (Baecher-Allan et al. 2001). Since there is no uniformly defined consensus as to where the boundary between $CD25^{high}$ and $CD25^{low}$ expression should be drawn, the comparison of data deriving from human samples has been difficult, particularly in the case of chronic inflammatory conditions where high numbers of activated effector cells are present.

Akin to CD25, other surface markers, including GITR, CD62L, LAG-3 and CTLA-4, initially thought to be specifically expressed by Tregs, have been subsequently found to be expressed also on effector T cells (Tang et al. 2004a, Ronchetti et al. 2004, Banham 2006). Despite being still considered one the most specific Treg markers, the lineage specific transcription factor FOXP3 cannot be used to purify Tregs because of its intracellular location (Sakaguchi et al. 2010).

Recent literature has indicated that the absence or low expression of CD127 is a marker of *bona fide* Tregs that can be readily utilised for their purification in humans. A recent study has shown that CD127 was down-regulated on all human T cells after activation. Interestingly, while CD127 was then re-expressed on the majority of effector and memory T cells, $FOXP3^{pos}$ T cells remained $CD127^{neg}$. Moreover expression of CD127 negatively correlated with that of FOXP3, as result of FOXP3 binding to the CD127 promoter (Liu et al. 2006). Simultaneously, Seddiki et al. reported that the surface expression of CD127, in combination with CD25, can distinguish between human regulatory and activated effector $CD4^{pos}$ T cells (Seddiki et al. 2006). The same evidence has since been reproduced in other disease states classically associated with impaired or enhanced $CD4^{pos}CD25^{high}$ Treg number and function, such as MS (Michel et al. 2008, McKay et al. 2008), allograft rejection (Codarri et al. 2007) and cancer (Shen et al. 2009).

Given the pivotal role of Tregs in controlling responder cells, my initial hypothesis is that unrestrained effector mechanisms involved in autoimmune liver damage result from defective Treg number. To test this hypothesis I have performed a phenotypic and functional characterisation of circulating $CD4^{pos}CD25^{pos}CD127^{neg}$ cells ("$CD127^{neg}$ Tregs") in patients with autoimmune liver disease (AILD).

Specific objectives of the current chapter are: 1) to determine the frequency of circulating $CD127^{neg}$ Tregs and activated $CD127^{pos}$ T cells ("$CD127^{pos}$ T cells") present within the conventional Treg population in AILD patients; 2) to assess the cytokine and the transcription factor profile of $CD127^{neg}$ Tregs, $CD4^{pos}CD25^{pos}$ Tregs and $CD127^{pos}$ T cells and 3) to purify $CD127^{low}$ Tregs and to test their ability to suppress proliferation of responder CD4 T cells.

2. Subjects

For this part of the study thirty-four patients with ANA and/or SMA positive AILD were investigated. Seventeen (50%) patients were female. The median age at the time of study was 13.4 years, range 5.6-26.7 years. Nineteen patients were studied during drug-induced remission (ie, with normal transaminase levels; [R] patients) while 15 patients had active disease ([A] patients) at the time of investigation. From 6 of 15 [A] patients, blood was obtained at diagnosis before treatment was started; the remaining 9 [A] patients were studied at relapse during immunosuppression tapering.

A liver biopsy performed at the time or close to diagnosis showed histological features of interface hepatitis in all patients. Viral, metabolic, and genetic causes of liver disease were excluded by appropriate investigations, all patients being negative for anti-hepatitis C virus, hepatitis B surface antigen, Epstein-Barr virus, and cytomegalovirus serological markers of active infection. In the group of [A] patients, ANA were present in 9 patients and SMA were present in 8 (Table 4.1); Concomitant presence of ANA and SMA occurred in 4 individuals. At the time of diagnosis, all 19 [R] patients tested positive for ANA and/or SMA whereas at the time of study 11 remained either ANAs and/or SMAs positive (one patient tested positive for both) and 8 were autoantibody negative. All patients were negative for anti-LKM-1 and anti-LC-1. Relevant demographic, clinical and laboratory data are summarised in Table 4.1.

Fourteen patients had bile duct changes characteristic of sclerosing cholangitis on retrograde cholangiography and were diagnosed as having autoimmune hepatitis/sclerosing cholangitis (AISC) overlap syndrome (Gregorio et al. 2001); this group consisted of 7 [R] and 7 [A] patients, the latter including 2 at presentation. When considered together, AIH and AISC are henceforth indicated as autoimmune liver

disease (AILD). Biochemical and immunological differences between AIH-1 and AISC patients investigated in this chapter are depicted in Table 4.2.

Table 4.1. Clinical and laboratory features of AILD patients at the time of study

Parameter	AILD (n=34)	[A] patients (n=15)	[R] patients (n=19)	*p*[*]
Age in years	13.4 (5.6-26.7)	13.8 (5.6-16.8)	13.0 (8.2-26.7)	NS[†]
Female, n of patients (%)	17 (50%)	10 (67%)	7 (37%)	NS[†]
AST (nv<50 IU/L)	45 (18-1061)	93(51-1061)	28 (18-49)	<0.0001[‡]
GGT (nv<50 IU/L)	44 (8-503)	74 (17-503)	27 (8-164)	0.0002[‡]
Total bilirubin (nv<20 μmol/l)	8 (3-127)	13 (3-127)	6 (4-14)	0.009[‡]
AP (nv<350 IU/L)	189 (68-834)	181 (88-834)	196 (68-344)	NS[‡]
AP/AST ratio	4.31 (0.17-16.38)	2.20 (0.17-4.65)	6.45 (1.58-16.38)	<0.0001[‡]
INR (nv<1.2)	1.05 (0.86-1.78)	1.07 (0.90-1.78)	1.04 (0.86-1.23)	NS[‡]
Albumin (nv>35 g/L)	45 (32-50)	44 (32-50)	46 (40-50)	0.09[‡]
IgG (nv 6.5-17 g/L)	16.82 (7.51-43.96)	18 (11.66-43.96)	12.99(7.5127.14)	0.03[‡]
ANA, n of positive patients (%)	16 (47%)	9 (60%)	7 (37%)	NS[†]
SMA, n of positive patients (%)	13 (38%)	8 (53%)	5 (26%)	NS[†]
pANNA, n of positive patients (%)	16 (47%)	9 (60%)	7 (37%)	NS[†]
ASIC, n of positive patients (%)	14 (41%)	7 (47%)	7 (37%)	NS[†]
UC, n of positive patients (%)	12 (35%)	5 (33%)	7 (37%)	NS[†]

Data presented as median (range) unless noted otherwise.
AILD, autoimmune liver disease; *AST*, aspartate aminotransferase; *GGT*, gamma-glutamyl transpeptidase; *AP*, alkaline phosphatase; *INR*, international normalised ratio; *IgG*, immunoglobulin G; *ANA*, anti-nuclear antibodies; *SMA*, anti-smooth muscle antibodies; *pANNA*, peripheral anti-nuclear neutrophil antibodies; *AISC*, autoimmune sclerosing cholangitis; *UC*, ulcerative colitis; *nv*, normal value; *NS* non significant
[*] *p* value when comparing clinical parameters between [A] and [R] patients
[†] Fisher´s exact test
[‡] Mann-Whitney test

Patients were treated with prednisolone (2.5-5 mg daily at remission and 1-2 mg/kg/day at relapse) either alone or in combination with azathioprine (1-2 mg/kg/day) and/or mycophenolate mofetil (MMF, up to 40 mg/kg/day). In those patients diagnosed with AISC, ursodeoxycholic acid (UDCA) at a dose of 15-30 mg/kg/day was added to the immunosuppressive regimen. Dosage and duration of treatments for each patient are

shown in Appendix I. At the time of the study, 3 patients with AIH-1 and 9 with AISC had concomitant ulcerative colitis (UC).

Table 4.2. Clinical and laboratory data comparing AIH and AISC patients.

Parameter	AIH-1 patients (n=20)	AISC patients (n=14)	*p*[*]
Age in years	12.9 (5.6-17.8)	14.4 (8.2-26.7)	NS[†]
Female, n of patients (%)	10 (50%)	7 (50%)	NS[†]
AST (nv<50 IU/L)	38 (18-1061)	49 (21-534)	NS[‡]
GGT (nv<50 IU/L)	34 (8-492)	73 (12-503)	0.02[‡]
Total bilirubin (nv<20 μmol/l)	7 (4-127)	8 (3-47)	NS[‡]
AP (nv<350 IU/L)	178 (79-669)	209 (68-834)	NS[‡]
AP/AST ratio	4.31 (0.17-15.64)	3.99 (1.56-16.38)	NS[‡]
INR (nv<1.2)	1.08 (0.94-1.30)	1.00 (0.86-1.78)	NS[‡]
Albumin (nv>35 g/L)	45 (39-50)	45 (32-50)	NS[‡]
IgG (nv 6.5-17 g/L)	16.54 (7.51-43.96)	17.08 (11.29-27.14)	NS[‡]
ANA, n of positive patients (%)	8 (40%)	8 (57%)	NS[†]
SMA, n of positive patients (%)	8 (40%)	5 (36%)	NS[†]
pANNA, n of positive patients (%)	6 (30%)	10 (71%)	0.03[†]
UC, n of positive patients (%)	3 (15%)	9 (64%)	0.005[†]
Active disease, n of patients (%)	8 (40%)	7 (50%)	NS[†]

Data presented as median (range) unless noted otherwise.
AIH-1, autoimmune hepatitis type 1; *AISC*, autoimmune sclerosing cholangitis; *AST*, aspartate aminotransferase; *GGT*, gamma-glutamyl transpeptidase; *AP*, alkaline phosphatase; *INR*, international normalised ratio; *IgG*, immunoglobulin G; *ANA*, anti-nuclear antibodies; *SMA*, anti-smooth muscle antibodies; *pANNA*, peripheral anti-nuclear neutrophil antibodies; *UC*, ulcerative colitis; *nv*, normal value; *NS* non significant
[*] *p* value when comparing clinical parameters between AIH-1 and AISC patients
[†] Fisher´s exact test
[‡] Mann-Whitney test

Fifteen healthy subjects (HS, median age: 28.9 years, range 22.6-35.8, 66% female), recruited from King's College Hospital staff, served as normal controls. The disparity between patient and control age derives from ethical constraints in obtaining biological specimens from healthy children. To test whether age disparity may account for differences, patients were divided into 2 subgroups, (≤14 and >14 years old).

3. Results

3.1. Frequency of regulatory T cells

The frequency and phenotype of regulatory T cells was analysed in 32 AILD patients and 14 HS. Gating of PBMCs according to their forward (FSC) and side (SSC) scatter characteristics and antibody fluorescence patterns (Figure 4.1.), revealed a population of lymphocytes expressing CD4 and CD25 ("conventional Tregs", cTregs). The proportion of these cells was lower in AILD compared to HS (5.86±0.53% vs 9.94±0.80%; $p<0.001$). $CD4^{pos}CD25^{pos}$ cells were subsequently subdivided according to their CD25 expression into $CD25^{low}$, $CD25^{medium}$, and $CD25^{high}$ subsets and the expression of CD127 within those populations was evaluated.

The percentage of $CD127^{pos}$ cells within cTregs was higher in patients (28.18±1.99%) than in HS (14.91±1.34%, $p<0.00001$), this difference being evident also when the $CD25^{low}$ (32.49±2.32% vs 18.90±1.05%, $p<0.00001$), $CD25^{med}$ (18.99±1.18% vs 11.29±0.97%, $p<0.00001$) and $CD25^{high}$ (10.10±0.79% vs 2.09±0.47%, $p<0.0000000001$) fractions were analysed separately.

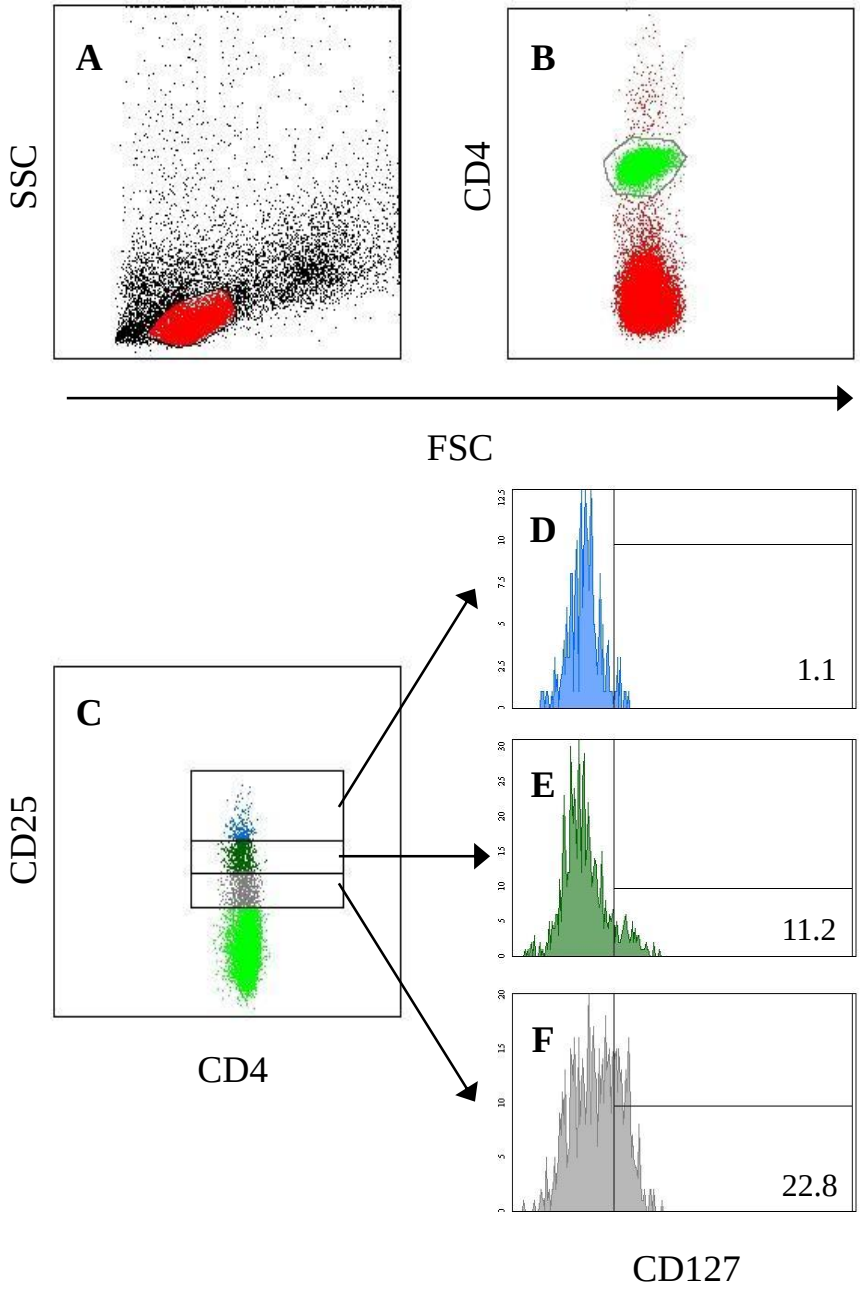
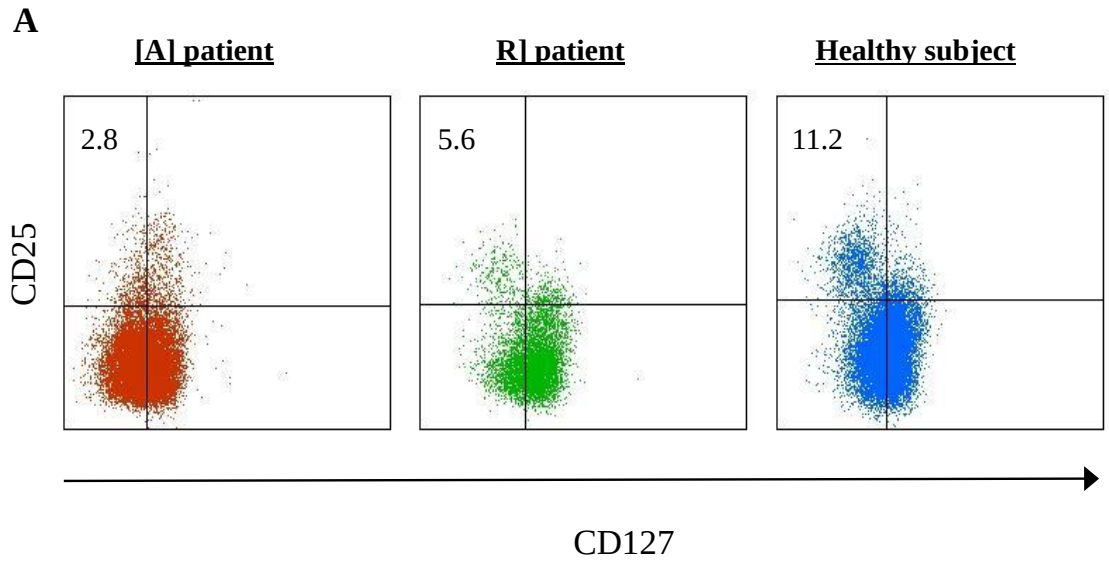


Figure 4.1. Gating strategy used to analyse CD127 expression. PBMCs were gated according to their forward (FSC) and side (SSC) scatter characteristics (*A*); lymphocytes were subsequently gated according to CD4 expression (*B*); a population of cells positive for CD25 was then identified within the CD4 cell population and subdivided according to its CD25 expression (*C*). CD127 expression, represented as mean fluorescence intensity on histogram plots, was evaluated within $CD25^{high}$(*D*), $CD25^{medium}$ (*E*) and $CD25^{low}$ (*F*) populations. Plots are from one representative healthy subject. Values represent the frequency of cells within each CD25 population.

Analysis of $CD127^{neg}$ Tregs showed that the frequency of these cells was markedly decreased in patients with AILD (4.35±0.45%) compared to HS (8.01±0.69%,

p<0.001), this difference being significant both in [A] (2.16±0.21%, *p*<0.000001) and [R] patients (6.06±0.13%, *p*=0.03; Figure 4.2.). $CD127^{neg}$ Tregs were significantly less numerous in [A] than in [R] patients (*p*<0.000001).



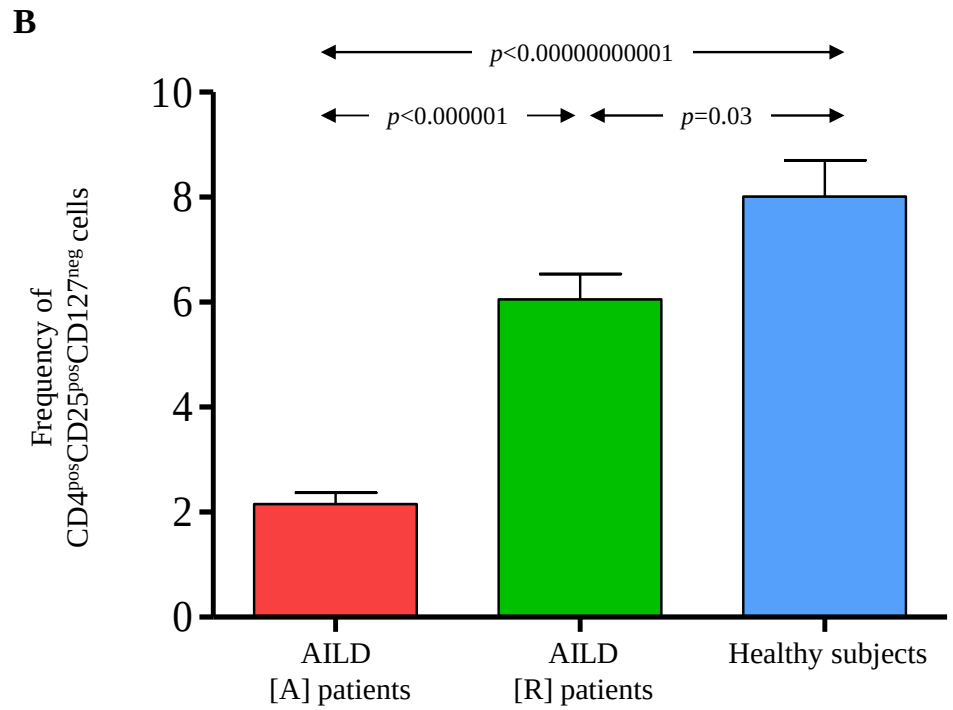


Figure 4.2. Frequency of $CD4^{pos}CD25^{pos}CD127^{neg}$ cells. Plots in *Panel A* show the frequency of $CD4^{pos}CD25^{pos}CD127^{neg}$ cells in one representative [A] patient, in one representative [R] patient and in one representative healthy subject. The graph in *Panel B* summarises data from 15 [A] patients, 19 [R] patients and 14 healthy subjects. Error bars represent SEM.

In line with this, the frequency of $CD127^{neg}$ Tregs in AILD inversely correlated with levels of AST and IgG, and to a lesser extent with SMA titres (Figure 4.3.).

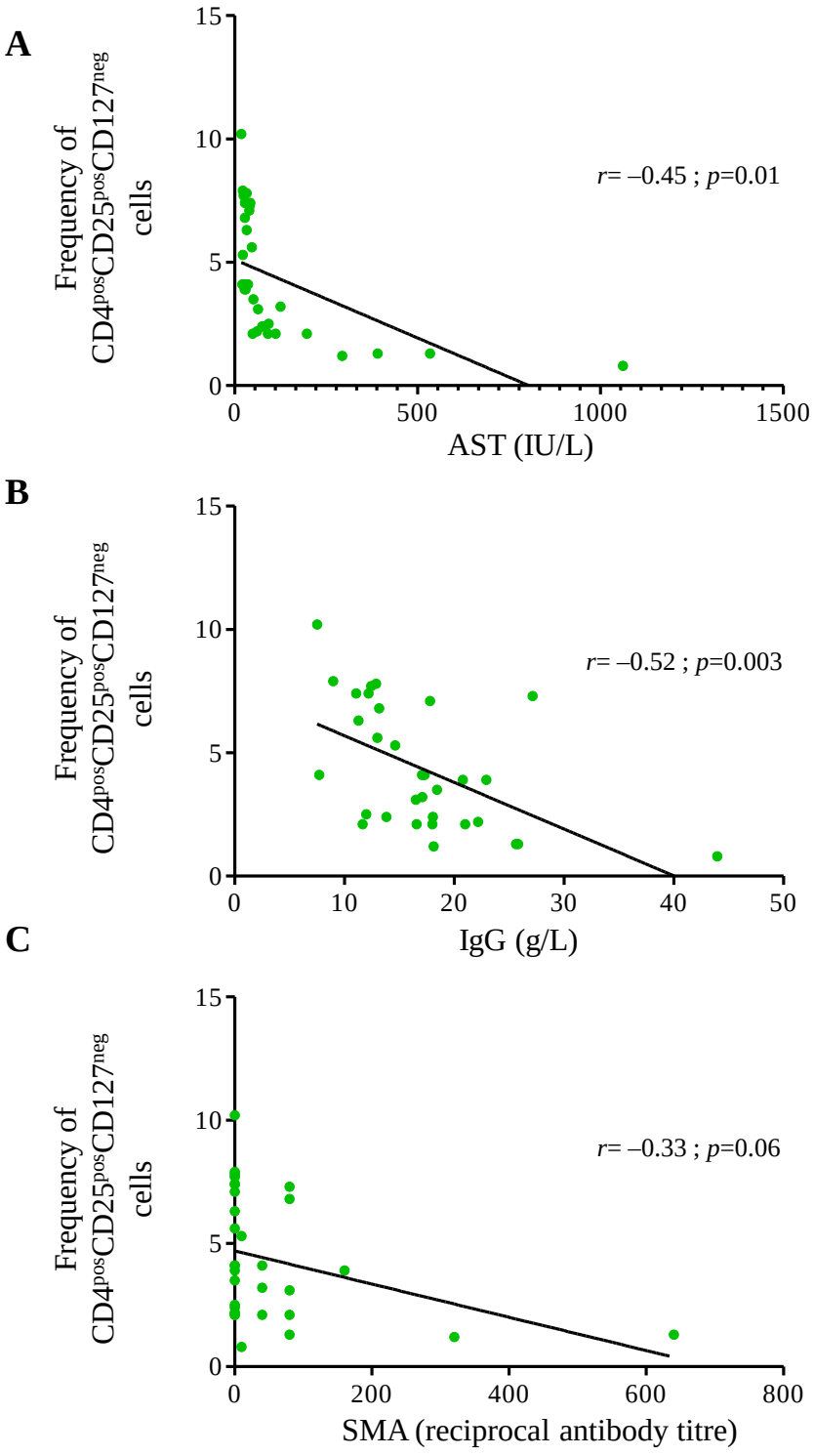


Figure 4.3. Correlation between the frequency of $CD4^{pos}CD25^{pos}CD127^{neg}$ cells and indices of disease activity. In AILD patients the percentage of $CD4^{pos}CD25^{pos}CD127^{neg}$ cells negatively correlates with levels of AST (*Panel A*), IgG (*Panel B*), and to a lesser extent SMA (*Panel C*).

Within [A] patients no differences in the frequency of $CD127^{neg}$ Tregs were observed between those studied at diagnosis and those studied during an episode of relapse. Within AILD patients, no differences were found between the two age groups, between AIH and AISC, or between those with or without concomitant UC.

The frequency of cTregs, $CD127^{neg}$ Tregs and $CD127^{pos}$ T cells positive for FOXP3 and CTLA-4, classical Treg markers that have been linked to the suppressive function of these cells, was investigated in 10 AILD patients and 5 HS.

The frequency of $FOXP3^{pos}$ cells was higher within $CD127^{neg}$ Tregs than within cTregs in both patients (77.83±4.91% vs 43.48±3.99%, $p<0.0001$), and HS (91.20±4.28% vs 66.30±10.53%, $p=0.001$). Compared to HS, AILD patients displayed a lower percentage of $FOXP3^{pos}$ cells both within cTregs ($p=0.004$) and $CD127^{neg}$ Tregs ($p=0.03$). In AILD, the frequency of $FOXP3^{pos}$ cells within $CD4^{pos}CD25^{pos}$ cells inversely correlated with the percentage of $CD127^{pos}$ cells within the same subset ($r= -0.66$, $p=0.04$, Figure 4.4.).

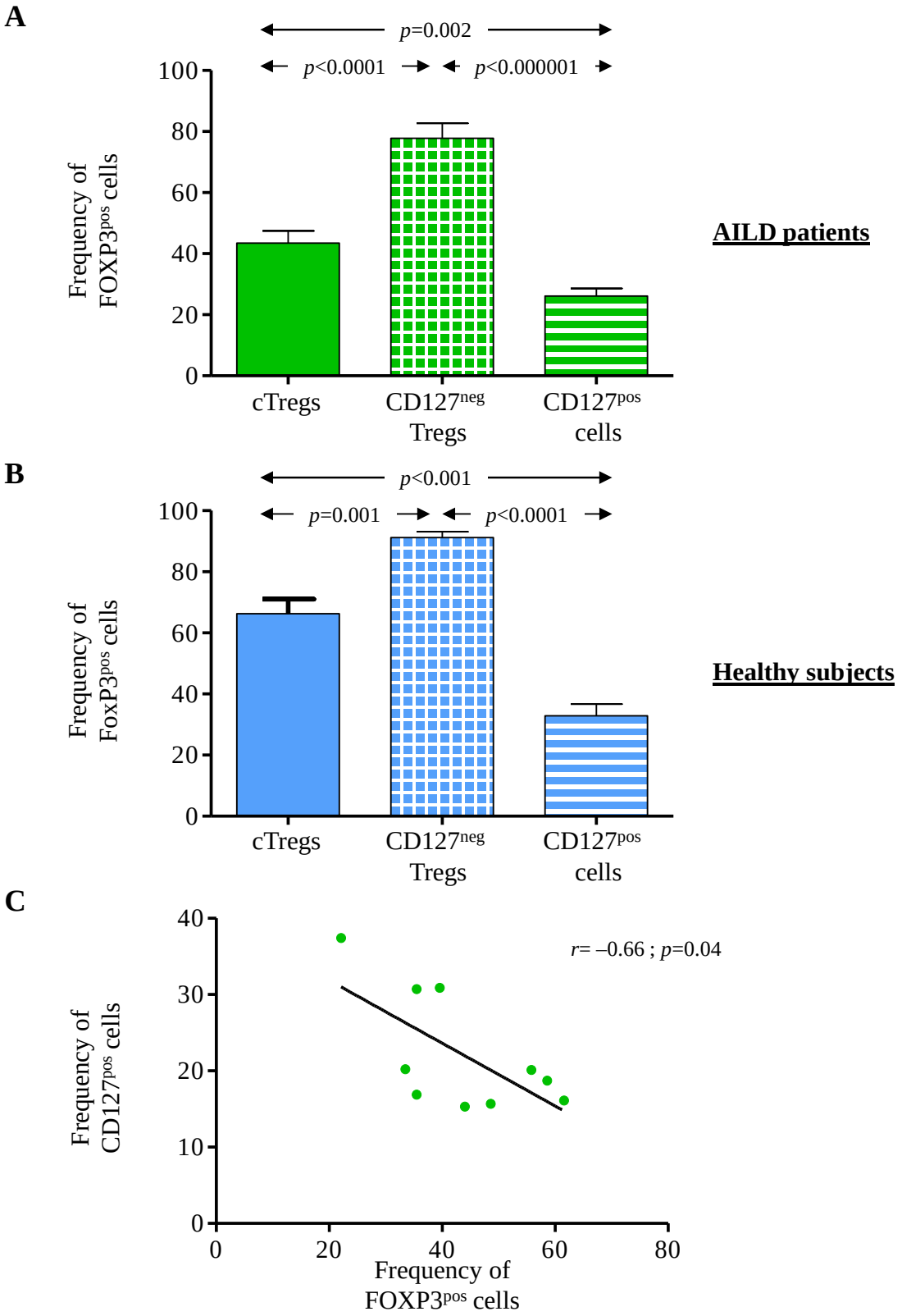


Figure 4.4. Frequency of FOXP3^{pos} cells within regulatory T cell populations. The plots show that the highest frequency of FOXP3^{pos} cells was within CD127^{neg} Tregs and the lowest within CD127^{pos} cells in AILD patients (*A*) and healthy subjects (*B*). In AILD the frequency of CD127^{pos} within CD4^{pos}CD25^{pos} cells inversely correlated with that of FOXP3^{pos} cells (*C*). Data from 5 healthy subjects and 10 AILD. Error bars represent SEM.

Akin to FOXP3, the frequency of CTLA-4^{pos} cells was higher in CD127^{neg} Tregs than in cTregs both in AILD (75.01±3.68% vs 51.6±5.69%, p=0.005) and HS (87.04±2.84% vs 74.88±2.89%, p=0.01), and lower in CD127^{neg} Tregs and cTregs isolated from AILD patients than in those obtained from HS (CD127^{neg} Tregs: p=0.03; cTregs: p=0.006). In both groups of subjects CD127^{pos} T cells expressed the lowest levels of FOXP3 (AILD: 26.10±2.51% vs HS: 32.90±8.54%, p=NS) and CTLA-4 (AILD: 38.34±7.59%; HS: 48.46±3.23%, p=NS; Figure 4.5.).

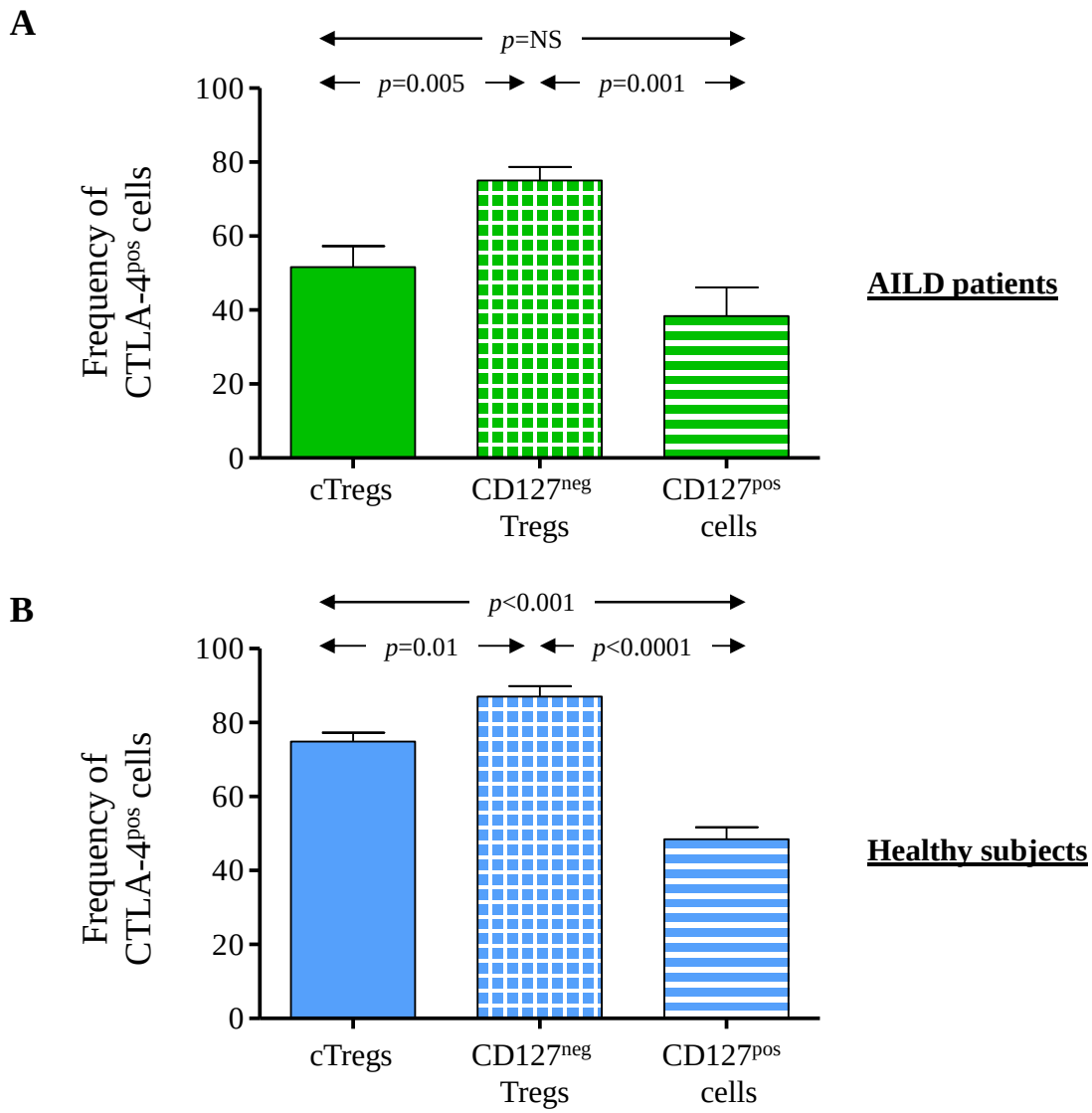


Figure 4.5. Frequency of CTLA-4^{pos} cells within regulatory T cell populations. FACS analysis of PBMCs from AILD patients (*A*) and healthy subjects (*B*) revealed that CD127^{neg} Tregs contained a higher frequency of CTLA-4^{pos} cells than cTregs and CD127^{pos} cells. The plots summarise data from 10 AILD patients 5 healthy subjects and. Error bars represent SEM.

The frequency of cells positive for CD45RO, a marker defining memory T cells, was also assessed. In AILD all subsets analysed contained a lower frequency of $CD45RO^{pos}$ cells ($CD127^{neg}$ Tregs: 68.88±4.29%; cTregs: 53.74±3.49%; $CD127^{pos}$ T cells: 78.73±63.69%) than in HS ($CD127^{neg}$ Tregs: 89.52±2.07%, p=0.001; cTregs: 75.68±5.38%, p=0.01; $CD127^{pos}$ T cells: 92.52±3.44%, p=0.02). Compared to cTregs, $CD127^{neg}$ Tregs displayed higher frequency of $CD45RO^{pos}$ cells both in AILD (p=0.01) and HS (p=0.05, Figure 4.6.).

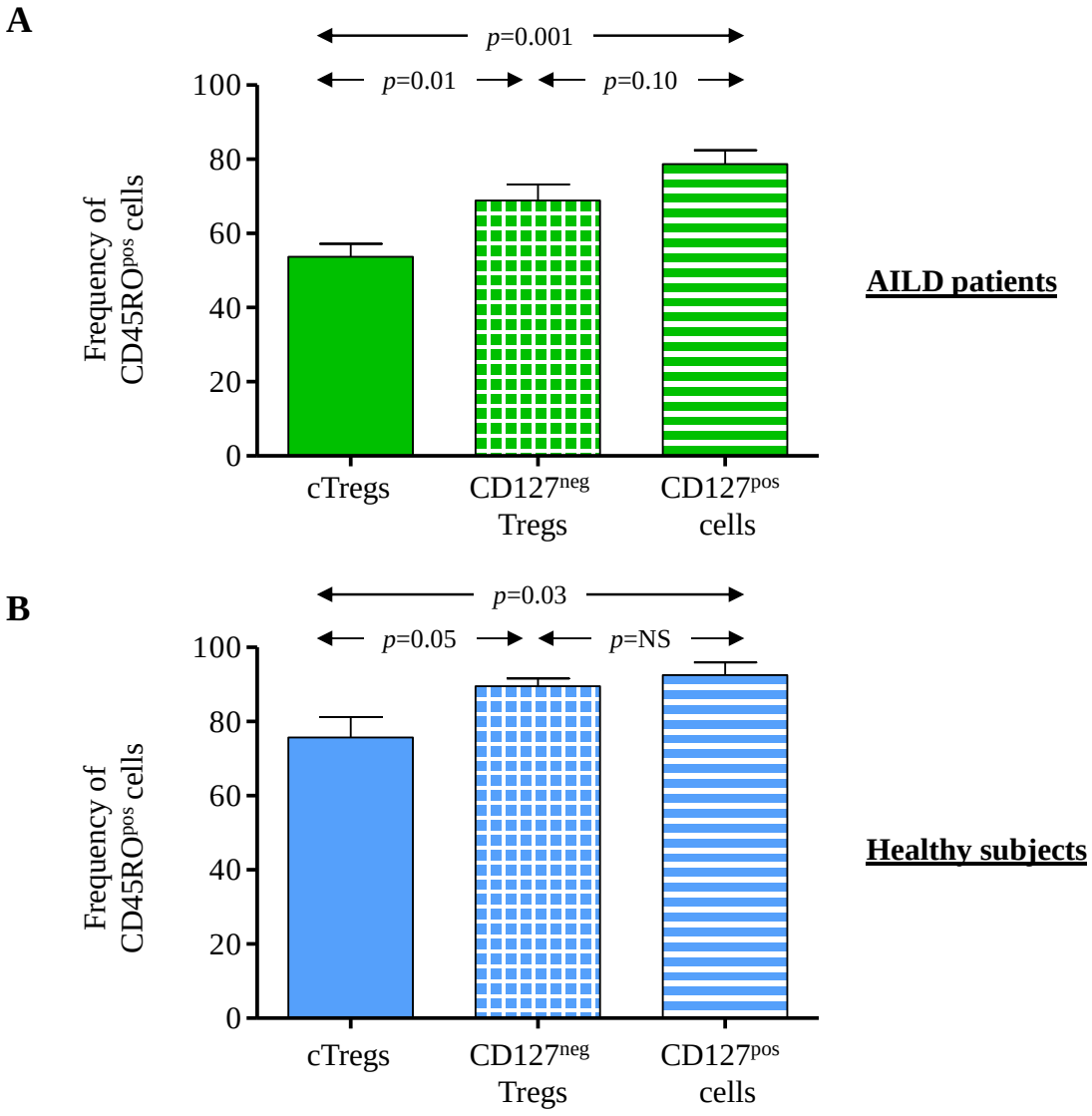


Figure 4.6. Frequency of $CD45RO^{pos}$ cells in regulatory T cell populations. FACS analysis revealed that $CD127^{neg}$ Tregs display a higher frequency of $CD45RO^{pos}$ cells compared to cTregs in AILD patients and healthy subjects. The plots summarise data from 5 healthy subjects 10 AILD patients. Error bars represent SEM.

3.2. Transcription factor profile

The frequency of cells positive for T-bet, GATA-3 and RORC - lineage-specific transcription factors for Th1, Th2, and Th17 cells respectively - was assessed in $CD127^{neg}$ Tregs, cTregs and $CD127^{pos}$ cells from 10 AILD patients and 5 HS.

Compared to cTregs, $CD127^{neg}$ Tregs contained fewer $Tbet^{pos}$ and $RORC^{pos}$ cells in AILD (Tbet: 8.75±1.15% vs 3.78±0.97%, p=0.004; RORC: 18.01±2.03% vs 6.83±0.58%, p<0.0001), and to a lesser extent in HS (Tbet: 4.54±1.18% vs 2.08±0.32%, p=0.08; RORC: 10.76±1.51% vs 4.78±0.89%, p=0.009). cTregs contained an increased frequency of $Tbet^{pos}$ and $RORC^{pos}$ cells in AIH compared to HS (Tbet: p=0.03; RORC: p=0.01), whereas such difference was not as evident when $CD127^{neg}$ Tregs from the two groups were analysed (Tbet: p=0.13; RORC: p=0.09). $CD127^{pos}$ T cells displayed the highest frequency of $Tbet^{pos}$ cells, the proportion of these being more elevated in AILD (16.89±2.59%) than in HS (6.64±1.87%, p=0.005; Figure 4.7.). Although $CD127^{pos}$ T cells were highly enriched for RORC, no statistically significant difference in the percentage of these cells was noted between health (33.86±5.23%) and disease (41.7±7.28%, p=NS).

No significant difference in the percentage of $GATA\text{-}3^{pos}$ cells was noted between $CD127^{pos}$ cells from AILD and health.

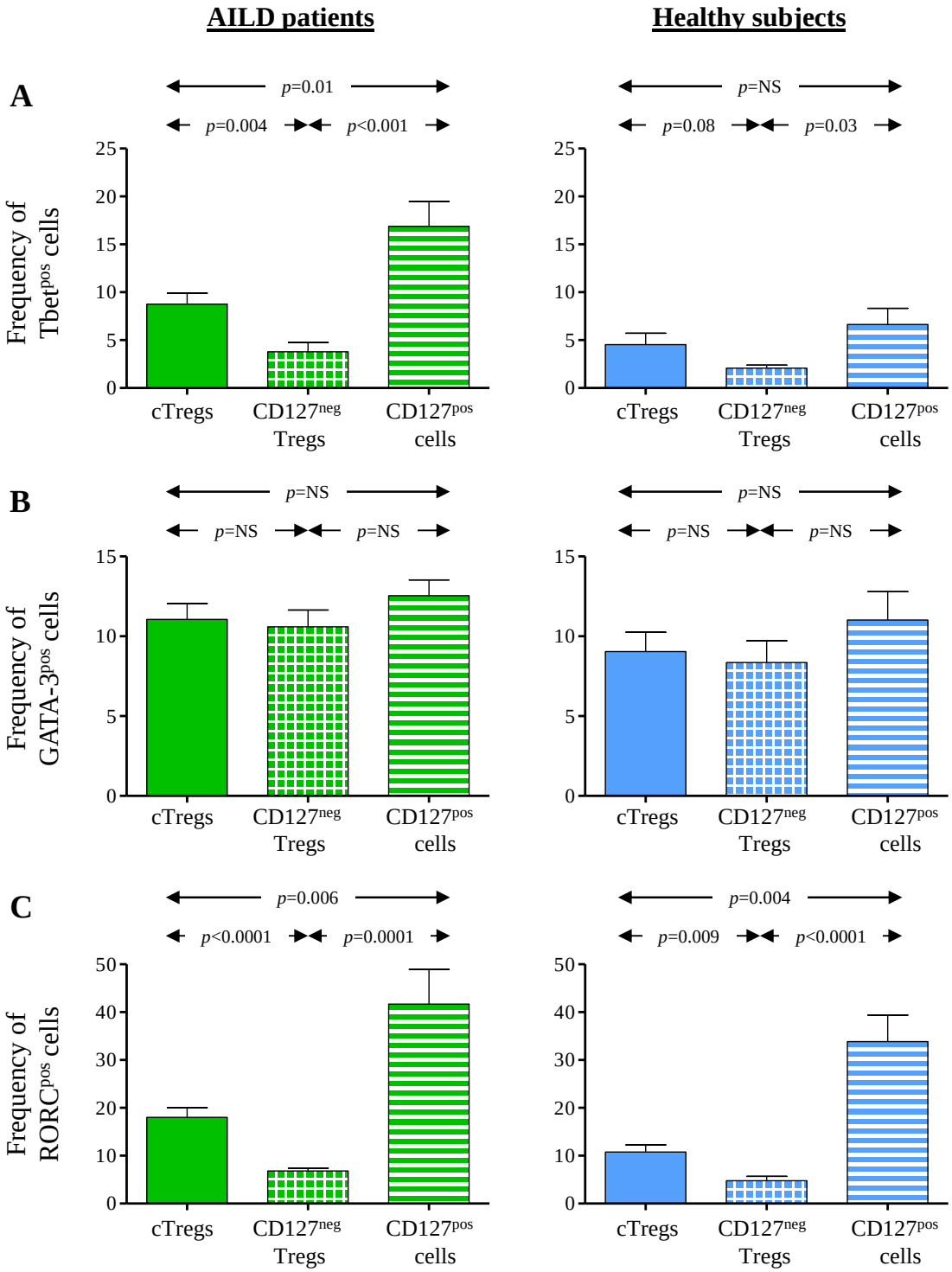


Figure 4.7. Transcription factor profile of cTregs, CD127^{neg} Tregs and CD127^{pos} T cells. Frequency of T-bet^{pos} (*A*), GATA-3^{pos} (*B*), and RORC^{pos} (*C*) cells within cTregs, CD127^{neg} Tregs and CD127^{pos} T cells in 10 AILD patients (*left plots*) and 5 healthy subjects (*right plots*). Error bars represent SEM.

3.3. Frequency of cytokine producing cells within regulatory T cell populations

The frequency of cytokine producing cells within Tregs was assessed in 10 patients and 5 HS. Compared to cTregs, $CD127^{neg}$ Tregs from AILD and HS contained more $IL\text{-}10^{pos}$ (AILD: 7.07±1.12% vs 9.77±0.78%, p=0.06; HS: 12.64±1.61% vs 18.66±2.02%, p=0.05), and similar numbers of $TGF\text{-}\beta^{pos}$ cells (AILD: 9.21±0.74% vs 10.45±1.86%, p=NS; HS: 12.48±1.43% vs 15.32±1.41%, p=NS). In AILD, cTregs and $CD127^{neg}$ Tregs contained fewer $IL\text{-}10^{pos}$ (cTregs: p=0.03; $CD127^{neg}$ Tregs: p=0.008), and $TGF\text{-}\beta^{pos}$ cells (cTregs: p=0.09; $CD127^{neg}$ Tregs: p=0.06) than in HS (Figure 4.8.)

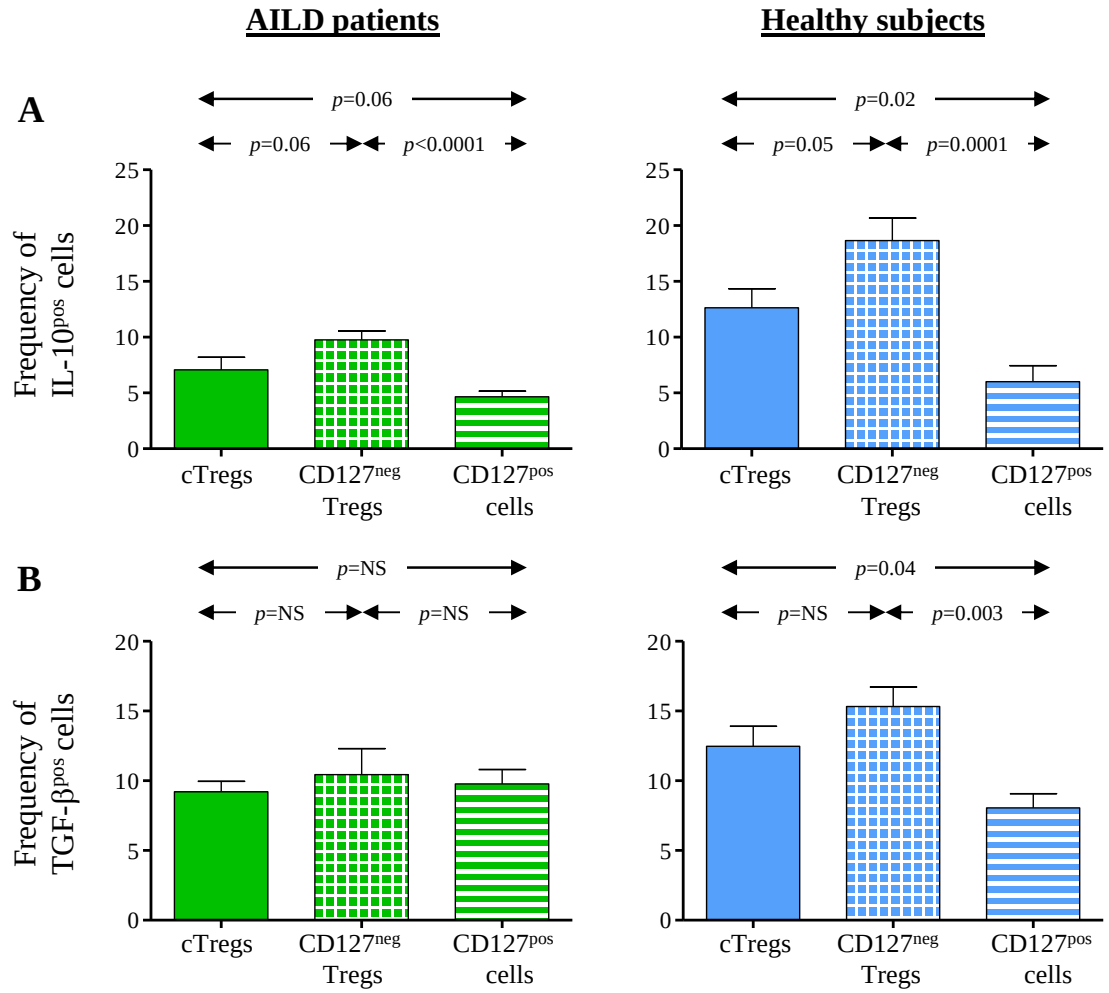


Figure 4.8. Frequency of anti-inflammatory cytokine-producing cells within different T cell subsets. Frequency of $IL\text{-}10^{pos}$ (*A*) and $TGF\text{-}\beta^{pos}$ (*B*) cells within cTregs, $CD127^{neg}$ Tregs and $CD127^{pos}$ T cells in 10 AILD patients (*left plots*) and 5 healthy subjects (*right plots*). Error bars represent SEM.

The assessment of pro-inflammatory cytokine profile showed that $CD127^{neg}$ Tregs from both groups contained a lower frequency of IFN-γ^{pos} (2.82±0.50% in AILD; 1.64±0.24% in HS) and IL-17^{pos} cells (3.83±0.63% in AILD; 1.80±0.79% in HS) than cTregs (IFN-γ: 7.45±0.94%, $p<0.001$ in AILD, 3.48±0.71%, $p=0.04$ in HS; IL-17: 10.05±1.41%, $p<0.001$ in AILD, 5.42±1.82%, $p=0.10$ for HS). Compared to HS, AILD patients had higher frequencies of IFN-γ^{pos} and IL-17^{pos} cells within cTregs (IFN-γ: $p=0.005$; IL-17: $p=0.05$) while this difference was less evident when $CD127^{neg}$ Tregs were considered (IFN-γ: $p=0.06$; IL-17: $p=0.07$). The $CD127^{pos}$ T cell fraction showed the highest frequency of IFN-γ (AILD: 13.38±2.55%; HS: 8.70±1.98%, $p=0.17$) and IL-17-producing cells (AILD: 17.11±2.76%; HS: 6.46±1.34, $p=0.005$) when compared with cTregs and $CD127^{neg}$ Tregs. Lastly, no differences were observed in terms of frequency of IL-2 producing cells amongst cTregs, $CD127^{neg}$ Tregs and $CD127^{pos}$ T cells in both groups of subjects (Figure 4.9.).

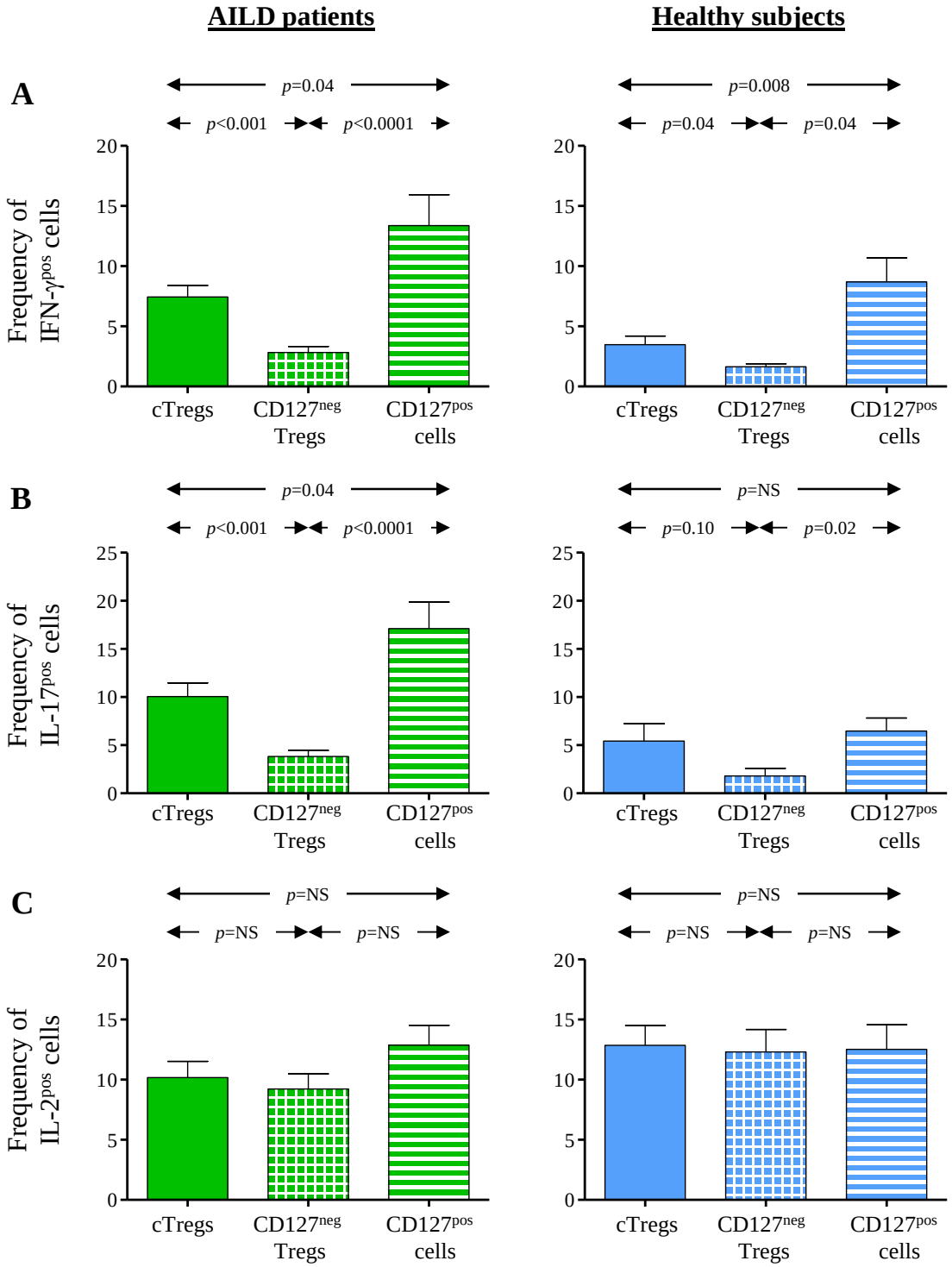


Figure 4.9. Frequency of pro-inflammatory cytokine producing cells within different T cell subsets. Frequency of IFN-γ^{pos} (*A*), IL-17^{pos} (*B*), and IL-2^{pos} (*C*) cells within cTregs, $CD127^{neg}$ Tregs and $CD127^{pos}$ T cells in 10 AILD patients (*left plots*) and 5 healthy subjects (*right plots*). Error bars represent SEM.

3.4. Suppressive function

The suppressive function of cTregs and CD127^{neg} Tregs, assessed as their ability to inhibit the proliferation of autologous CD4^{pos}CD25^{neg} T cells used as responders, was studied in 8 AILD patients and 6 HS.

Addition of cTregs reduced the mean CD4^{pos}CD25^{neg} T cell count per minute (cpm) by 26% in AILD patients (from 18,992±2,298 to 14,113±1,835 cpm, p=0.12) and by 56% in HS (from 32,253±2,463 to 14,170±911 cpm, p=0.0006; Figure 4.10).

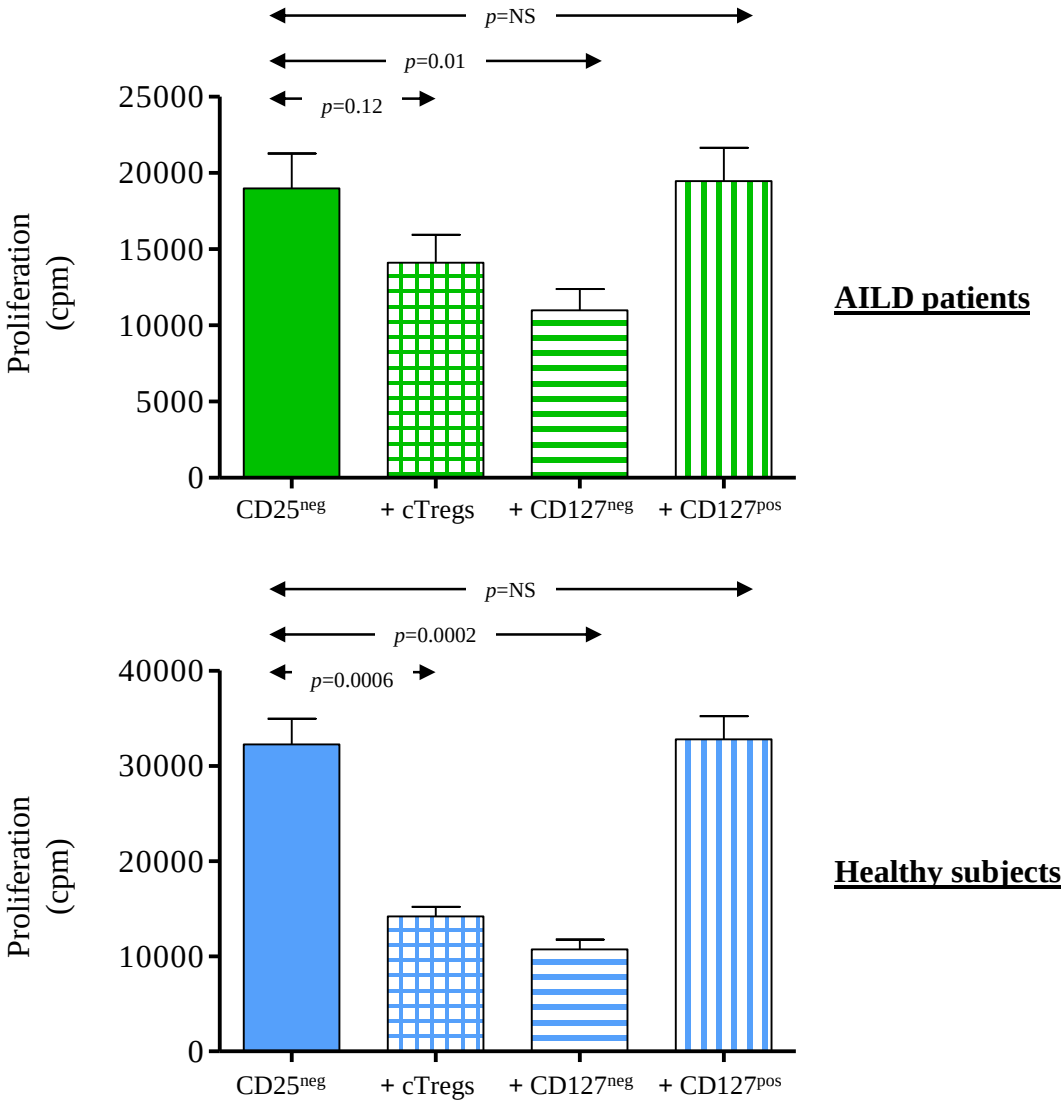


Figure 4.10. Suppressive function of cTregs, CD127^{neg} Tregs and CD127^{pos} T cells. The plots show the proliferation (expressed as counts per minute [cpm]) of CD25^{neg} responder cells cultured alone (*filled bars*) or in combination with cTregs (*square patterned bars*), CD127^{neg} Tregs (*horizontal striped bars*) or CD127^{pos} T cells (*vertical striped bars*). Error bars represent SEM. Eight AILD patients (*upper panel*) and six healthy subjects (*bottom panel*) were studied.

The percentage of inhibition was significantly lower in AILD compared to HS (p=0.002). Addition of CD127^{neg} Tregs reduced responder cell proliferation by 42% in AILD (p=0.01) and by 67% in HS (p=0.0002). In both AILD and HS CD127^{neg} Tregs inhibited responder cell proliferation more effectively than cTregs (AILD: 1.6 fold increase in the ability to suppress, p=0.002; HS: 1.2 fold increase, p=0.12, Figure 4.11.). Both in AILD and HS, addition of CD127^{pos} T cells failed to result in inhibition of responder cell proliferation.

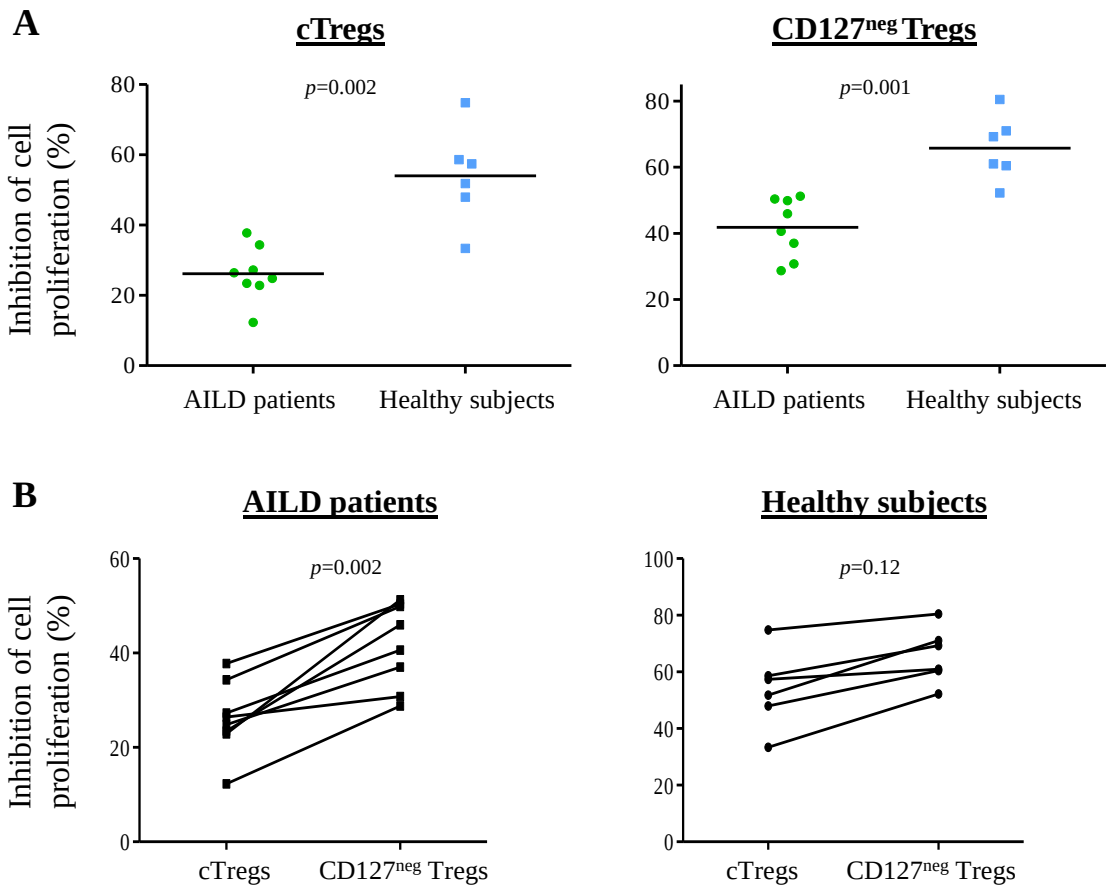


Figure 4.11. Suppressive activity of cTregs and CD127^{neg} Tregs. *Panel A* shows the suppressive function (expressed as percentage [%] of inhibition of CD25^{neg} responder cell proliferation) of cTregs (*left*) and CD127^{neg} Tregs (right). Comparison of suppressive activity between cTregs and CD127^{neg} Tregs is shown in *Panel B*. Data representative of 8 AILD patients and 6 healthy subjects.

3.5. Neutralisation assays

To explore whether the suppressive function of regulatory T cells is influenced by cytokine secretion, neutralising antibodies to IL-10, TGF-β, IFNγ and IL-17 were added to Tregs twelve hours prior to co-culture with responder cells obtained from 4 AILD patients and 4 HS.

Following treatment with anti-IL-10 neutralising mAb, cTreg and $CD127^{neg}$ Treg ability to suppress did not change in AILD but decreased from 56% to 36% (p=0.02) and from 67% to 43% (p=0.01) respectively in HS. Treatment of cTregs and $CD127^{neg}$ Tregs with anti-TGF-β neutralising antibody left unchanged their ability to suppress responder cell proliferation in both AILD and HS.

Anti-IFN-γ neutralising antibody treatment did not change the ability of cTregs to suppress in HS, but tended to decrease it in patients with AILD (p=0.06) and left unchanged $CD127^{neg}$ Treg suppressive function in both groups. No change was observed in cTreg and $CD127^{neg}$ Treg ability to suppress following exposure to anti-IL-17 neutralising antibodies in both patients and HS. Treatment with anti-IFN-γ and anti-IL-17 neutralising antibodies increased $CD127^{pos}$ T cell suppression in AILD (anti-IFN-γ: from –3% to 19%, p=0.02; anti-IL-17: from –3% to 9%, p=0.04) and in HS (anti-IFN-γ: from –2% to 18%, p=0.05; anti-IL-17: from –2% to 13%, p=0.04; Figure 4.12).

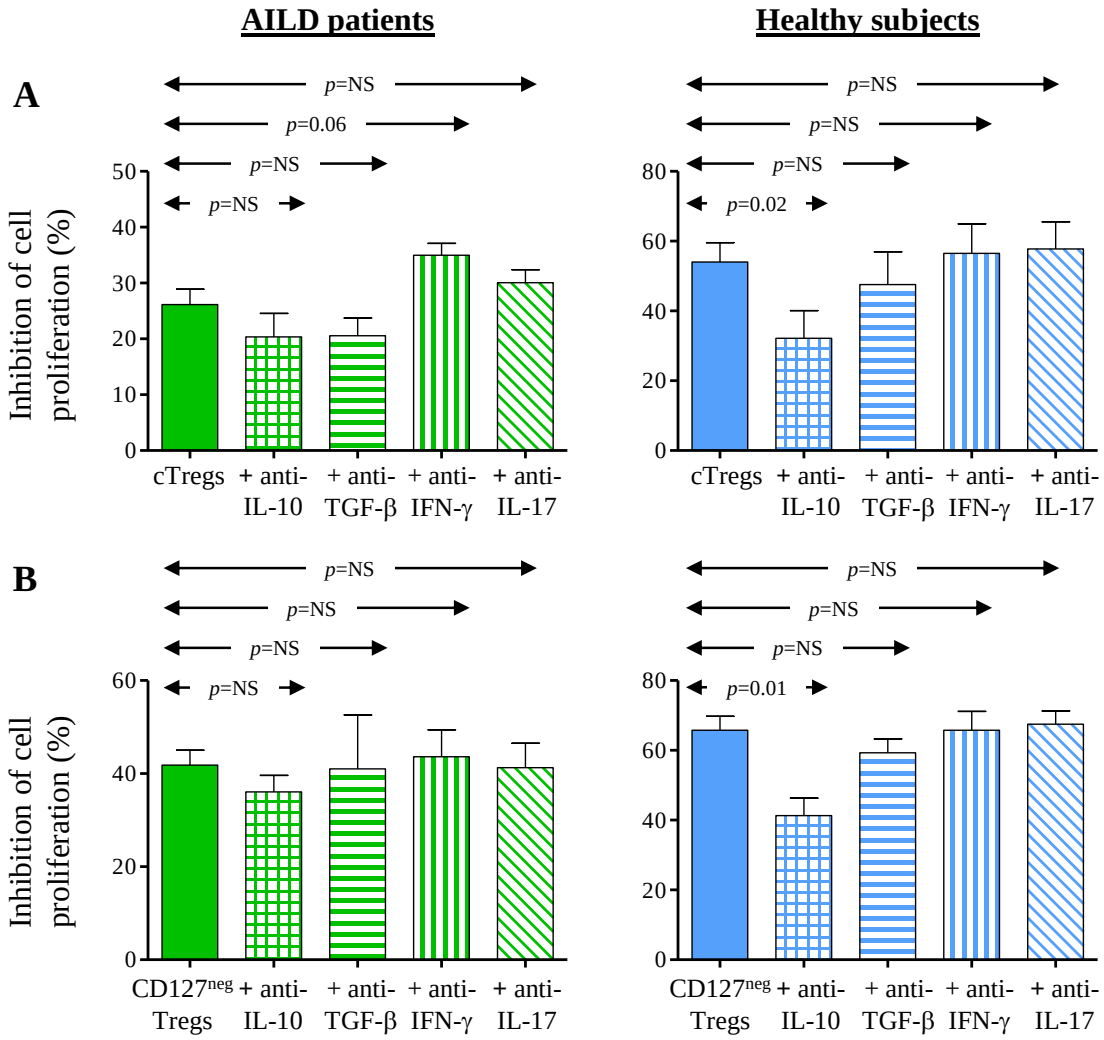


Figure 4.12. Effect of cytokine neutralisation on the ability of cTregs and CD127^{neg} T regs to suppress responder cell proliferation. Ability of cTregs (*Panel A*) and CD127^{neg} Tregs (*Panel B*) to suppress was tested in 8 AILD patients (*left plots*) and 6 healthy subjects (*right plots*) in the absence (*filled bars*) or presence of anti-IL-10 (*squared bars*), anti-TGF-β (*horizontal striped bars*), anti-IFN-γ (*vertical striped bars*) or anti-IL-17 (*diagonal striped bars*) neutralising antibodies. After 5-day co-culture, responder cell proliferation was assessed by ^{3}H-thymidine incorporation. Bars represent mean percentage (%) inhibition of responder cell proliferation by untreated or neutralising-antibody-treated responder cells. Error bars indicate SEM.

4. Discussion

This part of the study has extended recent work on regulatory T cells in AILD focusing on CD127^{neg} Tregs, a cell subset that, due to the lack of the activation molecule CD127, displays higher suppressor ability than cTregs (Seddiki et al. 2006,

Liu et al. 2006, Mazzucchelli and Durum 2007). In line with this, the results presented in this chapter provide evidence that $CD4^{pos}CD25^{pos}$ cells in AILD are highly enriched for CD127, whose expression allows the discrimination of a population of activated non-suppressive T cells within conventional regulatory cells.

While corroborating previous observations documenting a reduced frequency of circulating cTregs in AILD patients (Longhi et al. 2004, Ferri et al. 2010), this study shows that $CD127^{neg}$ Tregs are also numerically reduced in this condition. Given the pro-inflammatory phenotype of CD127 expressing cells, this finding implies that in AILD the impaired ability of cTregs to suppress is, at least in part, associated with high CD127 expression. Proof that $CD127^{neg}$ Tregs exert a suppressive control was supported by the observation of a strong negative correlation between the frequency of $CD127^{neg}$ Tregs and levels of transaminases and IgG and titres of autoantibodies, which are markers of disease activity.

That low levels of CD127 allow the identification of Tregs with highest ability to suppress was clearly shown in the current chapter by a series of experiments where cTregs and $CD127^{neg}$ cell subsets were tested for their ability to suppress the proliferation of $CD4^{pos}CD25^{neg}$ responder cells. The $CD127^{neg}$ fraction was the most capable of inhibiting responder T cell proliferation; suppressive capacity of $CD127^{neg}$ Tregs was however more evident in health than in AILD. Nevertheless, while in HS exclusion of CD127 only increased the suppressive activity of Tregs by 20%, in AILD it resulted in 60% increase when compared to cTregs, thus approximating its suppressive ability to that observed in HS and possibly reflecting the differential CD127 expression between the two groups of subjects.

Phenotypic analysis has shown that $CD127^{neg}$ Tregs isolated from both patients and HS display a more suppressive phenotype compared to cTregs, characterised by enhanced expression of FOXP3, CTLA-4 and IL-10. Interestingly, the difference in the level of FOXP3 and CTLA-4 expression between $CD127^{neg}$ Tregs and cTregs was more evident in AILD patients, once again approximating their levels to those observed in health. As previously reported (Liu et al. 2006, Codarri et al. 2007), we observed an inverse correlation between CD127 and FOXP3 expression, indicating that in AILD $CD127^{neg}$ cells, at variance with cTregs, contain high number of FOXP3-expressing cells. By showing that FOXP3 binds to the CD127 promoter region, Liu et al. proposed that CD127 down-regulation on Tregs may be consequent to direct repression of CD127 expression by FOXP3 (Liu et al. 2006).

Analysis of lymphocytes expressing CD45RO showed that $CD127^{neg}$ cells express higher frequencies of this memory cell marker compared to cTregs. This echoes a previous study reporting that $CD4^{pos}CD25^{pos}CD127^{neg}CD45RO^{pos}$ cells are associated with a higher ability to suppress (Seddiki et al. 2006). Compared to AILD, HS had higher expression of CD45RO, suggesting that lower CD45RO expression could account for the lower ability of $CD127^{neg}$ cells to suppress in patients with AILD. However, since the expression of these marker increases steadily with age (Booth et al. 2010), it is possible that the discrepancy in CD45RO expression just reflects the age disparity between patients and HS.

The frequency of IL-10-producing cells was consistently higher in Treg populations isolated from HS compared to AILD patients. In addition, neutralising experiments showing reduced Treg suppression following treatment with anti-IL-10 antibodies in HS but not in AILD indicate that the ability to secrete this immune-

regulatory cytokine is impaired in AILD, while in health it has a positive impact in the ability of Tregs to suppress.

Further phenotypic and functional analysis performed in this study showed that compared to cTregs, $CD127^{neg}$ Tregs contained fewer cells expressing T-bet, IFN-γ, RORC and IL-17 – signature transcription factors and cytokines of Th1 and Th17 cells respectively – this difference being more evident in AILD than in HS. In sharp contrast, analysis of $CD127^{pos}$ T cell subset showed the highest percentage of cells positive for these Th1 and Th17 markers both in health and disease compared to other $CD25^{pos}$ populations. In agreement with recent studies (Codarri et al. 2007, Michel et al. 2008), $CD127^{pos}$ cells did not suppress but instead tended to increase T cell proliferation. Interestingly, treatment of these cells with anti-IFN-γ and anti-IL-17 neutralising antibodies partially restored their ability to suppress. Codarri et al. implicated $CD4^{pos}CD25^{pos}CD127^{pos}$ T cells in human allograft rejection, showing that this population contains allospecific CD4 T cells, secrete effector cytokines, and is significantly more expanded in patients with chronic humoral rejection (Codarri et al. 2007). Collectively, these results indicate that the $CD4^{pos}CD25^{pos}CD127^{pos}$ T cell population does not contain cells with regulatory function, and its expansion in patients with AILD suggests that they may be involved in the process of liver damage.

In conclusion, the results presented in this chapter show that $CD127^{low}$ Tregs bear the phenotypic and functional signature of *bona fide* Tregs, thus defining CD127 as a good marker of regulatory T cells in human peripheral blood. Although reduced in patients with AILD, $CD127^{neg}$ Tregs are capable of exerting a superior suppressive activity when compared to cTregs, approximating the functional ability of those observed in health. On the other hand, the counterpart $CD127^{pos}$ T cell population,

which is expanded in AILD, may proliferate and produce pro-inflammatory cytokines more vigorously, and therefore inflict hepatocyte damage. These findings show that negative selection to remove the activated CD127^{pos} T cell population contaminating the pool of Tregs may be a useful tool for the selection and expansion of Tregs for both research and therapeutic applications.

CHAPTER V

Role of galectin-9 regulatory T cells in autoimmune liver disease

1. Background

The last chapter has confirmed and expanded the evidence that in AILD the extent of self-reactive CD4 T cell effector immune responses, and therefore the magnitude of liver injury, is associated with a numerical impairment of regulatory T cells. The results presented also show that $CD127^{neg}$ cells, though characterised by a more suppressive phenotype and function than cTregs, are in AILD impaired in their ability to control the proliferation of effector cells.

Although a plethora of molecular and cellular mechanisms have been described to explain the function of Tregs, their precise mode of suppression is still matter of debate. It is likely that a combination of several mechanisms, mediated either by direct cell contact or by the production of inhibitory cytokines, operate together depending on the milieu and the type of immune response. In the context of AILD, and akin to cell number, whether the impaired immune-regulation is mainly due to a primary Treg defect, or to a low responsiveness of CD4 effector cells to Treg control, is unclear.

Recent work performed in mice has shown that galectin-9 (GAL-9), a β-galactosidase-binding animal lectin belonging to the galectin family, inhibits Th1 effector immune responses after binding to the T-cell-imunoglobulin-and-mucin-domain-3 (TIM-3), its receptor on CD4 effector cells (Zhu et al. 2005). The immune-modulatory properties of GAL-9 have been clearly demonstrated in the context of: a) experimental autoimmune encephalomyelitis, where injection of GAL-9 into mice previously immunised with MOG peptide led to a loss of MOG-specific IFN-γ producing effector cells (Zhu et al. 2005); b) collagen induced arthritis, where GAL-9 administration resulted in disease amelioration by inducing synoviocytes apoptosis and Treg function and by suppressing the generation of Th17 cells (Seki et al. 2008).

In the human setting, Mengshol et al. reported that culture of PBMCs in the presence of GAL-9 results in the expansion of $CD4^{pos}CD25^{pos}FOXP3^{pos}CD127^{neg}$ Tregs, and is accompanied by contraction of $CD4^{pos}$ effector T cells and apoptosis of HCV-specific CTLs in both HCV infected patients and healthy subjects (Mengshol et al. 2010).

Within the non-activated immune system, GAL-9 is predominantly expressed on naïve $CD4^{pos}$ effector T cells and Tregs. Upon activation, GAL-9 is down-regulated on the effector T cell compartment, but importantly is maintained on Tregs (Sabatos et al. 2003, Sanchez-Fueyo et al. 2003). On this basis I have hypothesed that GAL-9 may be a mechanism of suppression employed by Tregs and that their functional impairment in AILD may be the result of defective GAL-9 expression.

Specific objectives of the current chapter are: 1) to determine the frequency of circulating and liver infiltrating $GAL\text{-}9^{pos}$ Tregs; 2) to provide a transcription factor and cytokine profiles of $GAL\text{-}9^{pos}$ and $GAL\text{-}9^{neg}$ T cell populations and 3) to explore to what extent GAL-9 impacts Treg ability to suppress by repressing its translation using small-interfering-RNA.

2. Subjects

The experiments included in this chapter were performed on PBMCs obtained from 58 AILD patients. Paraffin-embedded liver sections, available from 10 of those patients were also studied.

Thirty three (57%) patients were female. The median age at the time of study was 13.9 years, range 8.8-21.1 years (Table 5.1.). At the time of diagnosis, all 58 patients were positive for ANA and/or anti-SMA autoantibodies. A liver biopsy

performed at the time or close to diagnosis showed histological features of interface hepatitis in all patients. Viral, metabolic, and genetic causes of liver disease were excluded by appropriate investigations.

Table 5.1. Demographic, clinical and laboratory features of AILD patients at the time of the study.

Parameter	AILD (n=58)	[A] patients (n=20)	[R] patients (n=38)	*p**
Age in years	13.9 (8.8-21.1)	13.6 (9.3-19.5)	14.8 (8.8-21.1)	NS†
Female, n of patients (%)	33 (57%)	14 (70%)	19 (50%)	NS†
AST (nv<50 IU/L)	39 (16-1061)	88 (51-1061)	28 (16-49)	<0.0001‡
GGT (nv<50 IU/L)	39 (4-503)	85 (17-503)	25 (4-196)	<0.0001‡
Total bilirubin (nv<20 μmol/l)	9 (3-705)	11 (3-705)	7 (4-32)	0.008‡
AP (nv<350 IU/L)	211 (49-834)	225 (73-834)	197 (49-388)	NS‡
AP/AST ratio	4.46 (0.17-16.38)	2.45 (0.17-4.65)	6.32 (2.12-16.38)	<0.0001‡
INR (nv<1.2)	1.05 (0.89-2.06)	1.07 (0.90-2.06)	1.04 (0.89-1.31)	0.08‡
Albumin (nv>35 g/L)	45 (32-50)	44 (32-50)	46 (40-50)	NS‡
IgG (nv 6.5-17 g/L)	17.01 (5.70-43.96)	17.34 (6.83-43.96)	12.65(5.70-27.14)	0.007‡
ANA, n of positive patients (%)	21 (36%)	11 (55%)	10 (26%)	0.04†
SMA, n of positive patients (%)	18 (31%)	9 (45%)	9 (24%)	0.14†
pANNA, n of positive patients (%)	29 (50%)	10 (50%)	19 (50%)	NS†
AISC, n of positive patients (%)	29 (50%)	11 (55%)	18 (47%)	NS†
IBD, n of positive patients (%)	22 (38%)	9 (45%)	13 (34%)	NS†

Data presented as median (range) unless noted otherwise.
AILD, autoimmune liver disease; *AST*, aspartate aminotransferase; *GGT*, gamma-glutamyl transpeptidase; *AP*, alkaline phosphatase; *INR*, international normalised ratio; *IgG*, immunoglobulin G; *ANA*, anti-nuclear antibodies; *SMA*, anti-smooth muscle antibodies; *pANNA*, peripheral anti-nuclear neutrophil antibodies; *AISC*, autoimmune sclerosing cholangitis; *IBD*, inflammatory bowel disease; *nv*, normal value; *NS* non significant
* *p* value when comparing clinical parameters between [A] and [R] patients
† Fisher´s exact test
‡ Mann-Whitney test

Twenty-nine patients had bile duct changes characteristic of sclerosing cholangitis on retrograde cholangiography and were diagnosed as having AISC (Gregorio et al. 2001; Table 5.2.)

Table 5.2. Clinical and laboratory data comparing AIH and AISC patients.

Parameter	AIH-1 patients (n=29)	AISC patients (n=29)	*p**
Age in years	13.6 (8.8-21.1)	13.9 (9.1-17.6)	NS†
Female, n of patients (%)	21 (72%)	12 (41%)	0.06†
AST (nv<50 IU/L)	36 (16-1061)	38 (17-534)	NS‡
GGT (nv<50 IU/L)	35 (9-215)	38 (4-503)	0.10‡
Total bilirubin (nv<20 μmol/l)	8 (4-705)	9 (3-47)	NS‡
AP (nv<350 IU/L)	177 (49-340)	201 (68-834)	0.03‡
AP/AST ratio	4.13 (0.17-13.04)	4.44 (1.26-16.38)	0.15‡
INR (nv<1.2)	1.06 (0.89-2.06)	1.05 (0.89-1.78)	NS‡
Albumin (nv>35 g/L)	45 (38-50)	45 (32-50)	NS‡
IgG (nv 6.5-17 g/L)	13.50 (5.70-43.96)	14.63 (7.99-27.14)	NS‡
ANA, n of positive patients (%)	11 (38%)	10 (34%)	NS†
SMA, n of positive patients (%)	10 (34%)	8 (28%)	NS†
pANNA, n of positive patients (%)	10 (34%)	19 (66%)	0.02†
Active disease, n of patients(%)	10 (34%)	10 (34%)	NS†
IBD, n of patients (%)	5 (17%)	17 (59%)	0.001†

Data presented as median (range) unless noted otherwise.
AIH-1, autoimmune hepatitis type 1; *AISC*, autoimmune sclerosing cholangitis; *AST*, aspartate aminotransferase; *GGT*, gamma-glutamyl transpeptidase; *AP*, alkaline phosphatase; *INR*, international normalised ratio; *IgG*, immunoglobulin G; *ANA*, anti-nuclear antibodies; *SMA*, anti-smooth muscle antibodies*; pANNA*, peripheral anti-nuclear neutrophil antibodies; *IBD*, inflammatory bowel disease; *nv*, normal value; *NS* non significant
* *p* value when comparing clinical parameters between AIH-1 and AISC patients
† Fisher´s exact test
‡ Mann-Whitney test

Thirty-eight patients were in drug-induced remission ([R] patients), while 20 were studied during active disease ([A] patients). From 6 of 20 [A] patients, blood was obtained at diagnosis before the treatment was started; the remaining 14 [A] patients were studied during relapse. In one [R] patient blood was collected one year after successful treatment withdraw. All other patients were on immunosuppressive treatment, consisting of prednisolone either alone or in combination with azathioprine and/or MMF. In those diagnosed with AISC, UDCA was added to the treatment regimen.

Epidemiological, clinical and laboratory data for each patient are shown in Appendix I.

At the time of study, 22 AILD patients, including 5 with AIH-1 and 17 with AISC, had concomitant inflammatory bowel disease (IBD). Three of the 5 AIH-1 patients had ulcerative colitis (UC) while 2 had Crohn´s disease (CD); all the 17 AISC patients had UC.

Twenty-four HS, recruited from King's College Hospital staff members, served as normal controls; 16 were females and the median age was 29 years (range 22.6 to 23.7 years). The disparity between patients and HS age derives from ethical constraints in obtaining blood from healthy children. To test whether age disparity accounted for differences, patients were divided into 2 subgroups, (≤14 and >14 years old).

3. Results

3.1. Enumeration of circulating GAL-9^{pos} cells

The frequency of GAL-9^{pos} cells was analysed in 34 AILD patients and 20 HS. The percentage of GAL-9^{pos} cells within CD4^{pos}CD25^{pos} cells (cTregs) was lower in patients (32.57±2.86%) than in HS (53.85.4±3.13%, p<0.0001), this difference being evident also when the CD25^{low} (22.43±2.09% vs 38.42±2.87%, p<0.0001), CD25^{med} (29.69±2.49% vs 55.02±2.10%, p<0.0001) and CD25^{high} (37.68±2.88% vs 70.51±2.61%, p<0.0001) fractions were analysed separately (Figure 5.1.).

Analysis of CD4^{pos}CD25^{pos}CD127^{neg} cells showed that this subset contained higher percentage of GAL-9^{pos} cells (AILD: 44.43±2.61%; HS: 74.18±2.13%) compared to cTregs (AILD: p=0.005; HS: p<0.001). This percentage was however lower in AILD than in HS (p<0.001; Figure 5.2.).

The percentage of GAL-9^{pos} cells within cTregs inversely correlated with the titre of IgG (r= –0.49, p=0.003) and, to a lesser extent, with that of ANA (r= –0.31, p=0.07; Figure 5.3.).

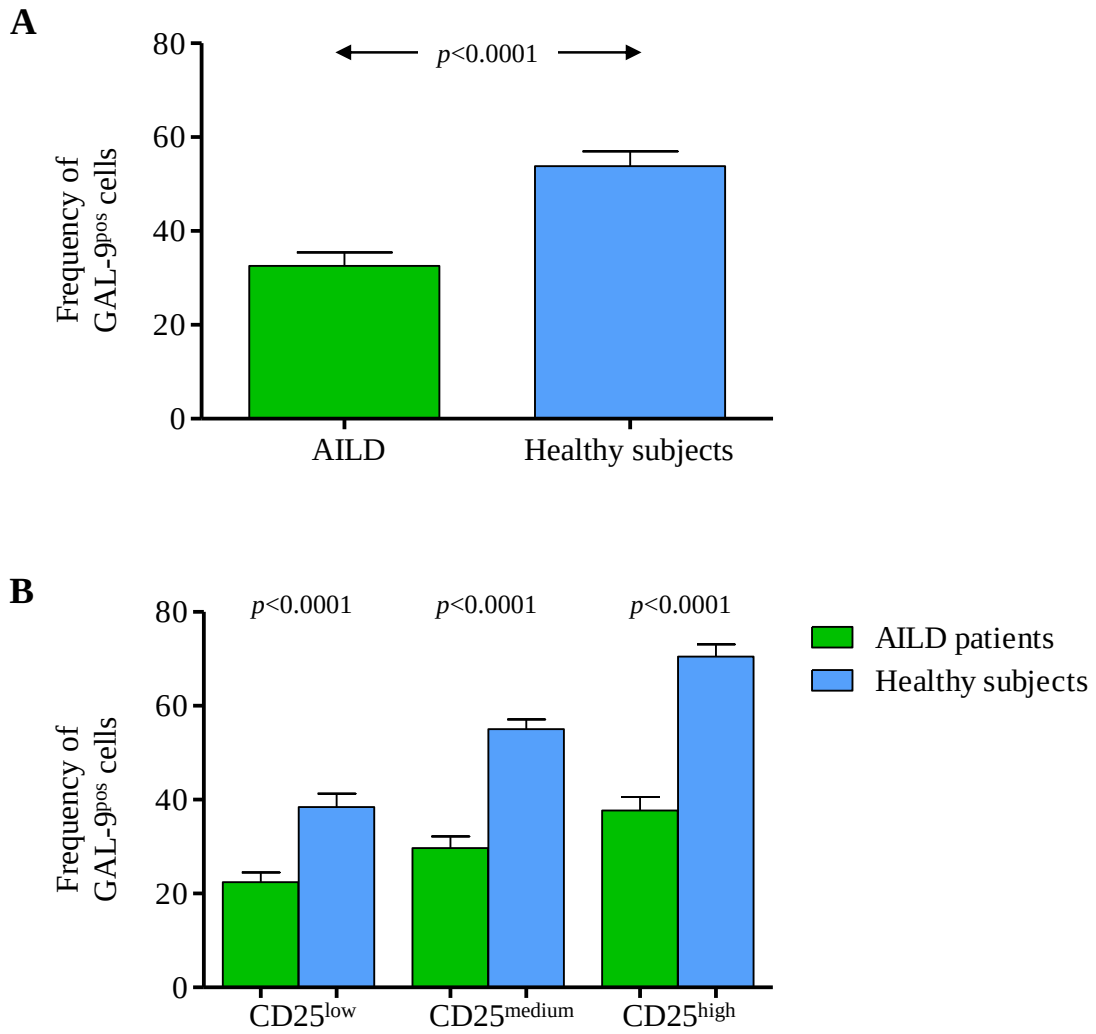


Figure 5.1. Percentage of GAL-9^{pos} lymphocytes within Treg subsets. Percentage of GAL-9^{pos} lymphocytes within unfractionated $CD4^{pos}CD25^{pos}$ cells (*Panel A*) and within the $CD4^{pos}CD25^{low}$, $CD4^{pos}CD25^{medium}$ and $CD4^{pos}CD25^{high}$ subsets (*Panel B*) in AILD patients (*green bars*, n=34) and healthy subjects (*blue bars*, n=20). GAL-9^{pos} cells were lower in AILD than in health both when undivided $CD25^{pos}$ cells and when individual CD25 low, medium and high cell fractions were analysed. Error bars represent SEM.

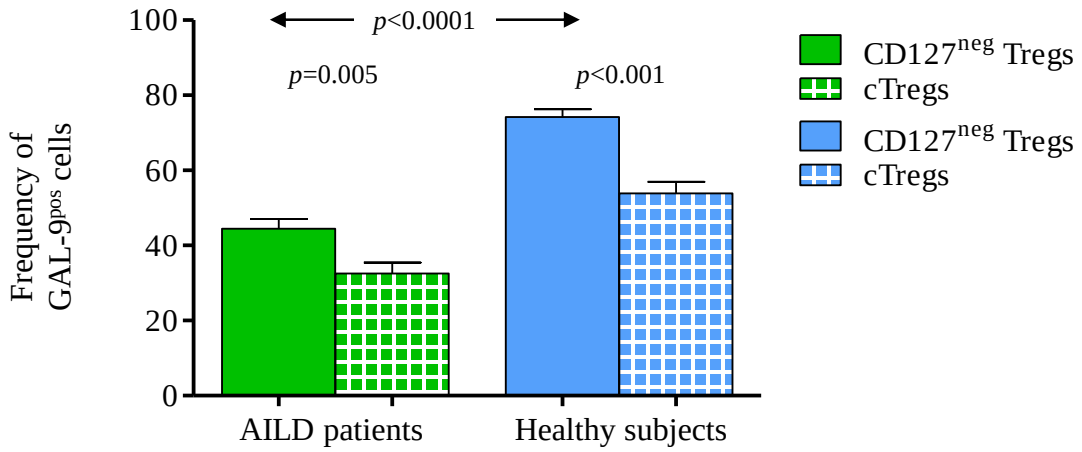


Figure 5.2. Frequency of GAL-9^{pos} cells within CD127^{neg} Tregs and cTregs. FACS analysis of PBMCs from healthy subjects (*A*) and AILD patients (*B*) revealed a higher GAL-9 expression within CD127^{neg} Tregs compared to cTregs. The plots summarise data from 34 AILD patients and 20 healthy subjects. Error bars depict SEM.

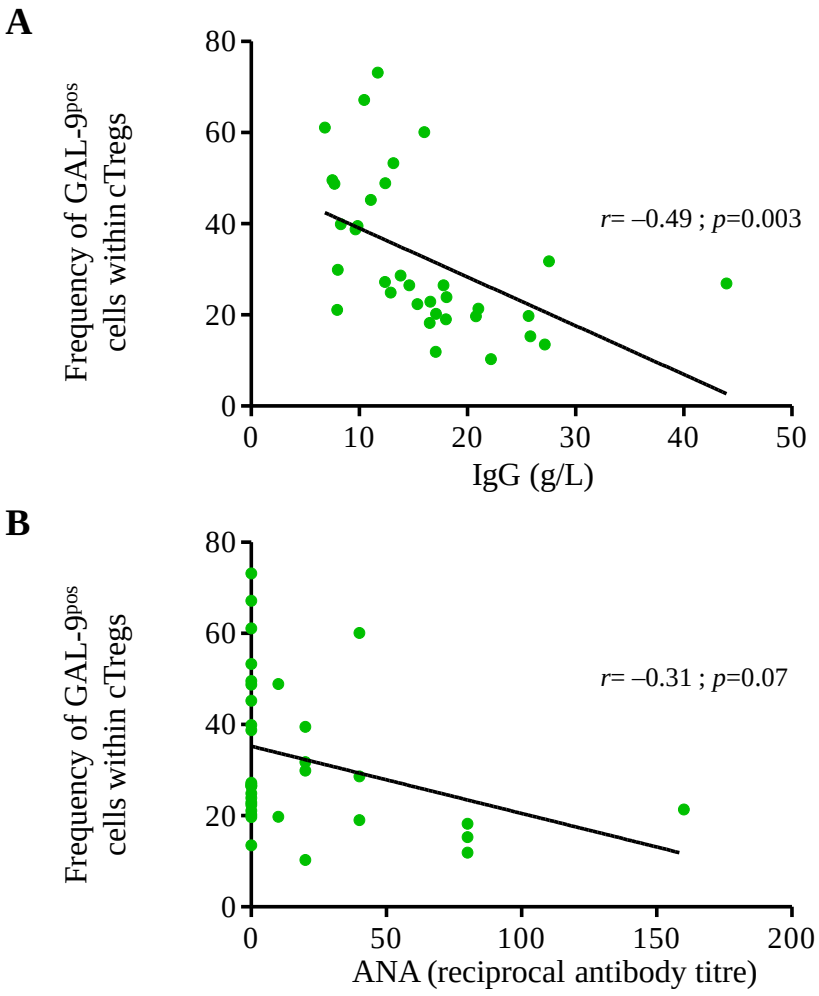


Figure 5.3. Correlation between the frequency of GAL-9^{pos} cells and indices of disease activity. In AILD patients the frequency of GAL-9^{pos} cells within cTregs negatively correlated with IgG levels (*Panel A*), and to a lesser extent ANA titres (*Panel B*).

The frequency of GAL-9^{pos} cells was lower in [A] patients (31.8±3.71%) compared to [R] patients (36.78±3.68%, *p*=0.03). Within [A] patients no differences were observed between those studied at diagnosis and those studied during an episode of relapse. Within AILD patients, no differences in terms of GAL-9^{pos} cells within cTregs were observed between the two age groups (≤14 years: 30.80±4.52%; >14 years: 34.94±3.90%, *p*=NS), between AIH and AISC (32.45±3.18% vs 32.69±5.69%, *p*=NS), and between those with or without concomitant IBD (37.75±7.26 vs 30.42±2.71%, *p*=NS, Figure 5.4.).

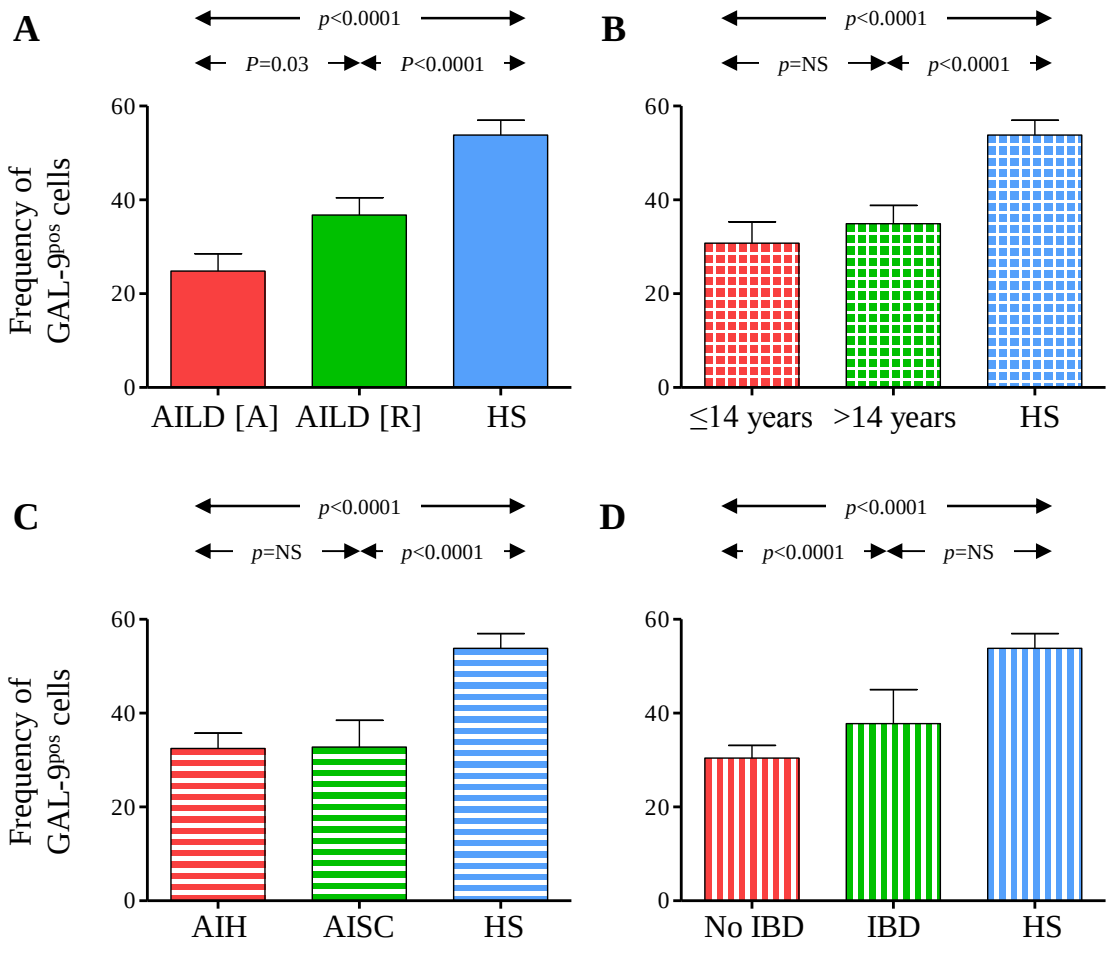


Figure 5.4. Frequency of GAL-9^{pos} cells within cTregs. The frequency of GAL-9^{pos} cells within cTregs was higher in patients with active disease compared to those studied during drug-induced remission (*Panel A*). No differences in the frequency of GAL-9^{pos} lymphocytes were observed between age groups (*Panel B*), between AIH and AISC (*Panel C*), and between those with and without concomitant IBD (*Panel D*). The plots summarise data from 20 healthy subjects and 34 AILD patients. Error bars represent SEM.

3.2. Phenotype of GAL-9^{pos} T cells

Transcription factor and cytokine profile of CD4^{pos}CD25^{pos}GAL-9^{pos} ("GAL-9^{pos} Tregs") and CD4^{pos}CD25^{pos}GAL-9^{neg} ("GAL-9^{neg} Tregs") populations was performed in 15 AILD patients and 15 HS.

Both in AILD and HS the frequency of FOXP3^{pos} cells was higher within GAL-9^{pos} (HS: 42.85±3.03%; AILD: 14.41±2.01%) than GAL-9^{neg} Tregs (HS: 16.45±1.48%, p<0.001; AILD: 2.62±0.51, p<0.001). In AILD the GAL-9^{pos} and the GAL-9^{neg} Treg subsets contained fewer FOXP3^{pos} cells than in HS (p<0.001 for both).

The frequency of RORC^{pos} cells was lower in the GAL-9^{pos} (HS: 0.92±0.38%; AILD: 1.08±0.60%) than in the GAL-9^{neg} Treg cell fraction (HS: 4.42±0.85%, p=0.02; AILD: 8.62±0.80%, p=0.005). GAL-9^{neg} Tregs contained higher frequencies of RORC^{pos} cells in AILD than in HS (p=0.04), while such a difference was not observed in the GAL-9^{pos} compartment.

GAL-9^{pos} and GAL-9^{neg} Treg populations had similar proportions of Tbet^{pos} and GATA-3^{pos} lymphocytes, with no difference observed between health and disease (Figure 5.5.).

Analysis of the cytokine profile (Table 5.3.) showed that the frequency of IL-10^{pos} cells was higher in the GAL-9^{pos} than in the GAL-9^{neg} cell fraction (HS: p<0.001; AILD: p=0.001). The GAL-9^{pos} fraction contained less IL-10^{pos} cells in AILD than in HS (p<0.001) whereas such a difference between the two groups was not evident when the GAL-9^{neg} subset was analysed. The percentage of TGF-β-producing cells was lower in the GAL-9^{pos} cells isolated from AILD patients than in those from HS (p=0.04). While in HS TGF-β^{pos} cells tended to be more frequent within GAL-9^{pos} than within GAL-9^{neg} fraction (p=0.07), such a trend was not observed in patients.

Regarding pro-inflammatory cytokines, the percentage of IFN-γ^{pos} lymphocytes was higher in the GAL-9^{pos} cells isolated from AILD patients than in those obtained from HS (p=0.002). The same tendency between subjects was observed regarding IFN-γ production by GAL-9^{neg} population (p<0.001). However, when GAL-9^{pos} and GAL-9^{neg} populations were compared they showed similar numbers of IFN-γ^{pos} cells both in AILD and HS. The frequency of IL-17^{pos} cells was higher in AILD than in HS both when GAL-9^{pos} (p=0.002) and GAL-9^{neg} (p<0.001) populations were analysed. While in health the percentage of IL-17^{pos} cells did not differ between GAL-9^{pos} and GAL-9^{neg} subsets, in AILD it was significantly higher in the latter compared to the former (p=0.01).

No differences were observed in the frequency of IL-2-producing cells between GAL-9^{pos} and GAL-9^{neg} cells in both groups of subjects.

Table 5.3. Transcription factor and of cytokine profile of CD4^{pos}CD25^{pos}GAL-9^{pos} and GAL-9^{neg} cells.

% of cells	GAL-9^{pos} Tregs			GAL-9^{neg} Tregs				
	AILD	HS	p^1	AILD	HS	p^2	$p^{\dagger}$	$p^{\ddagger}$
IL-10	5.15±0.61	9.11±0.54	<0.001	2.35±0.16	3.29±0.39	NS	0.001	<0.001
TGF-β	6.35±0.66	8.03±0.44	0.04	5.29±0.53	5.51±1.15	NS	NS	0.07
IFN-γ	4.39±0.57	2.11±0.35	0.002	5.74±0.78	2.17±0.42	<0.001	NS	NS
IL-17	4.08±0.62	1.75±0.22	0.002	7.95±1.17	2.29±0.67	<0.001	0.01	NS
IL-2	8.80±0.70	10.92±1.21	NS	8.34±0.83	9.38±1.55	NS	NS	NS

HS, healthy subjects; *AILD*, autoimmune liver disease; *NS*, non significant
Data are represented as mean±SEM
p values comparing the percentage of IL-10^{pos}, TGF-β^{pos}, IFN-γ^{pos}, IL-17^{pos}, and IL-2^{pos} cells:
p^1: within the GAL-9^{pos} subset between AILD and healthy subjects
p^2: within the GAL-9^{neg} subset between healthy AILD and healthy subjects
$p^{\dagger}$: between the GAL-9^{pos} and the GAL-9^{neg} subset in AILD
$p^{\ddagger}$: between the GAL-9^{pos} and the GAL-9^{neg} subset in healthy subjects
Data referring to 20 healthy subjects and 34 AILD patients and 20 healthy subjects

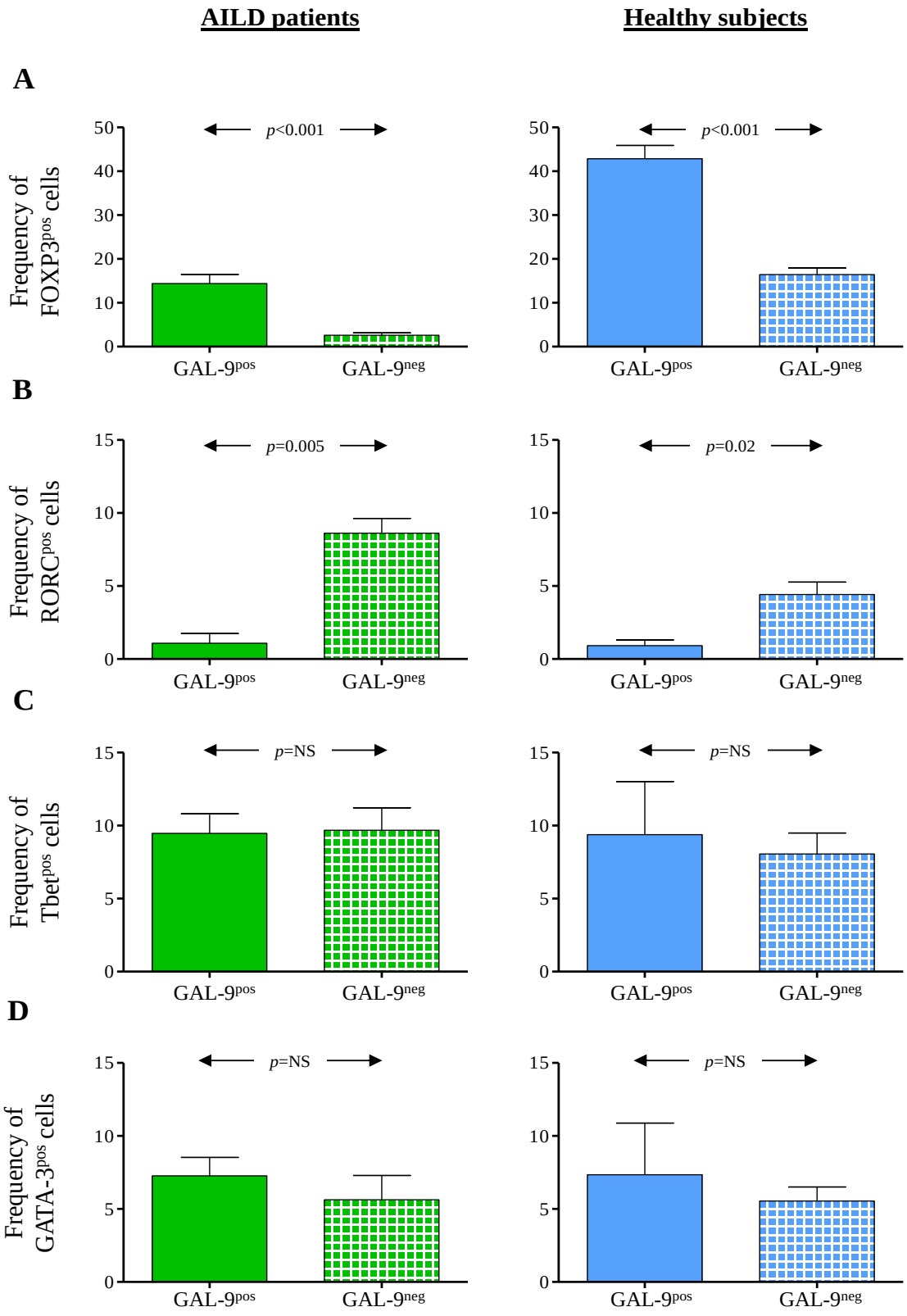


Figure 5.5. Transcription factor profile of GAL-9^{pos} and GAL-9^{neg} Treg subsets. Frequency of FOXP3^{pos} (*A*), RORC^{pos} (*B*), Tbet^{pos} (*C*), and GATA-3^{pos} (*D*) cells within CD4^{pos}CD25^{pos}GAL-9^{pos} (*filled bars*) and GAL-9^{neg} (*squared pattern bars*) cell subsets in 15 patients (*left plots*) and healthy subjects (*right plots*). Error bars represent SEM.

3.3. *Galectin-9* gene knockdown

The effect of *GAL-9* gene silencing on the ability of cTregs and $CD127^{neg}$ Tregs to suppress was investigated in 20 AILD patients and 8 HS. The suppressor function of untreated and siRNA-treated Tregs was tested using autologous $CD25^{neg}$ cells as responders. Treatment of Tregs with GAL-9-RNAi led to a decrease in the expression of *GAL-9* gene by 86% in AILD and by 88% in HS.

Inhibition of cell proliferation observed with untreated cTregs was reduced by 44% in AILD (from 25% with untreated to 14% with siRNA-treated Tregs , $p<0.001$) and by 67% in HS (from 49% to 16%, $p<0.001$) . Treatment with GAL-9-RNAi, reduced the suppressor function of $CD127^{neg}$ Treg suppressor by 54% (from 46±3.14% to 21%, $p<0.001$) in AILD and 67% (from 60% to 21±1.76%, $p<0.001$) in HS (Figure 5.6.)

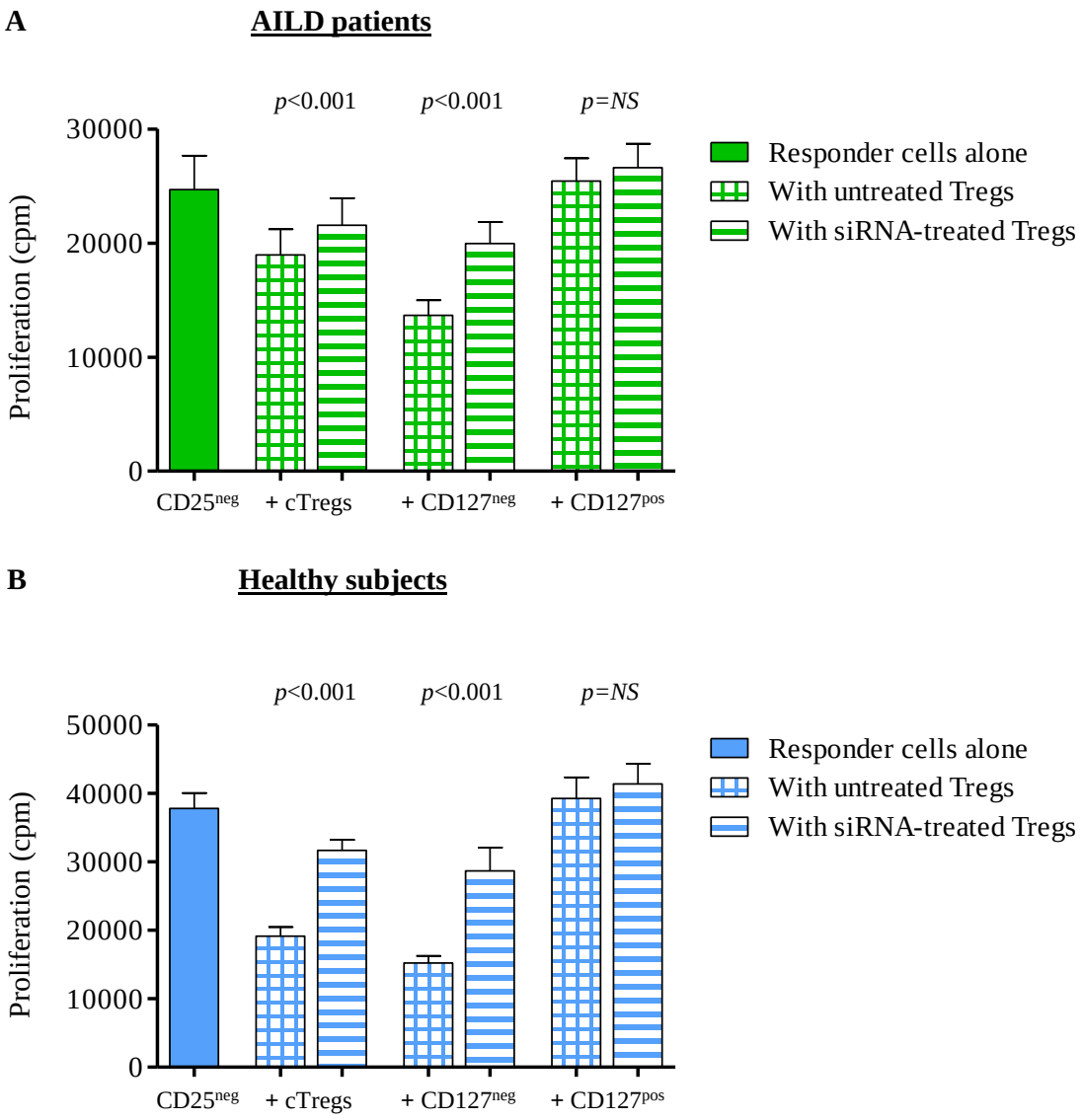


Figure 5.6. Suppressive function of untreated and GAL-9 siRNA-treated regulatory T cells. The plots show the proliferation (expressed as counts per minute [cpm]) of $CD25^{neg}$ responder cells cultured alone (*filled bars*) or in combination with untreated (*square patterned bars*) or siRNA-treated (*horizontal striped bars*) cTregs, $CD127^{neg}$ Tregs and $CD127^{pos}$ T cells in AILD (*Panel A*) and healthy subjects (*Panel B*). Error bars represent SEM. Data representative of 20 AILD patients and 8 healthy subjects.

In 2 HS, the effect of *GAL-9* gene knockdown on Treg suppression was also evaluated after FACS-sorting Tregs according to their CD25 expression. $CD25^{high}$ Treg suppressor function was markedly reduced by GAL-9 siRNA treatment: inhibition of $CD25^{neg}$ cell proliferation was 57% following addition of untreated and 14% after addition of GAL-9 siRNA-treated $CD25^{high}$ Tregs. No difference in the ability of

$CD25^{low}$ Tregs to suppress was noted before and after GAL-9 siRNA treatment (25% vs 27%, Figure 5.7.).

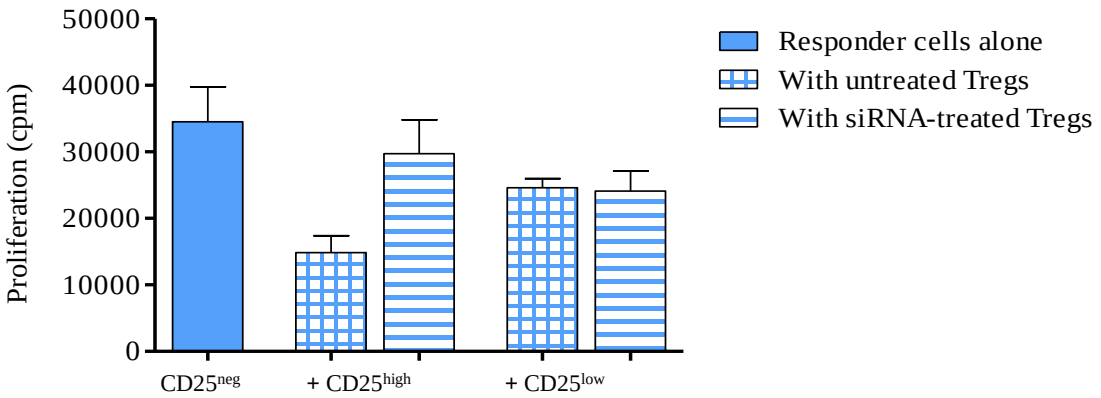


Figure 5.7. Effect of GAL-9 siRNA on $CD25^{high}$ and $CD25^{low}$ cells. The graph shows the proliferation (expressed as counts per minute [cpm]) of $CD25^{neg}$ responder cells cultured alone or in combination with untreated (square patterned bars) or siRNA-treated (horizontal striped bars) $CD4^{pos}CD25^{high}$ and $CD4^{pos}CD25^{low}$ cell subsets. Error bars represent SEM. Data representative of 2 healthy subjects.

3.4. Neutralisation assay

To explore whether Treg control over responder cell proliferation is influenced by secretion of inhibitory cytokines, anti-IL-10 or anti-TGF-β neutralising antibodies were added to Tregs prior co-culturing them with $CD25^{neg}$ as well as purified $TIM\text{-}3^{pos}$ and $TIM\text{-}3^{neg}$ target cells obtained from 4 AILD patients and 4 HS.

Following treatment with anti-IL-10 mAb, Treg inhibition of $CD25^{neg}$ cell proliferation did not change in AILD but was decreased from 51% to 34% (p=0.07) in HS. Anti-IL-10 neutralising antibody treatment resulted in no change in the ability of Tregs to suppress $TIM\text{-}3^{neg}$ cells, but decreased Treg inhibition over $TIM\text{-}3^{pos}$ cell proliferation from 42% to 36% (p=0.06) in AILD and from 56% to 48% (p=0.04) in HS. Treatment with anti-TGF-β did not change Treg inhibition of $CD25^{neg}$, $TIM\text{-}3^{neg}$ and $TIM\text{-}3^{pos}$ cell proliferation both in AILD patients and HS (Figure 5.8.).

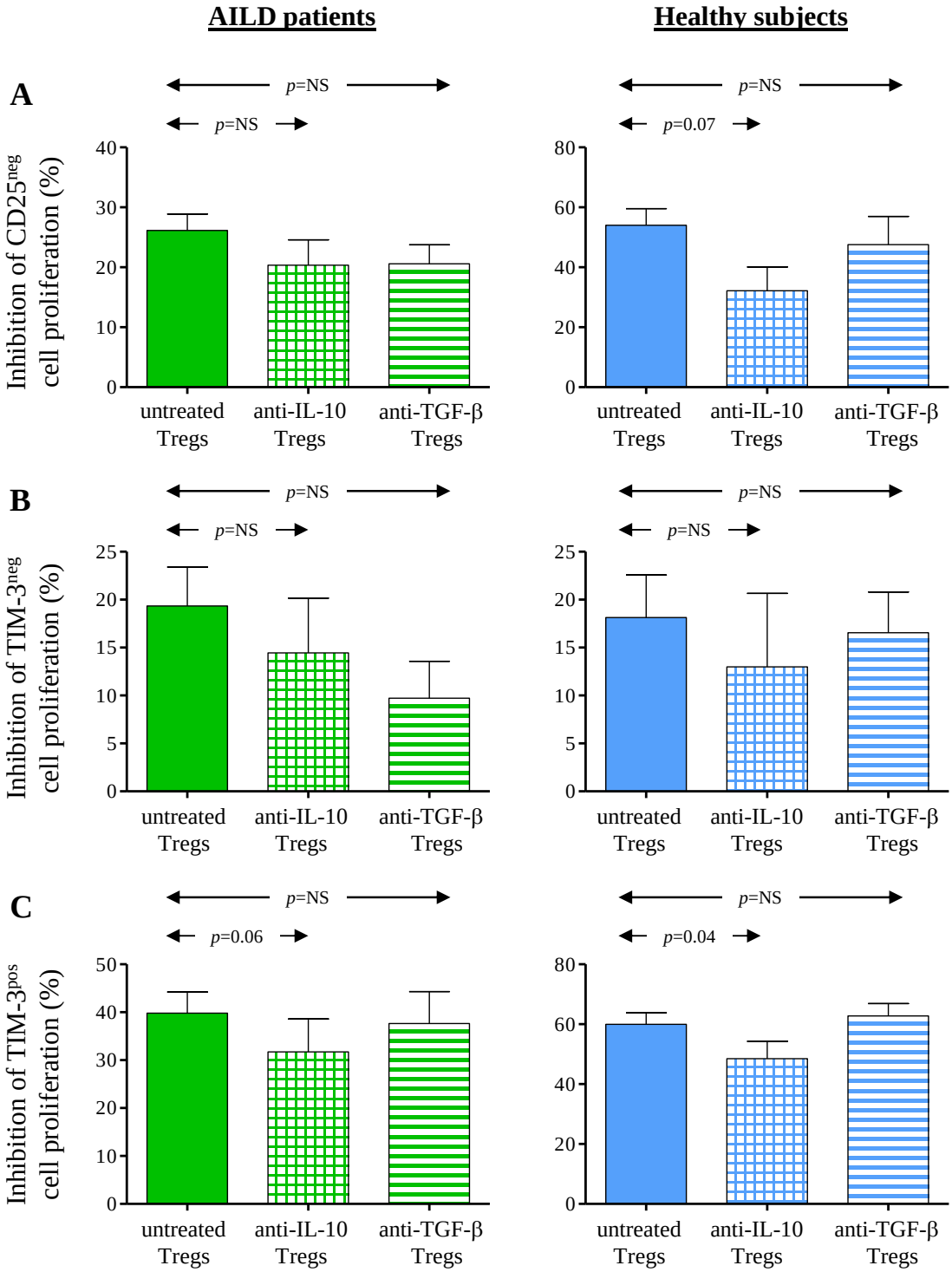


Figure 5.8. Effect of cytokine neutralisation on the ability of Tregs to inhibit proliferation of CD25^{neg}, TIM-3^{neg} and TIM-3^{pos} responder cells. CD25^{neg} (*Panel A*), TIM-3^{neg} (*Panel B*), and TIM-3^{pos} cells (*Panel C*) purified from 4 AILD patients (n=4, *left plots*) healthy subjects (n=4, *right plots*) were cultured with Tregs either untreated or treated with anti-IL-10 (*squared bars*) or anti-TGF-β (*horizontal striped bars*) neutralising antibodies. After 5-day co-culture, responder cell proliferation was assessed by ^{3}H-thymidine incorporation. Bars represent mean + SEM percentage (%) inhibition of responder cell. Error bars represent SEM.

3.5. Immunohistochemistry

The frequency of liver infiltrating GAL-9^{pos} lymphocytes was sought by immunohistochemistry in 13 liver biopsies available from 10 AILD patients. All patients were studied during a period of histologically active disease, and 3 of them were also investigated during inactive disease. The numbers of CD4^{pos}FOXP3^{pos}GAL-9^{pos} lymphocytes were counted at three high-power fields (x400) in each case

GAL-9^{pos} cells were detected in the portal tracts of 10 of 13 liver biopsy samples (Figure 5.9.).

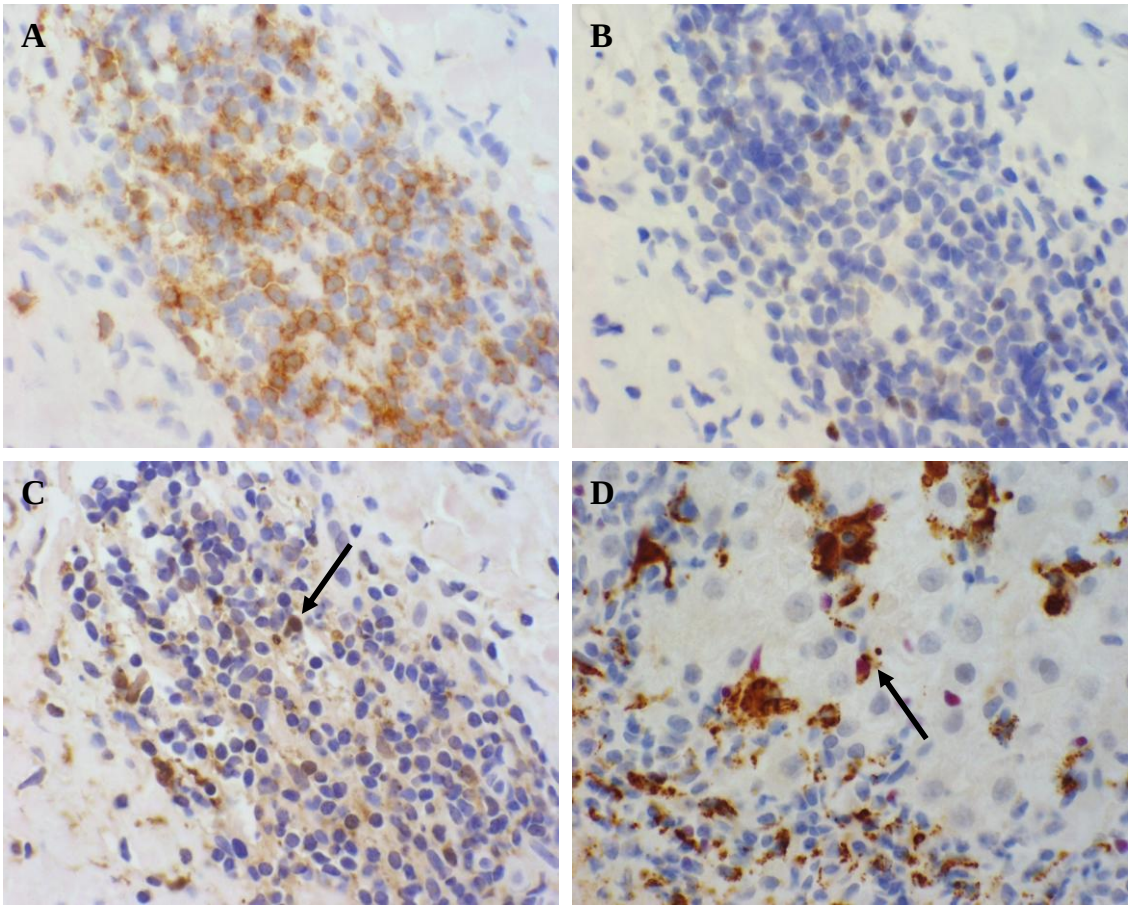


Figure 5.9. Liver infiltrating FOXP3^{pos} and GAL-9^{pos} lymphocytes. The number of CD4^{pos} lymphocytes was counted at three high-power fields (x400, *A*). Lymphocytes positive for FOXP3 and CD4 were counted at the same places (*B*). Similarly GAL-9^{pos} and CD4^{pos} lymphocytes were also counted at the same sample sections (*C*). Presence of lymphocytes positive for both GAL-9 and FOXP3 was confirmed by double immunostaining (*D*).

The percentage of $CD4^{pos}$ cells positive for GAL-9 was 4.70±2.15%, and that of $FOXP3^{pos}$ cells was 13.89±3.62%. The frequency of GAL-9^{pos} lymphocytes within $CD4^{pos}$ cells correlated with that of $FOXP3^{pos}$ cells (r=0.81, p=0.0008; Figure 5.10.) FOXP3/GAL-9 double staining showed that GAL-9 and FOXP3 are co-expressed in the lymphocytes infiltrating the portal areas. However, many $FOXP3^{pos}$ cells lack GAL-9 expression, thus mirroring the results obtained from the peripheral blood. In addition to lymphocytes, GAL-9 strongly stained Kupffer and endothelial cells; some spindle cells in the portal tracts were also positive for GAL-9.

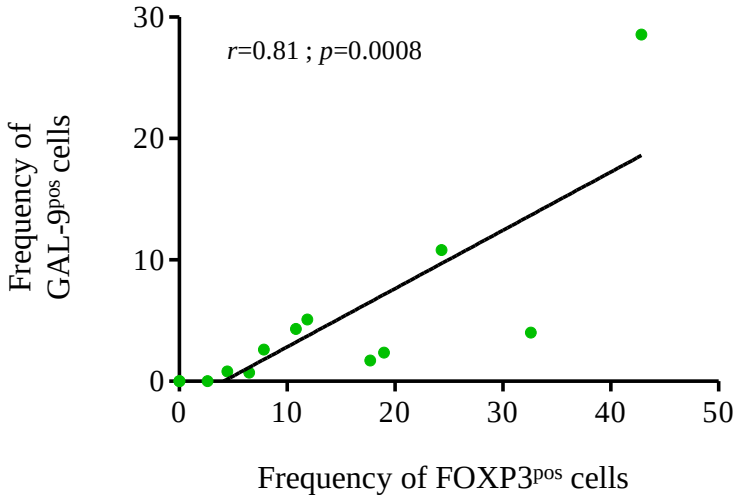


Figure 5.10. Correlation between the frequency of GAL-9^{pos} and $FOXP3^{pos}$ liver infiltrating lymphocytes. The frequency of GAL-9^{pos} cell correlates with that of $FOXP3^{pos}$ lymphocytes in patients with AILD.

4. Discussion

Although Tregs have the ability to inhibit the activation, proliferation and effector function of different immune cells, its exact mechanism of action and what causes its defect in autoimmunity is not yet known. This chapter explores the expression of GAL-9, a molecule known to inhibit Th1 immune responses, as a potential

mechanism of Treg-mediated suppression as well as its putative role in the defective immune-regulation in AILD.

The results presented in this chapter provide evidence that defective GAL-9 expression is one of the mechanisms responsible for Treg impairment in AILD. The role of GAL-9 in the ability of Tregs to exert their function has been highlighted by gene knockdown experiments where treatment of Tregs with a set of GAL-9-specific stealth RNAi led to a less effective control of responder cells´ proliferation, particularly in the case of treated-$CD25^{high}$ Tregs. The observed inverse correlation between the frequency of GAL-9^{pos} cells and the levels of IgG and titres of SMA, both serological markers of disease, suggests that GAL-9^{pos} Tregs exert control over disease activity. These results echo a murine study where in the context of autoimmune encephalomyelitis GAL-9 was shown to have a direct effect on disease severity, as mice immunised with a MOG immunodominant peptide and injected *in vivo* with GAL-9 had a less severe disease course and a lower mortality rate than mice that were not injected (Zhu et al. 2005).

Phenotypic analysis has shown that GAL-9^{pos} Tregs have a more suppressive phenotype compared to their negative counterparts, being characterised by higher FOXP3 expression and more IL-10 production. Indeed, the vast majority of IL-10^{pos} cells in both health and disease were found within GAL-9^{pos} Treg subset. Neutralisation experiments showing reduced Treg suppression following treatment with anti-IL-10 neutralising antibodies indicate that GAL-9^{pos} Tregs partly act through IL-10 production/secretion. Taken together, these findings suggest that GAL-9^{pos} Tregs, in addition to regulating effectors *via* induction of their apoptosis by binding to TIM-3 (Kashio et al. 2003, Zhu et al. 2005), can deliver suppression *via* IL-10 secretion.

Relatively recently, it has been shown that Treg and Th17 cells originate from the same progenitor, having a reciprocal induction during differentiation (Bettelli et al. 2006). While the concomitant presence of TGF-β and IL-6 leads to the differentiation of naïve $CD4^{pos}$ cells into Th17 cells, the presence of TGF-β alone leads to differentiation into Tregs, but prevents that into Th17 cells (Chen et al. 2003, Veldhoen et al. 2006). Seki et al. showed in the context of CIA that administration of exogenous GAL-9, by decreasing IL-6 production, leads to complete suppression of IL-17 expression at both gene and protein levels, even upon stimulation with high levels of TGF-β and IL-6 (Seki et al. 2008). In line with this, the results presented in this chapter suggest that the loss of GAL-9 expression within $CD4^{pos}CD25^{pos}$ T cells in AILD is mirrored by a reciprocal increase in RORC and IL-17 expression by the now GAL-$9^{neg}CD4^{pos}CD25^{pos}$ cells. Whether these GAL-9^{neg} cells correspond to the population of pathogenic effector "ex-FOXP3" cells, recently reported in a murine model of autoimmune diabetes as promoters of inflammation through the production of IL-17 (Zhou et al. 2009), is unknown. Nonetheless, GAL-9 seems then to play a key role in preventing Th17 differentiation both in mice and in humans. Thus, in view of the potential application of Tregs as an immunotherapeutic tool for the treatment/cure of AILD, the role of GAL-9 as a marker for the identification of stable, non-contaminated Tregs should be investigated. For that purpose, future studies should also focus on the phenotypic and functional stability of human GAL-9^{pos} Tregs over time and upon pro-inflammatory challenges.

Akin to the results in the circulation, in this study GAL-9^{pos} lymphocytes were detected in most liver biopsy samples from AILD patients, but they represented a very small component of the florid portal tract inflammatory infiltrate, hence mirroring the

picture observed in the peripheral blood. Although no healthy or pathological controls were studied, a recent report has demonstrated a high percentage of GAL-9^{pos} cells in the periportal area of patients with chronic HCV infection, and to a lesser extent in normal tissue obtained from donors evaluated for living-related liver transplantation or patients undergoing resection for metastatic adenocarcinoma (Mengshol et al. 2010).

Interestingly, in the present study the frequency of liver-infiltrating GAL-9^{pos} cells negatively correlated with that FOXP3^{pos} lymphocytes, suggesting that GAL-9, besides being pivotal to Tregs function, may also be involved in their induction. In line with this, studies performed in different murine models of autoimmunity (Seki et al. 2008, Sehrawat et al. 2009, Lv et al. 2011) consistently showed that treatment with GAL-9 resulted in up-regulation of FOXP3 mRNA expression and in expansion of TGF-β induced Tregs, leading to improvements in disease severity. Similar results have also been reported in the human setting, where culture of PBMCs in the presence of GAL-9 resulted in the expansion of CD4^{pos}CD25^{pos}FOXP3^{pos}CD127^{neg} Tregs both in HCV infected patients and healthy subjects (Mengshol et al. 2010). On the other hand, GAL-9 deficient mice had significantly decreased numbers of Tregs (Seki et al. 2008). Collectively, these data indicate that GAL-9 regulates, at least in part, the differentiation, maintenance and expansion of FOXP3^{pos} Tregs. Of interest, immunohistochemical studies presented in this chapter show that cells other than Tregs, particularly Kupffer and to a lesser extent endothelial cells, express GAL-9. Due to the absence of a control group, we do not know whether the frequencies of these GAL-9^{pos} cells are reduced or not in the livers of AILD patients. However, the study by Mengshol et al. showed that patients with chronic HCV, concomitantly to an enhanced Treg function, had a higher intensity of GAL-9 staining within Kupffer cells compared to

controls (Mengshol et al. 2010). In view of this, the contribution of GAL-9 either expressed by other immune cell types or given exogenously should be addressed in the context of AILD. If the link between macrophage-derived GAL-9 and a selective expansion of Tregs in the liver environment proves to be correct, GAL-9 may in the future be successful in restoring tolerance to liver self-antigens by enhancing the function and numbers of Tregs.

CHAPTER VI

Contribution of TIM-3 to the determination of effector T cell fate in autoimmune liver disease

1. Background

Whether the impaired immune-regulation in AILD is the result of a numerical and functional Treg defect, as highlighted in chapters IV and V, or is also due to low responsiveness of CD4 effector cells to Treg control, is unknown. Indeed, whereas many of the molecular and cellular mechanisms that regulate the function of regulatory T cells, at least in health, are understood, the molecules that determine the fate of effector T cell populations remain to be elucidated.

The immunoglobulin superfamily member TIM-3 was first identified as a specific cell marker of mouse Th1 CD4 cells (Monney et al. 2002) and was shown to be selectively expressed on the surface of fully differentiated Th1 but not Th2 cells (Rodriguez-Manzanet et al. 2009).

A number of studies conducted in animals have provided evidence of the role of TIM-3 in the modulation of Th1 immune responses For example, in the context of experimental allergic encephalomyelitis, *in vivo* administration of TIM-3 monoclonal antibody worsens the disease leading to severe brain inflammation (Monney et al. 2002). Moreover, blockade of TIM-3 on Th1 cells through the administration of the same anti-TIM-3 antibody accelerated diabetes development in NOD mice and prevented transplantation tolerance in a model of islet allograft (Sanchez-Fueyo et al. 2003, Sabatos et al. 2003). Further studies demonstrated that TIM-3, upon interaction with its ligand GAL-9, promotes termination of Th1 cell responses (Zhu et al. 2005).

Down-regulation of TIM-3 has been reported in T cell clones established from the cerebrospinal fluid of patients with multiple sclerosis and found to be associated with high levels of IFN-γ secretion (Koguchi et al. 2006, Li Yang et al. 2008).

Collectively, all these studies indicate that TIM-3 plays a role in modulating Th1 immune responses and that its expression has a role in limiting effector cell pathogenicity and therefore in promoting tolerance. The recent observation that effector Th17 cells express low levels of TIM-3 (Nakae et al. 2007b) raises the possibility that TIM-3 plays a role in the regulation of this subset. In view of this, I hypothesise that impaired expression of TIM-3 renders CD4 effector cells less amenable to Treg control in AILD.

Specific objectives of the current chapter are 1) to provide a phenotypic characterisation of effector CD4 T cells in AILD; 2) to determine the frequency of circulating and liver infiltrating TIM-3^{pos} effector cells; 3) to assess the transcription factor and cytokine profiles of TIM-3^{pos} and TIM-3^{neg} T cell subsets and 4) to test the susceptibility of TIM-3^{pos} and TIM-3^{neg} effector T cells to Treg control.

2. Subjects

For this part of the study fifty four patients with ANA and/or SMA positive AILD were investigated. Thirty one (57%) patients were female. The median age at the time of study was 14.3 years (range 8.2-21.1). Thirty seven patients were studied during drug-induced remission ([R] patients) while 17 patients had active disease ([A] patients) at the time of investigation. Five [A] patients was studied at diagnosis before treatment was started; the remaining 12 [A] patients were studied during an episode of relapse.

A liver biopsy performed at the time or close to diagnosis showed histological features of interface hepatitis in all patients. Viral, metabolic, and genetic causes of liver disease were excluded by appropriate investigations. In the group of [A] patients, ANA were present in 8 patients and SMA were present in 7 (Table 6.1); concomitant presence

of ANA and SMA occurred in 4 individuals. At the time of diagnosis, all 37 [R] patients tested positive for ANA and/or SMA whereas at the time of study 18 remained either ANAs and/or SMAs positive (one patient tested positive for both) and 19 were autoantibody negative. All patients were negative for anti-LKM-1 and anti-LC-1. Relevant demographic, clinical and laboratory data are summarised in Table 6.1.

Table 6.1. Demographic, clinical and laboratory features of AILD patients at the time of the study.

Parameter	AILD (n=54)	[A] patients (n=17)	[R] patients (n=37)	*p**
Age in years	14.3 (8.2-21.1)	14.4 (9.3-19.5)	14.2 (8.2-21.1)	NS†
Female, n of patients (%)	31 (57%)	12 (70%)	19 (51%)	NS†
AST (nv<50 IU/L)	36 (16-1061)	91 (61-1061)	30 (16-49)	<0.0001‡
GGT (nv<50 IU/L)	35 (4-503)	55 (17-503)	20 (4-169)	<0.0001‡
Total bilirubin (nv<20 μmol/l)	9 (4-705)	13 (7-705)	7 (4-32)	0.0007‡
AP (nv<350 IU/L)	198 (49-834)	222 (88-834)	186 (49-411)	NS‡
AP/AST ratio	4.38 (0.17-16.38)	2.20 (0.17-6.55)	6.03 (2.12-16.38)	<0.0001‡
INR (nv<1.2)	1.07 (0.89-2.06)	1.10 (0.92-2.06)	1.05 (0.89-1.27)	0.03‡
Albumin (nv>35 g/L)	46 (32-50)	44 (32-50)	46 (40-50)	0.09‡
IgG (nv 6.5-17 g/L)	13.03 (5.70-43.96)	17.10 (6.83-43.96)	12.38(5.70-27.14)	0.002‡
ANA, n of positive patients (%)	19 (35%)	9 (53%)	10 (27%)	0.08†
SMA, n of positive patients (%)	16 (30%)	8 (47%)	8 (22%)	0.11†
pANNA, n of positive patients (%)	21 (39%)	7 (41%)	14 (38%)	NS†
AISC, n of positive patients (%)	21 (39%)	8 (47%)	13 (35%)	NS†
IBD, n of positive patients (%)	16 (30%)	6 (35%)	10 (27%)	NS†

Data presented as median (range) unless noted otherwise.
AILD, autoimmune liver disease; *AST*, aspartate aminotransferase; *GGT*, gamma-glutamyl transpeptidase; *AP*, alkaline phosphatase; *INR*, international normalised ratio; *IgG*, immunoglobulin G; *ANA*, anti-nuclear antibodies; *SMA*, anti-smooth muscle antibodies; *pANNA*, peripheral anti-nuclear neutrophil antibodies; *AISC*, autoimmune sclerosing cholangitis; *IBD*, inflammatory bowel disease; *nv*, normal value; *NS* non significant
* *p* value when comparing clinical parameters between [A] and [R] patients
† Fisher´s exact test
‡ Mann-Whitney test

Twenty one patients had bile duct changes characteristic of sclerosing cholangitis on retrograde cholangiography and were diagnosed as having AISC

(Gregorio et al. 2001). Biochemical and immunological differences between AIH-1 and AISC patients investigated in this chapter are depicted in Table 6.2.

Table 6.2. Clinical and laboratory data comparing AIH and AISC patients.

Parameter	AIH-1 patients (n=33)	AISC patients (n=21)	*p*[*]
Age in years	13.9 (8.2-21.1)	13.9 (9.1-17.6)	NS[†]
Female, n of patients (%)	21 (64%)	10 (48%)	NS[†]
AST (nv<50 IU/L)	35 (16-1061)	40 (17-534)	NS[‡]
GGT (nv<50 IU/L)	31 (8-215)	52 (4-503)	0.04[‡]
Total bilirubin (nv<20 μmol/l)	8 (4-705)	9 43-47)	NS[‡]
AP (nv<350 IU/L)	178 (49-344)	232 (68-834)	0.05[‡]
AP/AST ratio	4.29 (0.17-15.64)	5.09 (1.56-16.38)	NS[‡]
INR (nv<1.2)	1.07 (0.92-2.06)	1.05 (0.89-1.78)	NS[‡]
Albumin (nv>35 g/L)	46 (38-50)	45 (32-50)	NS[‡]
IgG (nv 6.5-17 g/L)	13.50 (5.70-43.96)	12.44 (8.01-27.14)	NS[‡]
ANA, n of positive patients (%)	12 (36%)	7 (33%)	NS[†]
SMA, n of positive patients (%)	11 (33%)	5 (24%)	NS[†]
pANNA, n of positive patients (%)	11 (33%)	10 (48%)	NS[†]
Iactive disease, n of patients (%)	12 (36%)	7 (33%)	0.004[†]
IBD, n of patients (%)	4 (12%)	12 (57%)	NS[†]

Data presented as median (range) unless noted otherwise.
AIH-1, autoimmune hepatitis type 1; *AISC*, autoimmune sclerosing cholangitis; *AST*, aspartate aminotransferase; *GGT*, gamma-glutamyl transpeptidase; *AP*, alkaline phosphatase; *INR*, international normalised ratio; *IgG*, immunoglobulin G; *ANA*, anti-nuclear antibodies; *SMA*, anti-smooth muscle antibodies*; pANNA*, peripheral anti-nuclear neutrophil antibodies; *IBD*, inflammatory bowel disease; *nv*, normal value; *NS* non significant
[*] *p* value when comparing clinical parameters between AIH-1 and AISC patients
[†] Fisher´s exact test
[‡] Mann-Whitney test

Patients were treated with prednisolone (2.5-5 mg daily at remission and 1-2 mg/kg/day at relapse) either alone or in combination with azathioprine (1-2 mg/kg/day) and/or MMF (up to 40 mg/kg/day). In those patients diagnosed with AISC, UDCA at a dose of 15-30 mg/kg/day was added to the immunosuppressive regimen. Epidemiological, clinical and laboratory data for each patient are shown in Appendix I.

At the time of study, 4 patients with AIH-1 and 12 with AISC had concomitant IBD: one AIH-1 patient had CD, while the remaining AIH-1 and all the AISC patients had UC.

Controls were fifteen HS (median age: 29.0 years, range 22.6-39.0 years, 75% female), recruited from hospital staff, as for ethical reasons blood could not be obtained from healthy children. To test whether age disparity may account for differences, patients were divided into 2 subgroups (≤14 and >14 years old).

3. Results

3.1. Characterisation of CD4 effector cells in AILD

The frequency and the phenotype of circulating $CD4^{pos}$ T cells was analysed in 18 AILD patients (9 AIH-1 and 9 AISC) and 10 HS. Gating of PBMCs according to their forward (FSC) and side (SSC) scatter characteristics and antibody fluorescence patterns (Figure 6.1.), revealed a population of lymphocytes expressing CD3 and CD4 in AILD and HS patients, the proportion of these cells being similar in the two groups (49.16±3.80% vs 56.62±2.39%; *p*=NS). When patients were subdivided into AIH-1 and AISC frequency of CD4 T cells was found to be higher in the former (62.50±5.51%) than in the latter (42.48±1.37%, *p*=0.001), and lower in AISC than in HS (*p*=0.002). No differences were observed between AIH-1 patients and HS (Figure 6.2.).

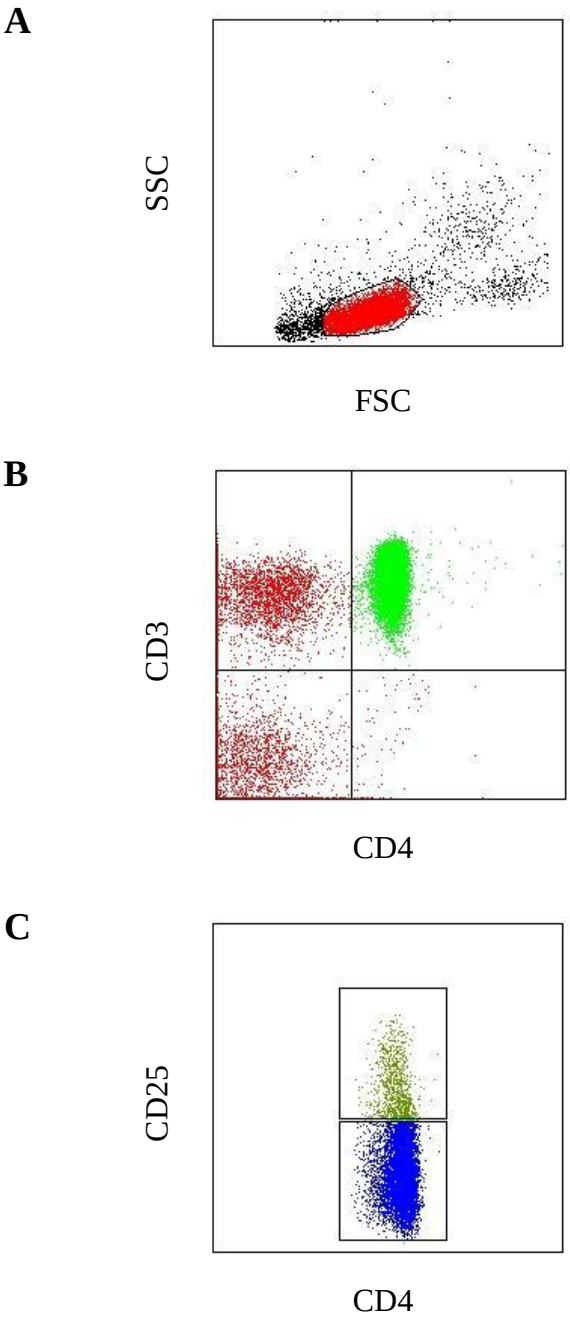


Figure 6.1. Gating strategy used to analyse effector T cells. PBMCs were gated according to their forward (FSC) and side (SSC) scatter characteristics (*A*); lymphocytes were subsequently gated according to their CD3 and CD4 expression (*B*); a population of cells positive for CD3 and CD4 was then identified and subdivided according to the expression CD25 (*C*) into $CD25^{neg}$ and $CD25^{pos}$ populations.

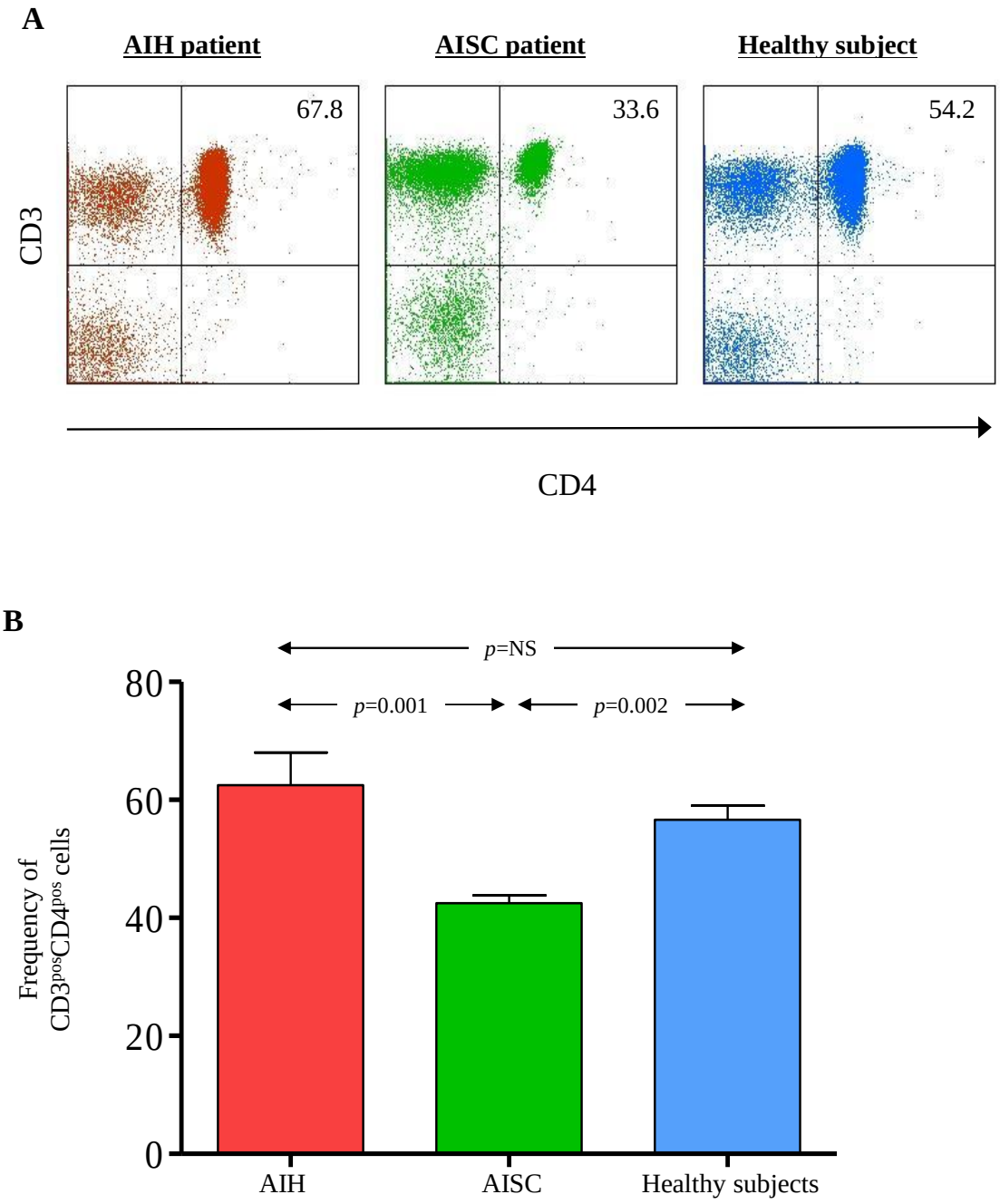


Figure 6.2. Frequency of $CD4^{pos}$ T cells. Plots in *Panel A* show the frequency of $CD3^{pos}CD4^{pos}$ cells in one representative AIH patient, in one representative AISC patient and in one representative healthy subject. The graph in *Panel B* summarises data from 9 AIH patients, 9 AISC patients and 10 healthy subjects. Error bars represent SEM.

$CD4^{pos}$ cells were subsequently gated according to their CD25 expression and subdivided into $CD25^{neg}$ and $CD25^{pos}$ T cell subsets (Figure 6.1.). AILD patients had a higher frequency of $CD25^{neg}$ cells than HS (93.52±0.61% vs 89.42±1.72%: p=0.01;

Figure 6.3.). There was no difference in the percentage of $CD25^{neg}$ cells between AIH-1 and AISC.

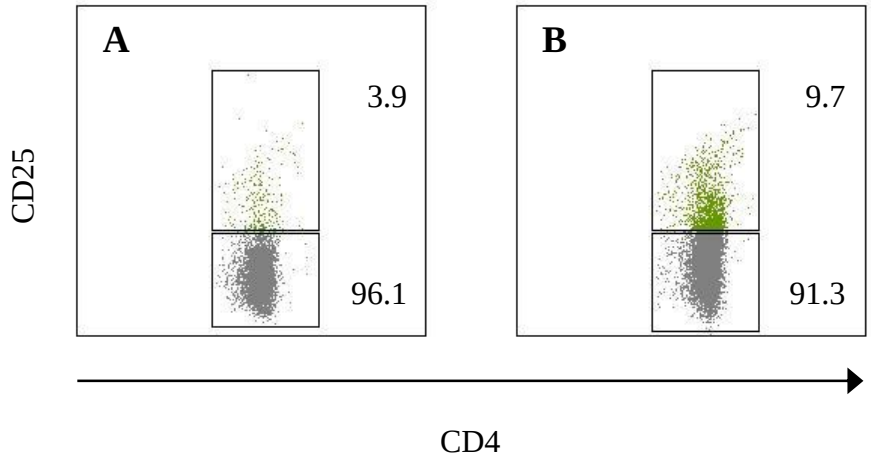


Figure 6.3. Percentage of $CD4^{pos}CD25^{neg}$ and $CD4^{pos}CD25^{pos}$ T lymphocytes. Percentage of $CD4^{pos}CD25^{neg}$ (bottom) and $CD4^{pos}CD25^{pos}$ (top) cells in one representative AILD patient (A) in one representative healthy subject (B). Dot plots of CD4(x axis) versus CD25.

Analysis of the $CD4^{pos}CD25^{neg}$ cell transcription factor showed that $Tbet^{pos}$ and $RORC^{pos}$ cells were higher in AILD (Tbet: 10.29±0.88%; RORC: 10.15±1.44%) than HS (Tbet: 6.52±0.91%, *p*=0.01; RORC: 2.58±0.54%, *p*=0.007). The frequency of $Tbet^{pos}$ cells did not differ between AIH-1 (10.92±1.35%) and AISC patients (9.66±1.16%, *p*=NS) being in both cases higher than in HS (AIH-1: p=0.01; AISC: *p*=0.05); in contrast the frequency of $RORC^{pos}$ cells was higher in AISC (12.90±2.46%) than in AIH-1 (7.40±0.93%, *p*=0.05), being in both cases higher than in HS (AISC: *p*=0.005; AIH-1: *p*=0.02). The frequency of GATA-3^{pos} cells did not differ among different groups.

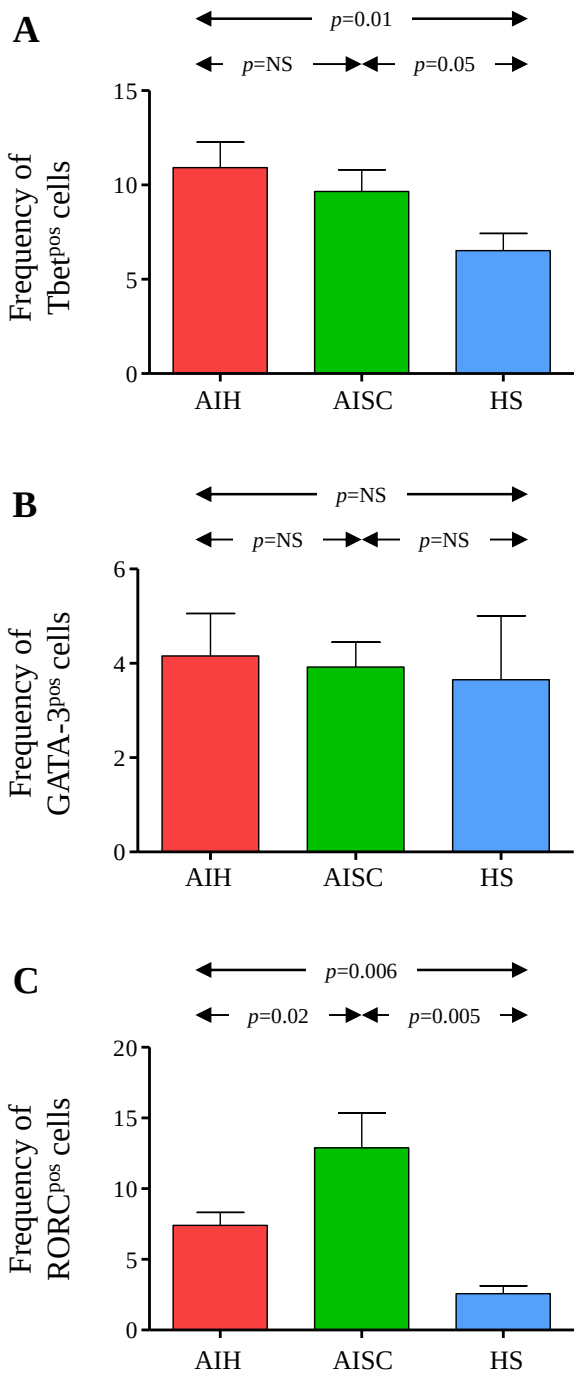


Figure 6.4. Transcription factor profile of CD4^{pos}CD25^{neg} T cells in autoimmune liver disease. Frequency of Tbet^{pos} (*A*), GATA-3^{pos} (*B*), and RORC^{pos} (*C*) cells within CD4^{pos}CD25^{neg} cells in 9 AIH patients (*red bars*), 9 AISC patients (*green bars*), and 10 healthy subjects (*blue bars*). Error bars represent SEM.

Analysis of the CD4^{pos}CD25^{neg} cytokine profile revealed that CD25^{neg} cells from AILD patients displayed higher frequencies of IFN-γ^{pos} (5.23±0.83%) and IL-17^{pos} (5.04±0.79%) cells when compared to HS (IFN-γ: 2.70±0.36%, p=0.03; IL-17:

2.06±0.27%, *p*=0.01). Similar to what observed for the transcription factors, while the frequency of IFN-γ^{pos} cells did not vary between AIH-1 and AISC, that of IL-17^{pos} cells was lower in the former (3.52±0.81%) than in the latter (6.56±0.87%, *p*=0.05). No differences in the frequency of $CD25^{neg}$ cells positive for IL-10 and TGF-β were noted among the different groups.

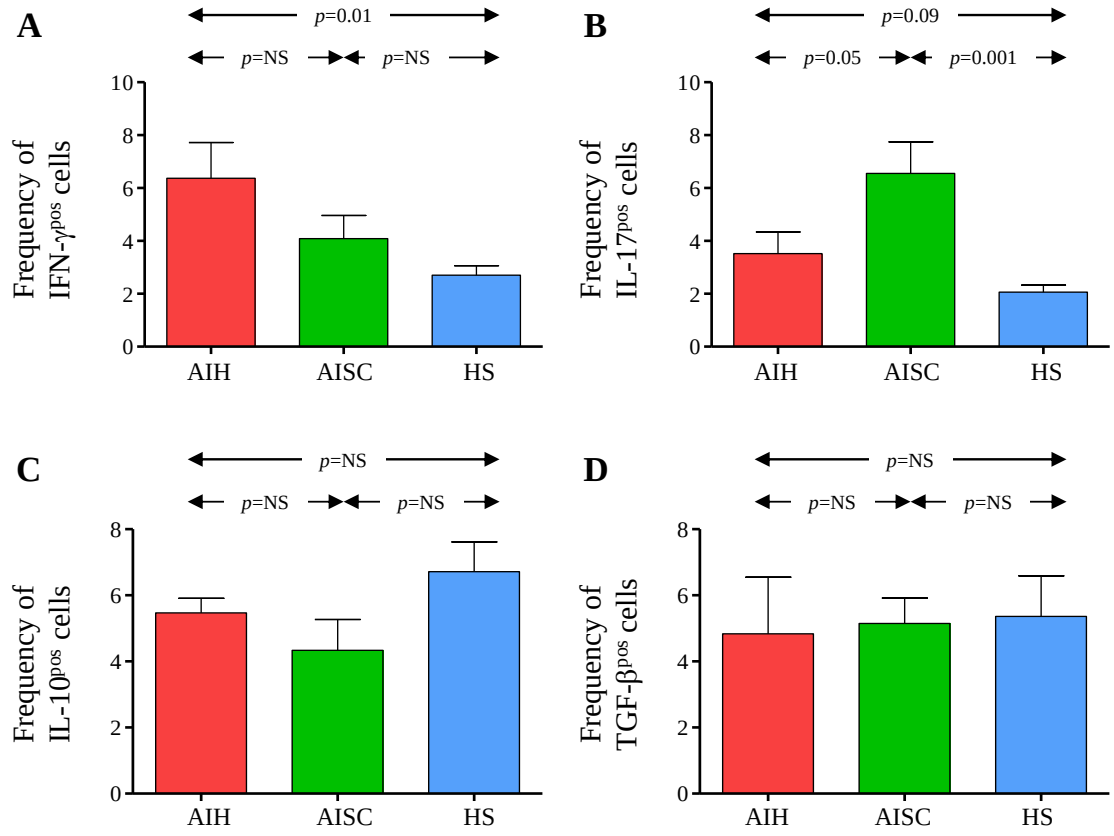


Figure 6.5. Frequency of cytokine producing cells within $CD4^{pos}CD25^{neg}$ effector T cells in autoimmune liver disease. Frequency of IFN-γ^{pos} (*A*), IL-17^{pos} (*B*), and IL-10^{pos} (*C*), and TGF-β^{pos} (*D*) cells within $CD4^{pos}CD25^{neg}$ T cells in 9 AIH patients (*red bars*), 9 AISC patients (*green bars*) and 10 healthy subjects (*blue bars*). Error bars represent SEM.

In AILD patients, the frequency of $CD25^{pos}Tbet^{pos}$ cells positively correlated with AST levels. When AISC patients were analysed separately, there was a positive correlation between the frequency of $CD25^{neg}$IL-17^{pos} cells and the levels of AP, and GGT.

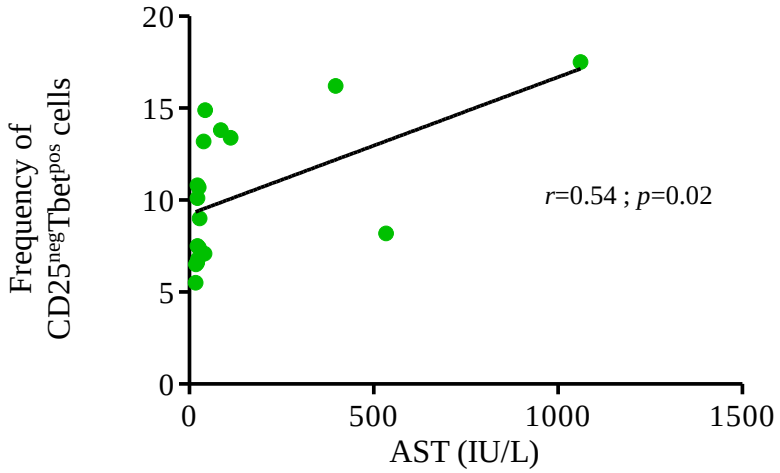


Figure 6.6. Correlation between the frequency of CD25^{neg}Tbet^{pos} cells and AST levels in AILD patients. In AILD patients the percentage of CD25^{neg}Tbet^{pos} cells positively correlates with levels of AST..

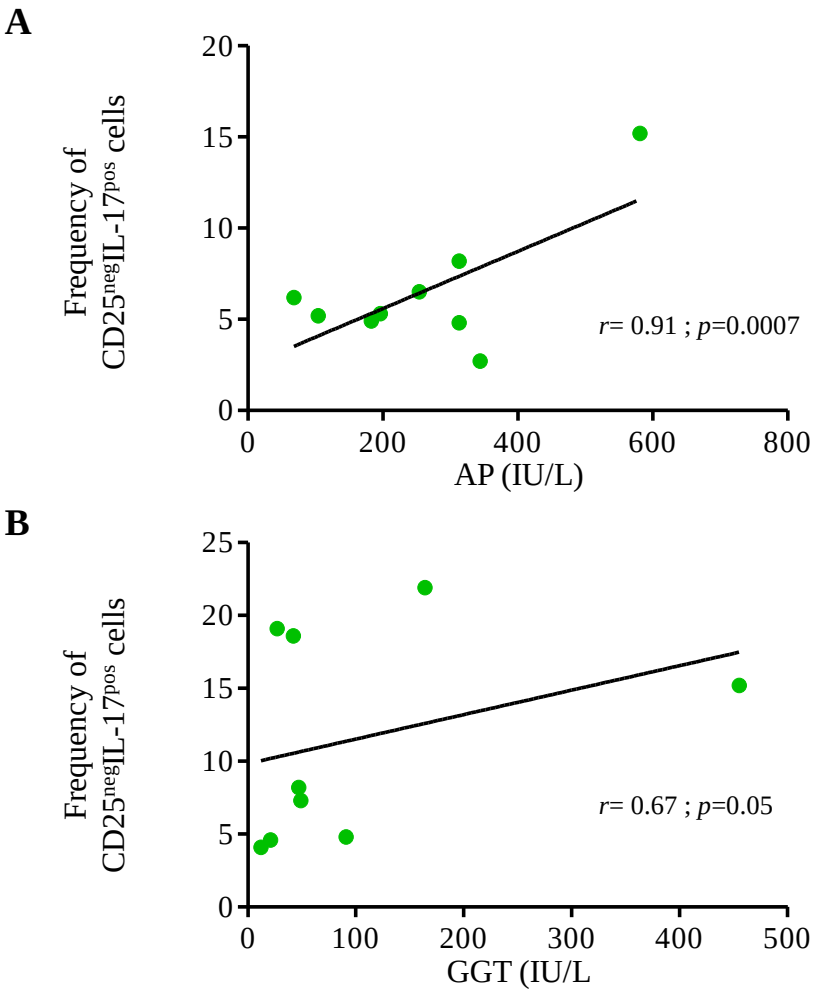


Figure 6.7. Correlation between the frequency of IL-17^{pos} cells and biochemical indices of cholestasis in AISC patients. In AISC patients the frequency of IL-17^{pos} cells within CD4^{pos}CD25^{neg} T cells positively correlated with AP (*Panel A*), and GGT levels (*Panel B*).

3.2. Frequency of circulating TIM-3^{pos} lymphocytes

The percentage of circulating TIM-3^{pos} lymphocytes was determined in 47 AILD patients and in 15 healthy subjects. In AILD patients, the mean percentage of TIM-3^{pos} cells within CD4^{pos} lymphocytes (3.66±0.30%) was lower than in HS (6.93±0.66%, p=0.0001), this difference being also significant when [R] (4.39±0.36%, p=0.0007) and [A] patients (1.92±0.26%, p<0.00001) were analysed separately. TIM-3^{pos}CD4^{pos} cells were significantly less numerous in [A] than in [R] patients (p=0.0001; Figure 6.8.).

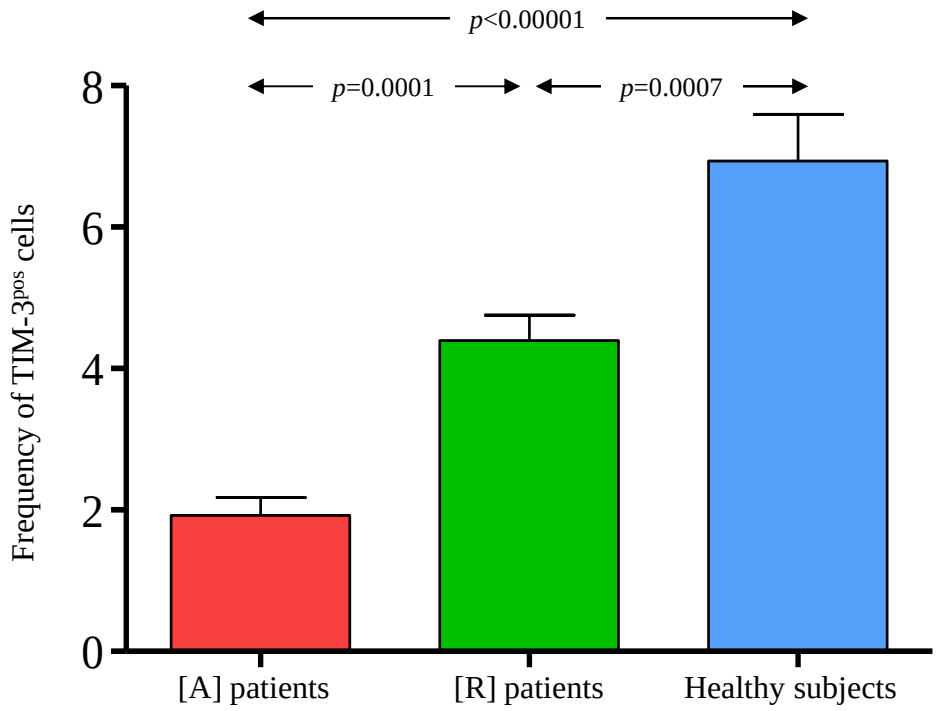


Figure 6.8. Percentage of TIM-3^{pos} lymphocytes within CD4^{pos} T cells. Percentage of TIM-3^{pos} lymphocytes within CD4^{pos} cells in 17 [A] patients, 30 [R] patients and 15 healthy subjects Error bars represent SEM

After CD4^{pos} cells were gated according to their CD25 expression, frequency of TIM-3^{pos} cells within the CD4^{pos}CD25^{pos} and the CD4^{pos}CD25^{neg} subsets was determined (Figure 6.9.). While the frequency of TIM-3^{pos} lymphocytes within the CD4^{pos}CD25^{pos} subset was negligible in both AILD (0.39±0.05%) and HS (0.45±0.11%, p=NS), that of TIM-3^{pos} cells within the CD4^{pos}CD25^{neg} subset was lower in patients

(4.02±0.31%) than in HS (7.95±0.82%, $p<0.00001$) whether patients were studied during active disease (2.66±0.35%, $p<0.00001$) or during remission (4.60±0.38%, $p=0.0001$). TIM-3^{pos} cells were lower in [A] than in [R] patients ($p=0.003$). Within AILD patients, no differences were observed between the two age groups, between AIH and AISC, or between those with or without concomitant UC.

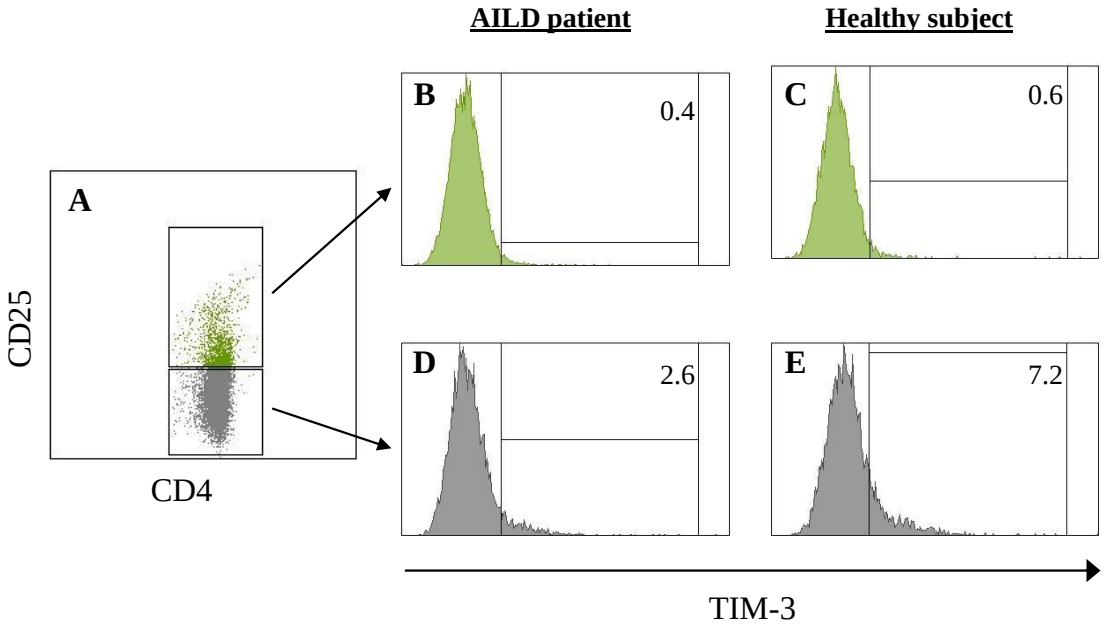


Figure 6.9. Frequency of TIM-3^{pos} cells within CD4^{pos}CD25^{pos} and CD4^{pos}CD25^{neg} cell subsets. PBMCs were gated according to CD4 and CD25 expression (*A*); TIM-3 expression, represented as mean fluorescence intensity on histogram plots, was evaluated within CD25^{pos} (*B & C*), and CD25^{neg} (*D & E*) populations. Plots are from one representative AILD patients (*B & C*), and one representative healthy subject (*A, D & F*). Values represent the frequency of cells within each CD25 population.

To determine the frequency of TIM-3^{pos} cells within CD4 Th subsets intracellular staining for Tbet, GATA-3 or RORC was performed in 15 AILD patients and in 15 HS. The percentage of TIM-3^{pos} cells within the Tbet^{pos} and the RORC^{pos} effector subsets was lower in AILD (Tbet: 5.13±0.69%; RORC: 4.07±0.64%) than in health (Tbet: 13.59±1.72%, $p<0.0001$; RORC: 8.23±1.13%, $p=0.02$) while that of GATA-3^{pos} cells was negligible in both groups (0.65±0.29% vs 0.72±0.15%, *p*=NS).

In AILD patients, the frequency of TIM-3^{pos} effector cells was negatively correlated with AST levels (r= –0.34, p=0.02), and with the frequency of $CD25^{neg}Tbet^{pos}$ cells (r= –0.81, p<0.0001).

3.3. Characterisation of TIM-3^{pos} and TIM-3^{neg} effector cells

The phenotypic profile of TIM-3^{pos} and TIM-3^{neg} cells was assessed in 10 AILD patients and 10 HS using mAbs to CD45RA and CCR7 to assign T cells into naïve, central memory (CM), and effector memory (EM) subsets (Sallusto et al. 1999, Sallusto and Lanzavecchia 2000). Co-expression of CD45RA and CCR7 defines naïve T cells, presence of CCR7 and absence of CD45RA indicates central memory (CM) cells, while absence of both markers defines effector memory (EM) cells (Figure 6.10.).

The frequency of naïve cells did not differ between TIM-3^{pos} and TIM-3^{neg} cell subsets whether they were obtained from AILD patients (12.77±2.11% vs 14.84±4.48%, p=NS) or HS (15.80±6.35% vs 16.88±6.66%, p=NS).

Compared to the TIM-3^{neg} fraction, TIM-3^{pos} cells showed a higher proportion of CM cells both in AILD (49.14±2.04% vs 32.98±4.88%, p=0.01) and in health (55.40±6.33% vs 32.31±6.06, p=0.03). Conversely, the proportion of EM cells was lower within TIM-3^{pos} cells compared to their negative counterparts (AILD: 10.43±1.43% vs 34.49±7.05%, p=0.007; HS: 17.22±3.16% vs 28.42±3.62%, p=0.05), and lower in the TIM-3^{pos} cells isolated from patients than in those obtained from HS (p=0.05).

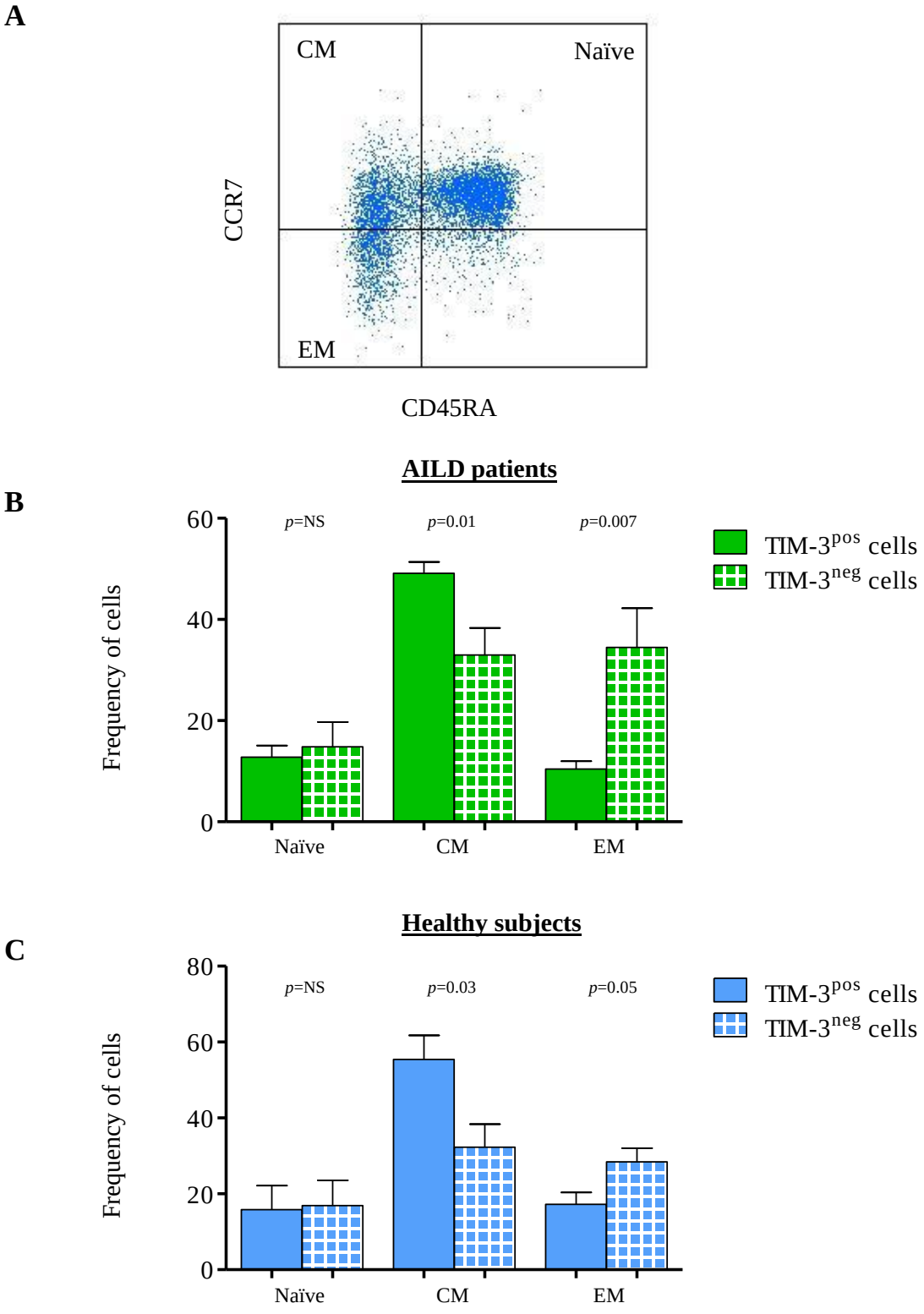


Figure 6.10. TIM-3 expression according to CD4 T cell maturation status. Naïve, central memory (CM), and effector memory (EM) CD4 T cell subsets can be defined by the pattern of expression of CD45RA and CCR7 as shown in *A.* A higher proportion of the TIM-3pos demonstrated a central memory (CM) phenotype, while fewer demonstrated an effector memory phenotype than their TIM-3neg counterparts in both AILD patients (*B*, n=10) and healthy subjects (*C*, n=10).

Analysis of the TIM-3^{pos} and TIM-3^{neg} transcription factor profile showed that the majority of TIM-3^{pos} cells expressed Tbet both in AILD and in health and that the percentage of Tbet^{pos} cells within the TIM-3^{neg} subset was higher in AILD than in HS (p=0.005). While the frequency of GATA-3^{pos} cells within the TIM-3^{pos} subset was similar in the two groups, when the TIM-3^{neg} subset was considered GATA-3^{pos} cells tended to be lower in AILD than in HS (p=0.08).The frequency of RORC-expressing cells did not differ between the TIM-3^{pos} and the TIM-3^{neg} subpopulation, but was higher in patients than in normal controls both within the positive (p=0.03) and the negative (p=0.02) fraction. The percentage of FOXP3^{pos} cells within TIM-3^{pos} subset was similar in AILD patients and in HS in patients, however, TIM-3^{pos} cells tended to contain more FOXP3^{pos} cells than their negative counterparts (p=0.06; Table 6.3.).

Table 6.3. Transcription factor and of cytokine profile of CD4^{pos}CD25^{neg}TIM-3^{pos} and TIM-3^{neg} cells.

% of cells	TIM-3^{pos} Tregs			TIM-3^{neg} Tregs				
	AILD	HS	p^1	AILD	HS	p^2	$p^{\dagger}$	$p^{\ddagger}$
T-bet	91.2±0.8	93.1±0.5	NS	10.1±0.9	6.1±0.9	0.005	<0.001	<0.001
GATA-3	0.71±0.23	0.78±0.24	NS	2±0.5	2.8±0.4	0.08	0.02	0.01
RORC	7.2±0.9	2.9±0.5	0.03	5.9±0.4	3.5±0.5	0.02	NS	NS
FOXP3	2.84±0.85	2.74±0.49	NS	0.5±0.2	1.6±0.8	NS	0.06	NS
IFN-γ	11.2±0.8	4.2±0.6	<0.001	6.09±0.7	2.4±0.3	0.001	<0.001	<0.001
IL-17	7.9±1.2	4.3±0.8	0.04	3.1±0.7	1.5±0.2	0.07	0.02	0.02
IL-10	7.1±1	9.9±1.2	NS	5.1±0.7	6.7±1	NS	0.11	0.09
TGF-β	6.8±0.4	9.8±1.9	NS	3.9±1.2	4.7±0.4	NS	0.03	0.03

HS, healthy subjects; *AILD*, autoimmune liver disease; *NS*, non significant
Data are represented as mean±SEM
p values comparing the percentage of Tbet^{pos}, GATA-3^{pos}, RORC^{pos}, FOXP3^{pos}, IFN-γ^{pos}, IL-17^{pos}, IL-10^{pos}, and TGF-β^{pos} cells:
p^1: within the TIM-3^{pos} subset between AILD and healthy subjects
p^2: within the TIM-3^{neg} subset between healthy AILD and healthy subjects
$p^{\dagger}$: between the TIM-3^{pos} and the TIM-3^{neg} subset in AILD
$p^{\ddagger}$: between the TIM-3^{pos} and the TIM-3^{neg} subset in healthy subjects
Data referring to 34 AILD patients and 20 healthy subjects

Analysis of the cytokine profile indicated that the frequency of IFN-γ and IL-17-producing cells was higher in the TIM-3^{pos} than in the TIM-3^{neg} cell fraction both in AILD patients (IFN-γ: p=0.004; IL-17: p=0.006) and in HS (IFN-γ: p=0.01; IL-17: p=0.006). In both effector subsets, AILD patients contained higher frequencies of IFN-γ^{pos} (TIM-3^{pos}: p<0.0001; TIM-3^{neg}: p<0.0001) and IL-17^{pos} cells (TIM-3^{pos}: p=0.02; TIM-3^{neg}: p=0.02) than HS (Table 6.3.). In both groups no differences were noted in the frequency of TGF-β and IL-10-producing cells between the two effector cell subsets.

3.4. Effect of stimulation with anti-CD3/anti-CD28 T cell expander and IL-2 on TIM-3 expression

Immunomagnetically isolated CD4^{pos} and CD4^{pos}CD25^{neg} cells from 4 HS and 4 AILD patients were stimulated with anti-CD3/anti-CD28 T cell expander and IL-2. The expression of TIM-3 on effector cells was then tested by flow cytometry after 12 hours and 5 days culture.

The frequency of TIM-3^{pos} cells within CD4^{pos} lymphocytes did not change after 12 hours-culture both in AILD patients (% of TIM-3^{pos} cells: 2.23±0.47 vs 2.93±0.38, p=NS; MFI: 390±25 vs 347±21, p=NS) and in HS (% TIM-3^{pos} cells: 6.70±0.49% at baseline vs 7.25±0.39% after 12 hours, p=NS; TIM-3 MFI: 639±25 vs 671±73, p=NS). After 5 days culture, there was an increase in the frequency of TIM-3^{pos}CD4^{pos} cells in HS (% of TIM-3^{pos} cells: 12.65±1.30%, p=0.01; MFI: 881±37, p=0.002), but not in AILD patients (%TIM-3^{pos} cells: 2.98±0.75%, p=NS; MFI: 344±50, p=NS; Figure 6.11.). Akin to what observed for CD4^{pos} cells, while in HS frequency of TIM-3^{pos} lymphocytes and TIM-3 MFI in CD25^{neg} cells were higher after 5 days compared to 12 hours culture and baseline, such an increase was not observed in AILD patients. The

frequency of TIM-3^{pos} cells and TIM-3 MFI were higher in HS than in AILD at all time points (baseline: % TIM-3^{pos} cells: p=0.0006; MFI: p=0.0004; 12 hours: % TIM-3^{pos} cells: p=0.0002; MFI: p=0.0006; 5 day culture: % of TIM-3^{pos} cells: p=0.0007; MFI: p=0.0001).

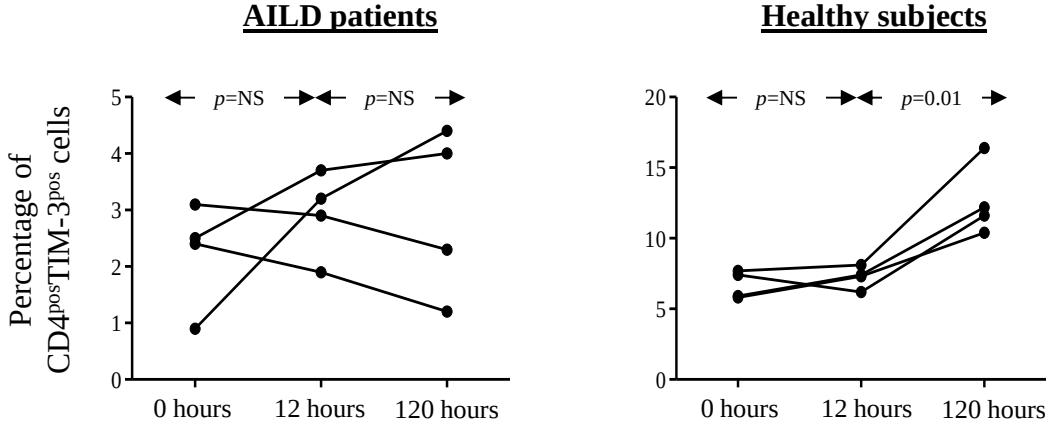


Figure 6.11. Effect of stimulation with anti-CD3/anti-CD28 T cell expander and IL-2 on TIM-3 expression CD4^{pos} cells were purified from PBMCs using immunomagnetic beads, and cultured in the presence of CD3/CD28 T cell expander and rIL-2. TIM-3 expression was assessed at baseline, after 12 hours and after 5 days in culture.

3.5. Proliferation of CD4^{pos}CD25^{neg}, TIM-3^{pos} and TIM-3^{neg} cells and responsiveness to Treg control

Purified CD25^{neg}, TIM-3^{pos} and TIM-3^{neg} cell populations obtained from 18 AILD patients and 9 HS were cultured with CD3/CD28 T cell expander and rIL-2 in the absence and presence of regulatory T cells. Cell proliferation was assessed after 5 days culture.

The proliferation of unfractionated CD25^{neg} cells (AILD: 6,160±714 cpm; HS: 37,745±5,015 cpm) was lower than in TIM-3^{neg} cells (AILD: 9,659±1,041 cpm, p=0.02; HS: 115,488±8348 cpm, p=0.001) and higher than in TIM-3^{pos} cells (AILD: 4,604±1,177 cpm; p=0.004; HS: 29,382±2,323 cpm, p=0.03; Figure 6.12.).

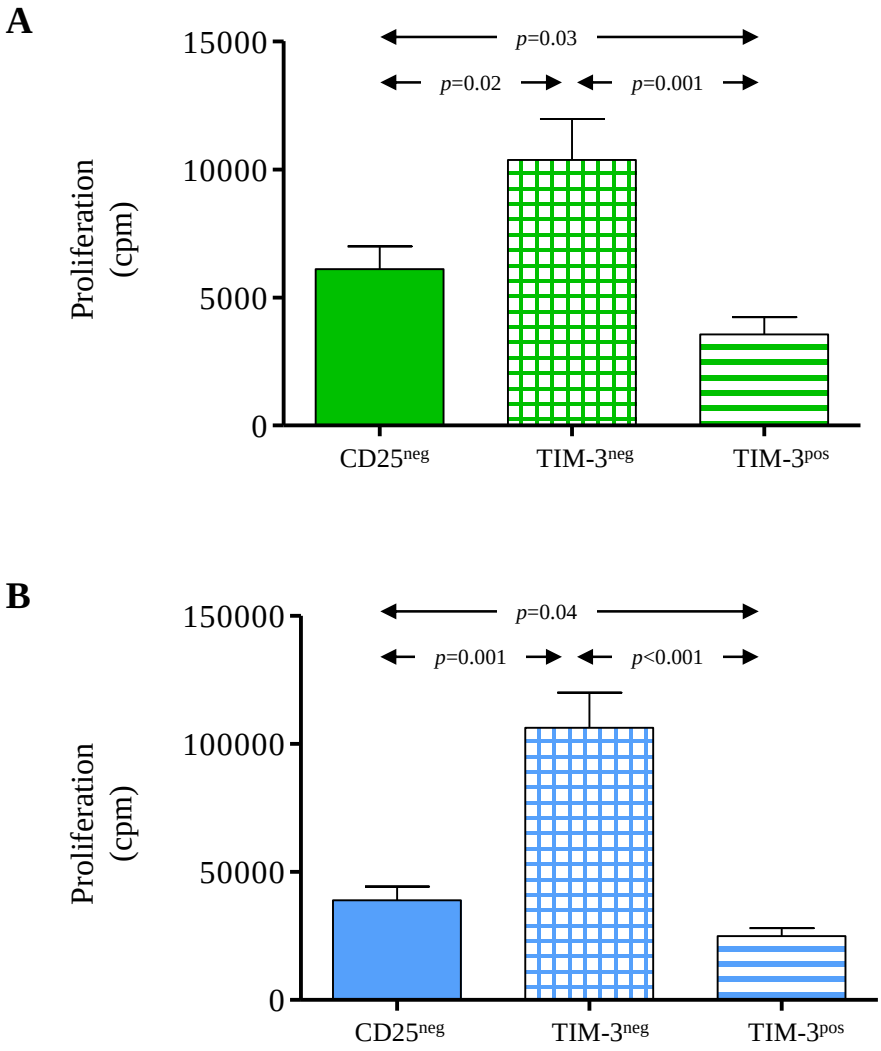


Figure 6.12. Proliferation of CD4^{pos}CD25^{neg}, TIM-3^{neg} and TIM-3^{pos} responder cells. CD4^{pos}CD25^{neg} cells were purified from PBMCs by two-step negative selection using immunomagnetic beads. TIM-3^{neg} and TIM-3^{pos} cell fractions were obtained following incubation of CD25^{neg} cells with PE-labeled TIM-3 mAbs and with anti-PE microbeads. Unfractionated CD25^{neg} cells (*filled bars*) and TIM-3^{neg} (*squared bars*) and TIM-3^{pos} (*horizontal stripped bars*) fractions from 18 AILD patients (*A*) and 9 healthy subjects (*B*) were cultured for 5 days in the presence of CD3/CD28 T cell expander and rIL-2. Results are expressed as mean + SEM cpm.

After addition of cTregs, the mean CD25^{neg} cell proliferation decreased by 26% in AILD patients (p=NS) by 53% in HS (p=0.007), being the percentage inhibition lower in the former than in the latter (p=0.0006). Addition of cTregs reduced cell proliferation by 23% (p=NS) in AILD by 25% (p=NS) in HS when TIM-3^{neg} cells were used as responders, the percentage inhibition of TIM-3^{neg} proliferation being similar in

the two groups (p=NS). While in HS the percentage inhibition of TIM-3^{neg} cells was significantly lower than that of CD25^{neg} responders (56% vs 25%, p=0.0003), such a difference was not observed in AILD patients (26% vs 23%, p=NS). When TIM-3^{pos} cells were used as responders, cTregs addition resulted in a decrease in cell proliferation by 47% (p=0.03) in AILD and by 62% in HS (p=0.001), the percentage inhibition being lower in patients than in HS (p=0.04). In AILD inhibition of TIM-3^{pos} cell proliferation was higher than that of CD25^{neg} responders (47% vs 26%, p<0.0001), whereas in HS there was no statistically significant difference in terms of cell proliferation inhibition between the two types of responders (62% vs 53%, p=NS).

Addition of CD127^{neg} Tregs reduced cell proliferation by 46% in AILD (p<0.001) and by 60% in HS (p<0.001) when CD25^{neg} were the targets; by 11% in AILD (p=NS) and by 13% in HS (p=NS) when the targets were TIM-3^{neg} cells; and by 56% in AILD (p=0.006) and by 69% in HS (p<0.001) when TIM-3^{pos} cells were used as responders. Compared to cTregs, addition of CD127^{neg} Tregs led to a higher suppression rate when CD25^{neg} (AILD: p=0.0001; HS: p=0.11) and TIM-3^{pos} (AILD: p=0.02; HS: p=0.05) cells were used as responders, while leaving unchanged the percentage inhibition over TIM-3^{neg} cell proliferation (AILD: p=NS; HS: p=NS).

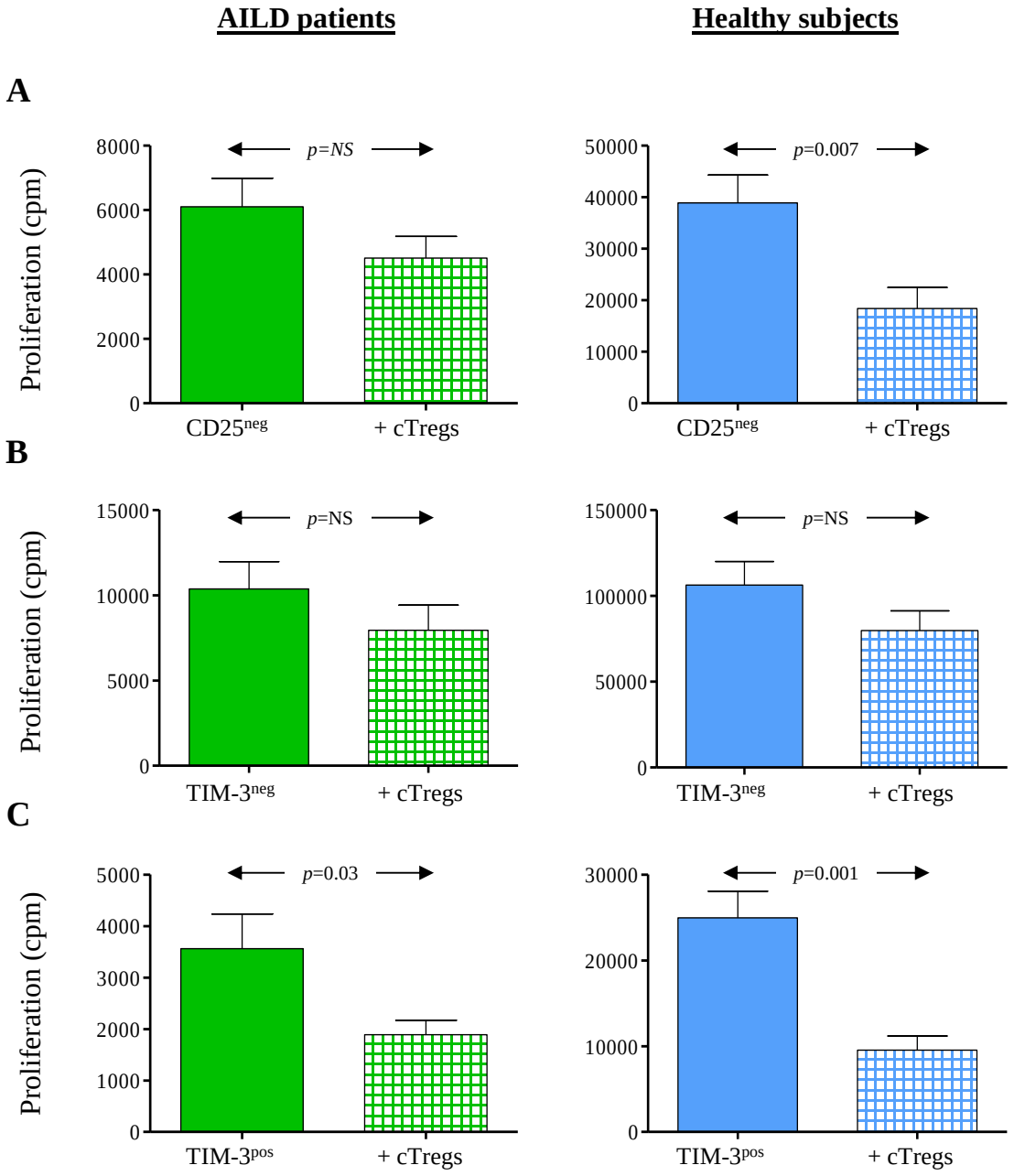


Figure 6.13. Suppression of CD4^{pos}CD25^{neg}, TIM-3^{neg} and TIM-3^{pos} responder cell proliferation by Tregs. Tregs purified from 18 AILD patients (*left plots*) and 9 healthy subjects (*right plots*) were added to CD4^{pos}CD25^{neg} (*A*), TIM-3^{neg} (*B*) or TIM-3^{pos} (*C*) responder cells. After 5-day co-culture, responder cell proliferation was assessed by ^{3}H-thymidine incorporation. Bars represent mean + SEM proliferation of before and after T-reg addition.

To determine whether the expression of GAL-9 by Tregs affects effector cell responsiveness to immune-regulation, TIM-3^{pos} cells were cultured in the presence of untreated and GAL-9 siRNA-treated Tregs. After addition of GAL-9-siRNA-treated

cTregs, inhibition of TIM-3^{pos} cell proliferation was reduced by 70% in AILD (from 46% to 13%, $p<0.0001$) and 74% in HS (from 62% to 16%, $p<0.0001$). After treatment with GAL-9-siRNA, $CD127^{neg}$ Treg suppressor function was reduced by 75% (from 56% to 14%, $p<0.0001$) in AILD patients and 71% (from 69% to 20%, $p<0.001$) in HS.

3.6. Neutralisation assay

Since the percentage of IFN-γ and, to a lesser extent, IL-17-producing cells was higher in TIM-3^{pos} than in TIM-3^{neg} cells, the effect of IFN-γ and IL-17 neutralisation on the ability of $CD25^{neg}$, TIM-3^{neg} and TIM-3^{pos} cells to be regulated by Tregs was investigated in 9 HS and 18 AILD patients.

Addition of anti-IFN-γ neutralising antibodies did not change $CD25^{neg}$ and TIM-3^{neg} cell responsiveness to Treg control in both AILD patients ($CD25^{neg}$: 33% of inhibition without vs 30% in the presence of anti-IFN-γ mAbs, p=NS; TIM-3^{neg}: 23% vs 25%, p=NS) and HS ($CD25^{neg}$: 53% vs 54%, p=NS; TIM-3^{neg}: 25% vs 31%, p=NS). In contrast, treatment with anti-IFN-γ abrogated TIM-3^{pos} responsiveness to Treg control (AILD: 46% vs –2%, $p<0.0001$; HS: 62% vs 10%, $p<0.0001$).

Treatment with anti-IL-17 neutralising antibodies did not change the responsiveness of $CD25^{neg}$, TIM-3^{neg} and TIM-3^{pos} responder cells to Treg control in both patients ($CD25^{neg}$: 26% vs 30%, p=NS; TIM-3^{neg}: 23% vs 28%, p=NS; TIM-3^{pos}: 47 vs 46%, p=NS) and HS ($CD25^{neg}$: 53% vs 54%, p=NS; TIM-3^{neg}: 25% vs 29%, p=NS; TIM-3^{pos}: 62% vs 59%, p=NS).

Addition of anti-IFN-γ and anti-IL-17 neutralising antibodies together reduced responsiveness of TIM-3^{pos} cells (AILD: 47% vs 3%, $p<0.0001$; HS: 62% to 13%, $p<0.0001$), while leaving unchanged that of $CD25^{neg}$ (AILD: 26% vs 34%, p=NS; HS:

53% vs 55%, *p*=NS) and TIM-3^{neg} cells (AILD: 23% vs 29%, *p*=NS; HS: 25% vs 33%, *p*=NS).

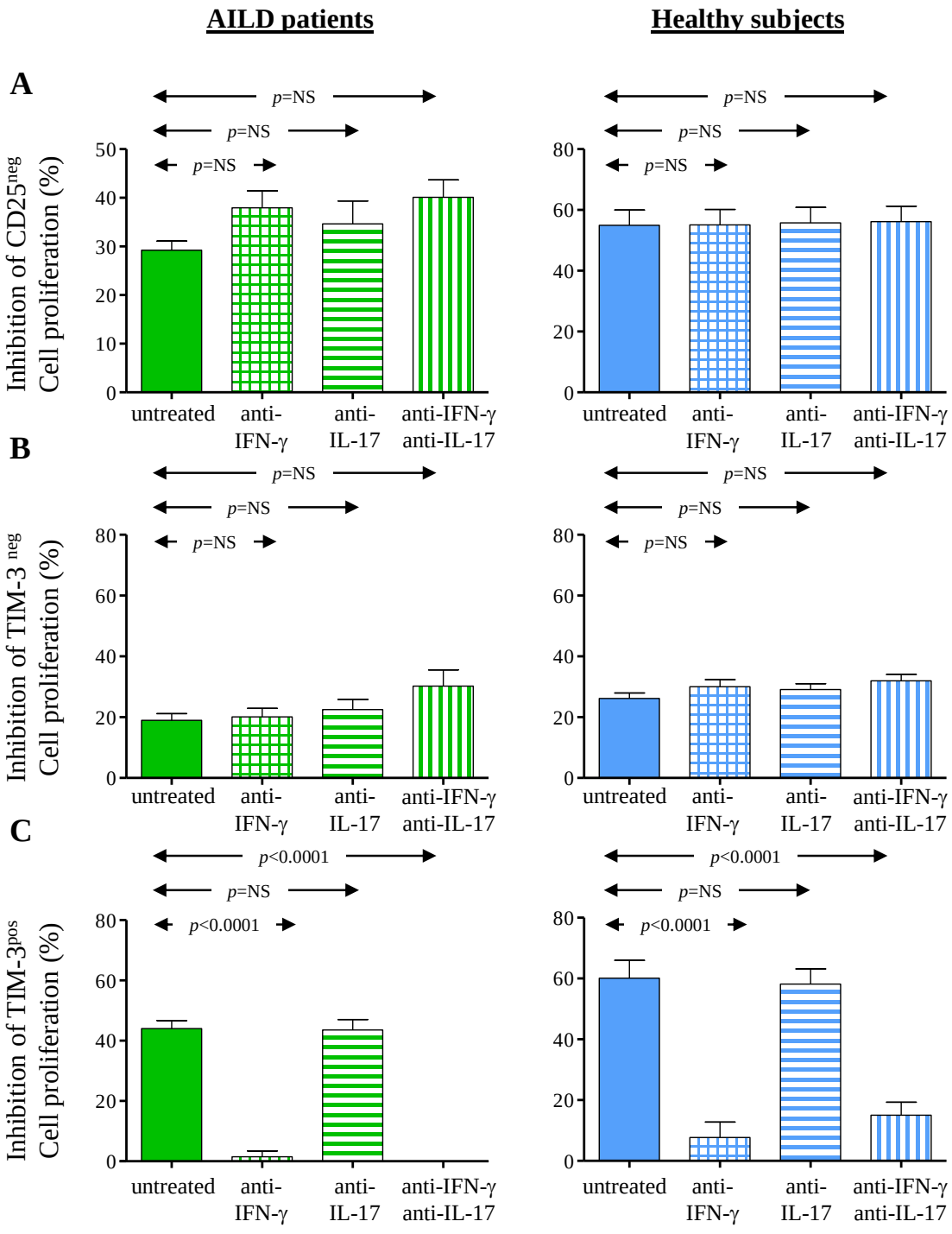


Figure 6.14. Inhibition of CD4^{pos}CD25^{neg}, TIM-3^{neg} and TIM-3^{pos} responder cell proliferation by T-regs in the presence of anti-IFN-γ and anti-IL-17 mAbs. Tregs from 18 AILD patients (*left plots*) and 9 HS(*right plots*) were added to CD4^{pos}CD25^{neg} (*A*), TIM-3^{neg} (*B*) or TIM-3^{pos} (*C*) responder cells, either untreated (*filled bars*) or treated with anti-IFN-γ (*squared bars*), anti-IL-17 (*horizontal striped bars*) or both (*vertical striped bars*) mAbs. After 5-day co-culture, responder cell proliferation was assessed by ^{3}H-thymidine incorporation.

Since the responsiveness of TIM-3^{pos} cells to Treg control was abrogated only in the presence of anti-IFN-γ neutralising antibodies, the effect of cytokine neutralisation on the frequency of TIM-3^{pos} lymphocytes was then tested after 12 hour and 5 days culture in 4 AILD patients and in 4 HS.

Treatment with anti-IFN-γ neutralising antibodies, compared to untreated effector cells, did not change the percentage of TIM-3^{pos} lymphocytes within $CD4^{pos}$ isolated cells after 12 hours (AILD: 2.93±0.38% vs 2.73±0.71%, *p*=NS; HS: 7.25±0.39% without vs 5.83±1.16% with anti-IFN-γ mAbs, *p*=NS) and 5 days (AILD: 2.98±0.75% vs 2.88±0.61%, *p*=NS; HS: 12.65±1.30% vs 9.58±0.90%, *p*=NS) culture. When $CD25^{neg}$ purified cell fraction was analysed, no difference in the frequency of TIM-3^{pos} cells between untreated and treated $CD25^{neg}$ cells were noted in AILD patients at any time point (12 hours: 2.63±0.53% vs 2.55±0.55%, *p*=NS; 5 days: 2.80±0.49% vs 3.03±0.29%, *p*=NS), whereas in HS anti-IFN-γ neutralising antibodies tended to decrease it (12 hours: 8.55±0.41% vs 6.25±1.21%, *p*=0.12; 5 hours: 15.55±1.64% vs 12.20±0.61%, *p*=0.10). However, when $CD25^{neg}$ cells were co-cultured with Tregs no difference in terms of TIM-3 expression was observed between untreated and IFN-γ treated effector cells in HS (7.40±1.18% vs 7.23±1.73%, *p*=NS), as well as in AILD patients (2.30±0.60% vs 2.75±0.78%, *p*=NS).

3.7. Immunohistochemistry

The frequency of liver infiltrating TIM-3^{pos} and $CD4^{pos}$ lymphocytes was determined by immunohistochemistry in 13 liver biopsies obtained from 10 AILD patients (5 AIH-1 and 5 AISC). All patients were studied while on histologically active disease; from 3 of these patients biopsies were also obtained during inactive disease.

The number of positive lymphocytes was counted at three high-power fields (x400) in each case. TIM-3^{pos} and CD4^{pos} lymphocytes were enumerated in the same areas within a biopsy specimen.

TIM-3^{pos} lymphocytes were detected in the portal tracts of all patients with histologically active disease, though representing a very small proportion of the portal tract inflammatory infiltrate (Figure 6.15.). While the numbers of TIM-3^{pos} cells did not differ between AIH-1 and AISC patients, the frequency of CD4^{pos} cells tended to be higher in the former (195.2±19.8) than in the latter (106.8±41.0, p=0.09).

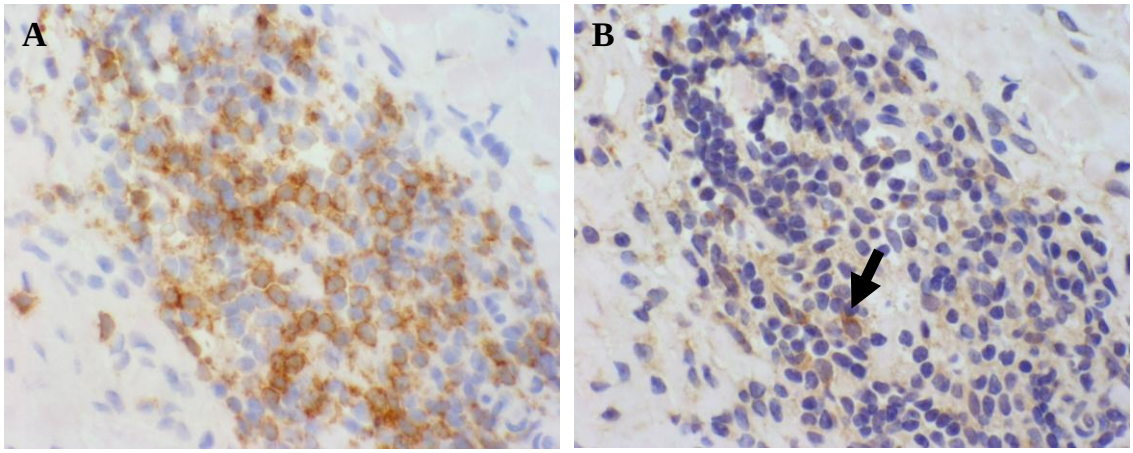


Figure 6.15. Liver infiltrating CD4^{pos} and TIM-3^{pos} lymphocytes. The number of CD4^{pos} lymphocytes was counted at three high-power fields (x400, *A*). Lymphocytes positive for TIM-33 and CD4 were counted at the same biopsy sample (*B*).

4. Discussion

This chapter has provided evidence that defective immune-regulation in AILD results not only from numerical and functional impairment of Tregs (see chapters IV and V) but also from low responsiveness of effector CD4 T cells to Treg control. The data presented clearly indicate that in AILD down-regulation of TIM-3 on the surface of responder CD4 T cells leads to lower control of their effector function by Tregs. That

TIM-3 renders effectors responsive to Treg control has been shown by a series of experiments where the TIM-3^{neg} and the TIM-3^{pos} cell fractions were tested for their ability to be regulated by Tregs. Effective control of cell proliferation was observed when TIM-3^{pos} cells were used as responders, confirming that the presence of TIM-3 on effectors confers responsivity to Treg control. Furthermore, TIM-3^{neg} cells were not only resistant to cTregs but also to the more suppressive *bona fide* $CD127^{neg}$ Tregs, thus demonstrating that the down-modulation of TIM-3 in AILD renders effector cells refractory to the control exerted by Tregs. That low levels of TIM-3 expression are associated with poor Treg control and therefore with more inflammation was further confirmed by the strong negative correlation between the percentage of TIM-3^{pos} effector lymphocytes and the levels of transaminases. Evidence supporting a relative resistance of effector cells to regulatory control had been previously shown in different animal models of autoimmunity, in which target organ inflammation progresses despite the presence of appropriate numbers of functional Tregs (Monk et al. 2005, Korn et al. 2007b, D'Alise et al. 2008).

Further phenotypic and functional analyses performed in the current study indicate that regulation of TIM-3^{pos} cells by Tregs depends on IFN-γ production, as blockade of IFN-γ resulted in a lower susceptibility of TIM-3^{pos} cells to Tregs. These data suggest that a pro-inflammatory signature is necessary for the effectors in order to be 'seen' and consequently regulated by Tregs *via* the TIM-3/GAL-9 pathway.

In AILD down-regulation of TIM-3 on the surface of effector cells is mirrored by a decreased percentage of GAL-9 expressing regulatory cells, as shown in Chapter V. Whether down-regulation of TIM-3 on effectors is the result of low GAL-9^{pos} Treg number or of a defective Th1 cell maturation/differentiation process, is unclear. The

results presented in this chapter support the second hypothesis. First, analysis of the maturation phenotype showed that TIM-3 expression is associated with markers of memory differentiation. Second, treatment of cells with IL-2 and T cell expander – a T cell activation protocol that mimics TCR and CD80/CD86-mediated co-stimulation (Bonyhadi et al. 2005) – induced an increase in the expression of TIM-3 in HS, whereas AILD patients failed to up-regulate it following 5 days in culture. Third, patients displayed a low percentage of TIM-3 on $Tbet^{pos}$ cells compared to HS. These data suggests that in AILD Th1 cells arrest at a Tbet positive status, acquiring effector properties typical of Th1 cells without further differentiating into the Tbet/TIM-3 double positive status, which would confer them susceptibility to Treg control. In line with this, Anderson et al. have recently documented that *Tbet*$^{-/-}$ mice show defective TIM-3 expression on CD4 T cells and that Tbet over-expression results in constitutive TIM-3 expression; moreover, by showing that *Tbet* binds directly to the *Tim-3* promoter, the authors suggest that *Tim-3* is a direct transcriptional target of *Tbet* (Anderson et al. 2010). As supported by the data presented in this chapter, it is therefore plausible that in AILD $Tbet^{pos}$/TIM-3^{neg} cells prevail over the $Tbet^{pos}$/TIM-3^{pos} cells, accounting for the high number of poorly controllable effectors that contribute to the unfolding of the liver damage.

It has recently been reported that TIM-3 is expressed also on other types of effectors; for example, its expression by Th17 cells has been documented in mice and in humans (Chen et al. 2006, Hastings et al. 2009). Interestingly, $RORC^{pos}$ cells in AILD not only displayed lower levels of TIM-3 compared to the same subset in health, as they expressed even lower levels compared to $Tbet^{pos}$ cells. Since low levels of TIM-3 are

associated with poor Treg control, it is plausible that Th17 cell pathogenicity is at least in part attributable to a lower susceptibility to the TIM-3/gal9 mediated suppression.

GAL-9 gene knock-down experiments showed that although treatment of Tregs with GAL-9 siRNA led to a significant reduction in the suppression of TIM-3^{pos} cell proliferation, it did not abrogate it completely. The evidence that after addition of siRNA-treated Tregs, some 20% and 15% inhibition of TIM-3^{pos} cell proliferation was still observed, indicates that TIM-3 mediated immune-regulation may operate in a GAL-9-dependent and -independent fashion. That TIM-3 may have more than one ligand was first suggested by crystallography studies demonstrating that, in addition to the β-galactosidase bound characteristic of the TIM-3/GAL-9 interaction (Zhu et al. 2005), non-glycosylated TIM-3-Ig was able to bind to ligands other than GAL-9 in various cell types (E. Cao et al. 2007). Although the nature of these additional ligands is still largely unknown, two different groups have recently reported that both in humans and mice TIM-3 is a receptor of phosphatidylserine (PtdSer), and that TIM-3^{pos} cells can bind apoptotic cells expressing PtdSer (Nakayama et al. 2009, DeKruyff et al. 2010). Whether this or other TIM-3 ligands are expressed by Tregs and/or have an impact in their ability to suppress is unknown.

The present chapter has also provided a phenotypic analysis of $CD4^{pos}$ effector cells and by showing an increased frequency of $Tbet^{pos}$, $RORC^{pos}$, IFN-γ^{pos} and IL-17^{pos} cells in AILD, implicates an involvement for both Th1 and Th17 cell subsets in the autoimmune liver damage. Interestingly while Th1 effectors are increased in both AIH and AISC, Th17 cells predominate in the latter. The correlation between frequency of 'type 1' immune responses and AST in both groups of patients suggests that IFN-γ orchestrates hepatocyte damage, whereas the correlation between 'type 17' immune

responses and biochemical indices of cholestasis observed in AISC only indicates that IL-17 is more likely to be involved in the bile duct damage characteristic of this condition. While the association between IFN-γ and tissue damage in AIH had been known for many years, the relationship between IL-17 and bile duct destruction was less evident. A putative pathogenic role for Th17 cells has been recently documented in primary biliary cirrhosis (PBC), another autoimmune liver disorder in which bile ducts are affected. According to Harada et al., IL-17^{pos} cells accumulate around the damaged interlobular bile ducts within areas of chronic non-suppurative destructive cholangitis (Harada et al. 2009). Collectively, it seems that the Th1/Th17 division of labor may correspond in terms of liver pathology to a similar hepatocyte vs bile duct damage dichotomy.

Although AISC patients displayed more prominent Th17 immune responses than AIH-1 patients, no differences in terms of TIM-3 expression were observed. However, both flow cytometric and immunohistochemical studies have revealed that CD4^{pos} cells are reduced in AISC, indicating that this condition may be also associated with more vigorous CD8 immune-response. Thus, future studies should focus on CD8 cells and on the role of TIM-3 in their responsiveness to Treg control in AISC.

In summary, the results presented in this chapter show that impaired immune-regulation in AILD also derives from poorly controllable effectors that are likely to have undergone a defective differentiation process. Importantly phenotypic analysis of CD4 effector cells has highlighted that while Th1 cells are elevated in both AIH and AISC, Th17 cells predominate in the latter and correlate with biochemical indices of cholestasis, suggesting that the biliary damage characteristic of AISC is driven by an immunologically distinct response.

CHAPTER IV

General discussion

Experimental work performed before this project commenced had shown that liver damage in AILD is orchestrated by $CD4^{pos}$ T cells that recognise autoantigenic liver epitopes (Ma et al. 2006). Compelling evidence deriving from both animal models and human disease had indicated that in AIH the extent of self-reactive CD4 T cell effector immune responses is associated with an impairment of $CD4^{pos}CD25^{pos}$ regulatory T cells (Tregs; Longhi et al. 2004, Longhi et al. 2005, Ferri et al. 2010, Kido et al. 2008), a cell subset that plays a central role in maintaining peripheral tolerance and controlling organ-specific autoimmunity (Sakaguchi 2000). Defects in Treg number and/or function had also been described in several other autoimmune diseases, including T1D, RA, SLE, and MS. Strategies to augment Treg activity are therefore being considered for treatment of these diseases.

Since Tregs are defective in AIH, they are unable to control the proliferation and the effector cytokine production of responder CD4 T cells, stimulated *in vitro* with polyclonal or antigen-specific stimuli (Longhi et al. 2011). However, before this Thesis had started the mechanisms responsible for this Treg impairment were largely unknown. This thesis has thus explored possible mechanisms governing the loss of immune tolerance and leading to the development of liver damage in patients with autoimmune liver disease. In particular this study aimed to understand whether the impaired immune-regulation is mainly due to a primary Treg defect, or to low responsiveness of effector cells to Treg control.

The most logical first objective was however to evaluate whether an inadequate number of functional Tregs accounts for the impaired immune-regulation observed in AILD patients (Chapter IV). Most previous studies had characterised Tregs on the basis of CD25 positivity, which defines a population frequently contaminated by effector T

cells. In this thesis, I have investigated the frequency of Tregs in patients with AILD by analysing the properties of Treg subsets taking into consideration the recent observations that activated effector cells within the $CD4^{pos}CD25^{pos}$ T cell population are characterised by the expression of CD127 (Seddiki et al. 2006, Liu et al. 2006). In this regard, the results presented in Chapter IV showed that $CD4^{pos}CD25^{pos}$ T cells deprived of CD127 expression ($CD127^{neg}$ Tregs) bear the phenotypic and functional signature of *bona fide* Tregs, being characterised by a higher suppressive activity than conventional Tregs. The frequency of $CD127^{neg}$ T cells is, however, reduced in patients with AILD compared to healthy subjects. Akin to cell number, functional analyses revealed that these cells are in patients less capable to suppress the proliferation of responder cells than in healthy subjects. Concomitantly, the counterpart $CD4^{pos}CD25^{pos}CD127^{pos}$ T cell subset did not suppress but rather proliferated and was characterised by the production of pro-inflammatory cytokines, indicating that physical removal of CD127 expressing cells may be a useful approach to facilitate the purification of highly functional Tregs. The role of the $CD4^{pos}CD25^{pos}CD127^{pos}$ T cell subset should be the subject of future investigations, since it might play a role in the inflammatory process observed in AILD.

After confirming and extending the previous observations of impaired Treg-mediated suppression in the context of AILD, this Thesis proceeded to analyse mechanistic causes that may account for that defect by studying the role of the TIM-3/GAL-9 pathway. GAL-9, a member of the galectin family expressed by Tregs, had been shown to inhibit Th1 effector immune responses after binding to TIM-3 (Sanchez-Fueyo et al. 2003, Zhu et al. 2005), demonstrating a role of this pathway in the inhibition of effector cells during normal immune responses and in the induction of peripheral tolerance.

This thesis clearly shows that in AILD an intrinsic Treg defect (Chapter V) is accompanied by low susceptibility of responder cells to the control exerted by Tregs (Chapter VI), indicating that there is a defect both at the regulatory and effector cell levels.

The results presented in Chapter V confirm previous observations of a reduced suppressor function of Tregs in AILD and provide evidence that low expression of GAL-9 is one of the mechanisms responsible for Treg impairment, as highlighted by gene knockdown experiments where Tregs treated with a set of GAL-9-specific siRNAs were less effective at controlling the proliferation of responder cells. To further validate this observation and to definitively establish GAL-9 as one physiological mechanism of action employed by Tregs, future studies will explore whether in the context of AILD Treg suppression can be restored by transfecting these cells with human GAL-9 cDNA. In addition, several studies have shown that *in vivo* and *in vitro* culture of Tregs in the presence of a soluble form of GAL-9 results in the boosting of their function (Zhu et al. 2005, Seki et al. 2008), by promoting their differentiation, maintenance and expansion. Immunohistochemical studies performed in this Thesis demonstrated that GAL-9 is also expressed by other liver cell populations, particularly by Kupffer and to a lesser extent by endothelial cells. Similarly, in the context of HCV infection macrophage-derived GAL-9 was associated with the expansion of Tregs that characterises this chronic condition (Mengshol et al. 2010). In view of this, the role of GAL-9, either expressed by Kupffer cells or given exogenously, should be investigated in the context of liver autoimmunity as a potential treatment aimed at restoring Treg number and function, and thus leading to the reconstitution of tolerance to liver self-antigens.

In AILD the decreased percentage of GAL-9^{pos} Tregs is mirrored by a down-modulation of TIM-3 on the surface of CD4^{pos}CD25^{neg} cells, as well as Th1 and Th17 subsets, rendering these cells less susceptible to the immune-regulatory control exerted by Tregs (Chapter VI). Functional analyses performed in this study showed that effective control of cell proliferation was achieved when TIM-3^{pos} cells were used as responders, indicating that the presence of this molecule on the surface of effector cells confers responsivity to Treg control. In contrast, Tregs were unable to control the proliferation of TIM-3^{neg} responder cells, clearly demonstrating that the down-regulation of TIM-3 in patients with AILD renders effector cells resistant to the action of Tregs. The information obtained in the present study is crucial for devising regulatory T cell based immunotherapy for the treatment of AILD: since TIM-3 expression is reduced when the disease is active, adoptive transfer of autologous Tregs may be more efficacious once inflammation is dampened by immunosuppression to render effectors more amenable to Treg inhibition.

Although no differences in terms of TIM-3 expression were noted between patients with AIH-1 and those with AISC overlap syndrome, phenotypic analysis of effector T cell populations showed that circulating and liver-infiltrating CD4^{pos} cells are reduced in AISC compared to AIH-1, indicating that AISC may be associated with a more vigorous CD8 immune-response. Thus, future studies will focus on CD8 cells and on the role of TIM-3 in their responsiveness to Treg control in AISC.

Further comparisons between the two conditions revealed that Th1 effector cells are associated with hepatocyte damage in both AIH-1 and AISC, whereas Th17 immune responses predominate in the latter where they correlate with biochemical indices of cholestasis, indicating that IL-17 is involved in the bile duct damage characteristic of

this condition. Since a substantial difference between these two pathologies is the frequent association of AISC with IBD (Gregorio et al. 2001), an observation also corroborated in this thesis, it can be speculated that lymphocytes of intestinal origin are present in patients with a diagnosis of AISC rather than those suffering from AIH. Interestingly, it has recently been suggested that IBD may also be linked with a vigorous Th17 immune response. Moreover, murine studies have shown that intestinal Th17 cells are characterised by the expression of α4β7 (Wang et al. 2010), an integrin that mediates adherence to tissues by binding to specific ligands such as vascular cell adhesion molecule (VCAM)-1 and mucosal vascular addressin cell adhesion molecule (MadCAM)-1 (Briskin et al. 1997, Hillan et al. 1999, Grant et al. 2001). It has been postulated that gut mucosa T cells primed during the active phases of IBD possess the ability to bind both hepatic and mucosal endothelium through the interaction between α4β7 and its ligands (Adams and Eksteen 2006). Some of these cells may persist as long-lived memory T cells after resolution of IBD, therefore providing an explanation for the independent courses of bowel and liver disease. Thus, future studies will investigate whether lymphocytes of gut origin, namely those expressing α4β7, are present in the liver inflammatory infiltrate of patients with AISC and whether they are also present in AIH. Since a humanised mAb that specifically recognises the α4β7 heterodimer is now available and has been shown to be effective for the induction of clinical and endoscopic remission in patients with active UC (Feagan et al. 2005), it is reasonable to hypothesise that such α4β7 antagonist, useful as anti-inflammatory agents in bowel disease, may in the future be successful in treating liver diseases such as AISC or adult PSC.

In conclusion, the results present in this thesis show that the immune-regulatory defects in AILD span multiple levels. Reduced numbers of $CD127^{neg}$ Tregs (Chapter IV), an intrinsic Treg inability to suppress (Chapter V), and also low susceptibility of effector cells to Treg control all contribute to the loss of immunological tolerance to liver autoantigens (Figure 7.1.). Additionally, this thesis shows that distinct patterns of autoimmune liver injury are likely mediated by different effector immune responses: Th1 immunity mediating hepatocyte injury, and Th17 cells responsible for the bile duct damage characteristic of the overlap syndrome.

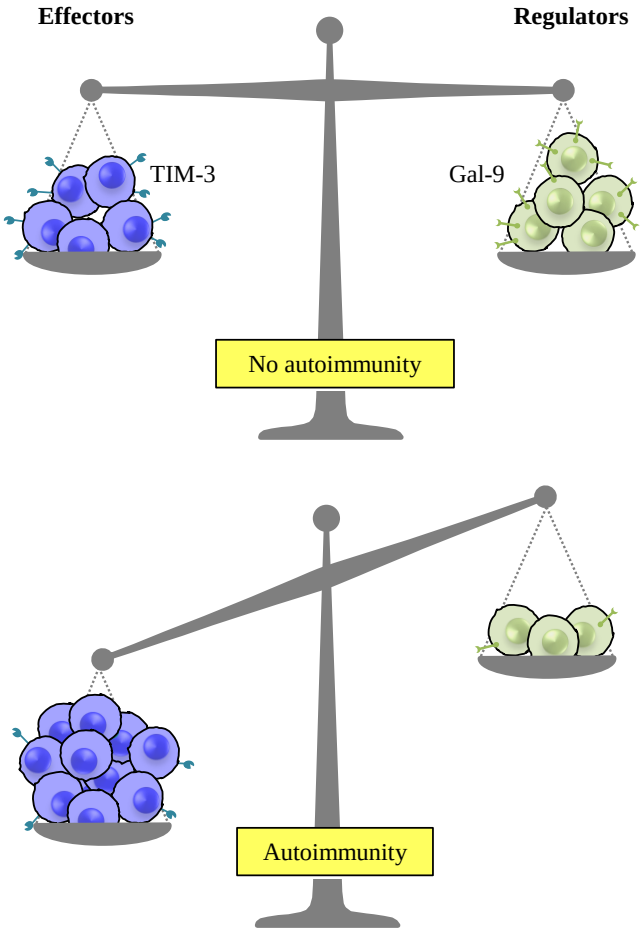


Figure 7.1. Effectors and regulators imbalance in autoimmune liver disease. In health, appropriate balance between Tregs and effector cell prevents autoimmunity (*top panel*). In autoimmune liver disease, this balance is destroyed. The immune-regulatory defect spans different levels: reduced numbers of Tregs, impaired intrinsic suppression, as well as resistance of effector cells to control of Tregs all contribute to the loss self tolerance with consequent ensuing of liver damage (*bottom panel*).

REFERENCES

Abdalian, R., Dhar, P., Jhaveri, K., Haider, M., Guindi, M. and Heathcote, E. J. (2008) 'Prevalence of sclerosing cholangitis in adults with autoimmune hepatitis: evaluating the role of routine magnetic resonance imaging', *Hepatology,* 47(3), 949-57.

Abdo, A. A., Bain, V. G., Kichian, K. and Lee, S. S. (2002) 'Evolution of autoimmune hepatitis to primary sclerosing cholangitis: A sequential syndrome', *Hepatology,* 36(6), 1393-9.

Abraham, C. and Cho, J. H. (2009) 'Inflammatory bowel disease', *N Engl J Med,* 361(21), 2066-78.

Abuaf, N., Johanet, C., Chretien, P., Martini, E., Soulier, E., Laperche, S. and Homberg, J. C. (1992) 'Characterization of the liver cytosol antigen type 1 reacting with autoantibodies in chronic active hepatitis', *Hepatology,* 16(4), 892-8.

Acosta-Rodriguez, E. V., Napolitani, G., Lanzavecchia, A. and Sallusto, F. (2007) 'Interleukins 1beta and 6 but not transforming growth factor-beta are essential for the differentiation of interleukin 17-producing human T helper cells', *Nat Immunol,* 8(9), 942-9.

Adams, L. A., Lindor, K. D. and Angulo, P. (2004) 'The prevalence of autoantibodies and autoimmune hepatitis in patients with nonalcoholic Fatty liver disease', *Am J Gastroenterol,* 99(7), 1316-20.

Afkarian, M., Sedy, J. R., Yang, J., Jacobson, N. G., Cereb, N., Yang, S. Y., Murphy, T. L. and Murphy, K. M. (2002) 'T-bet is a STAT1-induced regulator of IL-12R expression in naive CD4+ T cells', *Nat Immunol,* 3(6), 549-57.

Agarwal, K., Czaja, A. J. and Donaldson, P. T. (2007) 'A functional Fas promoter polymorphism is associated with a severe phenotype in type 1 autoimmune hepatitis characterized by early development of cirrhosis', *Tissue Antigens,* 69(3), 227-35.

Agarwal, K., Czaja, A. J., Jones, D. E. and Donaldson, P. T. (2000) 'Cytotoxic T lymphocyte antigen-4 (CTLA-4) gene polymorphisms and susceptibility to type 1 autoimmune hepatitis', *Hepatology,* 31(1), 49-53.

Aggarwal, S., Ghilardi, N., Xie, M. H., de Sauvage, F. J. and Gurney, A. L. (2003) 'Interleukin-23 promotes a distinct CD4 T cell activation state characterized by the production of interleukin-17', *J Biol Chem,* 278(3), 1910-4.

Agmon-Levin, N., Ram, M., Barzilai, O., Porat-Katz, B. S., Parikman, R., Selmi, C., Gershwin, M. E., Anaya, J. M., Youinou, P., Bizzaro, N., Tincani, A., Tzioufas, A. G., Cervera, R., Stojanovich, L., Martin, J., Gonzalez-Gay, M. A., Valentini, G., Blank, M., SanMarco, M., Rozman, B., Bombardieri, S., De Vita, S. and Shoenfeld, Y. (2009) 'Prevalence of hepatitis C serum antibody in autoimmune diseases', *J Autoimmun,* 32(3-4), 261-6.

Al-Chalabi, T., Boccato, S., Portmann, B. C., McFarlane, I. G. and Heneghan, M. A. (2006) 'Autoimmune hepatitis (AIH) in the elderly: a systematic retrospective analysis of a large group of consecutive patients with definite AIH followed at a tertiary referral centre', *J Hepatol,* 45(4), 575-83.

Alegre, M. L., Frauwirth, K. A. and Thompson, C. B. (2001) 'T-cell regulation by CD28 and CTLA-4', *Nat Rev Immunol,* 1(3), 220-8.

Aluvihare, V. R., Kallikourdis, M. and Betz, A. G. (2004) 'Regulatory T cells mediate maternal tolerance to the fetus', *Nat Immunol*, 5(3), 266-71.

Alvarado-Sanchez, B., Hernandez-Castro, B., Portales-Perez, D., Baranda, L., Layseca-Espinosa, E., Abud-Mendoza, C., Cubillas-Tejeda, A. C. and Gonzalez-Amaro, R. (2006) 'Regulatory T cells in patients with systemic lupus erythematosus', *J Autoimmun*, 27(2), 110-8.

Alvarez, F., Berg, P. A., Bianchi, F. B., Bianchi, L., Burroughs, A. K., Cancado, E. L., Chapman, R. W., Cooksley, W. G., Czaja, A. J., Desmet, V. J., Donaldson, P. T., Eddleston, A. L., Fainboim, L., Heathcote, J., Homberg, J. C., Hoofnagle, J. H., Kakumu, S., Krawitt, E. L., Mackay, I. R., MacSween, R. N., Maddrey, W. C., Manns, M. P., McFarlane, I. G., Meyer zum Buschenfelde, K. H., Zeniya, M. and et al. (1999) 'International Autoimmune Hepatitis Group Report: review of criteria for diagnosis of autoimmune hepatitis', *J Hepatol*, 31(5), 929-38.

Anderson, A. C. and Anderson, D. E. (2006) 'TIM-3 in autoimmunity', *Current Opinion in Immunology*, 18(6), 665-669.

Anderson, A. C., Anderson, D. E., Bregoli, L., Hastings, W. D., Kassam, N., Lei, C., Chandwaskar, R., Karman, J., Su, E. W., Hirashima, M., Bruce, J. N., Kane, L. P., Kuchroo, V. K. and Hafler, D. A. (2007) 'Promotion of tissue inflammation by the immune receptor Tim-3 expressed on innate immune cells', *Science*, 318(5853), 1141-3.

Anderson, A. C., Lord, G. M., Dardalhon, V., Lee, D. H., Sabatos-Peyton, C. A., Glimcher, L. H. and Kuchroo, V. K. (2010) 'T-bet, a Th1 transcription factor regulates the expression of Tim-3', *Eur J Immunol*, 40(3), 859-66.

Andersson, J., Tran, D. Q., Pesu, M., Davidson, T. S., Ramsey, H., O'Shea, J. J. and Shevach, E. M. (2008) 'CD4+ FoxP3+ regulatory T cells confer infectious tolerance in a TGF-beta-dependent manner', *J Exp Med*, 205(9), 1975-81.

Andries, S., Casamayou, L., Sempoux, C., Burlet, M., Reding, R., Bernard Otte, J., Buts, J. P. and Sokal, E. (2001) 'Posttransplant immune hepatitis in pediatric liver transplant recipients: incidence and maintenance therapy with azathioprine', *Transplantation*, 72(2), 267-72.

Asakura, H., Kashio, Y., Nakamura, K., Seki, M., Dai, S., Shirato, Y., Abedin, M. J., Yoshida, N., Nishi, N., Imaizumi, T., Saita, N., Toyama, Y., Takashima, H., Nakamura, T., Ohkawa, M. and Hirashima, M. (2002) 'Selective eosinophil adhesion to fibroblast via IFN-gamma-induced galectin-9', *J Immunol*, 169(10), 5912-8.

Asseman, C., Mauze, S., Leach, M. W., Coffman, R. L. and Powrie, F. (1999) 'An essential role for interleukin 10 in the function of regulatory T cells that inhibit intestinal inflammation', *J Exp Med*, 190(7), 995-1004.

Aujla, S. J., Chan, Y. R., Zheng, M., Fei, M., Askew, D. J., Pociask, D. A., Reinhart, T. A., McAllister, F., Edeal, J., Gaus, K., Husain, S., Kreindler, J. L., Dubin, P. J., Pilewski, J. M., Myerburg, M. M., Mason, C. A., Iwakura, Y. and Kolls, J. K. (2008) 'IL-22 mediates mucosal host defense against Gram-negative bacterial pneumonia', *Nat Med*, 14(3), 275-81.

Axelsson, S., Faresjo, M., Hedman, M., Ludvigsson, J. and Casas, R. (2008) 'Cryopreserved peripheral blood mononuclear cells are suitable for the assessment of immunological markers in type 1 diabetic children', *Cryobiology*, 57(3), 201-8.

Bachmann, M. F., Kohler, G., Ecabert, B., Mak, T. W. and Kopf, M. (1999) 'Cutting edge: lymphoproliferative disease in the absence of CTLA-4 is not T cell autonomous', *J Immunol*, 163(3), 1128-31.

Baecher-Allan, C., Brown, J. A., Freeman, G. J. and Hafler, D. A. (2001) 'CD4+CD25high regulatory cells in human peripheral blood', *J Immunol*, 167(3), 1245-53.

Bandin, O., Courvalin, J. C., Poupon, R., Dubel, L., Homberg, J. C. and Johanet, C. (1996) 'Specificity and sensitivity of gp210 autoantibodies detected using an enzyme-linked immunosorbent assay and a synthetic polypeptide in the diagnosis of primary biliary cirrhosis', *Hepatology*, 23(5), 1020-4.

Banham, A. H. (2006) 'Cell-surface IL-7 receptor expression facilitates the purification of FOXP3(+) regulatory T cells', *Trends Immunol*, 27(12), 541-4.

Barth, E. and Clawson, J. (2010) 'A Case of Autoimmune Hepatitis Treated with Rituximab', *Case Rep Gastroenterol*, 4(3), 502-509.

Baum, L. G., Pang, M., Perillo, N. L., Wu, T., Delegeane, A., Uittenbogaart, C. H., Fukuda, M. and Seilhamer, J. J. (1995) 'Human thymic epithelial cells express an endogenous lectin, galectin-1, which binds to core 2 O-glycans on thymocytes and T lymphoblastoid cells', *J Exp Med*, 181(3), 877-87.

Belkaid, Y., Piccirillo, C. A., Mendez, S., Shevach, E. M. and Sacks, D. L. (2002) 'CD4+CD25+ regulatory T cells control Leishmania major persistence and immunity', *Nature*, 420(6915), 502-7.

Belkaid, Y. and Rouse, B. T. (2005) 'Natural regulatory T cells in infectious disease', *Nat Immunol*, 6(4), 353-60.

Belladonna, M. L., Puccetti, P., Orabona, C., Fallarino, F., Vacca, C., Volpi, C., Gizzi, S., Pallotta, M. T., Fioretti, M. C. and Grohmann, U. (2007) 'Immunosuppression via tryptophan catabolism: the role of kynurenine pathway enzymes', *Transplantation*, 84(1 Suppl), S17-20.

Ben Ari, Z., Mehta, A., Lennard, L. and Burroughs, A. K. (1995) 'Azathioprine-induced myelosuppression due to thiopurine methyltransferase deficiency in a patient with autoimmune hepatitis', *J Hepatol*, 23(3), 351-4.

Benacerraf, B., Kapp, J. A., Debre, P., Pierce, C. W. and de la Croix, F. (1975) 'The stimulation of specific suppressor T cells in genetic non-responder mice by linear random copolymers of L-amino acids', *Transplant Rev*, 26, 21-38.

Benghiat, F. S., Graca, L., Braun, M. Y., Detienne, S., Moore, F., Buonocore, S., Flamand, V., Waldmann, H., Goldman, M. and Le Moine, A. (2005) 'Critical influence of natural regulatory CD25+ T cells on the fate of allografts in the absence of immunosuppression', *Transplantation*, 79(6), 648-54.

Bettelli, E., Carrier, Y., Gao, W., Korn, T., Strom, T. B., Oukka, M., Weiner, H. L. and Kuchroo, V. K. (2006) 'Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells', *Nature,* 441(7090), 235-8.

Bettelli, E., Dastrange, M. and Oukka, M. (2005) 'Foxp3 interacts with nuclear factor of activated T cells and NF-kappa B to repress cytokine gene expression and effector functions of T helper cells', *Proc Natl Acad Sci U S A,* 102(14), 5138-43.

Bettelli, E., Korn, T., Oukka, M. and Kuchroo, V. K. (2008) 'Induction and effector functions of T(H)17 cells', *Nature,* 453(7198), 1051-7.

Beuers, U. (2005) 'Hepatic overlap syndromes', *J Hepatol,* 42 Suppl(1), S93-9.

Beyer, M. and Schultze, J. L. (2006) 'Regulatory T cells in cancer', *Blood,* 108(3), 804-11.

Bhat, M., Guindi, M., Heathcote, E. J. and Hirschfield, G. M. (2009) 'Transient development of anti-mitochondrial antibodies accompanies autoimmune hepatitis-sclerosing cholangitis overlap', *Gut,* 58(1), 152-3.

Bianchi, T., Pincus, L. B., Wurbel, M. A., Rich, B. E., Kupper, T. S., Fuhlbrigge, R. C. and Boes, M. (2009) 'Maintenance of peripheral tolerance through controlled tissue homing of antigen-specific T cells in K14-mOVA mice', *J Immunol,* 182(8), 4665-74.

Billiard, F., Litvinova, E., Saadoun, D., Djelti, F., Klatzmann, D., Cohen, J. L., Marodon, G. and Salomon, B. L. (2006) 'Regulatory and effector T cell activation levels are prime determinants of in vivo immune regulation', *J Immunol,* 177(4), 2167-74.

Bittencourt, P. L., Palacios, S. A., Cancado, E. L., Porta, G., Carrilho, F. J., Laudanna, A. A., Kalil, J. and Goldberg, A. C. (2003) 'Cytotoxic T lymphocyte antigen-4 gene polymorphisms do not confer susceptibility to autoimmune hepatitis types 1 and 2 in Brazil', *Am J Gastroenterol,* 98(7), 1616-20.

Blaser, C., Kaufmann, M., Muller, C., Zimmermann, C., Wells, V., Mallucci, L. and Pircher, H. (1998) 'Beta-galactoside-binding protein secreted by activated T cells inhibits antigen-induced proliferation of T cells', *Eur J Immunol,* 28(8), 2311-9.

Bluestone, J. A., Herold, K. and Eisenbarth, G. (2010) 'Genetics, pathogenesis and clinical interventions in type 1 diabetes', *Nature,* 464(7293), 1293-300.

Boberg, K. M., Aadland, E., Jahnsen, J., Raknerud, N., Stiris, M. and Bell, H. (1998) 'Incidence and prevalence of primary biliary cirrhosis, primary sclerosing cholangitis, and autoimmune hepatitis in a Norwegian population', *Scand J Gastroenterol,* 33(1), 99-103.

Boberg, K. M., Chapman, R. W., Hirschfield, G. M., Lohse, A. W., Manns, M. P. and Schrumpf, E. (2011) 'Overlap syndromes: the International Autoimmune Hepatitis Group (IAIHG) position statement on a controversial issue', *J Hepatol,* 54(2), 374-85.

Bogdanos, D. P., Choudhuri, K. and Vergani, D. (2001) 'Molecular mimicry and autoimmune liver disease: virtuous intentions, malign consequences', *Liver,* 21(4), 225-32.

Bogdanos, D. P., Invernizzi, P., Mackay, I. R. and Vergani, D. (2008) 'Autoimmune liver serology: current diagnostic and clinical challenges', *World J Gastroenterol,* 14(21), 3374-87.

Bogdanos, D. P., Mieli-Vergani, G. and Vergani, D. (2009) 'Autoantibodies and their antigens in autoimmune hepatitis', *Semin Liver Dis,* 29(3), 241-53.

Bonelli, M., Savitskaya, A., von Dalwigk, K., Steiner, C. W., Aletaha, D., Smolen, J. S. and Scheinecker, C. (2008) 'Quantitative and qualitative deficiencies of regulatory T cells in patients with systemic lupus erythematosus (SLE)', *Int Immunol,* 20(7), 861-8.

Bonnevier, J. L., Zhang, R. and Mueller, D. L. (2005) 'E3 ubiquitin ligases and their control of T cell autoreactivity', *Arthritis Res Ther,* 7(6), 233-42.

Bonyhadi, M., Frohlich, M., Rasmussen, A., Ferrand, C., Grosmaire, L., Robinet, E., Leis, J., Maziarz, R. T., Tiberghien, P. and Berenson, R. J. (2005) 'In vitro engagement of CD3 and CD28 corrects T cell defects in chronic lymphocytic leukemia', *J Immunol,* 174(4), 2366-75.

Booth, N. J., McQuaid, A. J., Sobande, T., Kissane, S., Agius, E., Jackson, S. E., Salmon, M., Falciani, F., Yong, K., Rustin, M. H., Akbar, A. N. and Vukmanovic-Stejic, M. (2010) 'Different proliferative potential and migratory characteristics of human CD4+ regulatory T cells that express either CD45RA or CD45RO', *J Immunol,* 184(8), 4317-26.

Borsellino, G., Kleinewietfeld, M., Di Mitri, D., Sternjak, A., Diamantini, A., Giometto, R., Hopner, S., Centonze, D., Bernardi, G., Dell'Acqua, M. L., Rossini, P. M., Battistini, L., Rotzschke, O. and Falk, K. (2007) 'Expression of ectonucleotidase CD39 by Foxp3+ Treg cells: hydrolysis of extracellular ATP and immune suppression', *Blood,* 110(4), 1225-32.

Bottazzo, G. F., Florin-Christensen, A., Fairfax, A., Swana, G., Doniach, D. and Groeschel-Stewart, U. (1976) 'Classification of smooth muscle autoantibodies detected by immunofluorescence', *J Clin Pathol,* 29(5), 403-10.

Bouneaud, C., Kourilsky, P. and Bousso, P. (2000) 'Impact of negative selection on the T cell repertoire reactive to a self-peptide: a large fraction of T cell clones escapes clonal deletion', *Immunity,* 13(6), 829-40.

Brown, M. and Wittwer, C. (2000) 'Flow cytometry: principles and clinical applications in hematology', *Clin Chem,* 46(8 Pt 2), 1221-9.

Bruder, D., Probst-Kepper, M., Westendorf, A. M., Geffers, R., Beissert, S., Loser, K., von Boehmer, H., Buer, J. and Hansen, W. (2004) 'Neuropilin-1: a surface marker of regulatory T cells', *Eur J Immunol,* 34(3), 623-30.

Brunkow, M. E., Jeffery, E. W., Hjerrild, K. A., Paeper, B., Clark, L. B., Yasayko, S. A., Wilkinson, J. E., Galas, D., Ziegler, S. F. and Ramsdell, F. (2001) 'Disruption of a new forkhead/winged-helix protein, scurfin, results in the fatal lymphoproliferative disorder of the scurfy mouse', *Nat Genet,* 27(1), 68-73.

Brusko, T., Wasserfall, C., McGrail, K., Schatz, R., Viener, H. L., Schatz, D., Haller, M., Rockell, J., Gottlieb, P., Clare-Salzler, M. and Atkinson, M. (2007) 'No alterations in

the frequency of FOXP3+ regulatory T-cells in type 1 diabetes', *Diabetes,* 56(3), 604-12.

Brusko, T. M., Wasserfall, C. H., Clare-Salzler, M. J., Schatz, D. A. and Atkinson, M. A. (2005) 'Functional defects and the influence of age on the frequency of CD4+ CD25+ T-cells in type 1 diabetes', *Diabetes,* 54(5), 1407-14.

Buckner, J. H. (2010) 'Mechanisms of impaired regulation by CD4(+)CD25(+)FOXP3(+) regulatory T cells in human autoimmune diseases', *Nat Rev Immunol,* 10(12), 849-59.

Burak, K. W., Urbanski, S. J. and Swain, M. G. (1998) 'Successful treatment of refractory type 1 autoimmune hepatitis with methotrexate', *J Hepatol,* 29(6), 990-3.

Burchill, M. A., Yang, J., Vang, K. B., Moon, J. J., Chu, H. H., Lio, C. W., Vegoe, A. L., Hsieh, C. S., Jenkins, M. K. and Farrar, M. A. (2008) 'Linked T cell receptor and cytokine signaling govern the development of the regulatory T cell repertoire', *Immunity,* 28(1), 112-21.

Burlingame, R. W., Rubin, R. L. and Rosenberg, A. M. (1993) 'Autoantibodies to chromatin components in juvenile rheumatoid arthritis', *Arthritis Rheum,* 36(6), 836-41.

Buxbaum, J., Qian, P., Allen, P. M. and Peters, M. G. (2008) 'Hepatitis resulting from liver-specific expression and recognition of self-antigen', *J Autoimmun,* 31(3), 208-15.

Camby, I., Le Mercier, M., Lefranc, F. and Kiss, R. (2006) 'Galectin-1: a small protein with major functions', *Glycobiology,* 16(11), 137R-157R.

Cao, D., Malmstrom, V., Baecher-Allan, C., Hafler, D., Klareskog, L. and Trollmo, C. (2003) 'Isolation and functional characterization of regulatory CD25brightCD4+ T cells from the target organ of patients with rheumatoid arthritis', *Eur J Immunol,* 33(1), 215-23.

Cao, D., van Vollenhoven, R., Klareskog, L., Trollmo, C. and Malmstrom, V. (2004) 'CD25brightCD4+ regulatory T cells are enriched in inflamed joints of patients with chronic rheumatic disease', *Arthritis Res Ther,* 6(4), R335-46.

Cao, E., Zang, X., Ramagopal, U. A., Mukhopadhaya, A., Fedorov, A., Fedorov, E., Zencheck, W. D., Lary, J. W., Cole, J. L., Deng, H., Xiao, H., Dilorenzo, T. P., Allison, J. P., Nathenson, S. G. and Almo, S. C. (2007) 'T cell immunoglobulin mucin-3 crystal structure reveals a galectin-9-independent ligand-binding surface', *Immunity,* 26(3), 311-21.

Cao, X., Cai, S. F., Fehniger, T. A., Song, J., Collins, L. I., Piwnica-Worms, D. R. and Ley, T. J. (2007) 'Granzyme B and perforin are important for regulatory T cell-mediated suppression of tumor clearance', *Immunity,* 27(4), 635-46.

Cederbom, L., Hall, H. and Ivars, F. (2000) 'CD4+CD25+ regulatory T cells down-regulate co-stimulatory molecules on antigen-presenting cells', *Eur J Immunol,* 30(6), 1538-43.

Chabot, S., Kashio, Y., Seki, M., Shirato, Y., Nakamura, K., Nishi, N., Nakamura, T., Matsumoto, R. and Hirashima, M. (2002) 'Regulation of galectin-9 expression and release in Jurkat T cell line cells', *Glycobiology,* 12(2), 111-8.

Chae, S. C., Park, Y. R., Shim, S. C., Yoon, K. S. and Chung, H. T. (2004) 'The polymorphisms of Th1 cell surface gene Tim-3 are associated in a Korean population with rheumatoid arthritis', *Immunol Lett,* 95(1), 91-5.

Chang, S. H. and Dong, C. (2007) 'A novel heterodimeric cytokine consisting of IL-17 and IL-17F regulates inflammatory responses', *Cell Res,* 17(5), 435-40.

Chazouilleres, O., Wendum, D., Serfaty, L., Montembault, S., Rosmorduc, O. and Poupon, R. (1998) 'Primary biliary cirrhosis-autoimmune hepatitis overlap syndrome: clinical features and response to therapy', *Hepatology,* 28(2), 296-301.

Chen, M. L., Yan, B. S., Bando, Y., Kuchroo, V. K. and Weiner, H. L. (2008) 'Latency-associated peptide identifies a novel CD4+CD25+ regulatory T cell subset with TGFbeta-mediated function and enhanced suppression of experimental autoimmune encephalomyelitis', *J Immunol,* 180(11), 7327-37.

Chen, W., Jin, W., Hardegen, N., Lei, K. J., Li, L., Marinos, N., McGrady, G. and Wahl, S. M. (2003) 'Conversion of peripheral CD4+CD25- naive T cells to CD4+CD25+ regulatory T cells by TGF-beta induction of transcription factor Foxp3', *J Exp Med,* 198(12), 1875-86.

Chen, Y., Langrish, C. L., McKenzie, B., Joyce-Shaikh, B., Stumhofer, J. S., McClanahan, T., Blumenschein, W., Churakovsa, T., Low, J., Presta, L., Hunter, C. A., Kastelein, R. A. and Cua, D. J. (2006) 'Anti–IL-23 therapy inhibits multiple inflammatory pathways and ameliorates autoimmune encephalomyelitis', *The Journal of Clinical Investigation,* 116(5), 1317-1326.

Chilosi, M., Lestani, M., Pedron, S., Montagna, L., Benedetti, A., Pizzolo, G. and Menestrina, F. (1994) 'A rapid immunostaining method for frozen sections', *Biotech Histochem,* 69(4), 235-9.

Ciofani, M. and Zuniga-Pflucker, J. C. (2010) 'Determining gammadelta versus alphass T cell development', *Nat Rev Immunol,* 10(9), 657-63.

Codarri, L., Vallotton, L., Ciuffreda, D., Venetz, J. P., Garcia, M., Hadaya, K., Buhler, L., Rotman, S., Pascual, M. and Pantaleo, G. (2007) 'Expansion and tissue infiltration of an allospecific CD4+CD25+CD45RO+IL-7Ralphahigh cell population in solid organ transplant recipients', *J Exp Med,* 204(7), 1533-41.

Coffman, R. L., Seymour, B. W., Hudak, S., Jackson, J. and Rennick, D. (1989) 'Antibody to interleukin-5 inhibits helminth-induced eosinophilia in mice', *Science,* 245(4915), 308-10.

Collison, L. W., Workman, C. J., Kuo, T. T., Boyd, K., Wang, Y., Vignali, K. M., Cross, R., Sehy, D., Blumberg, R. S. and Vignali, D. A. (2007) 'The inhibitory cytokine IL-35 contributes to regulatory T-cell function', *Nature,* 450(7169), 566-9.

Cook, G. C., Mulligan, R. and Sherlock, S. (1971) 'Controlled prospective trial of corticosteroid therapy in active chronic hepatitis', *Q J Med,* 40(158), 159-85.

Coombes, J. L., Siddiqui, K. R., Arancibia-Carcamo, C. V., Hall, J., Sun, C. M., Belkaid, Y. and Powrie, F. (2007) 'A functionally specialized population of mucosal CD103+ DCs

induces Foxp3+ regulatory T cells via a TGF-beta and retinoic acid-dependent mechanism', *J Exp Med*, 204(8), 1757-64.

Cooper, D. N. and Barondes, S. H. (1999) 'God must love galectins; he made so many of them', *Glycobiology*, 9(10), 979-84.

Cooper, M. D., Raymond, D. A., Peterson, R. D., South, M. A. and Good, R. A. (1966) 'The functions of the thymus system and the bursa system in the chicken', *J Exp Med*, 123(1), 75-102.

Corey, D. R. (2007) 'Chemical modification: the key to clinical application of RNA interference?', *J Clin Invest*, 117(12), 3615-22.

Costa, M., Rodriguez-Sanchez, J. L., Czaja, A. J. and Gelpi, C. (2000) 'Isolation and characterization of cDNA encoding the antigenic protein of the human tRNP(Ser)Sec complex recognized by autoantibodies from patients withtype-1 autoimmune hepatitis', *Clin Exp Immunol*, 121(2), 364-74.

Crapper, R. M., Bhathal, P. S., Mackay, I. R. and Frazer, I. H. (1986) "Acute' autoimmune hepatitis', *Digestion*, 34(3), 216-25.

Crispe, I. N. (2009) 'The liver as a lymphoid organ', *Annu Rev Immunol*, 27, 147-63.

Crispin, J. C., Martinez, A. and Alcocer-Varela, J. (2003) 'Quantification of regulatory T cells in patients with systemic lupus erythematosus', *J Autoimmun*, 21(3), 273-6.

Csepregi, A., Nemesanszky, E., Luettig, B., Obermayer-Straub, P. and Manns, M. P. (2001) 'LKM3 autoantibodies in hepatitis C cirrhosis: a further phenomenon of the HCV-induced autoimmunity', *Am J Gastroenterol*, 96(3), 910-1.

Cua, D. J., Sherlock, J., Chen, Y., Murphy, C. A., Joyce, B., Seymour, B., Lucian, L., To, W., Kwan, S., Churakova, T., Zurawski, S., Wiekowski, M., Lira, S. A., Gorman, D., Kastelein, R. A. and Sedgwick, J. D. (2003) 'Interleukin-23 rather than interleukin-12 is the critical cytokine for autoimmune inflammation of the brain', *Nature*, 421(6924), 744-8.

Cuffari, C., Dassopoulos, T., Turnbough, L., Thompson, R. E. and Bayless, T. M. (2004) 'Thiopurine methyltransferase activity influences clinical response to azathioprine in inflammatory bowel disease', *Clin Gastroenterol Hepatol*, 2(5), 410-7.

Curotto de Lafaille, M. A. and Lafaille, J. J. (2009) 'Natural and adaptive foxp3+ regulatory T cells: more of the same or a division of labor?', *Immunity*, 30(5), 626-35.

Czaja, A. and Carpenter, H. A. (1996) 'Validation of scoring system for diagnosis of autoimmune hepatitis', *Dig Dis Sci*, 41(2), 305-14.

Czaja, A. J. (1996) 'The variant forms of autoimmune hepatitis', *Ann Intern Med*, 125(7), 588-98.

Czaja, A. J. (2008) 'Safety issues in the management of autoimmune hepatitis', *Expert Opin Drug Saf*, 7(3), 319-33.

Czaja, A. J. and Carpenter, H. A. (1997) 'Histological findings in chronic hepatitis C with autoimmune features', *Hepatology*, 26(2), 459-66.

Czaja, A. J. and Carpenter, H. A. (2006a) 'Distinctive clinical phenotype and treatment outcome of type 1 autoimmune hepatitis in the elderly', *Hepatology*, 43(3), 532-8.

Czaja, A. J. and Carpenter, H. A. (2006b) 'Thiopurine methyltransferase deficiency and azathioprine intolerance in autoimmune hepatitis', *Dig Dis Sci*, 51(5), 968-75.

Czaja, A. J., Carpenter, H. A. and Lindor, K. D. (1999a) 'Ursodeoxycholic acid as adjunctive therapy for problematic type 1 autoimmune hepatitis: a randomized placebo-controlled treatment trial', *Hepatology*, 30(6), 1381-6.

Czaja, A. J., Cassani, F., Cataleta, M., Valentini, P. and Bianchi, F. B. (1996) 'Frequency and significance of antibodies to actin in type 1 autoimmune hepatitis', *Hepatology*, 24(5), 1068-73.

Czaja, A. J., Cassani, F., Cataleta, M., Valentini, P. and Bianchi, F. B. (1997) 'Antinuclear antibodies and patterns of nuclear immunofluorescence in type 1 autoimmune hepatitis', *Dig Dis Sci*, 42(8), 1688-96.

Czaja, A. J., Cookson, S., Constantini, P. K., Clare, M., Underhill, J. A. and Donaldson, P. T. (1999b) 'Cytokine polymorphisms associated with clinical features and treatment outcome in type 1 autoimmune hepatitis', *Gastroenterology*, 117(3), 645-52.

Czaja, A. J., Doherty, D. G. and Donaldson, P. T. (2002) 'Genetic bases of autoimmune hepatitis', *Dig Dis Sci*, 47(10), 2139-50.

Czaja, A. J. and Donaldson, P. T. (2000) 'Genetic susceptibilities for immune expression and liver cell injury in autoimmune hepatitis', *Immunol Rev*, 174, 250-9.

Czaja, A. J. and Manns, M. P. (2010) 'Advances in the diagnosis, pathogenesis, and management of autoimmune hepatitis', *Gastroenterology*, 139(1), 58-72 e4.

Czaja, A. J., Nishioka, M., Morshed, S. A. and Hachiya, T. (1994) 'Patterns of nuclear immunofluorescence and reactivities to recombinant nuclear antigens in autoimmune hepatitis', *Gastroenterology*, 107(1), 200-7.

D'Alise, A. M., Auyeung, V., Feuerer, M., Nishio, J., Fontenot, J., Benoist, C. and Mathis, D. (2008) 'The defect in T-cell regulation in NOD mice is an effect on the T-cell effectors', *Proc Natl Acad Sci U S A*, 105(50), 19857-62.

D'Souza, M., Fontenot, A. P., Mack, D. G., Lozupone, C., Dillon, S., Meditz, A., Wilson, C. C., Connick, E. and Palmer, B. E. (2007) 'Programmed death 1 expression on HIV-specific CD4+ T cells is driven by viral replication and associated with T cell dysfunction', *J Immunol*, 179(3), 1979-87.

Dardalhon, V., Awasthi, A., Kwon, H., Galileos, G., Gao, W., Sobel, R. A., Mitsdoerffer, M., Strom, T. B., Elyaman, W., Ho, I. C., Khoury, S., Oukka, M. and Kuchroo, V. K. (2008) 'IL-4 inhibits TGF-beta-induced Foxp3+ T cells and, together with TGF-beta, generates IL-9+ IL-10+ Foxp3(-) effector T cells', *Nat Immunol*, 9(12), 1347-55.

Davis, M. M. and Bjorkman, P. J. (1988) 'T-cell antigen receptor genes and T-cell recognition', *Nature*, 334(6181), 395-402.

Day, C. L., Kaufmann, D. E., Kiepiela, P., Brown, J. A., Moodley, E. S., Reddy, S., Mackey, E. W., Miller, J. D., Leslie, A. J., DePierres, C., Mncube, Z., Duraiswamy, J., Zhu, B., Eichbaum, Q., Altfeld, M., Wherry, E. J., Coovadia, H. M., Goulder, P. J., Klenerman, P., Ahmed, R., Freeman, G. J. and Walker, B. D. (2006) 'PD-1 expression on HIV-specific T cells is associated with T-cell exhaustion and disease progression', *Nature*, 443(7109), 350-4.

De la Concha, E. G., Fernandez-Arquero, M., Gual, L., Vigil, P., Martinez, A., Urcelay, E., Ferreira, A., Garcia-Rodriguez, M. C. and Fontan, G. (2002) 'MHC susceptibility genes to IgA deficiency are located in different regions on different HLA haplotypes', *J Immunol*, 169(8), 4637-43.

de la Rosa, M., Rutz, S., Dorninger, H. and Scheffold, A. (2004) 'Interleukin-2 is essential for CD4+CD25+ regulatory T cell function', *Eur J Immunol*, 34(9), 2480-8.

De Rosa, S. C., Brenchley, J. M. and Roederer, M. (2003) 'Beyond six colors: a new era in flow cytometry', *Nat Med*, 9(1), 112-7.

Deaglio, S., Dwyer, K. M., Gao, W., Friedman, D., Usheva, A., Erat, A., Chen, J. F., Enjyoji, K., Linden, J., Oukka, M., Kuchroo, V. K., Strom, T. B. and Robson, S. C. (2007) 'Adenosine generation catalyzed by CD39 and CD73 expressed on regulatory T cells mediates immune suppression', *J Exp Med*, 204(6), 1257-65.

Deftos, M. L. and Bevan, M. J. (2000) 'Notch signaling in T cell development', *Curr Opin Immunol*, 12(2), 166-72.

Degauque, N., Mariat, C., Kenny, J., Zhang, D., Gao, W., Vu, M. D., Alexopoulos, S., Oukka, M., Umetsu, D. T., DeKruyff, R. H., Kuchroo, V., Zheng, X. X. and Strom, T. B. (2008) 'Immunostimulatory Tim-1-specific antibody deprograms Tregs and prevents transplant tolerance in mice', *J Clin Invest*, 118(2), 735-41.

DeKruyff, R. H., Bu, X., Ballesteros, A., Santiago, C., Chim, Y. L., Lee, H. H., Karisola, P., Pichavant, M., Kaplan, G. G., Umetsu, D. T., Freeman, G. J. and Casasnovas, J. M. (2010) 'T cell/transmembrane, Ig, and mucin-3 allelic variants differentially recognize phosphatidylserine and mediate phagocytosis of apoptotic cells', *J Immunol*, 184(4), 1918-30.

Delves, P. J. and Roitt, I. M. (2000a) 'The immune system. First of two parts', *N Engl J Med*, 343(1), 37-49.

Delves, P. J. and Roitt, I. M. (2000b) 'The immune system. Second of two parts', *N Engl J Med*, 343(2), 108-17.

Demetris, A. J., Adeyi, O., Bellamy, C. O., Clouston, A., Charlotte, F., Czaja, A., Daskal, I., El-Monayeri, M. S., Fontes, P., Fung, J., Gridelli, B., Guido, M., Haga, H., Hart, J., Honsova, E., Hubscher, S., Itoh, T., Jhala, N., Jungmann, P., Khettry, U., Lassman, C., Ligato, S., Lunz, J. G., 3rd, Marcos, A., Minervini, M. I., Molne, J., Nalesnik, M., Nasser, I., Neil, D., Ochoa, E., Pappo, O., Randhawa, P., Reinholt, F. P., Ruiz, P., Sebagh, M., Spada, M., Sonzogni, A., Tsamandas, A. C., Wernerson, A., Wu, T. and

Yilmaz, F. (2006) 'Liver biopsy interpretation for causes of late liver allograft dysfunction', *Hepatology*, 44(2), 489-501.

Derbinski, J., Gabler, J., Brors, B., Tierling, S., Jonnakuty, S., Hergenhahn, M., Peltonen, L., Walter, J. and Kyewski, B. (2005) 'Promiscuous gene expression in thymic epithelial cells is regulated at multiple levels', *J Exp Med*, 202(1), 33-45.

Dhirapong, A., Lleo, A., Leung, P., Gershwin, M. E. and Liu, F. T. (2009) 'The immunological potential of galectin-1 and -3', *Autoimmun Rev*, 8(5), 360-3.

Dinter, A. and Berger, E. G. (1998) 'Golgi-disturbing agents', *Histochem Cell Biol*, 109(5-6), 571-90.

Djilali-Saiah, I., Fakhfakh, A., Louafi, H., Caillat-Zucman, S., Debray, D. and Alvarez, F. (2006) 'HLA class II influences humoral autoimmunity in patients with type 2 autoimmune hepatitis', *J Hepatol*, 45(6), 844-50.

Djilali-Saiah, I., Renous, R., Caillat-Zucman, S., Debray, D. and Alvarez, F. (2004) 'Linkage disequilibrium between HLA class II region and autoimmune hepatitis in pediatric patients', *J Hepatol*, 40(6), 904-9.

Donaldson, P. T. (2002) 'Genetics in autoimmune hepatitis', *Semin Liver Dis*, 22(4), 353-64.

Donaldson, P. T. (2004a) 'Genetics of autoimmune and viral liver diseases; understanding the issues', *J Hepatol*, 41(2), 327-32.

Donaldson, P. T. (2004b) 'Genetics of liver disease: immunogenetics and disease pathogenesis', *Gut*, 53(4), 599-608.

Donaldson, P. T., Doherty, D. G., Hayllar, K. M., McFarlane, I. G., Johnson, P. J. and Williams, R. (1991) 'Susceptibility to autoimmune chronic active hepatitis: human leukocyte antigens DR4 and A1-B8-DR3 are independent risk factors', *Hepatology*, 13(4), 701-6.

Dorsett, Y. and Tuschl, T. (2004) 'siRNAs: applications in functional genomics and potential as therapeutics', *Nat Rev Drug Discov*, 3(4), 318-29.

Duarte-Rey, C., Pardo, A. L., Rodriguez-Velosa, Y., Mantilla, R. D., Anaya, J. M. and Rojas-Villarraga, A. (2009) 'HLA class II association with autoimmune hepatitis in Latin America: a meta-analysis', *Autoimmun Rev*, 8(4), 325-31.

Duhen, T., Geiger, R., Jarrossay, D., Lanzavecchia, A. and Sallusto, F. (2009) 'Production of interleukin 22 but not interleukin 17 by a subset of human skin-homing memory T cells', *Nat Immunol*, 10(8), 857-63.

Dwyer, K. M., Deaglio, S., Gao, W., Friedman, D., Strom, T. B. and Robson, S. C. (2007) 'CD39 and control of cellular immune responses', *Purinergic Signal*, 3(1-2), 171-80.

Eastaff-Leung, N., Mabarrack, N., Barbour, A., Cummins, A. and Barry, S. (2010) 'Foxp3+ regulatory T cells, Th17 effector cells, and cytokine environment in inflammatory bowel disease', *J Clin Immunol*, 30(1), 80-9.

Eggena, M. P., Walker, L. S., Nagabhushanam, V., Barron, L., Chodos, A. and Abbas, A. K. (2004) 'Cooperative roles of CTLA-4 and regulatory T cells in tolerance to an islet cell antigen', *J Exp Med*, 199(12), 1725-30.

Ehrenstein, M. R., Evans, J. G., Singh, A., Moore, S., Warnes, G., Isenberg, D. A. and Mauri, C. (2004) 'Compromised function of regulatory T cells in rheumatoid arthritis and reversal by anti-TNFalpha therapy', *J Exp Med*, 200(3), 277-85.

el-Shabrawi, M., Wilkinson, M. L., Portmann, B., Mieli-Vergani, G., Chong, S. K., Williams, R. and Mowat, A. P. (1987) 'Primary sclerosing cholangitis in childhood', *Gastroenterology*, 92(5 Pt 1), 1226-35.

Elbashir, S. M., Harborth, J., Lendeckel, W., Yalcin, A., Weber, K. and Tuschl, T. (2001) 'Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells', *Nature*, 411(6836), 494-8.

Encinas, J. A. and Kuchroo, V. K. (2000) 'Mapping and identification of autoimmunity genes', *Curr Opin Immunol*, 12(6), 691-7.

Fahlen, L., Read, S., Gorelik, L., Hurst, S. D., Coffman, R. L., Flavell, R. A. and Powrie, F. (2005) 'T cells that cannot respond to TGF-beta escape control by CD4(+)CD25(+) regulatory T cells', *J Exp Med*, 201(5), 737-46.

Fantini, M. C., Becker, C., Monteleone, G., Pallone, F., Galle, P. R. and Neurath, M. F. (2004) 'Cutting edge: TGF-beta induces a regulatory phenotype in CD4+CD25- T cells through Foxp3 induction and down-regulation of Smad7', *J Immunol*, 172(9), 5149-53.

Faria, A. M. and Weiner, H. L. (2005) 'Oral tolerance', *Immunol Rev*, 206, 232-59.

Fathman, C. G. and Lineberry, N. B. (2007) 'Molecular mechanisms of CD4+ T-cell anergy', *Nat Rev Immunol*, 7(8), 599-609.

Feger, U., Luther, C., Poeschel, S., Melms, A., Tolosa, E. and Wiendl, H. (2007) 'Increased frequency of CD4+ CD25+ regulatory T cells in the cerebrospinal fluid but not in the blood of multiple sclerosis patients', *Clin Exp Immunol*, 147(3), 412-8.

Feigelstock, D., Thompson, P., Mattoo, P., Zhang, Y. and Kaplan, G. G. (1998) 'The human homolog of HAVcr-1 codes for a hepatitis A virus cellular receptor', *J Virol*, 72(8), 6621-8.

Ferber, I. A., Brocke, S., Taylor-Edwards, C., Ridgway, W., Dinisco, C., Steinman, L., Dalton, D. and Fathman, C. G. (1996) 'Mice with a disrupted IFN-gamma gene are susceptible to the induction of experimental autoimmune encephalomyelitis (EAE)', *J Immunol*, 156(1), 5-7.

Fernandes, N. F., Redeker, A. G., Vierling, J. M., Villamil, F. G. and Fong, T. L. (1999) 'Cyclosporine therapy in patients with steroid resistant autoimmune hepatitis', *Am J Gastroenterol*, 94(1), 241-8.

Ferri, S., Longhi, M. S., De Molo, C., Lalanne, C., Muratori, P., Granito, A., Hussain, M. J., Ma, Y., Lenzi, M., Mieli-Vergani, G., Bianchi, F. B., Vergani, D. and Muratori, L. (2010) 'A multifaceted imbalance of T cells with regulatory function characterizes type 1 autoimmune hepatitis', *Hepatology*, 52(3), 999-1007.

Filipowicz, W. (2005) 'RNAi: the nuts and bolts of the RISC machine', *Cell*, 122(1), 17-20.

Fire, A., Xu, S., Montgomery, M. K., Kostas, S. A., Driver, S. E. and Mello, C. C. (1998) 'Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans', *Nature*, 391(6669), 806-11.

Fletcher, J. M., Lonergan, R., Costelloe, L., Kinsella, K., Moran, B., O'Farrelly, C., Tubridy, N. and Mills, K. H. (2009) 'CD39+Foxp3+ regulatory T Cells suppress pathogenic Th17 cells and are impaired in multiple sclerosis', *J Immunol*, 183(11), 7602-10.

Floreani, A., Rizzotto, E. R., Ferrara, F., Carderi, I., Caroli, D., Blasone, L. and Baldo, V. (2005) 'Clinical course and outcome of autoimmune hepatitis/primary sclerosing cholangitis overlap syndrome', *Am J Gastroenterol*, 100(7), 1516-22.

Fontenot, J. D., Gavin, M. A. and Rudensky, A. Y. (2003) 'Foxp3 programs the development and function of CD4+CD25+ regulatory T cells', *Nat Immunol*, 4(4), 330-6.

Fontenot, J. D., Rasmussen, J. P., Gavin, M. A. and Rudensky, A. Y. (2005) 'A function for interleukin 2 in Foxp3-expressing regulatory T cells', *Nat Immunol*, 6(11), 1142-51.

Freeman, G. J., Casasnovas, J. M., Umetsu, D. T. and DeKruyff, R. H. (2010) 'TIM genes: a family of cell surface phosphatidylserine receptors that regulate innate and adaptive immunity', *Immunol Rev*, 235(1), 172-89.

Freeman, G. J., Wherry, E. J., Ahmed, R. and Sharpe, A. H. (2006) 'Reinvigorating exhausted HIV-specific T cells via PD-1-PD-1 ligand blockade', *J Exp Med*, 203(10), 2223-7.

Fukumori, T., Takenaka, Y., Yoshii, T., Kim, H. R., Hogan, V., Inohara, H., Kagawa, S. and Raz, A. (2003) 'CD29 and CD7 mediate galectin-3-induced type II T-cell apoptosis', *Cancer Res*, 63(23), 8302-11.

Fusconi, M., Cassani, F., Zauli, D., Lenzi, M., Ballardini, G., Volta, U. and Bianchi, F. B. (1990) 'Anti-actin antibodies: a new test for an old problem', *J Immunol Methods*, 130(1), 1-8.

Garin, M. I., Chu, C. C., Golshayan, D., Cernuda-Morollon, E., Wait, R. and Lechler, R. I. (2007) 'Galectin-1: a key effector of regulation mediated by CD4+CD25+ T cells', *Blood*, 109(5), 2058-65.

Gautam, M., Cheruvattath, R. and Balan, V. (2006) 'Recurrence of autoimmune liver disease after liver transplantation: a systematic review', *Liver Transpl*, 12(12), 1813-24.

Gavin, M. A., Rasmussen, J. P., Fontenot, J. D., Vasta, V., Manganiello, V. C., Beavo, J. A. and Rudensky, A. Y. (2007) 'Foxp3-dependent programme of regulatory T-cell differentiation', *Nature*, 445(7129), 771-5.

Geng, H., Zhang, G. M., Li, D., Zhang, H., Yuan, Y., Zhu, H. G., Xiao, H., Han, L. F. and Feng, Z. H. (2006) 'Soluble form of T cell Ig mucin 3 is an inhibitory molecule in T cell-mediated immune response', *J Immunol*, 176(3), 1411-20.

Gershon, R. K. and Kondo, K. (1970) 'Cell interactions in the induction of tolerance: the role of thymic lymphocytes', *Immunology*, 18(5), 723-37.

Gill, J., Malin, M., Sutherland, J., Gray, D., Hollander, G. and Boyd, R. (2003) 'Thymic generation and regeneration', *Immunol Rev*, 195, 28-50.

Girardi, M. (2006) 'Immunosurveillance and immunoregulation by gammadelta T cells', *J Invest Dermatol*, 126(1), 25-31.

Gisbert, J. P., Gomollon, F., Cara, C., Luna, M., Gonzalez-Lama, Y., Pajares, J. M., Mate, J. and Guijarro, L. G. (2007) 'Thiopurine methyltransferase activity in Spain: a study of 14,545 patients', *Dig Dis Sci*, 52(5), 1262-9.

Gohlke, F., Lohse, A. W., Dienes, H. P., Lohr, H., Marker-Hermann, E., Gerken, G. and Meyer zum Buschenfelde, K. H. (1996) 'Evidence for an overlap syndrome of autoimmune hepatitis and primary sclerosing cholangitis', *J Hepatol*, 24(6), 699-705.

Golden-Mason, L., Palmer, B. E., Kassam, N., Townshend-Bulson, L., Livingston, S., McMahon, B. J., Castelblanco, N., Kuchroo, V., Gretch, D. R. and Rosen, H. R. (2009) 'Negative immune regulator Tim-3 is overexpressed on T cells in hepatitis C virus infection and its blockade rescues dysfunctional CD4+ and CD8+ T cells', *J Virol*, 83(18), 9122-30.

Goldrath, A. W. and Bevan, M. J. (1999) 'Selecting and maintaining a diverse T-cell repertoire', *Nature*, 402(6759), 255-62.

Gondek, D. C., Lu, L. F., Quezada, S. A., Sakaguchi, S. and Noelle, R. J. (2005) 'Cutting edge: contact-mediated suppression by CD4+CD25+ regulatory cells involves a granzyme B-dependent, perforin-independent mechanism', *J Immunol*, 174(4), 1783-6.

Goverman, J. M. (2011) 'Immune tolerance in multiple sclerosis', *Immunol Rev*, 241(1), 228-40.

Gowans, J. L. (1957) 'The effect of the continuous re-infusion of lymph and lymphocytes on the output of lymphocytes from the thoracic duct of unanaesthetized rats', *Br J Exp Pathol*, 38(1), 67-78.

Graca, L., Cobbold, S. P. and Waldmann, H. (2002) 'Identification of regulatory T cells in tolerated allografts', *J Exp Med*, 195(12), 1641-6.

Graf, T. (2008) 'Immunology: blood lines redrawn', *Nature*, 452(7188), 702-3.

Greenwald, R. J., Freeman, G. J. and Sharpe, A. H. (2005) 'The B7 family revisited', *Annu Rev Immunol*, 23, 515-48.

Gregori, S., Giarratana, N., Smiroldo, S. and Adorini, L. (2003) 'Dynamics of pathogenic and suppressor T cells in autoimmune diabetes development', *J Immunol*, 171(8), 4040-7.

Gregorio, G. V., Choudhuri, K., Ma, Y., Pensati, P., Iorio, R., Grant, P., Garson, J., Bogdanos, D. P., Vegnente, A., Mieli-Vergani, G. and Vergani, D. (2003) 'Mimicry between the hepatitis C virus polyprotein and antigenic targets of nuclear and smooth muscle antibodies in chronic hepatitis C virus infection', *Clin Exp Immunol*, 133(3), 404-13.

Gregorio, G. V., Choudhuri, K., Ma, Y., Vegnente, A., Mieli-Vergani, G. and Vergani, D. (1999) 'Mimicry between the hepatitis B virus DNA polymerase and the antigenic

targets of nuclear and smooth muscle antibodies in chronic hepatitis B virus infection', *J Immunol*, 162(3), 1802-10.

Gregorio, G. V., McFarlane, B., Bracken, P., Vergani, D. and Mieli-Vergani, G. (2002) 'Organ and non-organ specific autoantibody titres and IgG levels as markers of disease activity: a longitudinal study in childhood autoimmune liver disease', *Autoimmunity*, 35(8), 515-9.

Gregorio, G. V., Portmann, B., Karani, J., Harrison, P., Donaldson, P. T., Vergani, D. and Mieli-Vergani, G. (2001) 'Autoimmune hepatitis/sclerosing cholangitis overlap syndrome in childhood: a 16-year prospective study', *Hepatology*, 33(3), 544-53.

Gregorio, G. V., Portmann, B., Reid, F., Donaldson, P. T., Doherty, D. G., McCartney, M., Mowat, A. P., Vergani, D. and Mieli-Vergani, G. (1997) 'Autoimmune hepatitis in childhood: A 20-year experience', *Hepatology*, 25(3), 541-547.

Grohmann, U., Orabona, C., Fallarino, F., Vacca, C., Calcinaro, F., Falorni, A., Candeloro, P., Belladonna, M. L., Bianchi, R., Fioretti, M. C. and Puccetti, P. (2002) 'CTLA-4-Ig regulates tryptophan catabolism in vivo', *Nat Immunol*, 3(11), 1097-101.

Grossman, W. J., Verbsky, J. W., Tollefsen, B. L., Kemper, C., Atkinson, J. P. and Ley, T. J. (2004) 'Differential expression of granzymes A and B in human cytotoxic lymphocyte subsets and T regulatory cells', *Blood*, 104(9), 2840-8.

Groux, H., Bigler, M., de Vries, J. E. and Roncarolo, M. G. (1996) 'Interleukin-10 induces a long-term antigen-specific anergic state in human CD4+ T cells', *J Exp Med*, 184(1), 19-29.

Gueguen, M., Meunier-Rotival, M., Bernard, O. and Alvarez, F. (1988) 'Anti-liver kidney microsome antibody recognizes a cytochrome P450 from the IID subfamily', *J Exp Med*, 168(2), 801-6.

Gupta, P., Hart, J., Millis, J. M., Cronin, D. and Brady, L. (2001) 'De novo hepatitis with autoimmune antibodies and atypical histology: a rare cause of late graft dysfunction after pediatric liver transplantation', *Transplantation*, 71(5), 664-8.

Gurian, L. E., Rogoff, T. M., Ware, A. J., Jordan, R. E., Combes, B. and Gilliam, J. N. (1985) 'The immunologic diagnosis of chronic active "autoimmune" hepatitis: distinction from systemic lupus erythematosus', *Hepatology*, 5(3), 397-402.

Gutcher, I. and Becher, B. (2007) 'APC-derived cytokines and T cell polarization in autoimmune inflammation', *J Clin Invest*, 117(5), 1119-27.

Guy, C. S. and Vignali, D. A. (2009) 'Organization of proximal signal initiation at the TCR:CD3 complex', *Immunol Rev*, 232(1), 7-21.

Haas, J., Fritzsching, B., Trubswetter, P., Korporal, M., Milkova, L., Fritz, B., Vobis, D., Krammer, P. H., Suri-Payer, E. and Wildemann, B. (2007) 'Prevalence of newly generated naive regulatory T cells (Treg) is critical for Treg suppressive function and determines Treg dysfunction in multiple sclerosis', *J Immunol*, 179(2), 1322-30.

Haas, J., Hug, A., Viehover, A., Fritzsching, B., Falk, C. S., Filser, A., Vetter, T., Milkova, L., Korporal, M., Fritz, B., Storch-Hagenlocher, B., Krammer, P. H., Suri-Payer, E. and

Wildemann, B. (2005) 'Reduced suppressive effect of CD4+CD25high regulatory T cells on the T cell immune response against myelin oligodendrocyte glycoprotein in patients with multiple sclerosis', *Eur J Immunol,* 35(11), 3343-52.

Hafler, D. A. (2004) 'Multiple sclerosis', *J Clin Invest,* 113(6), 788-94.

Hafler, D. A. and Kuchroo, V. (2008) 'TIMs: central regulators of immune responses', *J Exp Med,* 205(12), 2699-701.

Hahn, M., Nicholson, M. J., Pyrdol, J. and Wucherpfennig, K. W. (2005) 'Unconventional topology of self peptide-major histocompatibility complex binding by a human autoimmune T cell receptor', *Nat Immunol,* 6(5), 490-6.

Hammerling, G. J., Schonrich, G., Momburg, F., Auphan, N., Malissen, M., Malissen, B., Schmitt-Verhulst, A. M. and Arnold, B. (1991) 'Non-deletional mechanisms of peripheral and central tolerance: studies with transgenic mice with tissue-specific expression of a foreign MHC class I antigen', *Immunol Rev,* 122, 47-67.

Han, G. M., O'Neil-Andersen, N. J., Zurier, R. B. and Lawrence, D. A. (2008) 'CD4+CD25high T cell numbers are enriched in the peripheral blood of patients with rheumatoid arthritis', *Cell Immunol,* 253(1-2), 92-101.

Han, S., Tredger, M., Gregorio, G. V., Mieli-Vergani, G. and Vergani, D. (1995) 'Anti-liver cytosolic antigen type 1 (LC1) antibodies in childhood autoimmune liver disease', *Hepatology,* 21(1), 58-62.

Hara, M., Kingsley, C. I., Niimi, M., Read, S., Turvey, S. E., Bushell, A. R., Morris, P. J., Powrie, F. and Wood, K. J. (2001) 'IL-10 is required for regulatory T cells to mediate tolerance to alloantigens in vivo', *J Immunol,* 166(6), 3789-96.

Harada, K., Shimoda, S., Sato, Y., Isse, K., Ikeda, H. and Nakanuma, Y. (2009) 'Periductal interleukin-17 production in association with biliary innate immunity contributes to the pathogenesis of cholangiopathy in primary biliary cirrhosis', *Clin Exp Immunol,* 157(2), 261-70.

Harada, Y., Elly, C., Ying, G., Paik, J. H., DePinho, R. A. and Liu, Y. C. (2010) 'Transcription factors Foxo3a and Foxo1 couple the E3 ligase Cbl-b to the induction of Foxp3 expression in induced regulatory T cells', *J Exp Med,* 207(7), 1381-91.

Harrington, L. E., Hatton, R. D., Mangan, P. R., Turner, H., Murphy, T. L., Murphy, K. M. and Weaver, C. T. (2005) 'Interleukin 17-producing CD4+ effector T cells develop via a lineage distinct from the T helper type 1 and 2 lineages', *Nat Immunol,* 6(11), 1123-32.

Harris, T. J., Grosso, J. F., Yen, H. R., Xin, H., Kortylewski, M., Albesiano, E., Hipkiss, E. L., Getnet, D., Goldberg, M. V., Maris, C. H., Housseau, F., Yu, H., Pardoll, D. M. and Drake, C. G. (2007) 'Cutting edge: An in vivo requirement for STAT3 signaling in TH17 development and TH17-dependent autoimmunity', *J Immunol,* 179(7), 4313-7.

Hastings, W. D., Anderson, D. E., Kassam, N., Koguchi, K., Greenfield, E. A., Kent, S. C., Zheng, X. X., Strom, T. B., Hafler, D. A. and Kuchroo, V. K. (2009) 'TIM-3 is expressed on activated human CD4+ T cells and regulates Th1 and Th17 cytokines', *European Journal of Immunology,* 39(9), 2492-2501.

Hawiger, D., Masilamani, R. F., Bettelli, E., Kuchroo, V. K. and Nussenzweig, M. C. (2004) 'Immunological unresponsiveness characterized by increased expression of CD5 on peripheral T cells induced by dendritic cells in vivo', *Immunity,* 20(6), 695-705.

Hempfling, W., Grunhage, F., Dilger, K., Reichel, C., Beuers, U. and Sauerbruch, T. (2003) 'Pharmacokinetics and pharmacodynamic action of budesonide in early- and late-stage primary biliary cirrhosis', *Hepatology,* 38(1), 196-202.

Heneghan, M. A. and McFarlane, I. G. (2002) 'Current and novel immunosuppressive therapy for autoimmune hepatitis', *Hepatology,* 35(1), 7-13.

Heneghan, M. A., Norris, S. M., O'Grady, J. G., Harrison, P. M. and McFarlane, I. G. (2001) 'Management and outcome of pregnancy in autoimmune hepatitis', *Gut,* 48(1), 97-102.

Hengartner, H., Odermatt, B., Schneider, R., Schreyer, M., Walle, G., MacDonald, H. R. and Zinkernagel, R. M. (1988) 'Deletion of self-reactive T cells before entry into the thymus medulla', *Nature,* 336(6197), 388-90.

Hennes, E. M., Zeniya, M., Czaja, A. J., Pares, A., Dalekos, G. N., Krawitt, E. L., Bittencourt, P. L., Porta, G., Boberg, K. M., Hofer, H., Bianchi, F. B., Shibata, M., Schramm, C., Eisenmann de Torres, B., Galle, P. R., McFarlane, I., Dienes, H. P. and Lohse, A. W. (2008) 'Simplified criteria for the diagnosis of autoimmune hepatitis', *Hepatology,* 48(1), 169-76.

Herbelin, A., Gombert, J. M., Lepault, F., Bach, J. F. and Chatenoud, L. (1998) 'Mature mainstream TCR alpha beta+CD4+ thymocytes expressing L-selectin mediate "active tolerance" in the nonobese diabetic mouse', *J Immunol,* 161(5), 2620-8.

Hernandez, H. M., Kovarik, P., Whitington, P. F. and Alonso, E. M. (2001) 'Autoimmune hepatitis as a late complication of liver transplantation', *J Pediatr Gastroenterol Nutr,* 32(2), 131-6.

Herzenberg, L. A., Parks, D., Sahaf, B., Perez, O. and Roederer, M. (2002) 'The history and future of the fluorescence activated cell sorter and flow cytometry: a view from Stanford', *Clin Chem,* 48(10), 1819-27.

Hilario, M. O., Len, C. A., Roja, S. C., Terreri, M. T., Almeida, G. and Andrade, L. E. (2004) 'Frequency of antinuclear antibodies in healthy children and adolescents', *Clin Pediatr (Phila),* 43(7), 637-42.

Hildeman, D. A., Zhu, Y., Mitchell, T. C., Kappler, J. and Marrack, P. (2002) 'Molecular mechanisms of activated T cell death in vivo', *Curr Opin Immunol,* 14(3), 354-9.

Hirabayashi, J., Hashidate, T., Arata, Y., Nishi, N., Nakamura, T., Hirashima, M., Urashima, T., Oka, T., Futai, M., Muller, W. E., Yagi, F. and Kasai, K. (2002) 'Oligosaccharide specificity of galectins: a search by frontal affinity chromatography', *Biochim Biophys Acta,* 1572(2-3), 232-54.

Hirashima, M., Kashio, Y., Nishi, N., Yamauchi, A., Imaizumi, T. A., Kageshita, T., Saita, N. and Nakamura, T. (2004) 'Galectin-9 in physiological and pathological conditions', *Glycoconj J,* 19(7-9), 593-600.

Ho, I. C., Tai, T. S. and Pai, S. Y. (2009) 'GATA3 and the T-cell lineage: essential functions before and after T-helper-2-cell differentiation', *Nat Rev Immunol,* 9(2), 125-35.

Hodgson, H. J., Wands, J. R. and Isselbacher, K. J. (1978) 'Alteration in suppressor cell activity in chronic active hepatitis', *Proc Natl Acad Sci U S A,* 75(3), 1549-53.

Hogquist, K. A., Baldwin, T. A. and Jameson, S. C. (2005) 'Central tolerance: learning self-control in the thymus', *Nat Rev Immunol,* 5(10), 772-82.

Holdener, M., Hintermann, E., Bayer, M., Rhode, A., Rodrigo, E., Hintereder, G., Johnson, E. F., Gonzalez, F. J., Pfeilschifter, J., Manns, M. P., Herrath, M. G. and Christen, U. (2008) 'Breaking tolerance to the natural human liver autoantigen cytochrome P450 2D6 by virus infection', *J Exp Med,* 205(6), 1409-22.

Hori, S., Nomura, T. and Sakaguchi, S. (2003) 'Control of regulatory T cell development by the transcription factor Foxp3', *Science,* 299(5609), 1057-61.

Horwitz, D. A., Zheng, S. G. and Gray, J. D. (2008) 'Natural and TGF-beta-induced Foxp3(+)CD4(+) CD25(+) regulatory T cells are not mirror images of each other', *Trends Immunol,* 29(9), 429-35.

Hoshino, K., Takeuchi, O., Kawai, T., Sanjo, H., Ogawa, T., Takeda, Y., Takeda, K. and Akira, S. (1999) 'Cutting edge: Toll-like receptor 4 (TLR4)-deficient mice are hyporesponsive to lipopolysaccharide: evidence for TLR4 as the Lps gene product', *J Immunol,* 162(7), 3749-52.

Hoskins, J. M., Meynell, G. G. and Sanders, F. K. (1956) 'A comparison of methods for estimating the viable count of a suspension of tumour cells', *Exp Cell Res,* 11(2), 297-305.

Hsu, D. K., Chen, H. Y. and Liu, F. T. (2009) 'Galectin-3 regulates T-cell functions', *Immunol Rev,* 230(1), 114-27.

Hsu, D. K., Yang, R. Y., Pan, Z., Yu, L., Salomon, D. R., Fung-Leung, W. P. and Liu, F. T. (2000) 'Targeted disruption of the galectin-3 gene results in attenuated peritoneal inflammatory responses', *Am J Pathol,* 156(3), 1073-83.

Hsu, S. M., Raine, L. and Fanger, H. (1981) 'Use of avidin-biotin-peroxidase complex (ABC) in immunoperoxidase techniques: a comparison between ABC and unlabeled antibody (PAP) procedures', *J Histochem Cytochem,* 29(4), 577-80.

Huang, C. T., Workman, C. J., Flies, D., Pan, X., Marson, A. L., Zhou, G., Hipkiss, E. L., Ravi, S., Kowalski, J., Levitsky, H. I., Powell, J. D., Pardoll, D. M., Drake, C. G. and Vignali, D. A. (2004) 'Role of LAG-3 in regulatory T cells', *Immunity,* 21(4), 503-13.

Hughes, R. C. (1999) 'Secretion of the galectin family of mammalian carbohydrate-binding proteins', *Biochim Biophys Acta,* 1473(1), 172-85.

Hurlburt, K. J., McMahon, B. J., Deubner, H., Hsu-Trawinski, B., Williams, J. L. and Kowdley, K. V. (2002) 'Prevalence of autoimmune liver disease in Alaska Natives', *Am J Gastroenterol,* 97(9), 2402-7.

Ichimura, T., Bonventre, J. V., Bailly, V., Wei, H., Hession, C. A., Cate, R. L. and Sanicola, M. (1998) 'Kidney injury molecule-1 (KIM-1), a putative epithelial cell adhesion molecule containing a novel immunoglobulin domain, is up-regulated in renal cells after injury', *J Biol Chem,* 273(7), 4135-42.

Imaizumi, T., Kumagai, M., Sasaki, N., Kurotaki, H., Mori, F., Seki, M., Nishi, N., Fujimoto, K., Tanji, K., Shibata, T., Tamo, W., Matsumiya, T., Yoshida, H., Cui, X. F., Takanashi, S., Hanada, K., Okumura, K., Yagihashi, S., Wakabayashi, K., Nakamura, T., Hirashima, M. and Satoh, K. (2002) 'Interferon-gamma stimulates the expression of galectin-9 in cultured human endothelial cells', *J Leukoc Biol,* 72(3), 486-91.

Ivanov, II, McKenzie, B. S., Zhou, L., Tadokoro, C. E., Lepelley, A., Lafaille, J. J., Cua, D. J. and Littman, D. R. (2006) 'The orphan nuclear receptor RORgammat directs the differentiation program of proinflammatory IL-17+ T helper cells', *Cell,* 126(6), 1121-33.

Ivanov, II, Zhou, L. and Littman, D. R. (2007) 'Transcriptional regulation of Th17 cell differentiation', *Semin Immunol,* 19(6), 409-17.

Janowski, B. A., Hu, J. and Corey, D. R. (2006) 'Silencing gene expression by targeting chromosomal DNA with antigene peptide nucleic acids and duplex RNAs', *Nat Protoc,* 1(1), 436-43.

Jenkins, M. K., Chen, C. A., Jung, G., Mueller, D. L. and Schwartz, R. H. (1990) 'Inhibition of antigen-specific proliferation of type 1 murine T cell clones after stimulation with immobilized anti-CD3 monoclonal antibody', *J Immunol,* 144(1), 16-22.

Jenkins, M. K. and Schwartz, R. H. (1987) 'Antigen presentation by chemically modified splenocytes induces antigen-specific T cell unresponsiveness in vitro and in vivo', *J Exp Med,* 165(2), 302-19.

Jensen, D. M., McFarlane, I. G., Portmann, B. S., Eddleston, A. L. and Williams, R. (1978) 'Detection of antibodies directed against a liver-specific membrane lipoprotein in patients with acute and chronic active hepatitis', *N Engl J Med,* 299(1), 1-7.

Jiang, H. and Chess, L. (2006) 'Regulation of immune responses by T cells', *N Engl J Med,* 354(11), 1166-76.

Johnson, P. J. and McFarlane, I. G. (1993) 'Meeting report: International Autoimmune Hepatitis Group', *Hepatology,* 18(4), 998-1005.

Johnson, P. J., McFarlane, I. G. and Williams, R. (1995) 'Azathioprine for long-term maintenance of remission in autoimmune hepatitis', *N Engl J Med,* 333(15), 958-63.

Jones, R. B., Ndhlovu, L. C., Barbour, J. D., Sheth, P. M., Jha, A. R., Long, B. R., Wong, J. C., Satkunarajah, M., Schweneker, M., Chapman, J. M., Gyenes, G., Vali, B., Hyrcza, M. D., Yue, F. Y., Kovacs, C., Sassi, A., Loutfy, M., Halpenny, R., Persad, D., Spotts, G., Hecht, F. M., Chun, T. W., McCune, J. M., Kaul, R., Rini, J. M., Nixon, D. F. and Ostrowski, M. A. (2008) 'Tim-3 expression defines a novel population of dysfunctional T cells with highly elevated frequencies in progressive HIV-1 infection', *J Exp Med,* 205(12), 2763-79.

Jordan, M. S., Boesteanu, A., Reed, A. J., Petrone, A. L., Holenbeck, A. E., Lerman, M. A., Naji, A. and Caton, A. J. (2001) 'Thymic selection of CD4+CD25+ regulatory T cells induced by an agonist self-peptide', *Nat Immunol,* 2(4), 301-6.

Josefowicz, S. Z. and Rudensky, A. (2009) 'Control of regulatory T cell lineage commitment and maintenance', *Immunity,* 30(5), 616-25.

Joske, R. A. and King, W. E. (1955) 'The L.E.-cell phenomenon in active chronic viral hepatitis', *Lancet,* 269(6888), 477-80.

Juszczynski, P., Ouyang, J., Monti, S., Rodig, S. J., Takeyama, K., Abramson, J., Chen, W., Kutok, J. L., Rabinovich, G. A. and Shipp, M. A. (2007) 'The AP1-dependent secretion of galectin-1 by Reed Sternberg cells fosters immune privilege in classical Hodgkin lymphoma', *Proc Natl Acad Sci U S A,* 104(32), 13134-9.

Kageshita, T., Kashio, Y., Yamauchi, A., Seki, M., Abedin, M. J., Nishi, N., Shoji, H., Nakamura, T., Ono, T. and Hirashima, M. (2002) 'Possible role of galectin-9 in cell aggregation and apoptosis of human melanoma cell lines and its clinical significance', *Int J Cancer,* 99(6), 809-16.

Kamradt, T. and Mitchison, N. A. (2001) 'Tolerance and autoimmunity', *N Engl J Med,* 344(9), 655-64.

Kanamaru, F., Youngnak, P., Hashiguchi, M., Nishioka, T., Takahashi, T., Sakaguchi, S., Ishikawa, I. and Azuma, M. (2004) 'Costimulation via glucocorticoid-induced TNF receptor in both conventional and CD25+ regulatory CD4+ T cells', *J Immunol,* 172(12), 7306-14.

Kanzler, S., Gerken, G., Dienes, H. P., Meyer zum Buschenfelde, K. H. and Lohse, A. W. (1997) 'Cyclophosphamide as alternative immunosuppressive therapy for autoimmune hepatitis--report of three cases', *Z Gastroenterol,* 35(7), 571-8.

Kao, J. K., Hsue, Y. T. and Lin, C. Y. (2007) 'Role of new population of peripheral CD11c(+)CD8(+) T cells and CD4(+)CD25(+) regulatory T cells during acute and remission stages in rheumatoid arthritis patients', *J Microbiol Immunol Infect,* 40(5), 419-27.

Kaplan, G., Totsuka, A., Thompson, P., Akatsuka, T., Moritsugu, Y. and Feinstone, S. M. (1996) 'Identification of a surface glycoprotein on African green monkey kidney cells as a receptor for hepatitis A virus', *EMBO J,* 15(16), 4282-96.

Kaplan, M. H., Schindler, U., Smiley, S. T. and Grusby, M. J. (1996a) 'Stat6 is required for mediating responses to IL-4 and for development of Th2 cells', *Immunity,* 4(3), 313-9.

Kaplan, M. H., Sun, Y. L., Hoey, T. and Grusby, M. J. (1996b) 'Impaired IL-12 responses and enhanced development of Th2 cells in Stat4-deficient mice', *Nature,* 382(6587), 174-7.

Kashio, Y., Nakamura, K., Abedin, M. J., Seki, M., Nishi, N., Yoshida, N., Nakamura, T. and Hirashima, M. (2003) 'Galectin-9 induces apoptosis through the calcium-calpain-caspase-1 pathway', *J Immunol,* 170(7), 3631-6.

Kaskas, B. A., Louis, E., Hindorf, U., Schaeffeler, E., Deflandre, J., Graepler, F., Schmiegelow, K., Gregor, M., Zanger, U. M., Eichelbaum, M. and Schwab, M. (2003) 'Safe treatment

of thiopurine S-methyltransferase deficient Crohn's disease patients with azathioprine', *Gut*, 52(1), 140-2.

Kaya, M., Angulo, P. and Lindor, K. D. (2000) 'Overlap of autoimmune hepatitis and primary sclerosing cholangitis: an evaluation of a modified scoring system', *J Hepatol*, 33(4), 537-42.

Keir, M. E., Liang, S. C., Guleria, I., Latchman, Y. E., Qipo, A., Albacker, L. A., Koulmanda, M., Freeman, G. J., Sayegh, M. H. and Sharpe, A. H. (2006) 'Tissue expression of PD-L1 mediates peripheral T cell tolerance', *J Exp Med*, 203(4), 883-95.

Kerkar, N., Choudhuri, K., Ma, Y., Mahmoud, A., Bogdanos, D. P., Muratori, L., Bianchi, F., Williams, R., Mieli-Vergani, G. and Vergani, D. (2003) 'Cytochrome P4502D6(193-212): a new immunodominant epitope and target of virus/self cross-reactivity in liver kidney microsomal autoantibody type 1-positive liver disease', *J Immunol*, 170(3), 1481-9.

Kerkar, N., Hadzic, N., Davies, E. T., Portmann, B., Donaldson, P. T., Rela, M., Heaton, N. D., Vergani, D. and Mieli-Vergani, G. (1998) 'De-novo autoimmune hepatitis after liver transplantation', *Lancet*, 351(9100), 409-13.

Kessler, W. R., Cummings, O. W., Eckert, G., Chalasani, N., Lumeng, L. and Kwo, P. Y. (2004) 'Fulminant hepatic failure as the initial presentation of acute autoimmune hepatitis', *Clin Gastroenterol Hepatol*, 2(7), 625-31.

Khademi, M., Illes, Z., Gielen, A. W., Marta, M., Takazawa, N., Baecher-Allan, C., Brundin, L., Hannerz, J., Martin, C., Harris, R. A., Hafler, D. A., Kuchroo, V. K., Olsson, T., Piehl, F. and Wallstrom, E. (2004) 'T Cell Ig- and mucin-domain-containing molecule-3 (TIM-3) and TIM-1 molecules are differentially expressed on human Th1 and Th2 cells and in cerebrospinal fluid-derived mononuclear cells in multiple sclerosis', *J Immunol*, 172(11), 7169-76.

Kido, M., Watanabe, N., Okazaki, T., Akamatsu, T., Tanaka, J., Saga, K., Nishio, A., Honjo, T. and Chiba, T. (2008) 'Fatal autoimmune hepatitis induced by concurrent loss of naturally arising regulatory T cells and PD-1-mediated signaling', *Gastroenterology*, 135(4), 1333-43.

Kingsley, C. I., Karim, M., Bushell, A. R. and Wood, K. J. (2002) 'CD25+CD4+ regulatory T cells prevent graft rejection: CTLA-4- and IL-10-dependent immunoregulation of alloresponses', *J Immunol*, 168(3), 1080-6.

Kirk, A. P., Jain, S., Pocock, S., Thomas, H. C. and Sherlock, S. (1980) 'Late results of the Royal Free Hospital prospective controlled trial of prednisolone therapy in hepatitis B surface antigen negative chronic active hepatitis', *Gut*, 21(1), 78-83.

Kitoh, A., Ono, M., Naoe, Y., Ohkura, N., Yamaguchi, T., Yaguchi, H., Kitabayashi, I., Tsukada, T., Nomura, T., Miyachi, Y., Taniuchi, I. and Sakaguchi, S. (2009) 'Indispensable role of the Runx1-Cbfbeta transcription complex for in vivo-suppressive function of FoxP3+ regulatory T cells', *Immunity*, 31(4), 609-20.

Klein, L., Hinterberger, M., Wirnsberger, G. and Kyewski, B. (2009) 'Antigen presentation in the thymus for positive selection and central tolerance induction', *Nat Rev Immunol*, 9(12), 833-44.

Klenerman, P. and Hill, A. (2005) 'T cells and viral persistence: lessons from diverse infections', *Nat Immunol,* 6(9), 873-9.

Kloppel, G., Seifert, G., Lindner, H., Dammermann, R., Sack, H. J. and Berg, P. A. (1977) 'Histopathological features in mixed types of chronic aggressive hepatitis and primary biliary cirrhosis. Correlations of liver histology with mitochondrial antibodies of different specificity', *Virchows Arch A Pathol Anat Histol,* 373(2), 143-60.

Kobayashi, N., Karisola, P., Pena-Cruz, V., Dorfman, D. M., Jinushi, M., Umetsu, S. E., Butte, M. J., Nagumo, H., Chernova, I., Zhu, B., Sharpe, A. H., Ito, S., Dranoff, G., Kaplan, G. G., Casasnovas, J. M., Umetsu, D. T., Dekruyff, R. H. and Freeman, G. J. (2007) 'TIM-1 and TIM-4 glycoproteins bind phosphatidylserine and mediate uptake of apoptotic cells', *Immunity,* 27(6), 927-40.

Kobie, J. J., Shah, P. R., Yang, L., Rebhahn, J. A., Fowell, D. J. and Mosmann, T. R. (2006) 'T regulatory and primed uncommitted CD4 T cells express CD73, which suppresses effector CD4 T cells by converting 5'-adenosine monophosphate to adenosine', *J Immunol,* 177(10), 6780-6.

Koch, M. A., Tucker-Heard, G., Perdue, N. R., Killebrew, J. R., Urdahl, K. B. and Campbell, D. J. (2009) 'The transcription factor T-bet controls regulatory T cell homeostasis and function during type 1 inflammation', *Nat Immunol,* 10(6), 595-602.

Koguchi, K., Anderson, D. E., Yang, L., O'Connor, K. C., Kuchroo, V. K. and Hafler, D. A. (2006) 'Dysregulated T cell expression of TIM3 in multiple sclerosis', *J Exp Med,* 203(6), 1413-8.

Kohm, A. P., Carpentier, P. A., Anger, H. A. and Miller, S. D. (2002) 'Cutting edge: CD4+CD25+ regulatory T cells suppress antigen-specific autoreactive immune responses and central nervous system inflammation during active experimental autoimmune encephalomyelitis', *J Immunol,* 169(9), 4712-6.

Kolls, J. K. and Linden, A. (2004) 'Interleukin-17 family members and inflammation', *Immunity,* 21(4), 467-76.

Kondo, M., Weissman, I. L. and Akashi, K. (1997) 'Identification of clonogenic common lymphoid progenitors in mouse bone marrow', *Cell,* 91(5), 661-72.

Konig, R. (2002) 'Interactions between MHC molecules and co-receptors of the TCR', *Curr Opin Immunol,* 14(1), 75-83.

Kopf, M., Le Gros, G., Bachmann, M., Lamers, M. C., Bluethmann, H. and Kohler, G. (1993) 'Disruption of the murine IL-4 gene blocks Th2 cytokine responses', *Nature,* 362(6417), 245-8.

Korn, T., Bettelli, E., Gao, W., Awasthi, A., Jager, A., Strom, T. B., Oukka, M. and Kuchroo, V. K. (2007a) 'IL-21 initiates an alternative pathway to induce proinflammatory T(H)17 cells', *Nature,* 448(7152), 484-7.

Korn, T., Bettelli, E., Oukka, M. and Kuchroo, V. K. (2009) 'IL-17 and Th17 Cells', *Annu Rev Immunol,* 27, 485-517.

Korn, T., Reddy, J., Gao, W., Bettelli, E., Awasthi, A., Petersen, T. R., Backstrom, B. T., Sobel, R. A., Wucherpfennig, K. W., Strom, T. B., Oukka, M. and Kuchroo, V. K. (2007b) 'Myelin-specific regulatory T cells accumulate in the CNS but fail to control autoimmune inflammation', *Nat Med*, 13(4), 423-31.

Kotenko, S. V., Izotova, L. S., Mirochnitchenko, O. V., Esterova, E., Dickensheets, H., Donnelly, R. P. and Pestka, S. (2001) 'Identification of the functional interleukin-22 (IL-22) receptor complex: the IL-10R2 chain (IL-10Rbeta) is a common chain of both the IL-10 and IL-22 (IL-10-related T cell-derived inducible factor, IL-TIF) receptor complexes', *J Biol Chem*, 276(4), 2725-32.

Kramer, S., Schimpl, A. and Hunig, T. (1995) 'Immunopathology of interleukin (IL) 2-deficient mice: thymus dependence and suppression by thymus-dependent cells with an intact IL-2 gene', *J Exp Med*, 182(6), 1769-76.

Krawitt, E. L. (2006) 'Autoimmune hepatitis', *N Engl J Med*, 354(1), 54-66.

Krawitt, E. L. (2008) 'Clinical features and management of autoimmune hepatitis', *World J Gastroenterol*, 14(21), 3301-5.

Kreslavsky, T., Gleimer, M., Garbe, A. I. and von Boehmer, H. (2010) 'alphabeta versus gammadelta fate choice: counting the T-cell lineages at the branch point', *Immunol Rev*, 238(1), 169-81.

Kubach, J., Lutter, P., Bopp, T., Stoll, S., Becker, C., Huter, E., Richter, C., Weingarten, P., Warger, T., Knop, J., Mullner, S., Wijdenes, J., Schild, H., Schmitt, E. and Jonuleit, H. (2007) 'Human CD4+CD25+ regulatory T cells: proteome analysis identifies galectin-10 as a novel marker essential for their anergy and suppressive function', *Blood*, 110(5), 1550-8.

Kuchroo, V. K., Umetsu, D. T., DeKruyff, R. H. and Freeman, G. J. (2003) 'The TIM gene family: emerging roles in immunity and disease', *Nat Rev Immunol*, 3(6), 454-62.

Kukreja, A., Cost, G., Marker, J., Zhang, C., Sun, Z., Lin-Su, K., Ten, S., Sanz, M., Exley, M., Wilson, B., Porcelli, S. and Maclaren, N. (2002) 'Multiple immuno-regulatory defects in type-1 diabetes', *J Clin Invest*, 109(1), 131-40.

Kurata, H., Lee, H. J., O'Garra, A. and Arai, N. (1999) 'Ectopic expression of activated Stat6 induces the expression of Th2-specific cytokines and transcription factors in developing Th1 cells', *Immunity*, 11(6), 677-88.

Lafferty, K. J., Andrus, L. and Prowse, S. J. (1980) 'Role of lymphokine and antigen in the control of specific T cell responses', *Immunol Rev*, 51, 279-314.

Lahm, H., Hoeflich, A., Andre, S., Sordat, B., Kaltner, H., Wolf, E. and Gabius, H. J. (2000) 'Gene expression of galectin-9/ecalectin, a potent eosinophil chemoattractant, and/or the insertional isoform in human colorectal carcinoma cell lines and detection of frame-shift mutations for protein sequence truncations in the second functional lectin domain', *Int J Oncol*, 17(3), 519-24.

Lam, K. C., Lai, C. L., Wu, P. C. and Todd, D. (1980) 'Etiological spectrum of liver cirrhosis in the Chinese', *J Chronic Dis*, 33(6), 375-81.

Langrish, C. L., Chen, Y., Blumenschein, W. M., Mattson, J., Basham, B., Sedgwick, J. D., McClanahan, T., Kastelein, R. A. and Cua, D. J. (2005) 'IL-23 drives a pathogenic T cell population that induces autoimmune inflammation', *J Exp Med*, 201(2), 233-40.

Lankisch, T. O., Mourier, O., Sokal, E. M., Habes, D., Lacaille, F., Bridoux-Henno, L., Hermeziu, B., Lenaerts, C., Strassburg, C. P. and Jacquemin, E. (2009) 'AIRE gene analysis in children with autoimmune hepatitis type I or II', *J Pediatr Gastroenterol Nutr*, 48(4), 498-500.

Lankisch, T. O., Strassburg, C. P., Debray, D., Manns, M. P. and Jacquemin, E. (2005) 'Detection of autoimmune regulator gene mutations in children with type 2 autoimmune hepatitis and extrahepatic immune-mediated diseases', *J Pediatr*, 146(6), 839-42.

Lapierre, P., Beland, K., Martin, C., Alvarez, F., Jr. and Alvarez, F. (2010) 'Forkhead box p3+ regulatory T cell underlies male resistance to experimental type 2 autoimmune hepatitis', *Hepatology*, 51(5), 1789-98.

Lapierre, P., Djilali-Saiah, I., Vitozzi, S. and Alvarez, F. (2004) 'A murine model of type 2 autoimmune hepatitis: Xenoimmunization with human antigens', *Hepatology*, 39(4), 1066-74.

Lapierre, P., Hajoui, O., Homberg, J. C. and Alvarez, F. (1999) 'Formiminotransferase cyclodeaminase is an organ-specific autoantigen recognized by sera of patients with autoimmune hepatitis', *Gastroenterology*, 116(3), 643-9.

Lawson, C. A., Brown, A. K., Bejarano, V., Douglas, S. H., Burgoyne, C. H., Greenstein, A. S., Boylston, A. W., Emery, P., Ponchel, F. and Isaacs, J. D. (2006) 'Early rheumatoid arthritis is associated with a deficit in the CD4+CD25high regulatory T cell population in peripheral blood', *Rheumatology (Oxford)*, 45(10), 1210-7.

Lawson, J. M., Tremble, J., Dayan, C., Beyan, H., Leslie, R. D., Peakman, M. and Tree, T. I. (2008) 'Increased resistance to CD4+CD25hi regulatory T cell-mediated suppression in patients with type 1 diabetes', *Clin Exp Immunol*, 154(3), 353-9.

Le Gros, G., Ben-Sasson, S. Z., Seder, R., Finkelman, F. D. and Paul, W. E. (1990) 'Generation of interleukin 4 (IL-4)-producing cells in vivo and in vitro: IL-2 and IL-4 are required for in vitro generation of IL-4-producing cells', *J Exp Med*, 172(3), 921-9.

Lecoeur, S., Andre, C. and Beaune, P. H. (1996) 'Tienilic acid-induced autoimmune hepatitis: anti-liver and-kidney microsomal type 2 autoantibodies recognize a three-site conformational epitope on cytochrome P4502C9', *Mol Pharmacol*, 50(2), 326-33.

Lee, Y. J., Kang, S. W., Song, J. K., Park, J. J., Bae, Y. D., Lee, E. Y., Lee, E. B. and Song, Y. W. (2007) 'Serum galectin-3 and galectin-3 binding protein levels in Behcet's disease and their association with disease activity', *Clin Exp Rheumatol*, 25(4 Suppl 45), S41-5.

Leffler, H., Carlsson, S., Hedlund, M., Qian, Y. and Poirier, F. (2004) 'Introduction to galectins', *Glycoconj J*, 19(7-9), 433-40.

Lennard, L., Van Loon, J. A. and Weinshilboum, R. M. (1989) 'Pharmacogenetics of acute azathioprine toxicity: relationship to thiopurine methyltransferase genetic polymorphism', *Clin Pharmacol Ther*, 46(2), 149-54.

Lenzi, M., Ballardini, G., Fusconi, M., Cassani, F., Selleri, L., Volta, U., Zauli, D. and Bianchi, F. B. (1990) 'Type 2 autoimmune hepatitis and hepatitis C virus infection', *Lancet*, 335(8684), 258-9.

Lenzi, M., Bellentani, S., Saccoccio, G., Muratori, P., Masutti, F., Muratori, L., Cassani, F., Bianchi, F. B. and Tiribelli, C. (1999) 'Prevalence of non-organ-specific autoantibodies and chronic liver disease in the general population: a nested case-control study of the Dionysos cohort', *Gut*, 45(3), 435-41.

Lenzi, M., Manotti, P., Muratori, L., Cataleta, M., Ballardini, G., Cassani, F. and Bianchi, F. B. (1995) 'Liver cytosolic 1 antigen-antibody system in type 2 autoimmune hepatitis and hepatitis C virus infection', *Gut*, 36(5), 749-54.

Li, Z., Arijs, I., De Hertogh, G., Vermeire, S., Noman, M., Bullens, D., Coorevits, L., Sagaert, X., Schuit, F., Rutgeerts, P., Ceuppens, J. L. and Van Assche, G. (2010) 'Reciprocal changes of Foxp3 expression in blood and intestinal mucosa in IBD patients responding to infliximab', *Inflamm Bowel Dis*, 16(8), 1299-310.

Liang, B., Workman, C., Lee, J., Chew, C., Dale, B. M., Colonna, L., Flores, M., Li, N., Schweighoffer, E., Greenberg, S., Tybulewicz, V., Vignali, D. and Clynes, R. (2008) 'Regulatory T cells inhibit dendritic cells by lymphocyte activation gene-3 engagement of MHC class II', *J Immunol*, 180(9), 5916-26.

Liang, S. C., Long, A. J., Bennett, F., Whitters, M. J., Karim, R., Collins, M., Goldman, S. J., Dunussi-Joannopoulos, K., Williams, C. M., Wright, J. F. and Fouser, L. A. (2007) 'An IL-17F/A heterodimer protein is produced by mouse Th17 cells and induces airway neutrophil recruitment', *J Immunol*, 179(11), 7791-9.

Liang, S. C., Tan, X. Y., Luxenberg, D. P., Karim, R., Dunussi-Joannopoulos, K., Collins, M. and Fouser, L. A. (2006) 'Interleukin (IL)-22 and IL-17 are coexpressed by Th17 cells and cooperatively enhance expression of antimicrobial peptides', *J Exp Med*, 203(10), 2271-9.

Liberal, R., Longhi, M. S., Grant, C. R., Mieli-Vergani, G. and Vergani, D. (2012) 'Autoimmune hepatitis after liver transplantation', *Clin Gastroenterol Hepatol*, 10(4), 346-53.

Lidman, K., Biberfeld, G., Fagraeus, A., Norberg, R., Torstensson, R., Utter, G., Carlsson, L., Luca, J. and Lindberg, U. (1976) 'Anti-actin specificity of human smooth muscle antibodies in chronic active hepatitis', *Clin Exp Immunol*, 24(2), 266-72.

Lighvani, A. A., Frucht, D. M., Jankovic, D., Yamane, H., Aliberti, J., Hissong, B. D., Nguyen, B. V., Gadina, M., Sher, A., Paul, W. E. and O'Shea, J. J. (2001) 'T-bet is rapidly induced by interferon-gamma in lymphoid and myeloid cells', *Proc Natl Acad Sci U S A*, 98(26), 15137-42.

Lin, S. C., Chen, K. H., Lin, C. H., Kuo, C. C., Ling, Q. D. and Chan, C. H. (2007) 'The quantitative analysis of peripheral blood FOXP3-expressing T cells in systemic lupus erythematosus and rheumatoid arthritis patients', *Eur J Clin Invest*, 37(12), 987-96.

Lindley, S., Dayan, C. M., Bishop, A., Roep, B. O., Peakman, M. and Tree, T. I. (2005) 'Defective suppressor function in CD4(+)CD25(+) T-cells from patients with type 1 diabetes', *Diabetes*, 54(1), 92-9.

Ling, E. M., Smith, T., Nguyen, X. D., Pridgeon, C., Dallman, M., Arbery, J., Carr, V. A. and Robinson, D. S. (2004) 'Relation of CD4+CD25+ regulatory T-cell suppression of allergen-driven T-cell activation to atopic status and expression of allergic disease', *Lancet,* 363(9409), 608-15.

Linsley, P. S., Brady, W., Urnes, M., Grosmaire, L. S., Damle, N. K. and Ledbetter, J. A. (1991) 'CTLA-4 is a second receptor for the B cell activation antigen B7', *J Exp Med,* 174(3), 561-9.

Linsley, P. S., Greene, J. L., Tan, P., Bradshaw, J., Ledbetter, J. A., Anasetti, C. and Damle, N. K. (1992) 'Coexpression and functional cooperation of CTLA-4 and CD28 on activated T lymphocytes', *J Exp Med,* 176(6), 1595-604.

Lio, C. W. and Hsieh, C. S. (2008) 'A two-step process for thymic regulatory T cell development', *Immunity,* 28(1), 100-11.

Lipkowitz, M. S., Leal-Pinto, E., Cohen, B. E. and Abramson, R. G. (2004) 'Galectin 9 is the sugar-regulated urate transporter/channel UAT', *Glycoconj J,* 19(7-9), 491-8.

Liston, A., Lesage, S., Gray, D. H., Boyd, R. L. and Goodnow, C. C. (2005) 'Genetic lesions in T-cell tolerance and thresholds for autoimmunity', *Immunol Rev,* 204, 87-101.

Liu, F. T., Patterson, R. J. and Wang, J. L. (2002) 'Intracellular functions of galectins', *Biochim Biophys Acta,* 1572(2-3), 263-73.

Liu, F. T. and Rabinovich, G. A. (2005) 'Galectins as modulators of tumour progression', *Nat Rev Cancer,* 5(1), 29-41.

Liu, M. F., Wang, C. R., Fung, L. L., Lin, L. H. and Tsai, C. N. (2005) 'The presence of cytokine-suppressive CD4+CD25+ T cells in the peripheral blood and synovial fluid of patients with rheumatoid arthritis', *Scand J Immunol,* 62(3), 312-7.

Liu, W., Putnam, A. L., Xu-Yu, Z., Szot, G. L., Lee, M. R., Zhu, S., Gottlieb, P. A., Kapranov, P., Gingeras, T. R., Fazekas de St Groth, B., Clayberger, C., Soper, D. M., Ziegler, S. F. and Bluestone, J. A. (2006) 'CD127 expression inversely correlates with FoxP3 and suppressive function of human CD4+ T reg cells', *J Exp Med,* 203(7), 1701-11.

Lobo-Yeo, A., Senaldi, G., Portmann, B., Mowat, A. P., Mieli-Vergani, G. and Vergani, D. (1990) 'Class I and class II major histocompatibility complex antigen expression on hepatocytes: a study in children with liver disease', *Hepatology,* 12(2), 224-32.

Lohr, H., Manns, M., Kyriatsoulis, A., Lohse, A. W., Trautwein, C., Meyer zum Buschenfelde, K. H. and Fleischer, B. (1991) 'Clonal analysis of liver-infiltrating T cells in patients with LKM-1 antibody-positive autoimmune chronic active hepatitis', *Clin Exp Immunol,* 84(2), 297-302.

Lohr, H., Treichel, U., Poralla, T., Manns, M. and Meyer zum Buschenfelde, K. H. (1992) 'Liver-infiltrating T helper cells in autoimmune chronic active hepatitis stimulate the production of autoantibodies against the human asialoglycoprotein receptor in vitro', *Clin Exp Immunol,* 88(1), 45-9.

Lohse, A. W. and Mieli-Vergani, G. (2011) 'Autoimmune hepatitis', *J Hepatol,* 55(1), 171-82.

Lohse, A. W., zum Buschenfelde, K. H., Franz, B., Kanzler, S., Gerken, G. and Dienes, H. P. (1999) 'Characterization of the overlap syndrome of primary biliary cirrhosis (PBC) and autoimmune hepatitis: evidence for it being a hepatitic form of PBC in genetically susceptible individuals', *Hepatology*, 29(4), 1078-84.

Longhi, M. S., Hussain, M. J., Bogdanos, D. P., Quaglia, A., Mieli-Vergani, G., Ma, Y. and Vergani, D. (2007) 'Cytochrome P450IID6-specific CD8 T cell immune responses mirror disease activity in autoimmune hepatitis type 2', *Hepatology*, 46(2), 472-84.

Longhi, M. S., Hussain, M. J., Kwok, W. W., Mieli-Vergani, G., Ma, Y. and Vergani, D. (2011) 'Autoantigen-specific regulatory T cells, a potential tool for immune-tolerance reconstitution in type-2 autoimmune hepatitis', *Hepatology*, 53(2), 536-47.

Longhi, M. S., Hussain, M. J., Mitry, R. R., Arora, S. K., Mieli-Vergani, G., Vergani, D. and Ma, Y. (2006) 'Functional study of CD4+CD25+ regulatory T cells in health and autoimmune hepatitis', *J Immunol*, 176(7), 4484-91.

Longhi, M. S., Liberal, R., Holder, B., Robson, S. C., Ma, Y., Mieli-Vergani, G. and Vergani, D. (2012) 'Inhibition of Interleukin-17 Promotes Differentiation of CD25- Cells into Stable T Regulatory Cells in Patients with Autoimmune Hepatitis', *Gastroenterology*.

Longhi, M. S., Ma, Y., Bogdanos, D. P., Cheeseman, P., Mieli-Vergani, G. and Vergani, D. (2004) 'Impairment of CD4(+)CD25(+) regulatory T-cells in autoimmune liver disease', *J Hepatol*, 41(1), 31-7.

Longhi, M. S., Ma, Y., Mieli-Vergani, G. and Vergani, D. (2010) 'Aetiopathogenesis of autoimmune hepatitis', *Journal of Autoimmunity*, 34(1), 7-14.

Longhi, M. S., Ma, Y., Mitry, R. R., Bogdanos, D. P., Heneghan, M., Cheeseman, P., Mieli-Vergani, G. and Vergani, D. (2005) 'Effect of CD4+ CD25+ regulatory T-cells on CD8 T-cell function in patients with autoimmune hepatitis', *J Autoimmun*, 25(1), 63-71.

Longhi, M. S., Meda, F., Wang, P., Samyn, M., Mieli-Vergani, G., Vergani, D. and Ma, Y. (2008) 'Expansion and de novo generation of potentially therapeutic regulatory T cells in patients with autoimmune hepatitis', *Hepatology*, 47(2), 581-91.

Longhi, M. S., Mitry, R. R., Samyn, M., Scalori, A., Hussain, M. J., Quaglia, A., Mieli-Vergani, G., Ma, Y. and Vergani, D. (2009) 'Vigorous activation of monocytes in juvenile autoimmune liver disease escapes the control of regulatory T-cells', *Hepatology*, 50(1), 130-42.

Lopez, E., del Pozo, V., Miguel, T., Sastre, B., Seoane, C., Civantos, E., Llanes, E., Baeza, M. L., Palomino, P., Cardaba, B., Gallardo, S., Manzarbeitia, F., Zubeldia, J. M. and Lahoz, C. (2006) 'Inhibition of chronic airway inflammation and remodeling by galectin-3 gene therapy in a murine model', *J Immunol*, 176(3), 1943-50.

Ludwig, J. (1995) 'Histopathological diagnosis and terminology of chronic hepatitis', *J Hepatol*, 23 Suppl 1, 49-53.

Lv, K., Xu, W., Wang, C., Niki, T., Hirashima, M. and Xiong, S. (2011) 'Galectin-9 administration ameliorates CVB3 induced myocarditis by promoting the proliferation of regulatory T cells and alternatively activated Th2 cells', *Clin Immunol*, 140(1), 92-101.

Ma, Y., Bogdanos, D. P., Hussain, M. J., Underhill, J., Bansal, S., Longhi, M. S., Cheeseman, P., Mieli-Vergani, G. and Vergani, D. (2006) 'Polyclonal T-cell responses to cytochrome P450IID6 are associated with disease activity in autoimmune hepatitis type 2', *Gastroenterology*, 130(3), 868-82.

Ma, Y., Okamoto, M., Thomas, M. G., Bogdanos, D. P., Lopes, A. R., Portmann, B., Underhill, J., Durr, R., Mieli-Vergani, G. and Vergani, D. (2002a) 'Antibodies to conformational epitopes of soluble liver antigen define a severe form of autoimmune liver disease', *Hepatology*, 35(3), 658-64.

Ma, Y., Thomas, M. G., Okamoto, M., Bogdanos, D. P., Nagl, S., Kerkar, N., Lopes, A. R., Muratori, L., Lenzi, M., Bianchi, F. B., Mieli-Vergani, G. and Vergani, D. (2002b) 'Key residues of a major cytochrome P4502D6 epitope are located on the surface of the molecule', *J Immunol*, 169(1), 277-85.

Mackay, I. R. (1993) 'Toward diagnostic criteria for autoimmune hepatitis', *Hepatology*, 18(4), 1006-8.

Mackay, I. R. (1994) 'The history of autoimmune hepatitis' in Nishioka, M., ed. *Autoimmune hepatitis*, Amesterdam: Elsevier.

Mackay, I. R. (2002) 'Hepatoimmunology: a perspective', *Immunol Cell Biol*, 80(1), 36-44.

Mackay, I. R., Cowling, D. C. and Taft, L. I. (1956) 'Lupoid hepatitis', *Lancet*, 271(6957), 1323-6.

Mackay, I. R. and Wood, I. J. (1963) 'The Course and Treatment of Lupoid Hepatitis', *Gastroenterology*, 45, 4-13.

Mackie, F. D., Peakman, M., Yun, M., Sallie, R., Smith, H., Davies, E. T., Mieli-Vergani, G. and Vergani, D. (1994) 'Primary and secondary liver/kidney microsomal autoantibody response following infection with hepatitis C virus', *Gastroenterology*, 106(6), 1672-5.

MacLennan, I. C., Toellner, K. M., Cunningham, A. F., Serre, K., Sze, D. M., Zuniga, E., Cook, M. C. and Vinuesa, C. G. (2003) 'Extrafollicular antibody responses', *Immunol Rev*, 194, 8-18.

Mallone, R., Kochik, S. A., Reijonen, H., Carson, B., Ziegler, S. F., Kwok, W. W. and Nepom, G. T. (2005) 'Functional avidity directs T-cell fate in autoreactive CD4+ T cells', *Blood*, 106(8), 2798-805.

Manetti, R., Parronchi, P., Giudizi, M. G., Piccinni, M. P., Maggi, E., Trinchieri, G. and Romagnani, S. (1993) 'Natural killer cell stimulatory factor (interleukin 12 [IL-12]) induces T helper type 1 (Th1)-specific immune responses and inhibits the development of IL-4-producing Th cells', *J Exp Med*, 177(4), 1199-204.

Mangan, P. R., Harrington, L. E., O'Quinn, D. B., Helms, W. S., Bullard, D. C., Elson, C. O., Hatton, R. D., Wahl, S. M., Schoeb, T. R. and Weaver, C. T. (2006) 'Transforming growth factor-beta induces development of the T(H)17 lineage', *Nature*, 441(7090), 231-4.

Manns, M., Gerken, G., Kyriatsoulis, A., Staritz, M. and Meyer zum Buschenfelde, K. H. (1987) 'Characterisation of a new subgroup of autoimmune chronic active hepatitis by autoantibodies against a soluble liver antigen', *Lancet*, 1(8528), 292-4.

Manns, M. P., Czaja, A. J., Gorham, J. D., Krawitt, E. L., Mieli-Vergani, G., Vergani, D. and Vierling, J. M. (2010a) 'Diagnosis and management of autoimmune hepatitis', *Hepatology*, 51(6), 2193-213.

Manns, M. P., Griffin, K. J., Sullivan, K. F. and Johnson, E. F. (1991) 'LKM-1 autoantibodies recognize a short linear sequence in P450IID6, a cytochrome P-450 monooxygenase', *J Clin Invest*, 88(4), 1370-8.

Manns, M. P. and Vogel, A. (2006) 'Autoimmune hepatitis, from mechanisms to therapy', *Hepatology*, 43(2 Suppl 1), S132-44.

Manns, M. P., Woynarowski, M., Kreisel, W., Lurie, Y., Rust, C., Zuckerman, E., Bahr, M. J., Gunther, R., Hultcrantz, R. W., Spengler, U., Lohse, A. W., Szalay, F., Farkkila, M., Prols, M. and Strassburg, C. P. (2010b) 'Budesonide induces remission more effectively than prednisone in a controlled trial of patients with autoimmune hepatitis', *Gastroenterology*, 139(4), 1198-206.

Marrack, P. and Kappler, J. (2004) 'Control of T cell viability', *Annu Rev Immunol*, 22, 765-87.

Marsh, D. G., Neely, J. D., Breazeale, D. R., Ghosh, B., Freidhoff, L. R., Ehrlich-Kautzky, E., Schou, C., Krishnaswamy, G. and Beaty, T. H. (1994) 'Linkage analysis of IL4 and other chromosome 5q31.1 markers and total serum immunoglobulin E concentrations', *Science*, 264(5162), 1152-6.

Marson, A., Kretschmer, K., Frampton, G. M., Jacobsen, E. S., Polansky, J. K., MacIsaac, K. D., Levine, S. S., Fraenkel, E., von Boehmer, H. and Young, R. A. (2007) 'Foxp3 occupancy and regulation of key target genes during T-cell stimulation', *Nature*, 445(7130), 931-5.

Martinez, C., Kersey, J., Papermaster, B. W. and Good, R. A. (1962) 'Skin homograft survival in thymectomized mice', *Proc Soc Exp Biol Med*, 109, 193-6.

Martini, E., Abuaf, N., Cavalli, F., Durand, V., Johanet, C. and Homberg, J. C. (1988) 'Antibody to liver cytosol (anti-LC1) in patients with autoimmune chronic active hepatitis type 2', *Hepatology*, 8(6), 1662-6.

Marwaha, A. K., Crome, S. Q., Panagiotopoulos, C., Berg, K. B., Qin, H., Ouyang, Q., Xu, L., Priatel, J. J., Levings, M. K. and Tan, R. (2010) 'Cutting edge: Increased IL-17-secreting T cells in children with new-onset type 1 diabetes', *J Immunol*, 185(7), 3814-8.

Mathis, D. and Benoist, C. (2009) 'Aire', *Annu Rev Immunol*, 27, 287-312.

Mathis, D. and Benoist, C. (2010) 'Levees of immunological tolerance', *Nat Immunol*, 11(1), 3-6.

Matsumoto, R., Matsumoto, H., Seki, M., Hata, M., Asano, Y., Kanegasaki, S., Stevens, R. L. and Hirashima, M. (1998) 'Human ecalectin, a variant of human galectin-9, is a novel

eosinophil chemoattractant produced by T lymphocytes', *J Biol Chem*, 273(27), 16976-84.

Maul, J., Loddenkemper, C., Mundt, P., Berg, E., Giese, T., Stallmach, A., Zeitz, M. and Duchmann, R. (2005) 'Peripheral and intestinal regulatory CD4+ CD25(high) T cells in inflammatory bowel disease', *Gastroenterology*, 128(7), 1868-78.

Mazzucchelli, R. and Durum, S. K. (2007) 'Interleukin-7 receptor expression: intelligent design', *Nat Rev Immunol*, 7(2), 144-54.

McFarland, H. F. and Martin, R. (2007) 'Multiple sclerosis: a complicated picture of autoimmunity', *Nat Immunol*, 8(9), 913-9.

McFarlane, B. M., McSorley, C. G., Vergani, D., McFarlane, I. G. and Williams, R. (1986) 'Serum autoantibodies reacting with the hepatic asialoglycoprotein receptor protein (hepatic lectin) in acute and chronic liver disorders', *J Hepatol*, 3(2), 196-205.

McFarlane, I. G. (2002) 'Definition and classification of autoimmune hepatitis', *Semin Liver Dis*, 22(4), 317-24.

McFarlane, I. G., McFarlane, B. M., Major, G. N., Tolley, P. and Williams, R. (1984) 'Identification of the hepatic asialo-glycoprotein receptor (hepatic lectin) as a component of liver specific membrane lipoprotein (LSP)', *Clin Exp Immunol*, 55(2), 347-54.

McFarlane, I. G. a. W., R. (1996) 'Historical overview' in McFarlane, I. G. a. W., R. , ed. *Molecular Basis of Autoimmune Hepatitis*, Austin: RG Landes, 1-21.

McGeachy, M. J., Bak-Jensen, K. S., Chen, Y., Tato, C. M., Blumenschein, W., McClanahan, T. and Cua, D. J. (2007) 'TGF-beta and IL-6 drive the production of IL-17 and IL-10 by T cells and restrain T(H)-17 cell-mediated pathology', *Nat Immunol*, 8(12), 1390-7.

McHugh, R. S., Whitters, M. J., Piccirillo, C. A., Young, D. A., Shevach, E. M., Collins, M. and Byrne, M. C. (2002) 'CD4(+)CD25(+) immunoregulatory T cells: gene expression analysis reveals a functional role for the glucocorticoid-induced TNF receptor', *Immunity*, 16(2), 311-23.

McInnes, I. B. and Schett, G. (2011) 'The pathogenesis of rheumatoid arthritis', *N Engl J Med*, 365(23), 2205-19.

McIntire, J. J., Umetsu, D. T. and DeKruyff, R. H. (2004) 'TIM-1, a novel allergy and asthma susceptibility gene', *Springer Semin Immunopathol*, 25(3-4), 335-48.

McIntire, J. J., Umetsu, S. E., Akbari, O., Potter, M., Kuchroo, V. K., Barsh, G. S., Freeman, G. J., Umetsu, D. T. and DeKruyff, R. H. (2001) 'Identification of Tapr (an airway hyperreactivity regulatory locus) and the linked Tim gene family', *Nat Immunol*, 2(12), 1109-16.

McIntire, J. J., Umetsu, S. E., Macaubas, C., Hoyte, E. G., Cinnioglu, C., Cavalli-Sforza, L. L., Barsh, G. S., Hallmayer, J. F., Underhill, P. A., Risch, N. J., Freeman, G. J., DeKruyff, R. H. and Umetsu, D. T. (2003) 'Immunology: hepatitis A virus link to atopic disease', *Nature*, 425(6958), 576.

McKay, F. C., Swain, L. I., Schibeci, S. D., Rubio, J. P., Kilpatrick, T. J., Heard, R. N., Stewart, G. J. and Booth, D. R. (2008) 'CD127 immunophenotyping suggests altered CD4+ T cell regulation in primary progressive multiple sclerosis', *J Autoimmun,* 31(1), 52-8.

McMahan, R. H., Golden-Mason, L., Nishimura, M. I., McMahon, B. J., Kemper, M., Allen, T. M., Gretch, D. R. and Rosen, H. R. (2010) 'Tim-3 expression on PD-1+ HCV-specific human CTLs is associated with viral persistence, and its blockade restores hepatocyte-directed in vitro cytotoxicity', *J Clin Invest,* 120(12), 4546-57.

McNair, A. N., Moloney, M., Portmann, B. C., Williams, R. and McFarlane, I. G. (1998) 'Autoimmune hepatitis overlapping with primary sclerosing cholangitis in five cases', *Am J Gastroenterol,* 93(5), 777-84.

Mellor-Pita, S., Citores, M. J., Castejon, R., Tutor-Ureta, P., Yebra-Bango, M., Andreu, J. L. and Vargas, J. A. (2006) 'Decrease of regulatory T cells in patients with systemic lupus erythematosus', *Ann Rheum Dis,* 65(4), 553-4.

Mengshol, J. A., Golden-Mason, L., Arikawa, T., Smith, M., Niki, T., McWilliams, R., Randall, J. A., McMahan, R., Zimmerman, M. A., Rangachari, M., Dobrinskikh, E., Busson, P., Polyak, S. J., Hirashima, M. and Rosen, H. R. (2010) 'A crucial role for Kupffer cell-derived galectin-9 in regulation of T cell immunity in hepatitis C infection', *PLoS One,* 5(3), e9504.

Meyers, J. H., Chakravarti, S., Schlesinger, D., Illes, Z., Waldner, H., Umetsu, S. E., Kenny, J., Zheng, X. X., Umetsu, D. T., DeKruyff, R. H., Strom, T. B. and Kuchroo, V. K. (2005a) 'TIM-4 is the ligand for TIM-1, and the TIM-1-TIM-4 interaction regulates T cell proliferation', *Nat Immunol,* 6(5), 455-64.

Meyers, J. H., Sabatos, C. A., Chakravarti, S. and Kuchroo, V. K. (2005b) 'The TIM gene family regulates autoimmune and allergic diseases', *Trends Mol Med,* 11(8), 362-9.

Micallef, M. J., Ohtsuki, T., Kohno, K., Tanabe, F., Ushio, S., Namba, M., Tanimoto, T., Torigoe, K., Fujii, M., Ikeda, M., Fukuda, S. and Kurimoto, M. (1996) 'Interferon-gamma-inducing factor enhances T helper 1 cytokine production by stimulated human T cells: synergism with interleukin-12 for interferon-gamma production', *Eur J Immunol,* 26(7), 1647-51.

Michel, L., Berthelot, L., Pettre, S., Wiertlewski, S., Lefrere, F., Braudeau, C., Brouard, S., Soulillou, J. P. and Laplaud, D. A. (2008) 'Patients with relapsing-remitting multiple sclerosis have normal Treg function when cells expressing IL-7 receptor alpha-chain are excluded from the analysis', *J Clin Invest,* 118(10), 3411-9.

Mieli-Vergani, G., Heller, S., Jara, P., Vergani, D., Chang, M. H., Fujisawa, T., Gonzalez-Peralta, R. P., Kelly, D., Mohan, N., Shah, U. and Murray, K. F. (2009) 'Autoimmune hepatitis', *J Pediatr Gastroenterol Nutr,* 49(2), 158-64.

Mieli-Vergani, G. and Vergani, D. (2002) 'Autoimmune hepatitis in children', *Clin Liver Dis,* 6(3), 623-34.

Miller, J. F. (1961) 'Immunological function of the thymus', *Lancet,* 2(7205), 748-9.

Miossec, P., Korn, T. and Kuchroo, V. K. (2009) 'Interleukin-17 and type 17 helper T cells', *N Engl J Med,* 361(9), 888-98.

Mitchell, S. A., Bansi, D. S., Hunt, N., Von Bergmann, K., Fleming, K. A. and Chapman, R. W. (2001) 'A preliminary trial of high-dose ursodeoxycholic acid in primary sclerosing cholangitis', *Gastroenterology,* 121(4), 900-7.

Miyagawa-Hayashino, A., Haga, H., Egawa, H., Hayashino, Y., Sakurai, T., Minamiguchi, S., Tanaka, K. and Manabe, T. (2004) 'Outcome and risk factors of de novo autoimmune hepatitis in living-donor liver transplantation', *Transplantation,* 78(1), 128-35.

Miyara, M., Amoura, Z., Parizot, C., Badoual, C., Dorgham, K., Trad, S., Nochy, D., Debre, P., Piette, J. C. and Gorochov, G. (2005) 'Global natural regulatory T cell depletion in active systemic lupus erythematosus', *J Immunol,* 175(12), 8392-400.

Mizui, M. and Kikutani, H. (2008) 'Neuropilin-1: the glue between regulatory T cells and dendritic cells?', *Immunity,* 28(3), 302-3.

Mizui, M., Shikina, T., Arase, H., Suzuki, K., Yasui, T., Rennert, P. D., Kumanogoh, A. and Kikutani, H. (2008) 'Bimodal regulation of T cell-mediated immune responses by TIM-4', *Int Immunol,* 20(5), 695-708.

Monk, C. R., Spachidou, M., Rovis, F., Leung, E., Botto, M., Lechler, R. I. and Garden, O. A. (2005) 'MRL/Mp CD4+,CD25- T cells show reduced sensitivity to suppression by CD4+,CD25+ regulatory T cells in vitro: a novel defect of T cell regulation in systemic lupus erythematosus', *Arthritis Rheum,* 52(4), 1180-4.

Monney, L., Sabatos, C. A., Gaglia, J. L., Ryu, A., Waldner, H., Chernova, T., Manning, S., Greenfield, E. A., Coyle, A. J., Sobel, R. A., Freeman, G. J. and Kuchroo, V. K. (2002) 'Th1-specific cell surface protein Tim-3 regulates macrophage activation and severity of an autoimmune disease', *Nature,* 415(6871), 536-41.

Moore, K. W., de Waal Malefyt, R., Coffman, R. L. and O'Garra, A. (2001) 'Interleukin-10 and the interleukin-10 receptor', *Annu Rev Immunol,* 19, 683-765.

Moore, M. A. and Owen, J. J. (1966) 'Experimental studies on the development of the bursa of Fabricius', *Dev Biol,* 14(1), 40-51.

Mora, J. R., Cheng, G., Picarella, D., Briskin, M., Buchanan, N. and von Andrian, U. H. (2005) 'Reciprocal and dynamic control of CD8 T cell homing by dendritic cells from skin- and gut-associated lymphoid tissues', *J Exp Med,* 201(2), 303-16.

Morgan, M. E., Sutmuller, R. P., Witteveen, H. J., van Duivenvoorde, L. M., Zanelli, E., Melief, C. J., Snijders, A., Offringa, R., de Vries, R. R. and Toes, R. E. (2003) 'CD25+ cell depletion hastens the onset of severe disease in collagen-induced arthritis', *Arthritis Rheum,* 48(5), 1452-60.

Mosmann, T. R., Cherwinski, H., Bond, M. W., Giedlin, M. A. and Coffman, R. L. (1986) 'Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins', *J Immunol,* 136(7), 2348-57.

Mosmann, T. R. and Coffman, R. L. (1989) 'TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties', *Annu Rev Immunol,* 7, 145-73.

Mottershead, M. and Neuberger, J. (2008) 'Transplantation in autoimmune liver diseases', *World J Gastroenterol,* 14(21), 3388-95.

Mottonen, M., Heikkinen, J., Mustonen, L., Isomaki, P., Luukkainen, R. and Lassila, O. (2005) 'CD4+ CD25+ T cells with the phenotypic and functional characteristics of regulatory T cells are enriched in the synovial fluid of patients with rheumatoid arthritis', *Clin Exp Immunol,* 140(2), 360-7.

Mueller, D. L. (2010) 'Mechanisms maintaining peripheral tolerance', *Nat Immunol,* 11(1), 21-7.

Munn, D. H., Shafizadeh, E., Attwood, J. T., Bondarev, I., Pashine, A. and Mellor, A. L. (1999) 'Inhibition of T cell proliferation by macrophage tryptophan catabolism', *J Exp Med,* 189(9), 1363-72.

Muratori, L., Cataleta, M., Muratori, P., Lenzi, M. and Bianchi, F. B. (1998) 'Liver/kidney microsomal antibody type 1 and liver cytosol antibody type 1 concentrations in type 2 autoimmune hepatitis', *Gut,* 42(5), 721-6.

Muratori, L., Cataleta, M., Muratori, P., Manotti, P., Lenzi, M., Cassani, F. and Bianchi, F. B. (1995) 'Detection of anti-liver cytosol antibody type 1 (anti-LC1) by immunodiffusion, counterimmunoelectrophoresis and immunoblotting: comparison of different techniques', *J Immunol Methods,* 187(2), 259-64.

Muratori, L., Parola, M., Ripalti, A., Robino, G., Muratori, P., Bellomo, G., Carini, R., Lenzi, M., Landini, M. P., Albano, E. and Bianchi, F. B. (2000) 'Liver/kidney microsomal antibody type 1 targets CYP2D6 on hepatocyte plasma membrane', *Gut,* 46(4), 553-61.

Muratori, P., Muratori, L., Agostinelli, D., Pappas, G., Veronesi, L., Granito, A., Cassani, F., Terlizzi, P., Lenzi, M. and Bianchi, F. B. (2002) 'Smooth muscle antibodies and type 1 autoimmune hepatitis', *Autoimmunity,* 35(8), 497-500.

Murphy, C. A., Langrish, C. L., Chen, Y., Blumenschein, W., McClanahan, T., Kastelein, R. A., Sedgwick, J. D. and Cua, D. J. (2003) 'Divergent pro- and antiinflammatory roles for IL-23 and IL-12 in joint autoimmune inflammation', *J Exp Med,* 198(12), 1951-7.

Murray-Lyon, I. M., Stern, R. B. and Williams, R. (1973) 'Controlled trial of prednisone and azathioprine in active chronic hepatitis', *Lancet,* 1(7806), 735-7.

Nakae, S., Iikura, M., Suto, H., Akiba, H., Umetsu, D. T., Dekruyff, R. H., Saito, H. and Galli, S. J. (2007a) 'TIM-1 and TIM-3 enhancement of Th2 cytokine production by mast cells', *Blood,* 110(7), 2565-8.

Nakae, S., Iwakura, Y., Suto, H. and Galli, S. J. (2007b) 'Phenotypic differences between Th1 and Th17 cells and negative regulation of Th1 cell differentiation by IL-17', *J Leukoc Biol,* 81(5), 1258-68.

Nakamura, K., Kitani, A., Fuss, I., Pedersen, A., Harada, N., Nawata, H. and Strober, W. (2004) 'TGF-beta 1 plays an important role in the mechanism of CD4+CD25+ regulatory T cell activity in both humans and mice', *J Immunol,* 172(2), 834-42.

Nakamura, K., Kitani, A. and Strober, W. (2001) 'Cell contact-dependent immunosuppression by CD4(+)CD25(+) regulatory T cells is mediated by cell surface-bound transforming growth factor beta', *J Exp Med,* 194(5), 629-44.

Nakayama, M., Akiba, H., Takeda, K., Kojima, Y., Hashiguchi, M., Azuma, M., Yagita, H. and Okumura, K. (2009) 'Tim-3 mediates phagocytosis of apoptotic cells and cross-presentation', *Blood*, 113(16), 3821-30.

Nangia-Makker, P., Raz, T., Tait, L., Hogan, V., Fridman, R. and Raz, A. (2007) 'Galectin-3 cleavage: a novel surrogate marker for matrix metalloproteinase activity in growing breast cancers', *Cancer Res*, 67(24), 11760-8.

Nemazee, D. and Hogquist, K. A. (2003) 'Antigen receptor selection by editing or downregulation of V(D)J recombination', *Curr Opin Immunol*, 15(2), 182-9.

Neuberger, J., Portmann, B., Calne, R. and Williams, R. (1984) 'Recurrence of autoimmune chronic active hepatitis following orthotopic liver grafting', *Transplantation*, 37(4), 363-5.

Ngu, J. H., Bechly, K., Chapman, B. A., Burt, M. J., Barclay, M. L., Gearry, R. B. and Stedman, C. A. (2010) 'Population-based epidemiology study of autoimmune hepatitis: a disease of older women?', *J Gastroenterol Hepatol*, 25(10), 1681-6.

Nishi, N., Itoh, A., Shoji, H., Miyanaka, H. and Nakamura, T. (2006) 'Galectin-8 and galectin-9 are novel substrates for thrombin', *Glycobiology*, 16(11), 15C-20C.

Nishimura, H., Nose, M., Hiai, H., Minato, N. and Honjo, T. (1999) 'Development of lupus-like autoimmune diseases by disruption of the PD-1 gene encoding an ITIM motif-carrying immunoreceptor', *Immunity*, 11(2), 141-51.

Nishioka, M. a. M., S. A. and McFarlane, I. G. et al. (1998) 'Geographical variation in the frequency and characteristics of autoimmune liver diseases' in Krawitt, E. L. a. W., R. and Nishioka, M., ed. *Autoimmune liver diseases*, 2nd ed., Amesterdam: Elsevier, 413-428.

Nishizuka, Y. and Sakakura, T. (1969) 'Thymus and reproduction: sex-linked dysgenesia of the gonad after neonatal thymectomy in mice', *Science*, 166(906), 753-5.

Nocentini, G., Giunchi, L., Ronchetti, S., Krausz, L. T., Bartoli, A., Moraca, R., Migliorati, G. and Riccardi, C. (1997) 'A new member of the tumor necrosis factor/nerve growth factor receptor family inhibits T cell receptor-induced apoptosis', *Proc Natl Acad Sci U S A*, 94(12), 6216-21.

Nouri-Aria, K. T., Hegarty, J. E., Alexander, G. J., Eddleston, A. L. and Williams, R. (1982) 'Effect of corticosteroids on suppressor-cell activity in "autoimmune" and viral chronic active hepatitis', *N Engl J Med*, 307(21), 1301-4.

Nurieva, R., Yang, X. O., Martinez, G., Zhang, Y., Panopoulos, A. D., Ma, L., Schluns, K., Tian, Q., Watowich, S. S., Jetten, A. M. and Dong, C. (2007) 'Essential autocrine regulation by IL-21 in the generation of inflammatory T cells', *Nature*, 448(7152), 480-3.

O'Shea, J. J., Ma, A. and Lipsky, P. (2002) 'Cytokines and autoimmunity', *Nat Rev Immunol*, 2(1), 37-45.

Ocklenburg, F., Moharregh-Khiabani, D., Geffers, R., Janke, V., Pfoertner, S., Garritsen, H., Groebe, L., Klempnauer, J., Dittmar, K. E., Weiss, S., Buer, J. and Probst-Kepper, M. (2006) 'UBD, a downstream element of FOXP3, allows the identification of LGALS3, a new marker of human regulatory T cells', *Lab Invest,* 86(7), 724-37.

Oettinger, R., Brunnberg, A., Gerner, P., Wintermeyer, P., Jenke, A. and Wirth, S. (2005) 'Clinical features and biochemical data of Caucasian children at diagnosis of autoimmune hepatitis', *J Autoimmun,* 24(1), 79-84.

Ohshima, S., Kuchen, S., Seemayer, C. A., Kyburz, D., Hirt, A., Klinzing, S., Michel, B. A., Gay, R. E., Liu, F. T., Gay, S. and Neidhart, M. (2003) 'Galectin 3 and its binding protein in rheumatoid arthritis', *Arthritis Rheum,* 48(10), 2788-95.

Okazawa, A., Kanai, T., Watanabe, M., Yamazaki, M., Inoue, N., Ikeda, M., Kurimoto, M., Ishii, H. and Hibi, T. (2002) 'Th1-mediated intestinal inflammation in Crohn's disease may be induced by activation of lamina propria lymphocytes through synergistic stimulation of interleukin-12 and interleukin-18 without T cell receptor engagement', *Am J Gastroenterol,* 97(12), 3108-17.

Okuno, T., Seto, Y., Okanoue, T. and Takino, T. (1987) 'Chronic active hepatitis with histological features of primary biliary cirrhosis', *Dig Dis Sci,* 32(7), 775-9.

Ollila, J. and Vihinen, M. (2005) 'B cells', *Int J Biochem Cell Biol,* 37(3), 518-23.

Ouyang, W., Lohning, M., Gao, Z., Assenmacher, M., Ranganath, S., Radbruch, A. and Murphy, K. M. (2000) 'Stat6-independent GATA-3 autoactivation directs IL-4-independent Th2 development and commitment', *Immunity,* 12(1), 27-37.

Pai, S. Y., Truitt, M. L. and Ho, I. C. (2004) 'GATA-3 deficiency abrogates the development and maintenance of T helper type 2 cells', *Proc Natl Acad Sci U S A,* 101(7), 1993-8.

Pala, P., Hussell, T. and Openshaw, P. J. (2000) 'Flow cytometric measurement of intracellular cytokines', *J Immunol Methods,* 243(1-2), 107-24.

Palioura, S., Sherrer, R. L., Steitz, T. A., Soll, D. and Simonovic, M. (2009) 'The human SepSecS-tRNASec complex reveals the mechanism of selenocysteine formation', *Science,* 325(5938), 321-5.

Palmer, E. (2003) 'Negative selection--clearing out the bad apples from the T-cell repertoire', *Nat Rev Immunol,* 3(5), 383-91.

Palmer, E. and Naeher, D. (2009) 'Affinity threshold for thymic selection through a T-cell receptor-co-receptor zipper', *Nat Rev Immunol,* 9(3), 207-13.

Pandiyan, P., Zheng, L., Ishihara, S., Reed, J. and Lenardo, M. J. (2007) 'CD4+CD25+Foxp3+ regulatory T cells induce cytokine deprivation-mediated apoptosis of effector CD4+ T cells', *Nat Immunol,* 8(12), 1353-62.

Park, H., Li, Z., Yang, X. O., Chang, S. H., Nurieva, R., Wang, Y. H., Wang, Y., Hood, L., Zhu, Z., Tian, Q. and Dong, C. (2005) 'A distinct lineage of CD4 T cells regulates tissue inflammation by producing interleukin 17', *Nat Immunol,* 6(11), 1133-41.

Parronchi, P., De Carli, M., Manetti, R., Simonelli, C., Sampognaro, S., Piccinni, M. P., Macchia, D., Maggi, E., Del Prete, G. and Romagnani, S. (1992) 'IL-4 and IFN (alpha and gamma) exert opposite regulatory effects on the development of cytolytic potential by Th1 or Th2 human T cell clones', *J Immunol,* 149(9), 2977-83.

Paul, W. E. and Seder, R. A. (1994) 'Lymphocyte responses and cytokines', *Cell,* 76(2), 241-51.

Pegg, D. E. (2007) 'Principles of cryopreservation', *Methods Mol Biol,* 368, 39-57.

Perfetto, S. P., Chattopadhyay, P. K. and Roederer, M. (2004) 'Seventeen-colour flow cytometry: unravelling the immune system', *Nat Rev Immunol,* 4(8), 648-55.

Perillo, N. L., Pace, K. E., Seilhamer, J. J. and Baum, L. G. (1995) 'Apoptosis of T cells mediated by galectin-1', *Nature,* 378(6558), 736-9.

Perillo, N. L., Uittenbogaart, C. H., Nguyen, J. T. and Baum, L. G. (1997) 'Galectin-1, an endogenous lectin produced by thymic epithelial cells, induces apoptosis of human thymocytes', *J Exp Med,* 185(10), 1851-8.

Petrie, H. T., Livak, F., Burtrum, D. and Mazel, S. (1995) 'T cell receptor gene recombination patterns and mechanisms: cell death, rescue, and T cell production', *J Exp Med,* 182(1), 121-7.

Philipp, T., Durazzo, M., Trautwein, C., Alex, B., Straub, P., Lamb, J. G., Johnson, E. F., Tukey, R. H. and Manns, M. P. (1994) 'Recognition of uridine diphosphate glucuronosyl transferases by LKM-3 antibodies in chronic hepatitis D', *Lancet,* 344(8922), 578-81.

Piccirillo, C. A., Letterio, J. J., Thornton, A. M., McHugh, R. S., Mamura, M., Mizuhara, H. and Shevach, E. M. (2002) 'CD4(+)CD25(+) regulatory T cells can mediate suppressor function in the absence of transforming growth factor beta1 production and responsiveness', *J Exp Med,* 196(2), 237-46.

Poupon, R., Chazouilleres, O., Corpechot, C. and Chretien, Y. (2006) 'Development of autoimmune hepatitis in patients with typical primary biliary cirrhosis', *Hepatology,* 44(1), 85-90.

Primo, J., Merino, C., Fernandez, J., Moles, J. R., Llorca, P. and Hinojosa, J. (2004) '[Incidence and prevalence of autoimmune hepatitis in the area of the Hospital de Sagunto (Spain)]', *Gastroenterol Hepatol,* 27(4), 239-43.

Putnam, A. L., Vendrame, F., Dotta, F. and Gottlieb, P. A. (2005) 'CD4+CD25high regulatory T cells in human autoimmune diabetes', *J Autoimmun,* 24(1), 55-62.

Qiu, D., Wang, Q., Wang, H., Xie, Q., Zang, G., Jiang, H., Tu, C., Guo, J., Zhang, S., Wang, J., Lu, Y., Han, Y., Shen, L., Chen, X., Hu, X., Wang, X., Chen, C., Fu, Q. and Ma, X. (2011) 'Validation of the simplified criteria for diagnosis of autoimmune hepatitis in Chinese patients', *J Hepatol,* 54(2), 340-7.

Rabinovich, G. A., Baum, L. G., Tinari, N., Paganelli, R., Natoli, C., Liu, F. T. and Iacobelli, S. (2002) 'Galectins and their ligands: amplifiers, silencers or tuners of the inflammatory response?', *Trends Immunol,* 23(6), 313-20.

Rabinovich, G. A., Iglesias, M. M., Modesti, N. M., Castagna, L. F., Wolfenstein-Todel, C., Riera, C. M. and Sotomayor, C. E. (1998) 'Activated rat macrophages produce a galectin-1-like protein that induces apoptosis of T cells: biochemical and functional characterization', *J Immunol,* 160(10), 4831-40.

Rabinovich, G. A., Liu, F. T., Hirashima, M. and Anderson, A. (2007a) 'An emerging role for galectins in tuning the immune response: lessons from experimental models of inflammatory disease, autoimmunity and cancer', *Scand J Immunol,* 66(2-3), 143-58.

Rabinovich, G. A. and Toscano, M. A. (2009) 'Turning 'sweet' on immunity: galectin-glycan interactions in immune tolerance and inflammation', *Nat Rev Immunol,* 9(5), 338-52.

Rabinovich, G. A., Toscano, M. A., Jackson, S. S. and Vasta, G. R. (2007b) 'Functions of cell surface galectin-glycoprotein lattices', *Curr Opin Struct Biol,* 17(5), 513-20.

Rabinovitz, M., Demetris, A. J., Bou-Abboud, C. F. and Van Thiel, D. H. (1992) 'Simultaneous occurrence of primary sclerosing cholangitis and autoimmune chronic active hepatitis in a patient with ulcerative colitis', *Dig Dis Sci,* 37(10), 1606-11.

Racanelli, V. and Rehermann, B. (2006) 'The liver as an immunological organ', *Hepatology,* 43(2 Suppl 1), S54-62.

Rahman, M. K., Midtling, E. H., Svingen, P. A., Xiong, Y., Bell, M. P., Tung, J., Smyrk, T., Egan, L. J. and Faubion, W. A., Jr. (2010) 'The pathogen recognition receptor NOD2 regulates human FOXP3+ T cell survival', *J Immunol,* 184(12), 7247-56.

Read, S., Malmstrom, V. and Powrie, F. (2000) 'Cytotoxic T lymphocyte-associated antigen 4 plays an essential role in the function of CD25(+)CD4(+) regulatory cells that control intestinal inflammation', *J Exp Med,* 192(2), 295-302.

Recktenwald, D. J. (1993) 'Introduction to flow cytometry: principles, fluorochromes, instrument set-up, calibration', *J Hematother,* 2(3), 387-94.

Reich, D. J., Fiel, I., Guarrera, J. V., Emre, S., Guy, S. R., Schwartz, M. E., Miller, C. M. and Sheiner, P. A. (2000) 'Liver transplantation for autoimmune hepatitis', *Hepatology,* 32(4 Pt 1), 693-700.

Ren, X., Ye, F., Jiang, Z., Chu, Y., Xiong, S. and Wang, Y. (2007) 'Involvement of cellular death in TRAIL/DR5-dependent suppression induced by CD4(+)CD25(+) regulatory T cells', *Cell Death Differ,* 14(12), 2076-84.

Richardson, P. D., James, P. D. and Ryder, S. D. (2000) 'Mycophenolate mofetil for maintenance of remission in autoimmune hepatitis in patients resistant to or intolerant of azathioprine', *J Hepatol,* 33(3), 371-5.

Rizzetto, M., Swana, G. and Doniach, D. (1973) 'Microsomal antibodies in active chronic hepatitis and other disorders', *Clin Exp Immunol,* 15(3), 331-44.

Robinson, D., Shibuya, K., Mui, A., Zonin, F., Murphy, E., Sana, T., Hartley, S. B., Menon, S., Kastelein, R., Bazan, F. and O'Garra, A. (1997) 'IGIF does not drive Th1 development but synergizes with IL-12 for interferon-gamma production and activates IRAK and NFkappaB', *Immunity,* 7(4), 571-81.

Robinson, R. T., Wang, J., Cripps, J. G., Milks, M. W., English, K. A., Pearson, T. A. and Gorham, J. D. (2009) 'End-organ damage in a mouse model of fulminant liver inflammation requires CD4+ T cell production of IFN-gamma but is independent of Fas', *J Immunol,* 182(5), 3278-84.

Rodewald, H. R. and Fehling, H. J. (1998) 'Molecular and cellular events in early thymocyte development', *Adv Immunol,* 69, 1-112.

Rodriguez-Manzanet, R., DeKruyff, R., Kuchroo, V. K. and Umetsu, D. T. (2009) 'The costimulatory role of TIM molecules', *Immunol Rev,* 229(1), 259-70.

Romagnani, S. (1991) 'Human TH1 and TH2 subsets: doubt no more', *Immunol Today,* 12(8), 256-7.

Roncarolo, M. G., Gregori, S., Battaglia, M., Bacchetta, R., Fleischhauer, K. and Levings, M. K. (2006) 'Interleukin-10-secreting type 1 regulatory T cells in rodents and humans', *Immunol Rev,* 212, 28-50.

Ronchetti, S., Zollo, O., Bruscoli, S., Agostini, M., Bianchini, R., Nocentini, G., Ayroldi, E. and Riccardi, C. (2004) 'GITR, a member of the TNF receptor superfamily, is costimulatory to mouse T lymphocyte subpopulations', *Eur J Immunol,* 34(3), 613-22.

Rowe, I. A., Webb, K., Gunson, B. K., Mehta, N., Haque, S. and Neuberger, J. (2008) 'The impact of disease recurrence on graft survival following liver transplantation: a single centre experience', *Transpl Int,* 21(5), 459-65.

Rudd, C. E., Taylor, A. and Schneider, H. (2009) 'CD28 and CTLA-4 coreceptor expression and signal transduction', *Immunol Rev,* 229(1), 12-26.

Sabatos, C. A., Chakravarti, S., Cha, E., Schubart, A., Sanchez-Fueyo, A., Zheng, X. X., Coyle, A. J., Strom, T. B., Freeman, G. J. and Kuchroo, V. K. (2003) 'Interaction of Tim-3 and Tim-3 ligand regulates T helper type 1 responses and induction of peripheral tolerance', *Nat Immunol,* 4(11), 1102-10.

Saita, N., Goto, E., Yamamoto, T., Cho, I., Tsumori, K., Kohrogi, H., Maruo, K., Ono, T., Takeya, M., Kashio, Y., Nakamura, K. and Hirashima, M. (2002) 'Association of galectin-9 with eosinophil apoptosis', *Int Arch Allergy Immunol,* 128(1), 42-50.

Sakaguchi, S. (2004) 'Naturally Arising CD4+ Regulatory T Cells for Immunologic Self-Tolerance and Negative Control of Immune Responses', *Annual Review of Immunology,* 22(1), 531-562.

Sakaguchi, S., Miyara, M., Costantino, C. M. and Hafler, D. A. (2010) 'FOXP3+ regulatory T cells in the human immune system', *Nat Rev Immunol,* 10(7), 490-500.

Sakaguchi, S. and Sakaguchi, N. (2005) 'Regulatory T cells in immunologic self-tolerance and autoimmune disease', *Int Rev Immunol,* 24(3-4), 211-26.

Sakaguchi, S., Sakaguchi, N., Asano, M., Itoh, M. and Toda, M. (1995) 'Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases', *J Immunol,* 155(3), 1151-64.

Sakaguchi, S., Wing, K., Onishi, Y., Prieto-Martin, P. and Yamaguchi, T. (2009a) 'Regulatory T cells: how do they suppress immune responses?', *Int Immunol,* 21(10), 1105-11.

Sakaguchi, S., Wing, K. and Yamaguchi, T. (2009b) 'Dynamics of peripheral tolerance and immune regulation mediated by Treg', *Eur J Immunol,* 39(9), 2331-6.

Sakuishi, K., Jayaraman, P., Behar, S. M., Anderson, A. C. and Kuchroo, V. K. (2011) 'Emerging Tim-3 functions in antimicrobial and tumor immunity', *Trends Immunol,* 32(8), 345-9.

Salcedo, M., Vaquero, J., Banares, R., Rodriguez-Mahou, M., Alvarez, E., Vicario, J. L., Hernandez-Albujar, A., Tiscar, J. L., Rincon, D., Alonso, S., De Diego, A. and Clemente, G. (2002) 'Response to steroids in de novo autoimmune hepatitis after liver transplantation', *Hepatology,* 35(2), 349-56.

Sallusto, F. and Lanzavecchia, A. (2000) 'Understanding dendritic cell and T-lymphocyte traffic through the analysis of chemokine receptor expression', *Immunol Rev,* 177, 134-40.

Sallusto, F., Lenig, D., Forster, R., Lipp, M. and Lanzavecchia, A. (1999) 'Two subsets of memory T lymphocytes with distinct homing potentials and effector functions', *Nature,* 401(6754), 708-12.

Salomon, B. and Bluestone, J. A. (2001) 'Complexities of CD28/B7: CTLA-4 costimulatory pathways in autoimmunity and transplantation', *Annu Rev Immunol,* 19, 225-52.

Salomon, B., Lenschow, D. J., Rhee, L., Ashourian, N., Singh, B., Sharpe, A. and Bluestone, J. A. (2000) 'B7/CD28 costimulation is essential for the homeostasis of the CD4+CD25+ immunoregulatory T cells that control autoimmune diabetes', *Immunity,* 12(4), 431-40.

Samuel, D., Riordan, S., Strasser, S., Kurtovic, J., Singh-Grewel, I. and Koorey, D. (2004) 'Severe autoimmune hepatitis first presenting in the early post partum period', *Clin Gastroenterol Hepatol,* 2(7), 622-4.

Sanchez-Fueyo, A., Tian, J., Picarella, D., Domenig, C., Zheng, X. X., Sabatos, C. A., Manlongat, N., Bender, O., Kamradt, T., Kuchroo, V. K., Gutierrez-Ramos, J. C., Coyle, A. J. and Strom, T. B. (2003) 'Tim-3 inhibits T helper type 1-mediated auto- and alloimmune responses and promotes immunological tolerance', *Nat Immunol,* 4(11), 1093-101.

Santiago, C., Ballesteros, A., Tami, C., Martinez-Munoz, L., Kaplan, G. G. and Casasnovas, J. M. (2007) 'Structures of T Cell immunoglobulin mucin receptors 1 and 2 reveal mechanisms for regulation of immune responses by the TIM receptor family', *Immunity,* 26(3), 299-310.

Santodomingo-Garzon, T. and Swain, M. G. (2011) 'Role of NKT cells in autoimmune liver disease', *Autoimmun Rev*.

Santori, F. R., Kieper, W. C., Brown, S. M., Lu, Y., Neubert, T. A., Johnson, K. L., Naylor, S., Vukmanovic, S., Hogquist, K. A. and Jameson, S. C. (2002) 'Rare, structurally homologous self-peptides promote thymocyte positive selection', *Immunity,* 17(2), 131-42.

Santucci, L., Fiorucci, S., Rubinstein, N., Mencarelli, A., Palazzetti, B., Federici, B., Rabinovich, G. A. and Morelli, A. (2003) 'Galectin-1 suppresses experimental colitis in mice', *Gastroenterology*, 124(5), 1381-94.

Saruta, M., Yu, Q. T., Fleshner, P. R., Mantel, P. Y., Schmidt-Weber, C. B., Banham, A. H. and Papadakis, K. A. (2007) 'Characterization of FOXP3+CD4+ regulatory T cells in Crohn's disease', *Clin Immunol*, 125(3), 281-90.

Schartner, J. M., Fathman, C. G. and Seroogy, C. M. (2007) 'Preservation of self: an overview of E3 ubiquitin ligases and T cell tolerance', *Semin Immunol*, 19(3), 188-96.

Scherer, A., Noest, A. and de Boer, R. J. (2004) 'Activation-threshold tuning in an affinity model for the T-cell repertoire', *Proc Biol Sci*, 271(1539), 609-16.

Schneider, A., Rieck, M., Sanda, S., Pihoker, C., Greenbaum, C. and Buckner, J. H. (2008) 'The effector T cells of diabetic subjects are resistant to regulation via CD4+ FOXP3+ regulatory T cells', *J Immunol*, 181(10), 7350-5.

Schramm, C., Herkel, J., Beuers, U., Kanzler, S., Galle, P. R. and Lohse, A. W. (2006) 'Pregnancy in autoimmune hepatitis: outcome and risk factors', *Am J Gastroenterol*, 101(3), 556-60.

Schwartz, R. H., Mueller, D. L., Jenkins, M. K. and Quill, H. (1989) 'T-cell clonal anergy', *Cold Spring Harb Symp Quant Biol*, 54 Pt 2, 605-10.

Seddiki, N., Santner-Nanan, B., Martinson, J., Zaunders, J., Sasson, S., Landay, A., Solomon, M., Selby, W., Alexander, S. I., Nanan, R., Kelleher, A. and Fazekas de St Groth, B. (2006) 'Expression of interleukin (IL)-2 and IL-7 receptors discriminates between human regulatory and activated T cells', *J Exp Med*, 203(7), 1693-700.

Sehrawat, S., Suryawanshi, A., Hirashima, M. and Rouse, B. T. (2009) 'Role of Tim-3/galectin-9 inhibitory interaction in viral-induced immunopathology: shifting the balance toward regulators', *J Immunol*, 182(5), 3191-201.

Seki, M., Oomizu, S., Sakata, K. M., Sakata, A., Arikawa, T., Watanabe, K., Ito, K., Takeshita, K., Niki, T., Saita, N., Nishi, N., Yamauchi, A., Katoh, S., Matsukawa, A., Kuchroo, V. and Hirashima, M. (2008) 'Galectin-9 suppresses the generation of Th17, promotes the induction of regulatory T cells, and regulates experimental autoimmune arthritis', *Clin Immunol*, 127(1), 78-88.

Sempere-Ortells, J. M., Perez-Garcia, V., Marin-Alberca, G., Peris-Pertusa, A., Benito, J. M., Marco, F. M., Zubcoff, J. J. and Navarro-Blasco, F. J. (2009) 'Quantification and phenotype of regulatory T cells in rheumatoid arthritis according to disease activity score-28', *Autoimmunity*, 42(8), 636-45.

Senaldi, G., Lobo-Yeo, A., Mowat, A. P., Mieli-Vergani, G. and Vergani, D. (1991) 'Class I and class II major histocompatibility complex antigens on hepatocytes: importance of the method of detection and expression in histologically normal and diseased livers', *J Clin Pathol*, 44(2), 107-14.

Senaldi, G., Portmann, B., Mowat, A. P., Mieli-Vergani, G. and Vergani, D. (1992) 'Immunohistochemical features of the portal tract mononuclear cell infiltrate in chronic aggressive hepatitis', *Arch Dis Child*, 67(12), 1447-53.

Setoguchi, R., Hori, S., Takahashi, T. and Sakaguchi, S. (2005) 'Homeostatic maintenance of natural Foxp3(+) CD25(+) CD4(+) regulatory T cells by interleukin (IL)-2 and induction of autoimmune disease by IL-2 neutralization', *J Exp Med*, 201(5), 723-35.

Sharpe, A. H. (2009) 'Mechanisms of costimulation', *Immunol Rev*, 229(1), 5-11.

Sharpe, A. H. and Abbas, A. K. (2006) 'T-cell costimulation--biology, therapeutic potential, and challenges', *N Engl J Med*, 355(10), 973-5.

Shen, L. S., Wang, J., Shen, D. F., Yuan, X. L., Dong, P., Li, M. X., Xue, J., Zhang, F. M., Ge, H. L. and Xu, D. (2009) 'CD4(+)CD25(+)CD127(low/-) regulatory T cells express Foxp3 and suppress effector T cell proliferation and contribute to gastric cancers progression', *Clin Immunol*, 131(1), 109-18.

Shevach, E. M. (2006) 'From vanilla to 28 flavors: multiple varieties of T regulatory cells', *Immunity*, 25(2), 195-201.

Shevach, E. M. (2009) 'Mechanisms of foxp3+ T regulatory cell-mediated suppression', *Immunity*, 30(5), 636-45.

Shimizu, J., Yamazaki, S., Takahashi, T., Ishida, Y. and Sakaguchi, S. (2002) 'Stimulation of CD25(+)CD4(+) regulatory T cells through GITR breaks immunological self-tolerance', *Nat Immunol*, 3(2), 135-42.

Sifuentes-Romero, I., Milton, S. L. and Garcia-Gasca, A. (2011) 'Post-transcriptional gene silencing by RNA interference in non-mammalian vertebrate systems: where do we stand?', *Mutat Res*, 728(3), 158-71.

Simmonds, M. J. and Gough, S. C. (2004) 'Genetic insights into disease mechanisms of autoimmunity', *Br Med Bull*, 71, 93-113.

Simonsen, M. (1957) 'The impact on the developing embryo and newborn animal of adult homologous cells', *Acta Pathol Microbiol Scand*, 40(6), 480-500.

Sitkovsky, M. V. and Ohta, A. (2005) 'The 'danger' sensors that STOP the immune response: the A2 adenosine receptors?', *Trends Immunol*, 26(6), 299-304.

Sizing, I. D., Bailly, V., McCoon, P., Chang, W., Rao, S., Pablo, L., Rennard, R., Walsh, M., Li, Z., Zafari, M., Dobles, M., Tarilonte, L., Miklasz, S., Majeau, G., Godbout, K., Scott, M. L. and Rennert, P. D. (2007) 'Epitope-dependent effect of anti-murine TIM-1 monoclonal antibodies on T cell activity and lung immune responses', *J Immunol*, 178(4), 2249-61.

Smeltz, R. B., Chen, J., Ehrhardt, R. and Shevach, E. M. (2002) 'Role of IFN-gamma in Th1 differentiation: IFN-gamma regulates IL-18R alpha expression by preventing the negative effects of IL-4 and by inducing/maintaining IL-12 receptor beta 2 expression', *J Immunol*, 168(12), 6165-72.

Soloway, R. D., Summerskill, W. H., Baggenstoss, A. H., Geall, M. G., Gitnick, G. L., Elveback, I. R. and Schoenfield, L. J. (1972) 'Clinical, biochemical, and histological remission of severe chronic active liver disease: a controlled study of treatments and early prognosis', *Gastroenterology*, 63(5), 820-33.

Spada, M., Bertani, A., Sonzogni, A., Petz, W., Riva, S., Torre, G., Melzi, M. L., Alberti, D., Colledan, M., Segalin, A., Lucianetti, A. and Gridelli, B. (2001) 'A cause of late graft dysfunction after liver transplantation in children: de-novo autoimmune hepatitis', *Transplant Proc*, 33(1-2), 1747-8.

Spits, H. (2002) 'Development of alphabeta T cells in the human thymus', *Nat Rev Immunol*, 2(10), 760-72.

Stephens, L. A., Mottet, C., Mason, D. and Powrie, F. (2001) 'Human CD4(+)CD25(+) thymocytes and peripheral T cells have immune suppressive activity in vitro', *Eur J Immunol*, 31(4), 1247-54.

Stoll, S., Jonuleit, H., Schmitt, E., Muller, G., Yamauchi, H., Kurimoto, M., Knop, J. and Enk, A. H. (1998) 'Production of functional IL-18 by different subtypes of murine and human dendritic cells (DC): DC-derived IL-18 enhances IL-12-dependent Th1 development', *Eur J Immunol*, 28(10), 3231-9.

Strassburg, C. P., Alex, B., Zindy, F., Gerken, G., Luttig, B., Meyer zum Buschenfelde, K. H., Brechot, C. and Manns, M. P. (1996a) 'Identification of cyclin A as a molecular target of antinuclear antibodies (ANA) in hepatic and non-hepatic autoimmune diseases', *J Hepatol*, 25(6), 859-66.

Strassburg, C. P. and Manns, M. P. (2002) 'Autoantibodies and autoantigens in autoimmune hepatitis', *Semin Liver Dis*, 22(4), 339-52.

Strassburg, C. P., Obermayer-Straub, P., Alex, B., Durazzo, M., Rizzetto, M., Tukey, R. H. and Manns, M. P. (1996b) 'Autoantibodies against glucuronosyltransferases differ between viral hepatitis and autoimmune hepatitis', *Gastroenterology*, 111(6), 1576-86.

Stravitz, R. T., Lefkowitch, J. H., Fontana, R. J., Gershwin, M. E., Leung, P. S., Sterling, R. K., Manns, M. P., Norman, G. L. and Lee, W. M. (2011) 'Autoimmune acute liver failure: proposed clinical and histological criteria', *Hepatology*, 53(2), 517-26.

Strettell, M. D., Donaldson, P. T., Thomson, L. J., Santrach, P. J., Moore, S. B., Czaja, A. J. and Williams, R. (1997) 'Allelic basis for HLA-encoded susceptibility to type 1 autoimmune hepatitis', *Gastroenterology*, 112(6), 2028-35.

Stumhofer, J. S., Silver, J. S., Laurence, A., Porrett, P. M., Harris, T. H., Turka, L. A., Ernst, M., Saris, C. J., O'Shea, J. J. and Hunter, C. A. (2007) 'Interleukins 27 and 6 induce STAT3-mediated T cell production of interleukin 10', *Nat Immunol*, 8(12), 1363-71.

Su, E. W., Lin, J. Y. and Kane, L. P. (2008) 'TIM-1 and TIM-3 proteins in immune regulation', *Cytokine*, 44(1), 9-13.

Suarez, A., Lopez, P., Gomez, J. and Gutierrez, C. (2006) 'Enrichment of CD4+ CD25high T cell population in patients with systemic lupus erythematosus treated with glucocorticoids', *Ann Rheum Dis*, 65(11), 1512-7.

Suen, J. L., Li, H. T., Jong, Y. J., Chiang, B. L. and Yen, J. H. (2009) 'Altered homeostasis of CD4(+) FoxP3(+) regulatory T-cell subpopulations in systemic lupus erythematosus', *Immunology*, 127(2), 196-205.

Sugimoto, N., Oida, T., Hirota, K., Nakamura, K., Nomura, T., Uchiyama, T. and Sakaguchi, S. (2006) 'Foxp3-dependent and -independent molecules specific for CD25+CD4+ natural regulatory T cells revealed by DNA microarray analysis', *Int Immunol,* 18(8), 1197-209.

Summerskill, W. H., Korman, M. G., Ammon, H. V. and Baggenstoss, A. H. (1975) 'Prednisone for chronic active liver disease: dose titration, standard dose, and combination with azathioprine compared', *Gut,* 16(11), 876-83.

Sun, J. B., Raghavan, S., Sjoling, A., Lundin, S. and Holmgren, J. (2006) 'Oral tolerance induction with antigen conjugated to cholera toxin B subunit generates both Foxp3+CD25+ and Foxp3-CD25- CD4+ regulatory T cells', *J Immunol,* 177(11), 7634-44.

Suzuki, Y., Orellana, M. A., Schreiber, R. D. and Remington, J. S. (1988) 'Interferon-gamma: the major mediator of resistance against Toxoplasma gondii', *Science,* 240(4851), 516-8.

Swain, S. L., Weinberg, A. D., English, M. and Huston, G. (1990) 'IL-4 directs the development of Th2-like helper effectors', *J Immunol,* 145(11), 3796-806.

Szabo, S. J., Kim, S. T., Costa, G. L., Zhang, X., Fathman, C. G. and Glimcher, L. H. (2000) 'A novel transcription factor, T-bet, directs Th1 lineage commitment', *Cell,* 100(6), 655-69.

Szabo, S. J., Sullivan, B. M., Stemmann, C., Satoskar, A. R., Sleckman, B. P. and Glimcher, L. H. (2002) 'Distinct effects of T-bet in TH1 lineage commitment and IFN-gamma production in CD4 and CD8 T cells', *Science,* 295(5553), 338-42.

Szostecki, C., Krippner, H., Penner, E. and Bautz, F. A. (1987) 'Autoimmune sera recognize a 100 kD nuclear protein antigen (sp-100)', *Clin Exp Immunol,* 68(1), 108-16.

Tai, X., Cowan, M., Feigenbaum, L. and Singer, A. (2005) 'CD28 costimulation of developing thymocytes induces Foxp3 expression and regulatory T cell differentiation independently of interleukin 2', *Nat Immunol,* 6(2), 152-62.

Takahama, Y. (2006) 'Journey through the thymus: stromal guides for T-cell development and selection', *Nat Rev Immunol,* 6(2), 127-35.

Takahashi, M., Nakamura, K., Honda, K., Kitamura, Y., Mizutani, T., Araki, Y., Kabemura, T., Chijiiwa, Y., Harada, N. and Nawata, H. (2006) 'An inverse correlation of human peripheral blood regulatory T cell frequency with the disease activity of ulcerative colitis', *Dig Dis Sci,* 51(4), 677-86.

Takahashi, T., Kuniyasu, Y., Toda, M., Sakaguchi, N., Itoh, M., Iwata, M., Shimizu, J. and Sakaguchi, S. (1998) 'Immunologic self-tolerance maintained by CD25+CD4+ naturally anergic and suppressive T cells: induction of autoimmune disease by breaking their anergic/suppressive state', *Int Immunol,* 10(12), 1969-80.

Takahashi, T., Tagami, T., Yamazaki, S., Uede, T., Shimizu, J., Sakaguchi, N., Mak, T. W. and Sakaguchi, S. (2000) 'Immunologic self-tolerance maintained by CD25(+)CD4(+) regulatory T cells constitutively expressing cytotoxic T lymphocyte-associated antigen 4', *J Exp Med,* 192(2), 303-10.

Tami, C., Silberstein, E., Manangeeswaran, M., Freeman, G. J., Umetsu, S. E., DeKruyff, R. H., Umetsu, D. T. and Kaplan, G. G. (2007) 'Immunoglobulin A (IgA) is a natural ligand of hepatitis A virus cellular receptor 1 (HAVCR1), and the association of IgA with HAVCR1 enhances virus-receptor interactions', *J Virol*, 81(7), 3437-46.

Tan, E. M. (1989) 'Antinuclear antibodies: diagnostic markers for autoimmune diseases and probes for cell biology', *Adv Immunol*, 44, 93-151.

Tan, E. M., Feltkamp, T. E., Smolen, J. S., Butcher, B., Dawkins, R., Fritzler, M. J., Gordon, T., Hardin, J. A., Kalden, J. R., Lahita, R. G., Maini, R. N., McDougal, J. S., Rothfield, N. F., Smeenk, R. J., Takasaki, Y., Wiik, A., Wilson, M. R. and Koziol, J. A. (1997) 'Range of antinuclear antibodies in "healthy" individuals', *Arthritis Rheum*, 40(9), 1601-11.

Tang, Q., Boden, E. K., Henriksen, K. J., Bour-Jordan, H., Bi, M. and Bluestone, J. A. (2004a) 'Distinct roles of CTLA-4 and TGF-beta in CD4+CD25+ regulatory T cell function', *Eur J Immunol*, 34(11), 2996-3005.

Tang, Q., Henriksen, K. J., Bi, M., Finger, E. B., Szot, G., Ye, J., Masteller, E. L., McDevitt, H., Bonyhadi, M. and Bluestone, J. A. (2004b) 'In vitro-expanded antigen-specific regulatory T cells suppress autoimmune diabetes', *J Exp Med*, 199(11), 1455-65.

Tay, S. P., Cheong, S. K., Hamidah, N. H. and Ainoon, O. (1999) 'Comparative analysis of three permeabilization methods for cytofluorometric evaluation of cytoplasmic myeloperoxidase', *Malays J Pathol*, 21(1), 37-43.

Thierfelder, W. E., van Deursen, J. M., Yamamoto, K., Tripp, R. A., Sarawar, S. R., Carson, R. T., Sangster, M. Y., Vignali, D. A., Doherty, P. C., Grosveld, G. C. and Ihle, J. N. (1996) 'Requirement for Stat4 in interleukin-12-mediated responses of natural killer and T cells', *Nature*, 382(6587), 171-4.

Thornton, A. M., Korty, P. E., Tran, D. Q., Wohlfert, E. A., Murray, P. E., Belkaid, Y. and Shevach, E. M. (2010) 'Expression of Helios, an Ikaros transcription factor family member, differentiates thymic-derived from peripherally induced Foxp3+ T regulatory cells', *J Immunol*, 184(7), 3433-41.

Thornton, A. M. and Shevach, E. M. (1998) 'CD4+CD25+ immunoregulatory T cells suppress polyclonal T cell activation in vitro by inhibiting interleukin 2 production', *J Exp Med*, 188(2), 287-96.

Tiscornia, G., Singer, O. and Verma, I. M. (2006) 'Design and cloning of lentiviral vectors expressing small interfering RNAs', *Nat Protoc*, 1(1), 234-40.

Tivol, E. A., Borriello, F., Schweitzer, A. N., Lynch, W. P., Bluestone, J. A. and Sharpe, A. H. (1995) 'Loss of CTLA-4 leads to massive lymphoproliferation and fatal multiorgan tissue destruction, revealing a critical negative regulatory role of CTLA-4', *Immunity*, 3(5), 541-7.

Toda, G., Zeniya, M., Watanabe, F., Imawari, M., Kiyosawa, K., Nishioka, M., Tsuji, T. and Omata, M. (1997) 'Present status of autoimmune hepatitis in Japan--correlating the characteristics with international criteria in an area with a high rate of HCV infection. Japanese National Study Group of Autoimmune Hepatitis', *J Hepatol*, 26(6), 1207-12.

Toh, B. H. (1979) 'Smooth muscle autoantibodies and autoantigens', *Clin Exp Immunol,* 38(3), 621-8.

Tran, D. Q., Andersson, J., Hardwick, D., Bebris, L., Illei, G. G. and Shevach, E. M. (2009) 'Selective expression of latency-associated peptide (LAP) and IL-1 receptor type I/II (CD121a/CD121b) on activated human FOXP3+ regulatory T cells allows for their purification from expansion cultures', *Blood,* 113(21), 5125-33.

Tree, T. I., Roep, B. O. and Peakman, M. (2004) 'Enhancing the sensitivity of assays to detect T cell reactivity: the effect of cell separation and cryopreservation media', *Ann N Y Acad Sci,* 1037, 26-32.

Tree, T. I., Roep, B. O. and Peakman, M. (2006) 'A mini meta-analysis of studies on CD4+CD25+ T cells in human type 1 diabetes: report of the Immunology of Diabetes Society T Cell Workshop', *Ann N Y Acad Sci,* 1079, 9-18.

Treichel, U., Gerken, G., Rossol, S., Rotthauwe, H. W., Meyer zum Buschenfelde, K. H. and Poralla, T. (1993) 'Autoantibodies against the human asialoglycoprotein receptor: effects of therapy in autoimmune and virus-induced chronic active hepatitis', *J Hepatol,* 19(1), 55-63.

Treichel, U., McFarlane, B. M., Seki, T., Krawitt, E. L., Alessi, N., Stickel, F., McFarlane, I. G., Kiyosawa, K., Furuta, S., Freni, M. A. and et al. (1994) 'Demographics of anti-asialoglycoprotein receptor autoantibodies in autoimmune hepatitis', *Gastroenterology,* 107(3), 799-804.

Trifari, S., Kaplan, C. D., Tran, E. H., Crellin, N. K. and Spits, H. (2009) 'Identification of a human helper T cell population that has abundant production of interleukin 22 and is distinct from T(H)-17, T(H)1 and T(H)2 cells', *Nat Immunol,* 10(8), 864-71.

Tsokos, G. C. (2011) 'Systemic lupus erythematosus', *N Engl J Med,* 365(22), 2110-21.

Tureci, O., Schmitt, H., Fadle, N., Pfreundschuh, M. and Sahin, U. (1997) 'Molecular definition of a novel human galectin which is immunogenic in patients with Hodgkin's disease', *J Biol Chem,* 272(10), 6416-22.

Tuschl, T., Zamore, P. D., Lehmann, R., Bartel, D. P. and Sharp, P. A. (1999) 'Targeted mRNA degradation by double-stranded RNA in vitro', *Genes Dev,* 13(24), 3191-7.

Uhlig, H. H., Coombes, J., Mottet, C., Izcue, A., Thompson, C., Fanger, A., Tannapfel, A., Fontenot, J. D., Ramsdell, F. and Powrie, F. (2006) 'Characterization of Foxp3+CD4+CD25+ and IL-10-secreting CD4+CD25+ T cells during cure of colitis', *J Immunol,* 177(9), 5852-60.

Umetsu, S. E., Lee, W. L., McIntire, J. J., Downey, L., Sanjanwala, B., Akbari, O., Berry, G. J., Nagumo, H., Freeman, G. J., Umetsu, D. T. and DeKruyff, R. H. (2005) 'TIM-1 induces T cell activation and inhibits the development of peripheral tolerance', *Nat Immunol,* 6(5), 447-54.

Urban, J. F., Jr., Noben-Trauth, N., Donaldson, D. D., Madden, K. B., Morris, S. C., Collins, M. and Finkelman, F. D. (1998) 'IL-13, IL-4Ralpha, and Stat6 are required for the expulsion of the gastrointestinal nematode parasite Nippostrongylus brasiliensis', *Immunity,* 8(2), 255-64.

Urbani, S., Amadei, B., Tola, D., Massari, M., Schivazappa, S., Missale, G. and Ferrari, C. (2006) 'PD-1 expression in acute hepatitis C virus (HCV) infection is associated with HCV-specific CD8 exhaustion', *J Virol,* 80(22), 11398-403.

Usui, T., Nishikomori, R., Kitani, A. and Strober, W. (2003) 'GATA-3 suppresses Th1 development by downregulation of Stat4 and not through effects on IL-12Rbeta2 chain or T-bet', *Immunity,* 18(3), 415-28.

Usui, T., Preiss, J. C., Kanno, Y., Yao, Z. J., Bream, J. H., O'Shea, J. J. and Strober, W. (2006) 'T-bet regulates Th1 responses through essential effects on GATA-3 function rather than on IFNG gene acetylation and transcription', *J Exp Med,* 203(3), 755-66.

Valencia, X., Yarboro, C., Illei, G. and Lipsky, P. E. (2007) 'Deficient CD4+CD25high T regulatory cell function in patients with active systemic lupus erythematosus', *J Immunol,* 178(4), 2579-88.

van Amelsfort, J. M., Jacobs, K. M., Bijlsma, J. W., Lafeber, F. P. and Taams, L. S. (2004) 'CD4(+)CD25(+) regulatory T cells in rheumatoid arthritis: differences in the presence, phenotype, and function between peripheral blood and synovial fluid', *Arthritis Rheum,* 50(9), 2775-85.

van der Leij, J., van den Berg, A., Blokzijl, T., Harms, G., van Goor, H., Zwiers, P., van Weeghel, R., Poppema, S. and Visser, L. (2004) 'Dimeric galectin-1 induces IL-10 production in T-lymphocytes: an important tool in the regulation of the immune response', *J Pathol,* 204(5), 511-8.

Van Thiel, D. H., Wright, H., Carroll, P., Abu-Elmagd, K., Rodriguez-Rilo, H., McMichael, J., Irish, W. and Starzl, T. E. (1995) 'Tacrolimus: a potential new treatment for autoimmune chronic active hepatitis: results of an open-label preliminary trial', *Am J Gastroenterol,* 90(5), 771-6.

Vargas-Rojas, M. I., Crispin, J. C., Richaud-Patin, Y. and Alcocer-Varela, J. (2008) 'Quantitative and qualitative normal regulatory T cells are not capable of inducing suppression in SLE patients due to T-cell resistance', *Lupus,* 17(4), 289-94.

Veldhoen, M., Hocking, R. J., Atkins, C. J., Locksley, R. M. and Stockinger, B. (2006) 'TGFbeta in the context of an inflammatory cytokine milieu supports de novo differentiation of IL-17-producing T cells', *Immunity,* 24(2), 179-89.

Veldhoen, M., Uyttenhove, C., van Snick, J., Helmby, H., Westendorf, A., Buer, J., Martin, B., Wilhelm, C. and Stockinger, B. (2008) 'Transforming growth factor-beta 'reprograms' the differentiation of T helper 2 cells and promotes an interleukin 9-producing subset', *Nat Immunol,* 9(12), 1341-6.

Venick, R. S., McDiarmid, S. V., Farmer, D. G., Gornbein, J., Martin, M. G., Vargas, J. H., Ament, M. E. and Busuttil, R. W. (2007) 'Rejection and steroid dependence: unique risk factors in the development of pediatric posttransplant de novo autoimmune hepatitis', *Am J Transplant,* 7(4), 955-63.

Venigalla, R. K., Tretter, T., Krienke, S., Max, R., Eckstein, V., Blank, N., Fiehn, C., Ho, A. D. and Lorenz, H. M. (2008) 'Reduced CD4+,CD25- T cell sensitivity to the suppressive

function of CD4+,CD25high,CD127 -/low regulatory T cells in patients with active systemic lupus erythematosus', *Arthritis Rheum,* 58(7), 2120-30.

Venkataramani, A., Jones, M. B. and Sorrell, M. F. (2001) 'Methotrexate therapy for refractory chronic active autoimmune hepatitis', *Am J Gastroenterol,* 96(12), 3432-4.

Venken, K., Hellings, N., Broekmans, T., Hensen, K., Rummens, J. L. and Stinissen, P. (2008a) 'Natural naive CD4+CD25+CD127low regulatory T cell (Treg) development and function are disturbed in multiple sclerosis patients: recovery of memory Treg homeostasis during disease progression', *J Immunol,* 180(9), 6411-20.

Venken, K., Hellings, N., Thewissen, M., Somers, V., Hensen, K., Rummens, J. L., Medaer, R., Hupperts, R. and Stinissen, P. (2008b) 'Compromised CD4+ CD25(high) regulatory T-cell function in patients with relapsing-remitting multiple sclerosis is correlated with a reduced frequency of FOXP3-positive cells and reduced FOXP3 expression at the single-cell level', *Immunology,* 123(1), 79-89.

Vento, S., Hegarty, J. E., Bottazzo, G., Macchia, E., Williams, R. and Eddleston, A. L. (1984) 'Antigen specific suppressor cell function in autoimmune chronic active hepatitis', *Lancet,* 1(8388), 1200-4.

Vergani, D., Alvarez, F., Bianchi, F. B., Cancado, E. L., Mackay, I. R., Manns, M. P., Nishioka, M. and Penner, E. (2004) 'Liver autoimmune serology: a consensus statement from the committee for autoimmune serology of the International Autoimmune Hepatitis Group', *J Hepatol,* 41(4), 677-83.

Vergani, D., Choudhuri, K., Bogdanos, D. P. and Mieli-Vergani, G. (2002) 'Pathogenesis of autoimmune hepatitis', *Clin Liver Dis,* 6(3), 727-37.

Vergani, D., Longhi, M. S., Bogdanos, D. P., Ma, Y. and Mieli-Vergani, G. (2009) 'Autoimmune hepatitis', *Semin Immunopathol,* 31(3), 421-35.

Vergani, D. and Mieli-Vergani, G. (2008) 'Aetiopathogenesis of autoimmune hepatitis', *World J Gastroenterol,* 14(21), 3306-12.

Vergani, D. and Mieli-Vergani, G. (2011a) 'Cutting Edge Issues in Autoimmune Hepatitis', *Clin Rev Allergy Immunol*.

Vergani, D. and Mieli-Vergani, G. (2011b) 'Pharmacological management of autoimmune hepatitis', *Expert Opin Pharmacother,* 12(4), 607-13.

Vergani, D., Mieli-Vergani, G., Mondelli, M., Portmann, B. and Eddleston, A. L. (1987) 'Immunoglobulin on the surface of isolated hepatocytes is associated with antibody-dependent cell-mediated cytotoxicity and liver damage', *Liver,* 7(6), 307-15.

Vergani, D., Wells, L., Larcher, V. F., Nasaruddin, B. A., Davies, E. T., Mieli-Vergani, G. and Mowat, A. P. (1985) 'Genetically determined low C4: a predisposing factor to autoimmune chronic active hepatitis', *Lancet,* 2(8450), 294-8.

Vergani, D. M.-V., G. (2007) 'Autoimmune hepatitis' in *Textbook of hepatology: from basic science to clinical practicde*, 3rd ed., London: Blackwell publishing, 1089-1101.

Vergani, G. M., Vergani, D., Jenkins, P. J., Portmann, B., Mowat, A. P., Eddleston, A. L. and Williams, R. (1979) 'Lymphocyte cytotoxicity to autologous hepatocytes in HBsAg-negative chronic active hepatitis', *Clin Exp Immunol,* 38(1), 16-21.

Vierling, J. M. (2004) 'Primary biliary cirrhosis and autoimmune cholangiopathy', *Clin Liver Dis,* 8(1), 177-94.

Viglietta, V., Baecher-Allan, C., Weiner, H. L. and Hafler, D. A. (2004) 'Loss of functional suppression by CD4+CD25+ regulatory T cells in patients with multiple sclerosis', *J Exp Med,* 199(7), 971-9.

Vignali, D. A., Collison, L. W. and Workman, C. J. (2008) 'How regulatory T cells work', *Nat Rev Immunol,* 8(7), 523-32.

Vila, M. R., Kaplan, G. G., Feigelstock, D., Nadal, M., Morote, J., Porta, R., Bellmunt, J. and Meseguer, A. (2004) 'Hepatitis A virus receptor blocks cell differentiation and is overexpressed in clear cell renal cell carcinoma', *Kidney Int,* 65(5), 1761-73.

Virgo, P. F. and Gibbs, G. J. (2012) 'Flow cytometry in clinical pathology', *Ann Clin Biochem,* 49(Pt 1), 17-28.

Vogel, A., Strassburg, C. P. and Manns, M. P. (2002) 'Genetic association of vitamin D receptor polymorphisms with primary biliary cirrhosis and autoimmune hepatitis', *Hepatology,* 35(1), 126-31.

von Boehmer, H. and Kisielow, P. (1990) 'Self-nonself discrimination by T cells', *Science,* 248(4961), 1369-73.

von Boehmer, H. and Melchers, F. (2010) 'Checkpoints in lymphocyte development and autoimmune disease', *Nat Immunol,* 11(1), 14-20.

von Freeden-Jeffry, U., Vieira, P., Lucian, L. A., McNeil, T., Burdach, S. E. and Murray, R. (1995) 'Lymphopenia in interleukin (IL)-7 gene-deleted mice identifies IL-7 as a nonredundant cytokine', *J Exp Med,* 181(4), 1519-26.

Vorechovsky, I., Webster, A. D., Plebani, A. and Hammarstrom, L. (1999) 'Genetic linkage of IgA deficiency to the major histocompatibility complex: evidence for allele segregation distortion, parent-of-origin penetrance differences, and the role of anti-IgA antibodies in disease predisposition', *Am J Hum Genet,* 64(4), 1096-109.

Wada, J. and Kanwar, Y. S. (1997) 'Identification and characterization of galectin-9, a novel beta-galactoside-binding mammalian lectin', *J Biol Chem,* 272(9), 6078-86.

Wada, J., Ota, K., Kumar, A., Wallner, E. I. and Kanwar, Y. S. (1997) 'Developmental regulation, expression, and apoptotic potential of galectin-9, a beta-galactoside binding lectin', *J Clin Invest,* 99(10), 2452-61.

Waldenström, J. (1950) 'Leber, Blutprotein und Nahrungseiweiss', *Deutsch Gesellshaff Z Verdan Stoffwechselkr,* 15, 113-119.

Walker, L. S. (2009) 'Regulatory T cells overturned: the effectors fight back', *Immunology,* 126(4), 466-74.

Walker, L. S. and Abbas, A. K. (2002) 'The enemy within: keeping self-reactive T cells at bay in the periphery', *Nat Rev Immunol*, 2(1), 11-9.

Wang, J. L., Gray, R. M., Haudek, K. C. and Patterson, R. J. (2004) 'Nucleocytoplasmic lectins', *Biochim Biophys Acta*, 1673(1-2), 75-93.

Wang, K. K., Czaja, A. J., Beaver, S. J. and Go, V. L. (1989) 'Extrahepatic malignancy following long-term immunosuppressive therapy of severe hepatitis B surface antigen-negative chronic active hepatitis', *Hepatology*, 10(1), 39-43.

Warner, N. L., Szenberg, A. and Burnet, F. M. (1962) 'The immunological role of different lymphoid organs in the chicken. I. Dissociation of immunological responsiveness', *Aust J Exp Biol Med Sci*, 40, 373-87.

Waterhouse, P., Penninger, J. M., Timms, E., Wakeham, A., Shahinian, A., Lee, K. P., Thompson, C. B., Griesser, H. and Mak, T. W. (1995) 'Lymphoproliferative disorders with early lethality in mice deficient in Ctla-4', *Science*, 270(5238), 985-8.

Watson, J. V. (1999) 'The early fluidic and optical physics of cytometry', *Cytometry*, 38(1), 2-14; discussion 1.

Weaver, C. T., Harrington, L. E., Mangan, P. R., Gavrieli, M. and Murphy, K. M. (2006) 'Th17: an effector CD4 T cell lineage with regulatory T cell ties', *Immunity*, 24(6), 677-88.

Weiner, H. L. (2001) 'Induction and mechanism of action of transforming growth factor-beta-secreting Th3 regulatory cells', *Immunol Rev*, 182, 207-14.

Wen, L., Ma, Y., Bogdanos, D. P., Wong, F. S., Demaine, A., Mieli-Vergani, G. and Vergani, D. (2001) 'Pediatric autoimmune liver diseases: the molecular basis of humoral and cellular immunity', *Curr Mol Med*, 1(3), 379-89.

Wen, L., Peakman, M., Lobo-Yeo, A., McFarlane, B. M., Mowat, A. P., Mieli-Vergani, G. and Vergani, D. (1990) 'T-cell-directed hepatocyte damage in autoimmune chronic active hepatitis', *Lancet*, 336(8730), 1527-30.

Werlen, G., Hausmann, B., Naeher, D. and Palmer, E. (2003) 'Signaling life and death in the thymus: timing is everything', *Science*, 299(5614), 1859-63.

Werlen, G. and Palmer, E. (2002) 'The T-cell receptor signalosome: a dynamic structure with expanding complexity', *Curr Opin Immunol*, 14(3), 299-305.

Werner, M., Almer, S., Prytz, H., Lindgren, S., Wallerstedt, S., Bjornsson, E., Bergquist, A., Sandberg-Gertzen, H., Hultcrantz, R., Sangfelt, P., Weiland, O. and Danielsson, A. (2009) 'Hepatic and extrahepatic malignancies in autoimmune hepatitis. A long-term follow-up in 473 Swedish patients', *J Hepatol*, 50(2), 388-93.

Wesierska-Gadek, J., Hohenuer, H., Hitchman, E. and Penner, E. (1996) 'Autoantibodies against nucleoporin p62 constitute a novel marker of primary biliary cirrhosis', *Gastroenterology*, 110(3), 840-7.

Whalley, S., Puvanachandra, P., Desai, A. and Kennedy, H. (2007) 'Hepatology outpatient service provision in secondary care: a study of liver disease incidence and resource costs', *Clin Med*, 7(2), 119-24.

Wherry, E. J., Ha, S. J., Kaech, S. M., Haining, W. N., Sarkar, S., Kalia, V., Subramaniam, S., Blattman, J. N., Barber, D. L. and Ahmed, R. (2007) 'Molecular signature of CD8+ T cell exhaustion during chronic viral infection', *Immunity,* 27(4), 670-84.

Wies, I., Brunner, S., Henninger, J., Herkel, J., Kanzler, S., Meyer zum Buschenfelde, K. H. and Lohse, A. W. (2000) 'Identification of target antigen for SLA/LP autoantibodies in autoimmune hepatitis', *Lancet,* 355(9214), 1510-5.

Wildin, R. S., Ramsdell, F., Peake, J., Faravelli, F., Casanova, J. L., Buist, N., Levy-Lahad, E., Mazzella, M., Goulet, O., Perroni, L., Bricarelli, F. D., Byrne, G., McEuen, M., Proll, S., Appleby, M. and Brunkow, M. E. (2001) 'X-linked neonatal diabetes mellitus, enteropathy and endocrinopathy syndrome is the human equivalent of mouse scurfy', *Nat Genet,* 27(1), 18-20.

Wildin, R. S., Smyk-Pearson, S. and Filipovich, A. H. (2002) 'Clinical and molecular features of the immunodysregulation, polyendocrinopathy, enteropathy, X linked (IPEX) syndrome', *J Med Genet,* 39(8), 537-45.

Williams, C. B., Vidal, K., Donermeyer, D., Peterson, D. A., White, J. M. and Allen, P. M. (1998) 'In vivo expression of a TCR antagonist: T cells escape central tolerance but are antagonized in the periphery', *J Immunol,* 161(1), 128-37.

Wing, K., Onishi, Y., Prieto-Martin, P., Yamaguchi, T., Miyara, M., Fehervari, Z., Nomura, T. and Sakaguchi, S. (2008) 'CTLA-4 control over Foxp3+ regulatory T cell function', *Science,* 322(5899), 271-5.

Wolk, K. and Sabat, R. (2006) 'Interleukin-22: a novel T- and NK-cell derived cytokine that regulates the biology of tissue cells', *Cytokine Growth Factor Rev,* 17(5), 367-80.

Wynn, T. A. (2003) 'IL-13 effector functions', *Annu Rev Immunol,* 21, 425-56.

Xiao, S., Najafian, N., Reddy, J., Albin, M., Zhu, C., Jensen, E., Imitola, J., Korn, T., Anderson, A. C., Zhang, Z., Gutierrez, C., Moll, T., Sobel, R. A., Umetsu, D. T., Yagita, H., Akiba, H., Strom, T., Sayegh, M. H., DeKruyff, R. H., Khoury, S. J. and Kuchroo, V. K. (2007) 'Differential engagement of Tim-1 during activation can positively or negatively costimulate T cell expansion and effector function', *J Exp Med,* 204(7), 1691-702.

Yang, J., Zhu, H., Murphy, T. L., Ouyang, W. and Murphy, K. M. (2001) 'IL-18-stimulated GADD45 beta required in cytokine-induced, but not TCR-induced, IFN-gamma production', *Nat Immunol,* 2(2), 157-64.

Yang, L., Anderson, D. E., Kuchroo, J. and Hafler, D. A. (2008) 'Lack of TIM-3 Immunoregulation in Multiple Sclerosis', *The Journal of Immunology,* 180(7), 4409-4414.

Yang, P. C., Xing, Z., Berin, C. M., Soderholm, J. D., Feng, B. S., Wu, L. and Yeh, C. (2007) 'TIM-4 expressed by mucosal dendritic cells plays a critical role in food antigen-specific Th2 differentiation and intestinal allergy', *Gastroenterology,* 133(5), 1522-33.

Yang, R. Y., Hsu, D. K. and Liu, F. T. (1996) 'Expression of galectin-3 modulates T-cell growth and apoptosis', *Proc Natl Acad Sci U S A,* 93(13), 6737-42.

Yang, R. Y., Rabinovich, G. A. and Liu, F. T. (2008) 'Galectins: structure, function and therapeutic potential', *Expert Rev Mol Med*, 10, e17.

Yang, X. O., Panopoulos, A. D., Nurieva, R., Chang, S. H., Wang, D., Watowich, S. S. and Dong, C. (2007) 'STAT3 regulates cytokine-mediated generation of inflammatory helper T cells', *J Biol Chem*, 282(13), 9358-63.

Yang, X. O., Pappu, B. P., Nurieva, R., Akimzhanov, A., Kang, H. S., Chung, Y., Ma, L., Shah, B., Panopoulos, A. D., Schluns, K. S., Watowich, S. S., Tian, Q., Jetten, A. M. and Dong, C. (2008) 'T helper 17 lineage differentiation is programmed by orphan nuclear receptors ROR alpha and ROR gamma', *Immunity*, 28(1), 29-39.

Yang, Y., Ochando, J. C., Bromberg, J. S. and Ding, Y. (2007) 'Identification of a distant T-bet enhancer responsive to IL-12/Stat4 and IFNgamma/Stat1 signals', *Blood*, 110(7), 2494-500.

Yeoman, A. D., Al-Chalabi, T., Karani, J. B., Quaglia, A., Devlin, J., Mieli-Vergani, G., Bomford, A., O'Grady, J. G., Harrison, P. M. and Heneghan, M. A. (2008) 'Evaluation of risk factors in the development of hepatocellular carcinoma in autoimmune hepatitis: Implications for follow-up and screening', *Hepatology*, 48(3), 863-70.

Yeoman, A. D., Longhi, M. S. and Heneghan, M. A. (2010) 'Review article: the modern management of autoimmune hepatitis', *Aliment Pharmacol Ther*, 31(8), 771-87.

Yoshizawa, K., Ota, M., Katsuyama, Y., Ichijo, T., Matsumoto, A., Tanaka, E. and Kiyosawa, K. (2005) 'Genetic analysis of the HLA region of Japanese patients with type 1 autoimmune hepatitis', *J Hepatol*, 42(4), 578-84.

You, S., Alyanakian, M. A., Segovia, B., Damotte, D., Bluestone, J., Bach, J. F. and Chatenoud, L. (2008) 'Immunoregulatory pathways controlling progression of autoimmunity in NOD mice', *Ann N Y Acad Sci*, 1150, 300-10.

You, S., Belghith, M., Cobbold, S., Alyanakian, M. A., Gouarin, C., Barriot, S., Garcia, C., Waldmann, H., Bach, J. F. and Chatenoud, L. (2005) 'Autoimmune diabetes onset results from qualitative rather than quantitative age-dependent changes in pathogenic T-cells', *Diabetes*, 54(5), 1415-22.

You, S., Leforban, B., Garcia, C., Bach, J. F., Bluestone, J. A. and Chatenoud, L. (2007) 'Adaptive TGF-beta-dependent regulatory T cells control autoimmune diabetes and are a privileged target of anti-CD3 antibody treatment', *Proc Natl Acad Sci U S A*, 104(15), 6335-40.

Zenewicz, L. A., Yancopoulos, G. D., Valenzuela, D. M., Murphy, A. J., Karow, M. and Flavell, R. A. (2007) 'Interleukin-22 but not interleukin-17 provides protection to hepatocytes during acute liver inflammation', *Immunity*, 27(4), 647-59.

Zhao, L., Tang, Y., You, Z., Wang, Q., Liang, S., Han, X., Qiu, D., Wei, J., Liu, Y., Shen, L., Chen, X., Peng, Y., Li, Z. and Ma, X. (2011) 'Interleukin-17 contributes to the pathogenesis of autoimmune hepatitis through inducing hepatic interleukin-6 expression', *PLoS One*, 6(4), e18909.

Zheng, S. G., Wang, J., Wang, P., Gray, J. D. and Horwitz, D. A. (2007) 'IL-2 is essential for TGF-beta to convert naive CD4+CD25- cells to CD25+Foxp3+ regulatory T cells and for expansion of these cells', *J Immunol,* 178(4), 2018-27.

Zheng, S. G., Wang, J. H., Gray, J. D., Soucier, H. and Horwitz, D. A. (2004) 'Natural and induced CD4+CD25+ cells educate CD4+CD25- cells to develop suppressive activity: the role of IL-2, TGF-beta, and IL-10', *J Immunol,* 172(9), 5213-21.

Zheng, W. and Flavell, R. A. (1997) 'The transcription factor GATA-3 is necessary and sufficient for Th2 cytokine gene expression in CD4 T cells', *Cell,* 89(4), 587-96.

Zheng, Y., Chaudhry, A., Kas, A., deRoos, P., Kim, J. M., Chu, T. T., Corcoran, L., Treuting, P., Klein, U. and Rudensky, A. Y. (2009) 'Regulatory T-cell suppressor program co-opts transcription factor IRF4 to control T(H)2 responses', *Nature,* 458(7236), 351-6.

Zheng, Y., Danilenko, D. M., Valdez, P., Kasman, I., Eastham-Anderson, J., Wu, J. and Ouyang, W. (2007a) 'Interleukin-22, a T(H)17 cytokine, mediates IL-23-induced dermal inflammation and acanthosis', *Nature,* 445(7128), 648-51.

Zheng, Y., Josefowicz, S. Z., Kas, A., Chu, T. T., Gavin, M. A. and Rudensky, A. Y. (2007b) 'Genome-wide analysis of Foxp3 target genes in developing and mature regulatory T cells', *Nature,* 445(7130), 936-40.

Zhou, L., Ivanov, II, Spolski, R., Min, R., Shenderov, K., Egawa, T., Levy, D. E., Leonard, W. J. and Littman, D. R. (2007) 'IL-6 programs T(H)-17 cell differentiation by promoting sequential engagement of the IL-21 and IL-23 pathways', *Nat Immunol,* 8(9), 967-74.

Zhou, X., Bailey-Bucktrout, S. L., Jeker, L. T., Penaranda, C., Martinez-Llordella, M., Ashby, M., Nakayama, M., Rosenthal, W. and Bluestone, J. A. (2009) 'Instability of the transcription factor Foxp3 leads to the generation of pathogenic memory T cells in vivo', *Nat Immunol,* 10(9), 1000-7.

Zhu, C., Anderson, A. C., Schubart, A., Xiong, H., Imitola, J., Khoury, S. J., Zheng, X. X., Strom, T. B. and Kuchroo, V. K. (2005) 'The Tim-3 ligand galectin-9 negatively regulates T helper type 1 immunity', *Nat Immunol,* 6(12), 1245-52.

Zhu, J., Cote-Sierra, J., Guo, L. and Paul, W. E. (2003) 'Stat5 activation plays a critical role in Th2 differentiation', *Immunity,* 19(5), 739-48.

Zhu, J., Guo, L., Watson, C. J., Hu-Li, J. and Paul, W. E. (2001) 'Stat6 is necessary and sufficient for IL-4's role in Th2 differentiation and cell expansion', *J Immunol,* 166(12), 7276-81.

Zhu, J., Min, B., Hu-Li, J., Watson, C. J., Grinberg, A., Wang, Q., Killeen, N., Urban, J. F., Jr., Guo, L. and Paul, W. E. (2004) 'Conditional deletion of Gata3 shows its essential function in T(H)1-T(H)2 responses', *Nat Immunol,* 5(11), 1157-65.

Zhu, J. and Paul, W. E. (2008) 'CD4 T cells: fates, functions, and faults', *Blood,* 112(5), 1557-69.

Zhu, J. and Paul, W. E. (2010) 'Peripheral CD4+ T-cell differentiation regulated by networks of cytokines and transcription factors', *Immunol Rev,* 238(1), 247-62.

Zhu, J., Yamane, H., Cote-Sierra, J., Guo, L. and Paul, W. E. (2006) 'GATA-3 promotes Th2 responses through three different mechanisms: induction of Th2 cytokine production, selective growth of Th2 cells and inhibition of Th1 cell-specific factors', *Cell Res*, 16(1), 3-10.

Zimmermann, H. (1992) '5'-Nucleotidase: molecular structure and functional aspects', *Biochem J*, 285 (Pt 2), 345-65.

Zuberi, R. I., Hsu, D. K., Kalayci, O., Chen, H. Y., Sheldon, H. K., Yu, L., Apgar, J. R., Kawakami, T., Lilly, C. M. and Liu, F. T. (2004) 'Critical role for galectin-3 in airway inflammation and bronchial hyperresponsiveness in a murine model of asthma', *Am J Pathol*, 165(6), 2045-53.

Zuklys, S., Balciunaite, G., Agarwal, A., Fasler-Kan, E., Palmer, E. and Hollander, G. A. (2000) 'Normal thymic architecture and negative selection are associated with Aire expression, the gene defective in the autoimmune-polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED)', *J Immunol*, 165(4), 1976-83.

Zuniga, E., Rabinovich, G. A., Iglesias, M. M. and Gruppi, A. (2001) 'Regulated expression of galectin-1 during B-cell activation and implications for T-cell apoptosis', *J Leukoc Biol*, 70(1), 73-9.

APPENDIX I

Table A. Clinical and laboratory features of all AILD patients included in this thesis

Parameter	AILD (n=77)	[A] patients (n=23)	[R] patients (n=54)	*p**
Age in years	14.0 (5.6-26.7)	13.6 (5.6-19.5)	14.4 (8.2-26.7)	NS†
Female, n of patients (%)	41 (53%)	15 (65%)	26 (48%)	NS†
AST (nv<50 IU/L)	36 (16-1061)	91(51-1061)	30 (16-49)	<0.0001‡
GGT (nv<50 IU/L)	38 (4-503)	78 (17-503)	25 (4-196)	<0.0001‡
Total bilirubin (nv<20 μmol/l)	8 (3-705)	10 (3-705)	7 (4-32)	0.003‡
AP (nv<350 IU/L)	136 (39-834)	228 (39-834)	190 (49-111)	0.09‡
AP/AST ratio	4.48 (0.17-16.38)	2.38 (0.17-6.54)	6.21 (1.58-16.38)	<0.001‡
INR (nv<1.2)	1.05 (0.86-20.6)	1.07 (0.90-2.06)	1.04 (0.86-1.31)	0.12‡
Albumin (nv>35 g/L)	45 (32-50)	44 (32-50)	46 (40-50)	0.03‡
IgA (nv 0.8-4.8 g/L)	1.75 (<0.03-4.21)	1.84 (0.67-4.21)	1.65 (<0.03-3.96)	NS‡
IgG (nv 6.5-17 g/L)	13.16 (5.70-43.96)	17.57 (6.83-43.96)	12.42 (5.70-27.14)	0.002‡
IgM (nv 0.5-2 g/L)	1.39 (0.54-3.92)	1.35 (0.85-3.92)	1.40 (0.54-3.73)	NS‡
ANA, n of positive patients (%)	32 (42%)	13 (57%)	19 (33%)	0.13†
SMA, n of positive patients (%)	21 (27%)	10 (43%)	11 (20%)	0.05†
pANNA, n of positive patients (%)	38 (49%)	12 (52%)	26 (48%)	NS†
AISC, n of positive patients (%)	38 (49%)	12 (52%)	26 (48%)	NS†
IBD, n of positive patients (%)	30 (39%)	10 (43%)	20 (37%)	NS†

Data presented as median (range) unless noted otherwise.
AILD, autoimmune liver disease; *AST*, aspartate aminotransferase; *GGT*, gamma-glutamyl transpeptidase; *AP*, alkaline phosphatase; *INR*, international normalised ratio; *IgG*, immunoglobulin G; *ANA*, anti-nuclear antibodies; *SMA*, anti-smooth muscle antibodies; *pANNA*, peripheral anti-nuclear neutrophil antibodies; *AISC*, autoimmune sclerosing cholangitis; *UC*, ulcerative colitis; *nv*, normal value; *NS* non significant
* *p* value when comparing clinical parameters between [A] and [R] patients
† Fisher´s exact test
‡ Mann-Whitney test

Table B. .Clinical and laboratory data comparing all AIH and AISC patients included in this thesis

Parameter	AIH-1 patients (n=39)	AISC patients (n=38)	*p*[*]
Age in years	13.7 (5.6-21.1)	14.8 (8.2-26.7)	NS[†]
Female, n of patients (%)	24 (62%)	17 (45%)	NS[†]
AST (nv<50 IU/L)	34 (16-1061)	41 (17-534)	NS[‡]
GGT (nv<50 IU/L)	33 (8-492)	50 (4-503)	0.04[‡]
Total bilirubin (nv<20 μmol/l)	4 (8-705)	9 (3-47)	NS[‡]
AP (nv<350 IU/L)	178 (49-669)	228 (68-834)	0.07[‡]
AP/AST ratio	4.37 (0.17-15.64)	5.12 (1.26-16.38)	NS[‡]
INR (nv<1.2)	1.07 (0.89-2.06)	1.05 (0.86-1.78)	NS[‡]
Albumin (nv>35 g/L)	45 (38-50)	45 (32-50)	NS[‡]
IgA (nv 0.8-4.8 g/L)	1.67 (<0.03-3.89)	1.83 (<0.03-4.21)	NS[‡]
IgG (nv 6.5-17 g/L)	13.16 (5.70-43.96)	14.21 (7.99-27.14)	NS[‡]
IgM (nv 0.5-2 g/L)	1.12 (0.54-3.15)	1.56 (0.83-3.92)	NS[‡]
ANA, n of positive patients (%)	16 (41%)	16 (42%)	NS[†]
SMA, n of positive patients (%)	12 (31%)	9 (24%)	NS[†]
pANNA, n of positive patients (%)	14 (36%)	24 (63%)	0.03[†]
Active disease, n of patients (%)	11 (28%)	12 (32%)	NS[†]
IBD, n of patients (%)	7 (18%)	23 (61%)	<0.0001[†]

Data presented as median (range) unless noted otherwise.
AIH-1, autoimmune hepatitis type 1; *AISC*, autoimmune sclerosing cholangitis; *AST*, aspartate aminotransferase; *GGT*, gamma-glutamyl transpeptidase; *AP*, alkaline phosphatase; *INR*, international normalised ratio; *IgG*, immunoglobulin G; *ANA*, anti-nuclear antibodies; *SMA*, anti-smooth muscle antibodies*; pANNA*, peripheral anti-nuclear neutrophil antibodies; *UC*, ulcerative colitis; *nv*, normal value; *NS* non significant
[*] *p* value when comparing clinical parameters between AIH-1 and AISC patients
[†] Fisher´s exact test
[‡] Mann-Whitney test

Table C. Epidemiological, clinical, and laboratory data for each individual patient

Patient	Chapter	Diagnosis	Sex	Age	AST nv<50 IU/L	GGT nv<50 IU/L	Bil tot nv<20 µmol/l	AP nv<350 IU/L	INR nv<1.2	Alb nv>35 g/L	IgG nv 6.5-17 g/L	ANA reciprocal	SMA reciprocal	ANCA	IBD	Other diseases	Treatment
1	IV	AISC	M	16.1	47	91	14	313	1.06	46	12.99	160	Neg	pANNA	UC	No	P + A + UDCA
2	IV, V	AISC	F	16.7	59	169	24	262	1.05	42	17.57	160	Neg	pANNA	UC	No	P + A + UDCA
3	IV, V, VI	AIH-1	M	10.4	36	14	5	274	1.08	44	7.71	Neg	Neg	Neg	No	IgA def	P + MMF
4	IV, V, VI	AIH-1	F	12.1	64	17	10	126	1.07	45	16.5	80	80	Neg	No	No	P + A
5	IV, V, VI	AISC	F	16.4	125	455	12	581	0.92	50	17.1	Neg	40	pANNA	UC	No	P + MMF + UDCA
6	IV, V, VI	AIH-1	M	17.8	18	35	11	79	1.04	50	7.51	Neg	Neg	Neg	No	No	P + MMF
7	IV, V, VI	AIH-1	F	16.8	197	74	8	179	1.05	48	18	40	80	pANNA	No	No	P + A
8	IV, V, VI	AIH-1	F	13.2	27	10	6	114	1.03	44	20.8	Neg	Neg	Neg	No	No	A
9	IV, V, VI	AIH-1	F	16.5	33	22	6	148	1.23	44	12.89	Neg	Neg	Neg	No	T1D, IgA def	P + A
10	IV, V, VI	AISC	M	14.9	91	47	15	313	1.3	47	16.58	Neg	40	pANNA	No	No	P + MMF + UDCA
11	IV, V, VI	AIH-1	F	10.5	28	33	6	340	0.98	47	13.16	Neg	80	pANNA	No	No	P +A
12	IV, V, VI	AISC	M	15.7	21	12	4	344	1.21	48	12.4	10	Neg	Neg	UC	No	A + UDCA
13	IV, V, VI	AIH-1	M	11.7	22	20	4	249	1.08	47	14.63	Neg	10	pANNA	No	No	P
14	IV, V, VI	AIH-1	F	11.1	61	55	73	178	1.26	45	22.17	20	Neg	pANNA	UC	No	P + A
15	IV, V, VI	AIH-1	F	12.7	49	45	6	104	0.98	40	21	160	Neg	Neg	No	No	P + A
16	IV, V, VI	AIH-1	M	17.1	28	28	13	158	1.08	48	11.06	Neg	Neg	Neg	No	No	P + A
17	IV, V, VI	AIH-1	M	10.1	40	26	7	258	0.99	45	17.8	Neg	Neg	Neg	No	No	P
18	IV, V, VI	AIH-1	F	15.2	391	54	13	140	1.1	47	25.83	80	640	Neg	No	No	At diagnosis
19	IV, V, VI	AISC	M	12.1	93	107	9	222	1.05	41	11.99	Neg	Neg	pANNA	UC	No	P + A + UDCA
20	IV, V, VI	AISC	F	14.5	81	503	24	178	1.19	40	18.07	Neg	Neg	Neg	No	No	P + MMF +UDCA

Table C (cont.)_. Epidemiological, clinical, and laboratory data for each individual patient

Patient	Chapter	Diagnosis	Sex	Age	AST nv<50 IU/L	GGT nv<50 IU/L	Bil tot nv<20 μmol/l	AP nv<350 IU/L	INR nv<1.2	Alb nv>35 g/L	IgG nv 6.5-17 g/L	ANA reciprocal	SMA Reciprocal	ANCA	IBD	Other diseases	Treatment
21	IV, V, VI	AIH-1	F	9.3	75	98	24	88	1.09	39	13.83	40	Neg	Neg	No	No	At diagnosis
22	IV, V, VI	AISC	M	10.3	534	50	47	834	1.78	32	25.64	10	80	Neg	No	No	At diagnosis
23	IV, V, VI	AIH-1	F	13.5	1061	38	127	178	1.16	40	43.96	Neg	10	Neg	No	T1D	At diagnosis
24	IV, VI	AIH-1	M	13	22	8	7	344	1.07	46	8.98	160	Neg	Neg	No	No	P + A
25	IV, V	AISC	M	13.8	51	226	3	181	0.9	45	18.44	40	Neg	pANNA	UC	No	At diagnosis
26	IV, V	AISC	F	9.5	27	21	4	183	0.93	46	17.06	Neg	40	pANNA	No	No	P + A
27	IV, V, VI	AISC	F	12.7	41	164	5	254	0.97	41	27.14	Neg	80	pANNA	UC	No	P + A + UDCA
28	IV	AISC	F	16.7	33	49	5	104	0.95	44	11.29	Neg	Neg	pANNA	UC	No	P + A + UDCA
29	IV	AISC	M	26.7	43	27	8	68	0.86	48	12.21	80	Neg	pANNA	UC	No	P + A + UDCA
30	IV	AIH-1	M	5.6	294	492	7	669	0.94	41	18.12	Neg	320	pANNA	No	No	At diagnosis
31	IV	AISC	M	8.2	30	42	8	196	0.96	47	22.93	40	160	Neg	No	IgA def	P + UDCA
32	IV, VI	AISC	F	14.4	112	54	7	194	1.02	44	11.66	40	Neg	pANNA	No	No	P + MMF + UDCA
33	IV, VI	AIH-1	M	14	24	18	7	260	1.05	47	12.44	Neg	Neg	Neg	UC	No	P
34	IV	AIH-1	M	8.9	21	39	7	123	1.09	44	17.3	40	Neg	Neg	UC	No	P + A
35	V, VI	AIH-1	M	14.5	23	36	28	201	1	45	5.7	Neg	Neg	Neg	CD	IgA def, nephritis	P + MMF
36	V, VI	AIH-1	F	17.4	39	50	8	104	1.08	47	11.75	Neg	Neg	Neg	No	No	P + MMF
37	V, VI	AISC	M	17.6	17	12	27	94	1.09	45	9.85	20	Neg	pANNA	UC	No	P + UDCA
38	V, VI	AIH-1	F	13.7	22	11	8	137	1.08	46	10.46	Neg	Neg	pANNA	No	No	P + A
39	V, VI	AIH-1	F	21.1	16	9	12	49	1	43	8.28	Neg	Neg	Neg	No	No	P
40	V, VI	AIH-1	F	12.3	85	215	9	254	0.92	49	6.83	Neg	Neg	Neg	UC	T1D	P + MMF

Table C (cont.)_. Epidemiological, clinical, and laboratory data for each individual patient

Patient	Chapter	Diagnosis	Sex	Age	AST nv<50 IU/L	GGT nv<50 IU/L	Bil tot nv<20 µmol/l	AP nv<350 IU/L	INR nv<1.2	Alb nv>35 g/L	IgG nv 6.5-17 g/L	ANA reciprocal	SMA Reciprocal	ANCA	IBD	Other diseases	Treatment
41	V, VI	AISC	F	16.4	25	4	32	68	0.92	46	11.7	Neg	Neg	Neg	UC	No	P + A + UDCA
42	V	AIH-1	F	13.9	28	196	9	113	0.98	42	13.16	80	Neg	pANNA	CD	No	P + Tac
43	V	AISC	M	11.6	120	169	9	460	1.14	47	11.67	Neg	Neg	pANNA	UC	No	P + UDCA
44	V, VI	AISC	M	15.1	26	17	10	388	1.07	47	15.38	Neg	20	Neg	No	IgA def	P + UDCA
45	V, VI	AISC	M	13.9	43	94	9	307	0.89	42	9.64	Neg	20	Neg	UC	No	P + A + UDCA
46	V	AIH-1	F	16.8	20	24	4	103	0.89	49	11.66	80	Neg	pANNA	UC	T1D, thyroiditis	P + A
47	V	AIH-1	F	10	24	12	5	313	0.95	47	11.74	80	Neg	pANNA	No	No	P + UDCA
48	V, VI	AISC	F	16.5	18	50	9	75	0.99	47	8.01	20	Neg	pANNA	No	SLE	P + A
49	V	AISC	M	15.3	30	6	7	308	1.16	45	15.3	Neg	Neg	pANNA	UC	T1D	P + A + UDCA
50	V, VI	AISC	F	9.1	23	20	5	221	0.93	47	9.1	Neg	Neg	pANNA	No	No	P + A + UDCA
51	V	AIH-1	M	15.7	36	39	12	105	1.27	48	15.7	Neg	Neg	pANNA	No	No	P + A
52	V, VI	AISC	M	15.9	39	123	12	235	1.05	47	15.9	40	Neg	pANNA	UC	No	P + A + UDCA
53	V	AIH-1	F	11.2	67	46	8	236	1.1	42	16.31	Pos	10	Neg	No	No	P + A
54	V	AISC	M	15.5	29	10	7	350	1.2	44	7.99	Neg	Neg	Neg	No	No	A + UDCA
55	V	AISC	M	12.1	41	59	9	193	1.13	41	19.93	Neg	Neg	pANNA	UC	No	P + A + UDCA
56	V	AISC	M	17.1	43	62	27	376	1.03	48	9.65	10	Neg	pANNA	UC	No	P + UDCA
57	V	AISC	F	15.9	42	42	30	129	1.31	43	23.93	Neg	Neg	pANNA	No	Sickle cell disease	P + A + UDCA
58	V, VI	AIH-1	F	19.5	397	46	705	235	2.06	38	27.54	20	Neg	neg	No	Sickle cell disease	At diagnosis
59	V	AISC	M	15.3	63	92	7	228	1.07	47	10.34	Neg	Neg	pANNA	UC	No	P + UDCA
60	V	AISC	F	13.1	177	196	7	446	1.04	44	13.03	40	Neg	pANNA	UC	No	P + MMF + UDCA

Table C (cont.)_. Epidemiological, clinical, and laboratory data for each individual patient

Patient	Chapter	Diagnosis	Sex	Age	AST nv<50 IU/L	GGT nv<50 IU/L	Bil tot nv<20 µmol/l	AP nv<350 IU/L	INR nv<1.2	Alb nv>35 g/L	IgG nv 6.5-17 g/L	ANA reciprocal	SMA Reciprocal	ANCA	IBD	Other diseases	Treatment
61	V	AISC	M	15.4	23	22	6	251	1	47	11.64	Neg	Neg	pANNA	No	No	P + A + UDCA
62	V	AISC	F	13.6	58	78	8	73	1.01	44	15.38	20	20	pANNA	No	No	P + A + UDCA
63	V	AISC	F	11.9	30	27	7	228	1.07	44	18.56	Neg	40	Neg	No	No	P + UDCA
64	V	AISC	M	12.5	27	25	20	173	0.96	44	17.08	80	Neg	pANNA	UC	No	P + A + UDCA
65	V	AIH-1	F	8.9	38	35	4	175	1.01	43	16.9	Neg	40	pANNA	No	No	P
66	V, VI	AIH-1	M	8.8	34	12	5	236	0.92	45	7.97	Neg	40	Neg	No	No	No*
67	V, VI	AIH-1	F	15.1	34	12	6	265	1.06	46	12.38	Neg	Neg	Neg	No	No	P
68	VI	AIH-1	F	14.9	34	19	17	141	1.1	47	15.62	20	Neg	pANNA	No	IgA def, vitiligo	P
69	VI	AISC	F	11.7	35	78	5	32	1.04	46	11.71	Neg	Neg	Neg	UC	No	P + A + UDCA
70	VI	AIH-1	M	8.2	30	9	4	107	0.98	45	9.92	Neg	Neg	Neg	No	Coeliac disease	P
71	VI	AISC	M	16.6	77	78	35	504	1.17	38	11.99	Neg	Neg	pANNA	UC	No	P + UDCA
72	VI	AISC	F	12.7	32	169	4	114	0.96	46	16.35	40	Neg	pANNA	No	No	P + A + UDCA
73	VI	AIH-1	F	14.2	36	59	19	186	1.1	44	14.68	Neg	Neg	pANNA	No	T1D	P + MMF + CyA
74	VI	AISC	M	12.3	30	23	5	319	1.17	45	11.08	Neg	Neg	Neg	No	No	P + UDCA
75	VI	AISC	F	10.6	41	26	6	411	1.09	46	12.47	Neg	Neg	Neg	UC	G6PD def	P + A + UDCA
76	VI	AIH-1	F	15.9	19	15	4	83	1.02	49	10.68	Neg	20	pANCA	No	Haemolytic anemia	P + A
77	VI	AIH-1	M	16.8	30	20	5	299	1.11	46	9.15	40	Neg	Neg	No	No	P + A

AISC, autoimmune sclerosing cholangitis; *AIH-1*, autoimmune hepatitis type 1; *M*, male; *F*, female; *AST*, aspartate aminotransferase; *GGT*, gamma-glutamyl transpeptidase; *Bil tot*, bilirubin total; *AP*, alkaline phosphatase; *INR*, international normalised ratio; *Alb*, albumin; *Ig*, immunoglobulin; *ANA*, anti-nuclear antibodies; *SMA*, anti-smooth muscle antibodies; *ANCA*, anti-neutrophil cytoplasmic antibodies; *pANNA*, peripheral anti-nuclear neutrophil antibodies; *IBD*, inflammatory bowel disease; *UC*, ulcerative colitis; *CD*, Crohn´s disease; *def*, deficiency; *T1D*, type 1 diabetes; *G6PD*, glucose 6 phosphate dehydrogenage; *P*, prednisolone; *A*, azathioprine; *UDCA*, ursodeoxycholic acid; *MMF*, mycophenolate mofetil; *Tac*, tacrolimus; *CyA*, cyclosporine

APPENDIX II

De acordo com o Artigo 8º do Decreto-Lei nº 388/70, fazem parte integrante desta dissertação os seguintes trabalhos publicados ou em publicação:

1. **Liberal R**, Longhi MS, Mieli-Vergani G, Vergani D (2011) "Pathogenesis of autoimmune hepatitis" *Best Pract Res Clin Gastroenterol* 25(6):653-64
2. **Liberal R**, Longhi MS, Grant CR, Mieli-Vergani G, Vergani D (2012) "Autoimmune hepatitis after liver transplantation" *Clin Gastroenterol Hepatol* 10(4):346-53
3. **Liberal R**, Grant CR, Holder B, Ma Y, Mieli-Vergani G, Vergani D, Longhi MS (2012) "The impaired immune regulation of autoimmune hepatitis is linked to a defective Galectin-9/Tim-3 pathway." *Hepatology* Feb 28. doi: 10.1002/hep.25682. [Epub ahead of print]
4. Longhi MS, **Liberal R**, Holder B, Robson SC, Ma Y, Mieli-Vergani G, Vergani D (2012) "Inhibition of Interleukin-17 Promotes Differentiation of CD25- Cells into Stable T Regulatory Cells in Patients with Autoimmune Hepatitis". *Gastroenterology* 142(7): 1526-1535.e6.
5. **Liberal R**, Vergani D. "Effect of ethnicity on the clinical presentation and outcome of autoimmune hepatitis". *Expert Rev Gastroenterol and Hepatol* 6(3): 267-9

Em cumprimento do disposto no referido Decreto-Lei, declaro que participei activamente na recolha e estudo do material incluído em todos os trabalhos.

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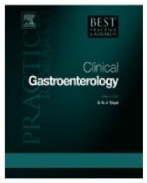

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Pathogenesis of autoimmune hepatitis

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The mechanisms underlying the pathogenesis of autoimmune hepatitis are not fully understood, though there is growing evidence that genetic predisposition, molecular mimicry and/or impairment of regulatory T-cells are involved in the initiation and perpetuation of the autoimmune liver attack. The histological picture of interface hepatitis, characterized by a dense portal mononuclear cell infiltrate, was the first to suggest an autoaggressive cellular immune attack in the pathogenesis of this condition. Liver damage is likely to be orchestrated by $CD4^{pos}$ T-cells recognizing an autoantigenic liver peptide. For autoimmunity to arise, the peptide must be presented by antigen-presenting cells to naïve $CD4^{pos}$ T-helper (Th0) cells. Once activated, Th0-cells can differentiate into Th1-, Th2-, or Th17-cells, initiating a cascade of immune reactions that are determined by the cytokines they produce. Autoantigen recognition and the above effector mechanisms are opposed by regulatory T-cells, a cell subset numerically and functionally impaired in autoimmune hepatitis.

Introduction

Autoimmune hepatitis (AIH), an immune-mediated liver disorder with a strong female preponderance, is characterized by hypergammaglobulinaemia, seropositivity for autoantibodies and interface hepatitis on histology. AIH responds to immunosuppressive treatment, which should be instituted as soon as the diagnosis is made. AIH can be divided in two subsets according to the autoantibodies detected at diagnosis: type 1 autoimmune hepatitis (AIH-1), associated with anti-nuclear antibody (ANA) and/or anti-smooth muscle antibody (SMA), and type 2 autoimmune hepatitis (AIH-2) characterized by positivity for anti-liver/kidney-microsomal-antibody-type-1 (anti-LKM-1) or anti-liver-

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cytosol-type-1 (anti-LC1). In spite of its global distribution, AIH prevalence and behaviour varies according to ethnicity. The aetiology of AIH is unknown, though both genetic and environmental factors are involved. Immune reactions against liver host antigens not adequately controlled by impaired regulatory T-cells are believed to be the major mechanism of liver damage.

Genetics

AIH is a "complex trait" disease that does not follow a Mendelian pattern of inheritance. Its mode of inheritance is unknown, though like other human complex trait disorders, it involves one or more genes, that acting alone or in concert, and interacting with environmental factors, increase or reduce the risk of the trait [1].

In AIH, the strongest genetic associations is with genes located within the major histocompatibility complex (MHC) – the human leukocyte antigen (HLA) region – on the short arm of chromosome 6, particularly those encoding the HLA class II *DRB1* alleles [1,2]. The role of class II MHC molecules is to present peptide antigens to CD4 T-cells, suggesting that HLA class II antigen presentation and T-cell activation are involved in the pathogenesis of AIH [3].

In Europe and North America, the alleles conferring susceptibility to AIH-1 are *DRB1*0301* and *DRB1*0401* which encode the HLA DR3 and DR4 antigens respectively [4,5]. Both heterodimers contain a K (lysine) residue at position 71 of the DRB1 polypeptide and the hexameric amino acid sequence L (leucin) L (leucin) E (glutamic acid) Q (glutamine) K (lysine) R (arginine) at position 67–72 [2,6]. Amongst the many associations reported to date, only possession of either HLA-DR3 or -DR4 are considered strong enough to point positively towards a diagnosis of AIH according to the revised diagnostic scoring system elaborated by the International Autoimmune Hepatitis Group (IAIHG). In Japan, Argentina and Mexico, susceptibility is linked to *DRB1*0405* and *DRB1*0404*, alleles encoding R (arginine) rather than K (lysine) at position 71, but sharing the motif LLEQ-R with *DRB1*0401* and *DRB1*0301* [7]. Thus, K or R at position 71 in the context of LLEQ-R may be critical for susceptibility to AIH, favouring the binding of autoantigenic peptides, complementary to this hexameric sequence [8]. However, an alternative model based on valine/glycine dimorphism at position 86 of the DR-β polypeptide has been proposed, better representing the key HLA associations in patients from Argentina and Brazil [2,6]. In a study from Japan, AIH-1 patients were found to have *DRB1* alleles, which encode histidine at position 13 [1]. Taken together, these data point to the existence of at least three potential models, indicating that different populations show distinct genetic associations and that the peptides presented by MHC class II molecules to the T-cell receptors are different and may derive from different antigens. Thus, these HLA associations are potentially indicative of the prevailing environmental insults triggering AIH-1 in different environments. In this regard, it is interesting that in South America possession of HLA *DRB1*1301* allele, which predisposes to paediatric AIH-1 in this population, is also associated with persistent infection with the endemic hepatitis A virus [9].

Recently, a meta-analysis study, aiming to identify common HLA class II alleles conferring susceptibility to AIH-1 in Latin America, reported that DQ2 imparts risk to the disease, whilst the possession of DR5 or DQ3 is protective [10].

Susceptibility to AIH-2 is conferred by the possession of HLA DR7 (*DRB1*0701*) and DR3 (*DRB1*0301*), patients positive for *DRB1*0701* having a more aggressive disease and severe prognosis [11]. Another study has suggested that the possession of HLA-*DQB1*0201* is the major genetic determinant for children with AIH-2, although HLA-*DQB1*0201* is in linkage disequilibrium with *DRB1*0701* and *DRB1*0301*, both associated with AIH-2 [12]. In AIH-2, HLA alleles influence the autoantigenic humoural response, *DRB1*0301* being associated with seropositivity for both anti-LKM-1 and anti-LC-1 antibodies and *DRB1*0701* being predominant amongst patients positive only for anti-LKM-1. Additionally, children positive for *DRB1*0701* have a more restricted anti-LKM-1 epitopic repertoire compared to those positive for *DRB1*0301* [13].

Other genes within the HLA locus shown to be associated with AIH susceptibility include the IgA and complement factor 4A genes [14]. IgA deficiency, frequently found in patients with AIH-2, is genetically linked to the MHC locus, particularly with HLA-DR1 and -DR7 [15]. Also, low levels of complement factor 4a (C4a) are present in 69% of paediatric children with AIH, this deficiency being genetically determined [16].

A number of genes outside the MHC may also be involved in the susceptibility to AIH. Cytotoxic- T-lymphocyte-antigen-4 (CTLA-4), also known as CD152, is a molecule present on the surface of T-cells that interacts, in competition with CD28, with the ligands B7-1 and B7-2 on antigen presenting cells, transmitting an inhibitory signal to T-cells. A transition from adenine (A) to guanine (G) in exon 1 of the *CTLA-4* gene confers susceptibility to several autoimmune diseases, including AIH-1 in caucasians from North America [17]; however, this finding was not confirmed in Brazilian patients [18]. A polymorphism at position 308 in the tumour necrosis factor α gene promoter (TNFA) occurs more frequently in patients with AIH-1 from Europe and North America than in healthy subjects and is associated with a poorer response to steroids [19], a finding, however, not observed in Japanese patients [20]. A polymorphism at position 670 in the FAS gene promoter was found to influence AIH susceptibility and progression, leading to a more aggressive disease with an early development of cirrhosis [21]. Polymorphisms in the vitamin D receptor – capable of activating macrophages and monocytes, preventing dendritic cell differentiation, and inhibiting Th1-cell function – were reported to contribute to the development of autoimmune liver disease [22].

A form of AIH resembling AIH-2 has been described in some 20% of patients with autoimmune polyendocrinopathy-candidiasis-ectodermal-dystrophy (APECED). This condition is a monogenic autosomal recessive disorder characterized by a variety of organ-specific autoimmune diseases, the most common of which are hypoparathyroidism and primary adrenocortical failure, accompanied by chronic mucocutaneous candidiasis [23]. At variance with many other autoimmune diseases, APECED is associated with mutations of a single gene – designated autoimmune regulator 1 (*AIRE1*). The *AIRE1* gene sequence consists of 14 exons containing 45 different mutations, with a 13-bp deletion at nucleotide 964 in exon 8 accounting for more than 70% of APECED alleles in the United Kingdom [24]. *AIRE1*, which codifies a transcription factor, is highly expressed in medullary epithelial cells and other thymic stromal cells involved in clonal deletion of self-reactive T-cells. Interestingly, APECED has a high level of variability of symptoms, especially between populations. Since in animal models different *AIRE* mutations result in the same phenotype, it is likely that the clinical variability across human populations relates to environmental or genetic modifiers [25,26]. Of the latter, the most likely to synergize with *AIRE* mutations are polymorphisms in the HLA region. HLA molecules are not only highly variable and strongly associated with multiple autoimmune diseases but are also able to affect thymic repertoire selection of autoreactive T-cell clones [27].

Immune mechanisms

The liver is regarded as a unique anatomical and immunological organ, continuously exposed to antigen-rich blood (containing pathogens, toxins, tumour cells and self-antigens) from the gastrointestinal tract [28]. The liver is highly enriched in phagocytic cells, antigen-presenting cells (APC) and lymphocytes and is an active site for the production of cytokines, complement components and acute phase proteins [29]. Within the liver, many cell populations can act as APCs, namely endothelial cells, subendothelial stellate cells, hepatocytes, and dendritic cells [30]. The liver lymphocytes are found scattered throughout the parenchyma, as well as in the portal tracts; these include cells of the innate [natural killer (NK) and NKT-cells] and adaptive immune systems (B- and T-cells) [28]. Immune-regulatory mechanisms are required to determine whether an antigen encounter will result in immunological tolerance or reactivity. Liver autoimmunity ensues when self-tolerance is lost [18].

Several mechanisms may account for the onset of an autoimmune liver response. Though no single initiating event is able to explain all instances of liver autoimmunity, two general conditions should prevail: self-reactive B- and T-lymphocytes must exist in the immunological repertoire and autoantigens must be presented by APC [18].

Impairment of regulatory T-cells

The development of autoimmune diseases is characterized by the breakdown of immune-regulatory mechanisms that in health are responsible for maintaining immunological tolerance against self-antigens. In the context of AIH, seminal studies conducted during the 1980s were able to a) demonstrate impairment of cells with "suppressor" function, which recovers following in vitro

exposure of therapeutic doses of steroids [31,32]; and b) show that this defect specifically involved a subpopulation of T-lymphocytes controlling immune responses against a liver-specific membrane autoantigen [33]. Those were the seeds for a series of subsequent studies, that almost 20 years later, clearly indicated that impairment in regulatory T-cells is central to the loss of immune tolerance in AIH and to the emergence of uncontrolled effector immune responses [34–39].

Among the multiple T-cell subsets with suppressive function, the regulatory T-cells (T-regs), defined by the expression of CD4, the IL-2 receptor α chain (CD25), and the transcription factor forkhead box P3 (FOXP3), have emerged as having a central role in protecting from autoimmunity [40]. These T-regs can be subdivided into thymus-derived natural T-regs (nT-regs) and peripherally-induced adaptive T-regs (iT-regs). Both subpopulations exert their suppressive function either through a contact-dependent mechanism – by targeting directly the function of effector T-cells as well as modulating the maturation and/or function of dendritic cells, which in turn are required for the activation of the former – and/or through contact-independent mechanisms, i.e. by the production of soluble factors, such as the cytokines TGF-β, IL-10 and IL-35 [40,41]. Thymus-derived nT-regs retain their regulatory function and FOXP3 expression in the periphery, being characterized by the demethylation of their *FOXP3* promotor [42], and express the regulatory transcription factor Helios [43]. In the presence of TGF-β, iT-regs can be induced in the periphery from a $CD4^{pos}FOXP3^{neg}$ T-cell population. Though iT-regs express the same cell surface markers as nT-regs, they can be distinguished from them based on their *FOXP3* DNA methylation patterns and lack of Helios expression [43].

Compared to healthy subjects, both children and adults with AIH display lower numbers of $CD4^{pos}CD25^{high}$ T-regs that also express lower levels of FOXP3 [34,35,38]. This defect relates to liver disease activity, being more evident at disease presentation than during drug-induced remission. The frequency of T-regs is inversely correlated with the levels of autoantibodies, such as anti-soluble liver antigen (anti-SLA) and anti-LKM-1, suggesting that a numerical reduction of T-regs favours the serologic manifestations of AIH [34].

In AIH a functional impairment of T-regs has also been demonstrated: T-regs not only fail to restrain the proliferation and IFN-γ production by CD4 and CD8 effector T-cells [34,35], but also to create the regulatory milieu that would support and enhance their own function [36]. In addition, T-regs in AIH paradoxically enhance the activation of monocytes, cells of the innate immune system abundantly present in the portal-periportal inflammatory infiltrate [44].

Because T-regs are highly effective at controlling auto-aggression, adoptive T-reg cell therapy constitutes a promising candidate for immune intervention aiming at reconstituting self-tolerance in autoimmune conditions [45]. This approach, however, has been heretofore hindered by the limited ability of T-regs to expand and by their propensity to apoptosis [46]. The observation that corticosteroid therapy can partially restore T-reg number and function indicates that, though impaired, T-regs in AIH have the potential to expand and regain their function [34,35]. Using a polyclonal T-cell stimulation strategy (that engages the T-cell receptor via CD3 and the co-stimulatory molecule CD28, while providing exogenous IL-2, a key cytokine for T-reg survival and growth), T-regs can be expanded from circulating $CD4^{pos}CD25^{pos}$ T-regs, and also generated *de novo* from non-regulatory $CD4^{pos}CD25^{neg}$ T-cells in healthy subjects, and to a lesser extent, in patients with AIH [37]. Interestingly, expanded T-regs express higher levels of FOXP3 and suppress more efficiently when compared to freshly isolated T-regs [37].

There are several challenges to be tackled before therapeutic application of T-regs. These include the search for a T-reg cell surface marker, as specific as the intracellular FOXP3, to facilitate cell purification; and the silencing or physical removal of effector T-cells contaminating the human T-reg $CD4^{pos}CD25^{high}$ population [47], in particular Th17 cells that share a common progenitor with nT-regs and produce the highly pro-inflammatory cytokine IL-17 [48].

Studies in experimental animals have shown that T-regs with autoantigen specificity suppress more efficiently than their non-antigen-specific counterparts. In contrast to AIH-1, in which auto-antigens are less well defined, in AIH-2 not only is the key autoantigen known (CYP2D6) [49], but also the antigenic regions ($CYP2D6_{217–260}$ and $CYP2D6_{305–348}$), targeted by B-cells, CD4 and CD8 T-cells [50,51]. Thus, AIH-2 is an ideal model for attempting reconstitution of self-tolerance by specific immunological intervention [27]. In this regard, antigen-specific T-regs from patients with AIH-2 suppressed CD4 and CD8 T-cell responses more potently than polyclonal expanded T-regs [39]. The most efficient

suppression of autoreactive T-cells is achieved when T-regs exposed to CYP2D6 peptides are co-cultured with semi-mature dendritic cells loaded with the same peptide [39].

Among other cells with suppressive potential, NKT-cells, a subset of T-cells that co-express NK-cell markers and that are abundant in the liver, have been suggested to play an important role in regulating immune responses in the context of autoimmune liver diseases [52]. NKT-cells are numerically reduced in the peripheral blood of AIH patients, particularly during the active phases of disease and this number is partially restored during drug-induced remission; their behaviour, therefore, mirrors that of $CD4^{pos}CD25^{high}$ regulatory T-cells [38]. In addition, NKT-cells from AIH patients produce lower quantities of the regulatory cytokine IL-4 compared to healthy controls [38].

Molecular mimicry

Several lines of evidence have shown that "molecular mimicry", a process whereby immune responses to external pathogens become directed towards structurally similar self-components, is implicated in the initiation of autoimmune damage [53]. Autoimmunity is a common feature during chronic hepatitis B virus (HBV) and hepatitis C virus (HCV) infections, where 50% of the patients eventually develop autoantibodies such as ANA and SMA [54,55]. Also, 10% of chronic HCV patients are positive for anti-LKM-1, this positivity being correlated with disease severity and adverse reactions to interferon treatment [56]. Thus, HBV and HCV infection may play a role in the initiation of the autoimmune process; however, a strong link between autoimmunity and viral hepatitis has been shown only for HCV. Interestingly, there is a high amino acid sequence homology between the HCV polyprotein and CYP2D6, target of anti-LKM-1, indicating that molecular mimicry may be a trigger for the production of anti-LKM-1 in HCV patients [50,57].

Mechanisms leading to autoimmune liver damage

The mechanisms leading to autoimmune liver damage have been the focus of intense investigation over the past three decades. However, the initial trigger remains elusive. The histological picture of interface hepatitis (Fig. 1), the hallmark of the disease, with its dense infiltration of lymphocytes, plasma cells, and macrophages was the first to suggest that an autoaggressive cellular immune attack is at the basis of AIH. Whatever the initial trigger, this massive recruitment of activated inflammatory cells is likely to cause damage. Immunohistochemical studies of interface hepatitis have identified a predominance of T-lymphocytes expressing the α/β T-cell receptor [58]. Amongst the T-cells, the majority are positive for the CD4 helper/inducer phenotype, and a sizable minority for the CD8 cytotoxic phenotype. Lymphocytes of a non T-cell lineage are fewer and include NK-cells, macrophages, B-cells and plasma cells [58].

Irrespective of the trigger, the pathogenic mechanisms leading to liver damage are complex and involve the intervention of both innate and adaptive arms of the immune system (Fig. 2).

Liver damage is likely to be orchestrated by CD4 positive lymphocytes that recognise a self-antigenic peptide. The autoimmune response is initiated after the peptide is embraced by an HLA class II molecule and presented to a naïve CD4 positive T helper cell (Th0) by an APC, in the presence of the appropriate co-stimulatory signals, induced by the interaction of CD28 on Th0 and CD80 on APC. Once activated, depending on the cytokines present in the microenvironment and the type of antigen, Th0-cells can differentiate into: Th1-, Th2- or Th17-cells. These effector cells initiate a cascade of immune reactions largely determined by the cytokines they produce:

- Th1 cells secrete mainly IL-2 and IFN-γ. IFN-γ is considered the main mediator of tissue damage since it not only stimulates CD8 cells, enhances the expression of HLA class I, and induces expression of HLA class II molecules on hepatocytes [59,60], but also activates monocytes/macrophages, which in turn release IL-1 and TNF-α.
- Th2-cells produce IL-4, IL-10 and IL-13, cytokines that induce the maturation of B-cells into plasma cells, with consequent production of autoantibodies.

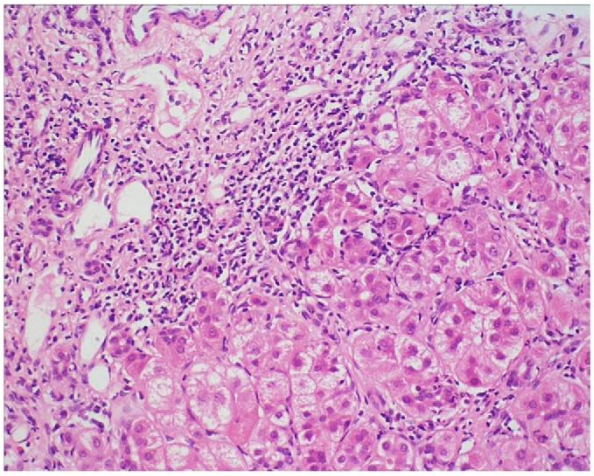

Fig. 1. The portal and periportal inflammatory infiltrate characteristic of autoimmune hepatitis is composed of lymphocytes, macrophages, and plasma cells – interface hepatitis (picture kindly provided by Dr. Yoh Zen).

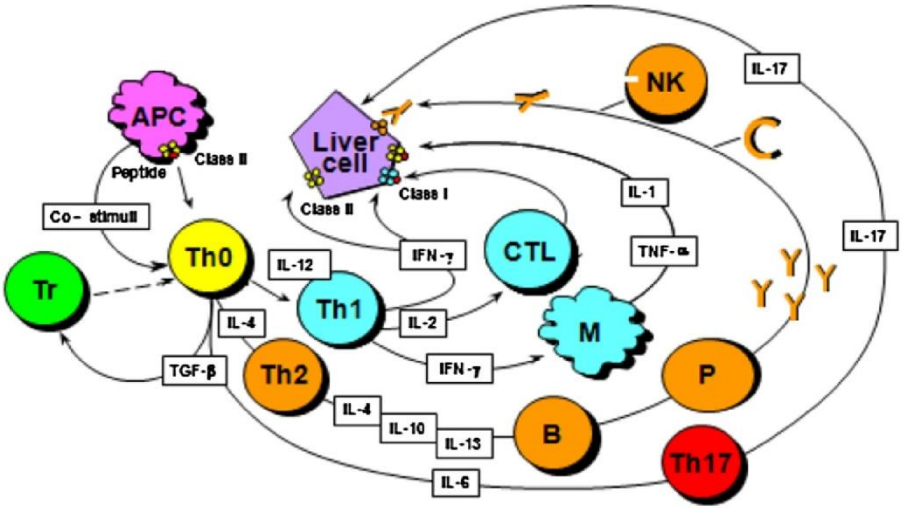

Fig. 2. Autoimmune attack to the liver cell. An autoantigenic peptide is presented to an uncommitted T helper (Th0) lymphocyte within the HLA class II molecule of an antigen-presenting cell (APC). Th0-cells become activated and, according to the cytokines present in the microenvironment and the nature of the antigen, differentiate into Th1-, Th2-, or Th17-cells, initiating a series of immune reactions determined by the cytokines they produce: Th2 secrete mainly IL-4, IL-10 and IL-13, and direct autoantibody production by B lymphocytes; Th1 secrete IL-2 and IFN-γ, which stimulate T cytotoxic lymphocytes (CTL), enhance expression of class I and induce expression of class II HLA molecules on hepatocytes and activate macrophages; activated macrophages release IL-1 and tumour necrosis factor alpha (TNF-α). If regulatory T cells do not oppose, a variety of effector mechanisms are triggered: liver cell destruction could derive from the action of CTL; cytokines released by Th1 and recruited macrophages; complement activation or engagement of Fc receptor-bearing cells such as natural killer (NK) lymphocytes by the autoantibody bound to the hepatocyte surface. The role of the recently described Th17 cells, which arise in the presence of transforming growth factor beta (TGF-β) and IL-6, is under investigation.

- Th17-cells, which arise in the presence of TGF-β and IL-6, produce IL-17, IL-22, TNF-α, and express CCL20 [48]. Th17 immunity has been recently shown to be involved in both experimental and human autoimmune disease [61,62]. Th17-cells have been implicated in the pathogenesis of another autoimmune liver disease, primary biliary cirrhosis [63]. Although their contribution to liver damage in AIH is still under investigation, a recent paper shows an increased number of Th17-cells in both the peripheral blood and liver of patients with AIH compared to healthy controls [64].

Since T-regs are numerically and functionally impaired in AIH, these effector responses are perpetuated with unremitting liver cell destruction through the action of cytotoxic T-lymphocytes (CTL), cytokines released by Th1-cells (and possibly Th17-cells) and by monocytes/macrophages, and complement activation or engagement of NK-cells by autoantibodies bound to the hepatocyte surface [27].

Humoural immunity

Several autoantibodies have been reported to contribute to the liver damage in AIH. The titres of anti-liver-specific membrane lipoprotein (LSP) antibody, as well as antibodies to the LSP components asialoglycoprotein receptor (ASGPR) and alcohol dehydrogenase (ADH) correlate with biochemical and histological indices of disease severity [65,66]. A role for these autoantibodies in the autoimmune liver attack has been suggested by the finding that hepatocytes, isolated from patients with AIH, are coated with immunoglobulins and are susceptible to cytotoxicity when exposed to autologous Fc receptor bearing mononuclear cells [67,68]. More recently, anti-SLA antibodies, targeting the UGA suppressor tRNP-associated antigenic protein ($tRNP^{(ser)sec}$, SEPSECS), were found to be present in 50% of AIH-1 and AIH-2 patients and to define a more severe disease course compared to seronegative patients [69].

In AIH-2, the demonstration that CYP2D6 is expressed on the surface of hepatocyte suggests a direct role of anti-LKM1 antibodies in liver damage [70]. In AIH-2 anti-LKM-1 antibodies recognize linear regions (autoepitopes) of CYP2D6 in a hierarchical manner. Thus, the principal linear B-cell epitope, $CYP2D6_{193-212}$ is recognized by 93% of the patients, $CYP2D6_{257-269}$ by 85%, $CYP2D6_{321-351}$ by 53%, and two additional minor epitopes, $CYP2D6_{363-389}$ and $CYP2D6_{410-429}$, are recognized by 7% and 13% respectively [50]. Expressing both full-length and a series of truncated CYP2D6 proteins in a eukaryotic system, Ma et al have provided a conformational epitope mapping of CYP2D6, showing that antigenicity is confined to the C terminal portion of the molecule, increasing stepwise towards the C terminal [71].

Cellular immunity

Early investigations of the cellular immune mechanisms involved in the pathogenesis of autoimmune liver damage concluded that AIH patients have circulating lymphocytes 'sensitized' to liver antigens and able to kill target cells *in vitro*. Studies performed using separated T- and non-T- cell subsets from the peripheral blood of AIH patients and xenogenic target cells demonstrated that cytotoxic cells were present in the non-T-cell subpopulation [67]. Involvement of an antibody-dependent cell-mediated cytotoxicity (ADCC) in the autoimmune liver attack has been suggested by experiments showing that the cytotoxic cells were located within the non-T-cell compartment and that aggregated IgG were able to block the cytotoxic activity. Such involvement was later confirmed by the observation that hepatocytes carrying IgG on their surface were susceptible to damage by lymphocytes from healthy individuals [68]. In the early 90s, clonal analysis studies showed that cytotoxicity against liver-specific antigens could also be detected within the T-cell compartment [72].

Studies conducted later by Wen et al demonstrated that AIH children have a 10-fold higher frequency of liver antigen-specific precursors in their circulation when compared to healthy subjects [73]. Clonal analysis experiments revealed that CD4 T-cells were capable of inducing autoantibody production by autologous B-lymphocytes, a process that could be blocked by neutralizing antibodies against HLA-DR, CD4 and IL-2R, leading to the conclusion that these clones follow the classical rules of immune recognition [72]. Subsequent investigations showed that in AIH the largest numbers of clones generated from the peripheral blood are $CD4^{pos}$ T-cells bearing the αβ T-cell receptor, while the highest proportion of clones obtained from liver biopsies of the same patients are $CD4^{neg}CD8^{neg}$ cells bearing T-the γδ T-cell receptor or $CD8^{pos}$ αβ T-cells [73].

based on immunizing C57BL/6 female mice with a plasmid containing cDNA for the antigenic region of human CYP2D6 and for formimino-transferase cyclodeaminase, targets of anti-LKM1 and anti-LC-1 respectively, together with the end of the terminal region of murine CTLA-4 [77]. Only after three immunizations and when a plasmid containing the cDNA encoding IL-12 was also used, antigen specific autoantibodies were produced, and a modest liver damage accompanied by portal and periportal infiltration of CD4, CD8 T- and, B-cells was demonstrated. When the same immunization protocol was used in different mouse strains, no inflammatory changes were observed highlighting the importance of a specific genetic background. Another model of AIH-2 uses CYP2D6 transgenic mice and aims at breaking tolerance with an Adenovirus-CYP2D6 vector [78]. While focal hepatocyte necrosis was seen both in mice treated with the Adenovirus-CYP2D6 vector and control mice treated with Adenovirus alone, only the former developed chronic histological changes, including fibrosis, reminiscent of AIH. The hepatic lesion was associated with a specific immune response to an immunodominant region of CYP2D6 and a cytotoxic T-cell response to Adenovirus-CYP2D6 vector infected target cells. Though these two experimental approaches provide useful information on the possible pathogenic mechanisms leading to AIH-2, a model mimicking closely AIH in humans is still missing.

Practice points

- The strongest genetic association in AIH relates to HLA genes, particularly those encoding *DRB1* alleles.
- In AIH, regulatory T-cells are numerically and functionally impaired, permitting the perpetuation of effector immune responses with ensuing persistent liver destruction.
- T-regs can be expanded from circulating $CD4^{pos}CD25^{pos}$ T-regs, and generated *de novo* from non-regulatory $CD4^{pos}CD25^{neg}$ T-cells in AIH patient.

Research agenda

- Deeper understanding of AIH pathogenesis, possibly through the development of animal models faithfully reproducing the human disease.
- Development of novel treatments for AIH, aiming at reconstituting self-tolerance by specific immunologic manoeuvres, such as adoptive transfer of autologous expanded T-regs. This requires investigation of T-reg stability, tolerability and half-life in human.

Conflict of interest

None declared.

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References

[1] Donaldson PT. Genetics of liver disease: immunogenetics and disease pathogenesis. Gut 2004;53:599–608.
[2] Donaldson PT. Genetics in autoimmune hepatitis. Semin Liver Dis 2002;22:353–64.

[3] Vergani D, Choudhuri K, Bogdanos DP, Mieli-Vergani G. Pathogenesis of autoimmune hepatitis. Clin Liver Dis 2002;6:727–37.
[4] Donaldson PT, Doherty DG, Hayllar KM, McFarlane IG, Johnson PJ, Williams R. Susceptibility to autoimmune chronic active hepatitis: human leukocyte antigens DR4 and A1-B8-DR3 are independent risk factors. Hepatology 1991;13:701–6.
[5] Strettell MD, Donaldson PT, Thomson LJ, et al. Allelic basis for HLA-encoded susceptibility to type 1 autoimmune hepatitis. Gastroenterology 1997;112:2028–35.
[6] Donaldson PT. Genetics of autoimmune and viral liver diseases; understanding the issues. J Hepatol 2004;41:327–32.
[7] Czaja AJ, Donaldson PT. Genetic susceptibilities for immune expression and liver cell injury in autoimmune hepatitis. Immunol Rev 2000;174:250–9.
[8] Czaja AJ, Doherty DG, Donaldson PT. Genetic bases of autoimmune hepatitis. Dig Dis Sci 2002;47:2139–50.
[9] Vergani D, Longhi MS, Bogdanos DP, Ma Y, Mieli-Vergani G. Autoimmune hepatitis. Semin Immunopathol 2009;31:421–35.
[10] Duarte-Rey C, Pardo AL, Rodriguez-Velosa Y, Mantilla RD, Anaya JM, Rojas-Villarraga A. HLA class II association with autoimmune hepatitis in Latin America: a meta-analysis. Autoimmun Rev 2009;8:325–31.
*[11] Ma Y, Bogdanos DP, Hussain MJ, et al. Polyclonal T-cell responses to cytochrome P450IID6 are associated with disease activity in autoimmune hepatitis type 2. Gastroenterology 2006;130:868–82.
[12] Djilali-Saiah I, Renous R, Caillat-Zucman S, Debray D, Alvarez F. Linkage disequilibrium between HLA class II region and autoimmune hepatitis in pediatric patients. J Hepatol 2004;40:904–9.
[13] Djilali-Saiah I, Fakhfakh A, Louafi H, Caillat-Zucman S, Debray D, Alvarez F. HLA class II influences humoral autoimmunity in patients with type 2 autoimmune hepatitis. J Hepatol 2006;45:844–50.
[14] Gregorio GV, Portmann B, Reid F, et al. Autoimmune hepatitis in childhood: a 20-year experience. Hepatology 1997;25:541–7.
[15] Vorechovsky I, Webster AD, Plebani A, Hammarstrom L. Genetic linkage of IgA deficiency to the major histocompatibility complex: evidence for allele segregation distortion, parent-of-origin penetrance differences, and the role of anti-IgA antibodies in disease predisposition. Am J Hum Genet 1999;64:1096–109.
[16] Vergani D, Wells L, Larcher VF, et al. Genetically determined low C4: a predisposing factor to autoimmune chronic active hepatitis. Lancet 1985;2:294–8.
[17] Agarwal K, Czaja AJ, Jones DE, Donaldson PT. Cytotoxic T lymphocyte antigen-4 (CTLA-4) gene polymorphisms and susceptibility to type 1 autoimmune hepatitis. Hepatology 2000;31:49–53.
[18] Vergani D, Mieli-Vergani G. Aetiopathogenesis of autoimmune hepatitis. World J Gastroenterol 2008;14:3306–12.
*[19] Czaja AJ, Cookson S, Constantini PK, Clare M, Underhill JA, Donaldson PT. Cytokine polymorphisms associated with clinical features and treatment outcome in type 1 autoimmune hepatitis. Gastroenterology 1999;117:645–52.
[20] Yoshizawa K, Ota M, Katsuyama Y, et al. Genetic analysis of the HLA region of Japanese patients with type 1 autoimmune hepatitis. J Hepatol 2005;42:578–84.
[21] Agarwal K, Czaja AJ, Donaldson PT. A functional Fas promoter polymorphism is associated with a severe phenotype in type 1 autoimmune hepatitis characterized by early development of cirrhosis. Tissue Antigens 2007;69:227–35.
[22] Vogel A, Strassburg CP, Manns MP. Genetic association of vitamin D receptor polymorphisms with primary biliary cirrhosis and autoimmune hepatitis. Hepatology 2002;35:126–31.
*[23] Mathis D, Benoist C. Aire Annu Rev Immunol 2009;27:287–312.
[24] Simmonds MJ, Gough SC. Genetic insights into disease mechanisms of autoimmunity. Br Med Bull 2004;71:93–113.
[25] Liston A, Gray DH, Lesage S, et al. Gene dosage–limiting role of Aire in thymic expression, clonal deletion, and organ-specific autoimmunity. J Exp Med 2004;200:1015–26.
[26] Liston A, Lesage S, Gray DH, Boyd RL, Goodnow CC. Genetic lesions in T-cell tolerance and thresholds for autoimmunity. Immunol Rev 2005;204:87–101.
[27] Longhi MS, Ma Y, Mieli-Vergani G, Vergani D. Aetiopathogenesis of autoimmune hepatitis. J Autoimmun 2010;34:7–14.
[28] Racanelli V, Rehermann B. The liver as an immunological organ. Hepatology 2006;43:S54–62.
[29] Mackay IR. Hepatoimmunology: a perspective. Immunol Cell Biol 2002;80:36–44.
[30] Crispe IN. The liver as a lymphoid organ. Annu Rev Immunol 2009;27:147–63.
[31] Nouri-Aria KT, Hegarty JE, Alexander GJ, Eddleston AL, Williams R. Effect of corticosteroids on suppressor-cell activity in "autoimmune" and viral chronic active hepatitis. N Engl J Med 1982;307:1301–4.
[32] Hodgson HJ, Wands JR, Isselbacher KJ. Alteration in suppressor cell activity in chronic active hepatitis. Proc Natl Acad Sci U S A 1978;75:1549–53.
*[33] Vento S, Hegarty JE, Bottazzo G, Macchia E, Williams R, Eddleston AL. Antigen specific suppressor cell function in autoimmune chronic active hepatitis. Lancet 1984;1:1200–4.
*[34] Longhi MS, Ma Y, Bogdanos DP, Cheeseman P, Mieli-Vergani G, Vergani D. Impairment of CD4(+)CD25(+)regulatory T-cells in autoimmune liver disease. J Hepatol 2004;41:31–7.
[35] Longhi MS, Ma Y, Mitry RR, et al. Effect of CD4 + CD25 + regulatory T-cells on CD8 T-cell function in patients with autoimmune hepatitis. J Autoimmun 2005;25:63–71.
[36] Longhi MS, Hussain MJ, Mitry RR, et al. Functional study of CD4 + CD25+regulatory T-cells in health and autoimmune hepatitis. J Immunol 2006;176:4484–91.
*[37] Longhi MS, Meda F, Wang P, et al. Expansion and de novo generation of potentially therapeutic regulatory T-cells in patients with autoimmune hepatitis. Hepatology 2008;47:581–91.
[38] Ferri S, Longhi MS, De Molo C, et al. A multifaceted imbalance of T-cells with regulatory function characterizes type 1 autoimmune hepatitis. Hepatology 2010;52:999–1007.
*[39] Longhi MS, Hussain MJ, Kwok WW, Mieli-Vergani G, Ma Y, Vergani D. Autoantigen-specific regulatory T-cells, a potential tool for immune-tolerance reconstitution in type-2 autoimmune hepatitis. Hepatology 2011;53:536–47.
[40] Sakaguchi S, Miyara M, Costantino CM, Hafler DA. FOXP3 + regulatory T cells in the human immune system. Nat Rev Immunol 2010;10:490–500.
[41] Vignali DA, Collison LW, Workman CJ. How regulatory T cells work. Nat Rev Immunol 2008;8:523–32.
[42] Janson PC, Winerdal ME, Marits P, Thorn M, Ohlsson R, Winqvist O. FOXP3 promoter demethylation reveals the committed Treg population in humans. PLoS One 2008;3:e1612.

[43] Thornton AM, Korty PE, Tran DQ, et al. Expression of Helios, an Ikaros transcription factor family member, differentiates thymic-derived from peripherally induced Foxp3 + T regulatory cells. J Immunol 2010;184:3433–41.
[44] Longhi MS, Mitry RR, Samyn M, et al. Vigorous activation of monocytes in juvenile autoimmune liver disease escapes the control of regulatory T-cells. Hepatology 2009;50:130–42.
[45] Riley JL, June CH, Blazar BR. Human T regulatory cell therapy: take a billion or so and call me in the morning. Immunity 2009;30:656–65.
[46] Akbar AN, Taams LS, Salmon M, Vukmanovic-Stejic M. The peripheral generation of CD4 + CD25 + regulatory T cells. Immunology 2003;109:319–25.
[47] Longhi MS, Liberal R, Mieli-Vergani G, Ma Y, Vergani D. Inhibition of Th17-cells enhances the suppressor function of de novo generated T-Reg in patients with autoimmune hepatitis. Hepatology 2011;50:1012a-a.
[48] Bettelli E, Carrier Y, Gao W, et al. Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells. Nature 2006;441:235–8.
[49] Gueguen M, Meunier-Rotival M, Bernard O, Alvarez F. Anti-liver-kidney-microsome-antibody recognizes a cytochrome P450 from the IID subfamily. J Exp Med 1988;168:801–6.
[50] Kerkar N, Choudhuri K, Ma Y, et al. Cytochrome P4502D6(193–212): a new immunodominant epitope and target of virus/self cross-reactivity in liver kidney microsomal autoantibody type 1-positive liver disease. J Immunol 2003; 170:1481–9.
[51] Longhi MS, Hussain MJ, Bogdanos DP, et al. Cytochrome P450IID6-specific CD8 T-cell immune responses mirror disease activity in autoimmune hepatitis type 2. Hepatology 2007;46:472–84.
[52] Santodomingo-Garzon T, Swain MG. Role of NKT cells in autoimmune liver disease. Autoimmun Rev; 2011.
[53] Bogdanos DP, Choudhuri K, Vergani D. Molecular mimicry and autoimmune liver disease: virtuous intentions, malign consequences. Liver 2001;21:225–32.
[54] Gregorio GV, Choudhuri K, Ma Y, Vegnente A, Mieli-Vergani G, Vergani D. Mimicry between the hepatitis B virus DNA polymerase and the antigenic targets of nuclear and smooth muscle antibodies in chronic hepatitis B virus infection. J Immunol 1999;162:1802–10.
[55] Gregorio GV, Choudhuri K, Ma Y, et al. Mimicry between the hepatitis C virus polyprotein and antigenic targets of nuclear and smooth muscle antibodies in chronic hepatitis C virus infection. Clin Exp Immunol 2003;133:404–13.
[56] Lenzi M, Bellentani S, Saccoccio G, et al. Prevalence of non-organ-specific autoantibodies and chronic liver disease in the general population: a nested case-control study of the Dionysos cohort. Gut 1999;45:435–41.
*[57] Manns MP, Griffin KJ, Sullivan KF, Johnson EF. LKM-1 autoantibodies recognize a short linear sequence in P450IID6, a cytochrome P-450 monooxygenase. J Clin Invest 1991;88:1370–8.
[58] Senaldi G, Portmann B, Mowat AP, Mieli-Vergani G, Vergani D. Immunohistochemical features of the portal tract mononuclear cell infiltrate in chronic aggressive hepatitis. Arch Dis Child 1992;67:1447–53.
[59] Lobo-Yeo A, Senaldi G, Portmann B, Mowat AP, Mieli-Vergani G, Vergani D. Class I and class II major histocompatibility complex antigen expression on hepatocytes: a study in children with liver disease. Hepatology 1990;12:224–32.
[60] Senaldi G, Lobo-Yeo A, Mowat AP, Mieli-Vergani G, Vergani D. Class I and class II major histocompatibility complex antigens on hepatocytes: importance of the method of detection and expression in histologically normal and diseased livers. J Clin Pathol 1991;44:107–14.
[61] Miossec P, Korn T, Kuchroo VK. Interleukin-17 and type 17 helper T cells. N Engl J Med 2009;361:888–98.
[62] Korn T, Bettelli E, Oukka M, Kuchroo VK. IL-17 and Th17 cells. Annu Rev Immunol 2009;27:485–517.
[63] Harada K, Shimoda S, Sato Y, Isse K, Ikeda H, Nakanuma Y. Periductal interleukin-17 production in association with biliary innate immunity contributes to the pathogenesis of cholangiopathy in primary biliary cirrhosis. Clin Exp Immunol 2009; 157:261–70.
[64] Zhao L, Tang Y, You Z, et al. Interleukin-17 contributes to the pathogenesis of autoimmune hepatitis through inducing hepatic interleukin-6 expression. PLoS One 2011;6:e18909.
[65] Jensen DM, McFarlane IG, Portmann BS, Eddleston AL, Williams R. Detection of antibodies directed against a liver-specific membrane lipoprotein in patients with acute and chronic active hepatitis. N Engl J Med 1978;299:1–7.
[66] McFarlane BM, McSorley CG, Vergani D, McFarlane IG, Williams R. Serum autoantibodies reacting with the hepatic asialoglycoprotein receptor protein (hepatic lectin) in acute and chronic liver disorders. J Hepatol 1986; 3:196–205.
[67] Vergani GM, Vergani D, Jenkins PJ, et al. Lymphocyte cytotoxicity to autologous hepatocytes in HBsAg-negative chronic active hepatitis. Clin Exp Immunol 1979;38:16–21.
[68] Vergani D, Mieli-Vergani G, Mondelli M, Portmann B, Eddleston AL. Immunoglobulin on the surface of isolated hepatocytes is associated with antibody-dependent cell-mediated cytotoxicity and liver damage. Liver 1987;7: 307–15.
*[69] Ma Y, Okamoto M, Thomas MG, et al. Antibodies to conformational epitopes of soluble liver antigen define a severe form of autoimmune liver disease. Hepatology 2002;35:658–64.
[70] Muratori L, Parola M, Ripalti A, Robino G, Muratori P, Bellomo G. Liver/kidney microsomal antibody type 1 targets CYP2D6 on hepatocyte plasma membrane. Gut 2000;46:553–61.
[71] Ma Y, Thomas MG, Okamoto M, et al. Key residues of a major cytochrome P4502D6 epitope are located on the surface of the molecule. J Immunol 2002;169:277–85.
[72] Wen L, Peakman M, Lobo-Yeo A, et al. T-cell-directed hepatocyte damage in autoimmune chronic active hepatitis. Lancet 1990;336:1527–30.
[73] Wen L, Ma Y, Bogdanos DP, et al. Pediatric autoimmune liver diseases: the molecular basis of humoral and cellular immunity. Curr Mol Med 2001;1:379–89.
*[74] Lohr H, Manns M, Kyriatsoulis A, et al. Clonal analysis of liver-infiltrating T cells in patients with LKM-1 antibody-positive autoimmune chronic active hepatitis. Clin Exp Immunol 1991;84:297–302.
[75] Kreslavsky T, Gleimer M, Garbe AI, von Boehmer H. alphabeta versus gammadelta fate choice: counting the T-cell lineages at the branch point. Immunol Rev 2010;238:169–81.

[76] Wen L, Peakman M, Mieli-Vergani G, Vergani D. Elevation of activated gamma delta T cell receptor bearing T lymphocytes in patients with autoimmune chronic liver disease. Clin Exp Immunol 1992;89:78–82.
[77] Lapierre P, Djilali-Saiah I, Vitozzi S, Alvarez F. A murine model of type 2 autoimmune hepatitis: Xenoimmunization with human antigens. Hepatology 2004;39:1066–74.
[78] Holdener M, Hintermann E, Bayer M, Rhode A, Rodrigo E, Hintereder G. Breaking tolerance to the natural human liver autoantigen cytochrome P450 2D6 by virus infection. J Exp Med 2008;205:1409–22.

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Autoimmune Hepatitis After Liver Transplantation

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Liver transplantation is an effective treatment for patients with end-stage acute and chronic autoimmune hepatitis. However, despite the good outcomes reported, disease recurrence is relatively common in the allograft. In addition, autoimmunity and autoimmune liver disease can arise de novo after transplantation for non-autoimmune liver disorders. Little is known about the mechanisms by which autoimmune diseases develop after liver transplantation, but genetic factors, molecular mimicry, impaired regulatory T-cell responses, and exposures to new alloantigens might be involved. Regardless of the pathogenic mechanisms, it is important to remain aware of the existence of recurrent and de novo autoimmune hepatitis after liver transplantation; these disorders are similar to classic autoimmune hepatitis and are therefore not treated with standard antirejection strategies.

Keywords: Autoimmune Hepatitis; Liver Transplantation; Recurrent Autoimmune Hepatitis; De Novo Autoimmune Hepatitis.

Liver transplantation (LT) is a standard therapeutic approach for end-stage acute and chronic liver diseases. It is the best treatment option for selected patients with end-stage liver disease caused by autoimmune hepatitis (AIH).[1–3] In these patients the current 5-year post-LT survival rate is 80%–90%.[4] Transplant recipients, however, can experience various complications, including chronic rejection, recurrence of primary disease, chronic hepatitis (recurrent or acquired viral hepatitis and idiopathic post-transplant hepatitis), cancer, and intrahepatic and/or extrahepatic autoimmunity.[5] AIH can recur after LT,[6,7] and AIH and autoimmunity can also arise de novo after LT for non-autoimmune liver diseases.[7,8]

Transplantation of organs between individuals that are not genetically identical can cause a T-cell–mediated immune response that leads to rejection and graft destruction.[9] The level of the alloimmune response is determined by the disparity of polymorphic antigens between individuals. Reactivity against nonpolymorphic antigens shared by individuals of the same species and transferred with the graft should be the exception,[8] yet autoimmunity and autoimmune liver disease do occur after LT.

After the initial description of recurrent AIH in a patient who received a liver allograft in 1984[6] and a subsequent report proposing that AIH can arise de novo after LT,[10] several advances have been made in the diagnosis and management of these conditions. However, the pathogenesis of de novo AIH is not known. We review the latest developments in determining the diagnosis and clinical course and treating these conditions; we then speculate about the pathogenic mechanisms of autoimmunity and autoimmune liver disease after LT.

Recurrence of Autoimmune Hepatitis

AIH accounts for 2%–3% of pediatric and 4%–6% of adult LTs performed in Europe and the United States.[3] LTs are performed on patients with AIH who present with fulminant hepatic failure, on the 10%–15% who progress to end-stage chronic liver disease despite immunosuppressive treatment, and on those with hepatocellular carcinoma who meet transplant criteria.[2,11] A combination of prednisolone and a calcineurin inhibitor is the most common immunosuppressive regimen used after LT[3]; outcomes are good, with reported 5-year and 10-year patient survival of 80%–90% and 75%, respectively.[4] Despite the use of immunosuppressive drugs after LT, AIH can recur.

The recurrence rate of AIH in transplant allografts varies. It ranges from 12%–46%, depending on diagnostic criteria, immunosuppressive regimens, length of follow-up, and performance of per protocol biopsies.[12–29] Recurrence has been reported in adult and pediatric[14] populations, with a mean time to recurrence of 4.6 years.[3] Although it can occur as early as 35 days after LT,[19] the rate seems to increase with the postsurgery interval. Table 1 summarizes reports on AIH recurrence.

Recurrence of AIH is diagnosed on the basis of several features (Table 2) because there is no specific marker; these include reappearance of clinical symptoms and signs, detection of autoantibodies, interface hepatitis based on histologic analysis, increased levels of transaminases and immunoglobulin G, and response to prednisolone and azathioprine.[3,24] Histologic changes can precede clinical and biochemical evidence of recurrence.[22] These diagnostic criteria for AIH are the same as those used in the International Autoimmune Hepatitis Group (IAIHG) scoring system[30–32] for patients who have not received LT, but the criteria have not been validated in patients who received allografts.

There are no consistent predictors of AIH recurrence, but some factors that confer predisposition have been reported. Although not confirmed in all patients, possession by the re-

Abbreviations used in this paper: ACR, acute cellular rejection; AIH, autoimmune hepatitis; ANA, antinuclear antibody; APC, antigen-presenting cell; GSTT1, glutathione-S-tranferase-T1; GVHD, graft-versus-host disease; HCV, hepatitis C virus; IAIHG, International Autoimmune Hepatitis Group; LKM-1, liver kidney microsomal antibody type-1; LT, liver transplantation; MHC, major histocompatibility complex; SMA, anti–smooth muscle antibody.

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Table 1. Reports on AIH Recurrence

Author (reference)	Year	Age (LT)[a]	AIH no.[b]	Recurrence, no. (%)[c]
Wright et al[12]	1992	Adult	43	11 (25.5)
Birnbaum et al[14]	1997	Pediatric	6	5 (83.3)
Prados et al[15]	1998	Adult	27	9 (33.3)
Ratziu et al[16]	1999	Adult	15	3 (20)
Milkiewicz et al[18]	1999	Adult	47	13 (27.7)
Narumi et al[29]	1999	Adult	40	5 (12)
Reich et al[11]	2000	Adult	32	6 (18.8)
Ayata et al[19]	2000	Adult	12	5 (41.7)
Gonzalez-Koch et al[21]	2001	Adult	41	7 (17.1)
Duclos-Vallée et al[22]	2003	Adult	17	7 (41.2)
Vogel et al[23]	2004	Adult	28	5 (17.9)
Campsen et al[26]	2008	Adult	66	23 (34.8)

[a]Age at liver transplantation.
[b]Number of patients transplanted for AIH.
[c]Number (and percentage) of patients with recurrent AIH after liver transplantation.

cipient of HLA-DR3 or HLA-DR4 has been associated with risk for recurrence.[12,21] Caution should be exercised in weaning patients off immunosuppressive therapy, because recurrence has been associated with discontinuation of corticosteroids.[15,33,34] Also, adherence to corticosteroid medication is as important in achieving remission of AIH as it is in maintaining good graft function after LT.[35,36] Physicians should watch carefully for nonadherence, particularly among adolescents and young adults, in whom corticosteroid-related side effects can have a devastating psychological impact, and address it promptly.

Despite the fact that type-2 AIH is associated with a more aggressive course of disease, a review of 89 cases of AIH who underwent LT reported a recurrence rate 7-fold higher in patients with type-1 AIH than in those with type-2 AIH.[20,37] Ayata et al[19] found that severe necroinflammatory activity in the native liver at the time of LT predicted AIH recurrence. In the same year, Reich et al[11] reported that AIH was less likely to recur in patients who initially presented with fulminant hepatic failure than in those who had chronic disease. These data led them to suggest that patients with chronic AIH who require LT have a propensity for resistance to immunosuppression, whereas patients with fulminant hepatitis who have never received immunosuppressive therapy are likely to respond. Other risk factors that were analyzed (pretransplant disease duration, sex of donors and recipients, and rejection episodes) did not correlate with AIH recurrence. In contradiction to studies that associated tacrolimus with development of recurrent AIH,[17,19] a systematic review reported that primary immunosuppression with either cyclosporine or tacrolimus did not influence risk of recurrence.[38]

Most transplant recipients with recurrent AIH respond to reintroduction or an increase in dose of corticosteroids and azathioprine, which should be implemented as soon as the diagnosis is made,[7,15,21] but recurrent AIH can follow an immunosuppressive-unresponsive course, ultimately leading to graft loss.[14,39] These patients can be treated with a combination of mycophenolate mofetil and the standard therapeutic regimen,[3] a replacement of tacrolimus with cyclosporine,[40] or a replacement of the calcineurin inhibitor with sirolimus.[41] Successful management of recurrent AIH relies on early diagnosis. Because histologic evidence can precede clinical evidence of recurrence, biopsy testing might be included in the protocol for management of patients with AIH.[22]

The consequences of recurrent disease on graft function are controversial. Some studies associated recurrent AIH with an aggressive course of disease after LT, reporting a higher rate of progression to cirrhosis, graft failure, and need for retransplantation.[37] These studies also found a high rate of re-recurrence after a second transplantation. In a retrospective study of the effects of disease recurrence on graft survival among all adult patients who underwent transplantation, Rowe et al[27] reported that the risk for graft loss after disease recurrence was higher among patients with AIH than patients with primary biliary cirrhosis. However, they observed a lower rate of recurrence and absence of graft loss caused by recurrent AIH among patients who received liver transplants from 2000–2004, compared with those who received them before 2000. The authors speculated that increased awareness of the existence of this condition, along with the maintenance of corticosteroid therapy in patients who received transplants for AIH, improved patient outcomes.[27] Therefore, weaning patients who received liver transplants for AIH off corticosteroids should be done with caution because it might increase the risk for recurrence and worsen the outcome.

Autoantibodies

Patients who received liver transplants for non-autoimmune liver diseases can develop autoantibodies.[10,42] Antinuclear antibodies (ANAs) are most frequently reported, followed by anti–smooth muscle antibodies (SMAs). An atypical form of the liver-kidney microsomal antibody type-1 (LKM-1) has also been detected in these patients; this antibody has been reported to react with an unidentified cytosolic antigen instead of the microsomal liver fraction that contains cytochrome P4502D6, the target of LKM-1 in classic AIH type 2.[10,43]

The reported prevalence of autoantibodies in patients who received LT varies. Dubel et al[42] detected autoantibodies in 71% of adult patients who received transplants for non-autoimmune liver disease during a follow-up period of at least 6 months, compared with 30% of cirrhotic patients who did not receive liver transplants (controls). In a study by Riva et al,[44] 24.3% of 247 children who received transplants for non-autoimmune liver disease developed autoantibodies. The probabilities for the new appearance of autoantibodies at 6, 12, and 36 months after liver transplantation were calculated to be 23%, 42%, and 66%, respectively.[45]

The presence of autoantibodies has been associated with poor outcomes after LT, including development of chronic hepatitis, severe graft dysfunction, chronic rejection, loss of

Table 2. Diagnosis of Recurrent AIH

Liver transplant for autoimmune hepatitis
Elevation of transaminases
Interface hepatitis
Elevation of immunoglobulin G
Presence of autoantibodies (ANA, SMA, and/or anti–LKM-1)
Corticosteroid dependency
Exclusion of other causes of graft dysfunction

graft, and death.[10,42–47] Autoantibodies were also associated with graft dysfunction from de novo AIH. Positivity for autoantibodies on its own, however, is not sufficient to diagnose de novo AIH, because autoantibodies have been detected in animal models of LT when liver function test results were normal,[48] in pediatric patients with stable liver function after LT,[49] and in adults who underwent chronic rejection.[42] Dubel et al[42] correlated the appearance of AIH-associated autoantibodies with chronic rejection of liver allografts. In this study, 71% of patients who underwent chronic rejection had positive test results for ANA and/or SMA, whereas only 7% of patients without chronic rejection tested positive for these antibodies. Although the treatment for chronic rejection was not specified in the report, 8 of 10 autoantibody-positive patients required retransplantation,[42] indicating that the presence of autoantibodies was associated with poor outcome. In a pediatric study by Riva et al,[44] 22 of 60 patients (37%) who had autoantibodies experienced graft dysfunction, including 9 who were diagnosed with de novo AIH and 13 with early chronic rejection. Although de novo AIH improved after standard treatment for AIH, the early chronic rejection group had a good response to increased doses of a calcineurin inhibitor. The authors concluded that an early differential diagnosis of these conditions in patients who test positive for autoantibodies after LT and appropriate treatment can improve overall results, which was demonstrated by a reported 90% graft survival. Richter et al[50] reported the presence of autoantibodies in 74% of children who received LT but a diagnosis of de novo AIH in only 1%. Although autoantibodies are frequently detected after LT and their presence, per se, cannot be used to diagnose de novo AIH or rejection, patients should be monitored regularly and tested for abnormal liver function. Autoantibody seropositivity and high levels of immunoglobulin G and transaminase indicate a need for prompt histologic assessment.

De Novo Autoimmune Hepatitis

Post-transplant de novo AIH was initially described in 1998 among patients who received liver transplants for disorders other than AIH (Table 3). In the first report, during a 5-year period, 4% of 180 children who underwent LT developed a form of graft dysfunction with features identical to those of classic AIH, including hypergammaglobulinemia; positive test results for ANA, SMA, a gastric parietal cell antibody, and atypical LKM-1; and histologic features of chronic hepatitis, with portal and periportal inflammation (Figure 1).[10] The index case did not respond to the standard antirejection regimen, but only to the standard treatment for AIH. None of the children received transplants for autoimmune liver disease or were infected with hepatitis C virus (HCV), and all had serum concentrations of the calcineurin inhibitor that were within therapeutic, antirejection range at the time of diagnosis with de novo AIH.[10] Since that report, several other groups have reported occurrences of de novo AIH after LT. Its prevalence in children ranges from 2.35%–6.2%[47,49,51–54]; reported indications for LT include extrahepatic biliary atresia, Alagille syndrome, acute liver failure, α_1-antitrypsin deficiency, primary familial intrahepatic cholestasis, primary sclerosing cholangitis, and Budd–Chiari syndrome.[10,47,49,51–54]

Table 3. Defining Features of De Novo AIH

Elevation of transaminases
Interface hepatitis
Elevation of immunoglobulin G
Presence of autoantibodies in decreasing frequency
Anti-smooth muscle
Anti-nuclear
Anti-LKM typical
Anti-LKM atypical (only kidney staining)
Anti-mitochondrial
Exclusion of other causes of graft dysfunction
Response to treatment according to protocol used for AIH

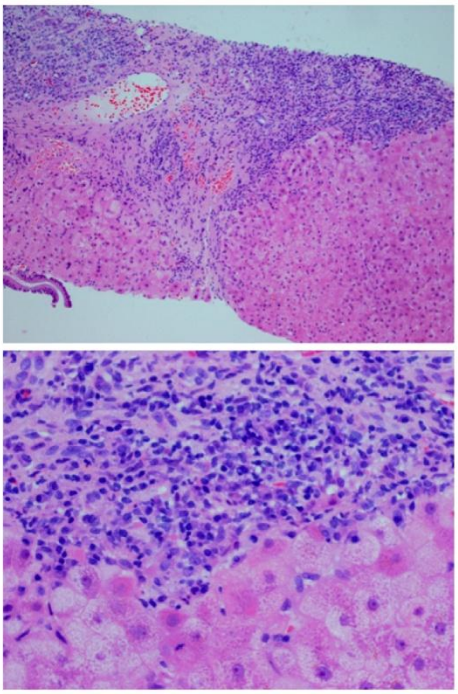

Figure 1. De novo AIH. The portal tract is densely infiltrated by mononuclear cells, with a clear presence of plasma cells, that invade the parenchyma, disrupting the limiting plate (Picture provided by Dr Yoh Zen, Institute of Liver Studies, King's College Hospital).

Miyagawa-Hayashino et al[53] reported that de novo AIH is a complication among recipients of livers (all younger than 18 years old) from living donors. Thirteen patients developed a form of graft dysfunction with serologic and histologic features of AIH at a median of 3.1 years after LT. In the largest and only case-control study of children published, 41 of the 788 patients (5.2%) who underwent LT developed de novo AIH; all had histologic features of classic AIH, and 24 (59%) tested positive for ANA and 25 (61%) for anti–double-stranded DNA at the time of diagnosis.[54]

Adults develop de novo AIH, although with lower prevalence than children. The original indications for LT were primary

sclerosing cholangitis, primary biliary cirrhosis, alcoholic cirrhosis, hepatitis C–associated cirrhosis, Wilson disease, and acute liver failure.[43,55-57] In a retrospective study, graft dysfunction similar to that observed in AIH was described in 7 of more than 1000 adult patients at a median time of 4.3 years after LT.[55] All patients fulfilled IAIHG criteria for either definite or probable AIH and responded to treatment with prednisolone and azathioprine. Salcedo et al[43] reported 12 cases of de novo AIH (3.4% of 350 adults who received LT).

De novo AIH is diagnosed by using IAIHG scoring systems that have not been validated in patients who underwent LT. When applied to de novo AIH, however, most published cases were scored as probable or definite AIH. Because autoantibodies can be detected in patients with de novo AIH or allograft rejection, liver biopsy is important for the differential diagnosis. Histologically, de novo AIH is similar to classic AIH, with presence of interface hepatitis and infiltration of plasma cells.[37]

As physicians have become aware that treatment with prednisolone alone or in combination with azathioprine or mycophenolate mofetil by using a protocol similar to that for standard AIH is successful for patients with de novo AIH, there have been improvements in graft and patient survival. Children should be given a starting dose of 1–2 mg/kg predniso(lo)ne, without exceeding a daily dose of 60 mg, in combination with azathioprine (1–2 mg/kg); the steroid dose should then be reduced during 4–8 weeks to a maintenance dose of 5–10 mg per day, depending on age and weight. Adults are generally given an initial dose of 30 mg predniso(lo)ne, combined with 1–2 mg of azathioprine per day. The dose of steroid is reduced according to response to a maintenance dose of 5–10 mg daily. In the absence of response, azathioprine should be substituted with mycophenolate mofetil (up to 40 mg/kg/d in 2 divided daily doses for children; 1 g twice daily for adults). The importance of maintenance therapy with steroids was shown in a study that compared treatment with and without steroids; all the patients who did not receive steroids developed cirrhosis and either died or required retransplantation, whereas none of the steroid-treated patients had progressive disease.[43] However, some studies have reported poor outcomes among patients who received steroid-based treatment.[51]

Several studies have aimed to identify risk factors for the development of de novo AIH. In the first study, 5 of the 7 children received livers from donors who were HLA-DR3 or HLA-DR4 positive.[10] In adults, Heneghan et al[55] detected HLA-DR3 or HLA-DR4 in all donors and recipients, and Salcedo et al[43] reported an over-representation of HLA-DR3 among recipients. However, larger studies are needed to establish the immunogenetic influence on the development of de novo AIH. In an attempt to define possible risk factors, Miyagawa-Hayashino et al[53] observed that of 69% of patients, at least 1 had had an episode of acute cellular rejection (ACR) before developing de novo AIH. However, other studies reported that de novo AIH was preceded by ACR in only 20%–50% of patients.[47,52,55] In a matched case-control study, Venick et al[54] found that previous episodes of ACR and steroid dependence were risk factors for the development of pediatric de novo AIH.

More recently, a high percentage of patients who received LT for HCV-related liver diseases were reported to develop de novo AIH. Berardi et al[58] reported that 9 patients receiving antiviral therapy (pegylated interferon-alpha-2b and ribavirin) for recurrent HCV infection developed de novo AIH. Despite HCV clearance and exclusion of concomitant infections or complications, these patients developed laboratory and histologic characteristics of AIH. Moreover, 3 developed other definite autoimmune disorders (autoimmune thyroiditis, antimitochondrial positive overlap syndrome, and systemic lupus erythematosus). In contrast, Fiel et al[59] identified 38 patients who received LT for HCV and had liver infiltration by plasma cells similar to that associated with de novo AIH. This occurred, however, after doses of immunosuppressive medications were lowered to subtherapeutic levels, so it might have resulted from graft dysfunction and allograft rejection instead of de novo AIH. Nevertheless, occurrence of de novo AIH in patients who received LT for HCV-related liver disease has been confirmed in other studies.[60-63] Because de novo AIH seems to be related to antiviral therapy, it is important to decide whether to increase doses of immunosuppressive medications when managing HCV-infected patients. Although addition of corticosteroids to therapy could reduce the severity of liver inflammation, it could increase viral replication, resulting in progressive HCV-related fibrosis and reduced viral clearance.[63]

Pathogenic Mechanisms

Although little is known about the pathogenesis of de novo AIH, the mechanisms of recurrence of AIH after LT are understood (Figure 2). The transplant recipient's immune system is sensitized to species-specific antigens and has a pool of memory T cells that are restimulated and re-expanded when antigens (and autoantigens) are presented, either by the recipient's antigen-presenting cells (APCs), which repopulate the grafted liver, or by the donor's APCs, which share histocompatibility antigens with the recipient.[7]

There are several pathways by which autoimmunity and AIH can arise, de novo, in patients who underwent LT for non-autoimmune conditions (Figure 3, Table 4). In addition to release of autoantigens from the damaged tissue, molecular mimicry (an initial immune response toward a non-self antigen that became directed toward a structurally similar self-antigen) might also be involved.[64,65] Salcedo et al[43] reported that all patients who developed de novo AIH had been infected with cytomegalovirus, Epstein-Barr virus, or parvovirus. Viral infections, which occur frequently after LT, can induce autoimmunity by other mechanisms, including polyclonal stimulation of immune cells, increasing the density of major histocompatibility complex (MHC) class I antigens on target cells and MHC class II antigens on immune cells and interfering with immunoregulatory cells.[7,66]

In animal studies, a calcineurin inhibitor interfered with the maturation of T cells and the function of regulatory T (Treg) cells, which resulted in emergence and activation of autoaggressive T-cell clones.[66-70] Calcineurin inhibitor–associated autoimmunity was described in animals whose immune system development was disrupted during the neonatal period by irradiation. Thus, patients who received LT and were treated with immunosuppressants such as prednisolone might be predisposed to autoimmunity through the effects of calcineurin inhibitors. Cyclosporine blocks activation-induced death of effector T cells and interferes with tolerance induction by costimulatory blockade.[71] Calcineurin inhibitors also reduce production of interleukin-2,[72] which is required for survival and proliferation of Treg cells[73]; absence of interleukin-2 is associated with impaired suppressor function. Zheng et al[74] reported

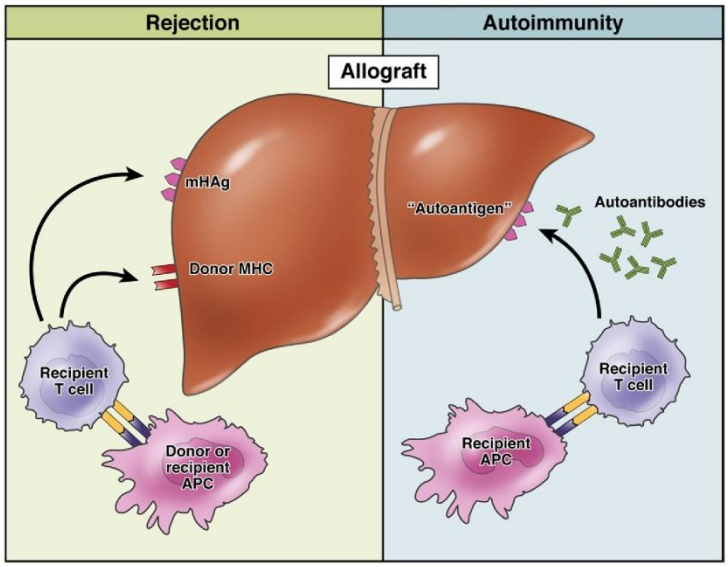

Figure 2. In recurrent AIH, the recipient's immune system is sensitized to species-specific antigens and has a pool of memory cells that are restimulated and re-expanded after presentation of antigens (or autoantigens) by the recipient's APCs, which repopulate the grafted liver. In rejection, the targets are primarily MHC molecules or minor histocompatibility antigens. APCs that promote rejection are of either donor or recipient origin; whereas the former are present in the graft as passenger leukocytes that cause direct activation of the recipient's T cells, the latter are located in draining lymphoid tissues, acquiring antigens that are shed from the allograft and presenting them to the recipient's T cells, causing their indirect activation.

that cyclosporine blocked the effects of donor-specific transfusion with anti-CD40 ligand (CD154) monoclonal antibody on Treg cell function and allograft tolerance; administration of a stable form of interleukin-2 reversed the effects of cyclosporine on Treg cell function.

In animal models, administration of cyclosporine after syngeneic or autologous bone marrow transplantation elicited a T-cell–dependent autoimmune disease that resembled graft-versus-host-disease (GVHD), termed syngeneic GVHD.[75] This autoaggression appeared to result from recognition of self-

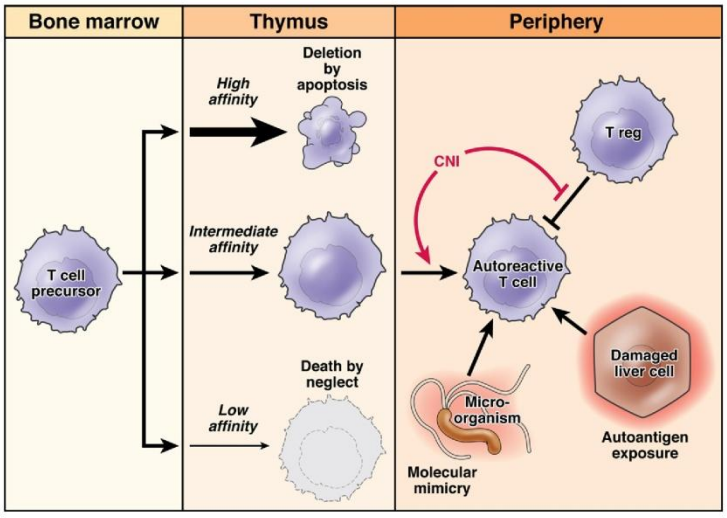

Figure 3. Bone marrow–derived T-cell precursors enter the thymus and are eliminated if their affinity for self is either too high or too low. Cells with an intermediate affinity for self are permitted into the periphery, where they are controlled by thymus-derived regulatory (Treg) cells. After liver transplantation, the release of self-antigens from the liver cell or an infection with microorganisms that are structurally similar to self activates quiescent autoreactive T cells. This process is facilitated by the administration of calcineurin inhibitors (CNI) such as cyclosporine and tacrolimus, which would promote efflux of self-reactive precursors from the thymus and impair the function of Treg cells.

Table 4. Hypotheses for the Pathogenesis of De Novo AIH

Molecular mimicry
Effect of calcineurin inhibitors in the maturation of T cells
Impairment of regulatory T cells
Mismatch for glutathione-S-tranferase-T1 (GSTT1)

MHC class II antigens by T cells. Monoclonal antibodies against MHC class II determinants prevented the adoptive transfer of syngeneic GVHD.[76] These experiments support the concept that calcineurin inhibitors have different effects in individuals after LT. Although calcineurin inhibitor–based immunosuppression is used to prevent rejection, in genetically predisposed individuals, it might induce or promote autoreactivity. Because children have immature immune systems (active thymus and immature T-cell–receptor repertoires) and develop many primary infections, they could be more vulnerable to develop autoimmunity as a consequence of calcineurin inhibitor therapy.[8] This would account for the higher incidence of de novo AIH among pediatric patients.

In addition to the negative effects of calcineurin inhibitors on Treg-cell functions, Treg-cell numbers have been reported to be reduced in recipients of LT who experienced ACR.[77] Reduced numbers and functions of Treg cells are inversely correlated with production of autoantibodies such as anti-soluble liver antigen and LKM-1 in patients with classic AIH.[78] Reduced function of Treg cells, from calcineurin inhibitor therapy or ACR, could perpetuate an autoimmune response and development of de novo AIH.

Some patients with de novo AIH have antibodies against glutathione-S-tranferase-T1 (GSTT1),[79] a drug-metabolizing enzyme that is produced in large quantities in liver and kidney cells. Twenty percent of whites do not carry the gene *GSTT1*. Organ transplantation between a donor who carries the gene and a recipient who does not could therefore cause production of antibodies against GSTT1.[80] *GSTT1* donor–recipient mismatch and the presence of anti-GSTT1 antibodies might be risk factors for the development of de novo AIH.[81] However, studies reported that lack of *GSTT1* and anti-GSTT1 antibodies did not account for the development of de novo AIH in most patients, and that mechanisms other than *GSTT1* mismatch could be involved.[46,82,83]

Conclusions

Although LT is a successful treatment for end-stage liver disease caused by AIH, primary disease recurs in about 30% of patients. Awareness of its existence and appropriate management have reduced the frequency and improved outcomes of AIH. It is important to be cautious in reducing dose of immunosuppressive therapy in patients who received LT for AIH because discontinuation of corticosteroid is a risk factor for recurrent disease. AIH can arise de novo in patients who received LT for disorders other than AIH. It is not clear whether de novo AIH is a distinct entity or a form of atypical rejection in individuals who are at risk for autoimmunity, leading to the use of different terminology. However, patients who develop de novo AIH do not have a satisfactory response to standard antirejection regimens, but they do respond to the standard treatment for AIH (steroids and azathioprine), in combination with a low dose of calcineurin inhibitor. This management strategy is graft-saving and life-saving. Therefore, as for recurrent disease, increased awareness of the existence and management of de novo AIH, regardless of the term used to describe it, could lead to earlier diagnoses and better patient outcomes.

References

1. Sanchez-Urdazpal L, Czaja AJ, van Hoek B, et al. Prognostic features and role of liver transplantation in severe corticosteroid-treated autoimmune chronic active hepatitis. Hepatology 1992; 15:215–221.
2. Mottershead M, Neuberger J. Transplantation in autoimmune liver diseases. World J Gastroenterol 2008;14:3388–3395.
3. Manns MP, Czaja AJ, Gorham JD, et al. Diagnosis and management of autoimmune hepatitis. Hepatology 2010;51:2193–2213.
4. Krawitt EL. Autoimmune hepatitis. N Engl J Med 2006;354:54–66.
5. Hubscher SG. What is the long-term outcome of the liver allograft? J Hepatol 2011;55:702–707.
6. Neuberger J, Portmann B, Calne R, et al. Recurrence of autoimmune chronic active hepatitis following orthotopic liver grafting. Transplantation 1984;37:363–365.
7. Vergani D, Mieli-Vergani G. Autoimmunity after liver transplantation. Hepatology 2002;36:271–276.
8. Czaja AJ. Autoimmune hepatitis after liver transplantation and other lessons of self-intolerance. Liver Transplant 2002;8:505–513.
9. Sánchez-Fueyo A, Strom TB. Immunologic basis of graft rejection and tolerance following transplantation of liver or other solid organs. Gastroenterology 2011;140:51–64.
10. Kerkar N, Hadzić N, Davies ET, et al. De-novo autoimmune hepatitis after liver transplantation. Lancet 1998;351:409–413.
11. Reich DJ, Fiel I, Guarrera JV, et al. Liver transplantation for autoimmune hepatitis. Hepatology 2000;32:693–700.
12. Wright HL, Bou-Abboud CF, Hassanein T, et al. Disease recurrence and rejection following liver transplantation for autoimmune chronic active liver disease. Transplantation 1992;53: 136–139.
13. Devlin J, Donaldson P, Portmann B, et al. Recurrence of autoimmune hepatitis following liver transplantation. Liver Transplant Surg 1995;1:162–165.
14. Birnbaum AH, Benkov KJ, Pittman NS, et al. Recurrence of autoimmune hepatitis in children after liver transplantation. J Pediatr Gastroenterol Nutr 1997;25:20–25.
15. Prados E, Cuervas-Mons V, de la Mata M, et al. Outcome of autoimmune hepatitis after liver transplantation. Transplantation 1998;66:1645–1650.
16. Ratziu V, Samuel D, Sebagh M, et al. Long-term follow-up after liver transplantation for autoimmune hepatitis: evidence of recurrence of primary disease. J Hepatol 1999;30:131–141.
17. Götz G, Neuhaus R, Bechstein WO, et al. Recurrence of autoimmune hepatitis after liver transplantation. Transplant Proc 1999; 31:430–431.
18. Milkiewicz P, Hubscher SG, Skiba G, et al. Recurrence of autoimmune hepatitis after liver transplantation. Transplantation 1999;68:253–256.
19. Ayata G, Gordon FD, Lewis WD, et al. Liver transplantation for autoimmune hepatitis: a long-term pathologic study. Hepatology 2000;32:185–192.
20. Manns MP, Bahr MJ. Recurrent autoimmune hepatitis after liver transplantation: when non-self becomes self. Hepatology 2000; 32:868–870.
21. Gonzalez-Koch A, Czaja AJ, Carpenter HA, et al. Recurrent autoimmune hepatitis after orthotopic liver transplantation. Liver Transplant 2001;7:302–310.
22. Duclos-Vallée JC, Sebagh M, Rifai K, et al. A 10 year follow up study of patients transplanted for autoimmune hepatitis: histo-

logical recurrence precedes clinical and biochemical recurrence. Gut 2003;52:893–897.
23. Vogel A, Heinrich E, Bahr MJ, et al. Long-term outcome of liver transplantation for autoimmune hepatitis. Clin Transpl 2004;18: 62–69.
24. Demetris AJ, Adeyi O, Bellamy CO, et al. Liver biopsy interpretation for causes of late liver allograft dysfunction. Hepatology 2006;44:489–501.
25. Kotlyar DS, Campbell MS, Reddy KR. Recurrence of diseases following orthotopic liver transplantation. Am J Gastroenterol 2006;101:1370–1378.
26. Campsen J, Zimmerman MA, Trotter JF, et al. Liver transplantation for autoimmune hepatitis and the success of aggressive corticosteroid withdrawal. Liver Transplant 2008;14:1281–1286.
27. Rowe IA, Webb K, Gunson BK, et al. The impact of disease recurrence on graft survival following liver transplantation: a single centre experience. Transpl Int 2008;21:459–465.
28. Schramm C, Bubenheim M, Adam R, et al. Primary liver transplantation for autoimmune hepatitis: a comparative analysis of the European Liver Transplant Registry. Liver Transplant 2010; 16:461–469.
29. Narumi S, Hakamada K, Sasaki M, et al. Liver transplantation for autoimmune hepatitis: rejection and recurrence. Transplant Proc 1999;31:1955–1956.
30. Johnson PJ, McFarlane IG. Meeting report: International Autoimmune Hepatitis Group. Hepatology 1993;18:998–1005.
31. Alvarez F, Berg PA, Bianchi FB, et al. International Autoimmune Hepatitis Group report: review of criteria for diagnosis of autoimmune hepatitis. J Hepatol 1999;31:929–938.
32. Hennes EM, Zeniya M, Czaja AJ, et al. Simplified criteria for the diagnosis of autoimmune hepatitis. Hepatology 2008;48:169–176.
33. Sempoux C, Horsmans Y, Lerut J, et al. Acute lobular hepatitis as the first manifestation of recurrent autoimmune hepatitis after orthotopic liver transplantation. Liver 1997;17:311–315.
34. Czaja AJ. The immunoreactive propensity of autoimmune hepatitis: is it corticosteroid-dependent after liver transplantation? Liver Transplant Surg 1999;5:460–463.
35. Shemesh E, Shneider BL, Savitzky JK, et al. Medication adherence in pediatric and adolescent liver transplant recipients. Pediatrics 2004;113:825–832.
36. Kerkar N, Annunziato RA, Foley L, et al. Prospective analysis of nonadherence in autoimmune hepatitis: a common problem. J Pediatr Gastroenterol Nutr 2006;43:629–634.
37. Hubscher SG. Recurrent autoimmune hepatitis after liver transplantation: diagnostic criteria, risk factors, and outcome. Liver Transplant 2001;7:285–291.
38. Gautam M, Cheruvattath R, Balan V. Recurrence of autoimmune liver disease after liver transplantation: a systematic review. Liver Transplant 2006;12:1813–1824.
39. Renz JF, Ascher NL. Liver transplantation for nonviral, nonmalignant diseases: problem of recurrence. World J Surg 2002;26: 247–256.
40. Hurtova M, Duclos-Vallee JC, Johanet C, et al. Successful tacrolimus therapy for a severe recurrence of type 1 autoimmune hepatitis in a liver graft recipient. Liver Transplant 2001;7:556–558.
41. Kerkar N, Dugan C, Rumbo C, et al. Rapamycin successfully treats post-transplant autoimmune hepatitis. Am J Transplant 2005;5:1085–1089.
42. Dubel L, Farges O, Johanet C, et al. High incidence of antitissue antibodies in patients experiencing chronic liver allograft rejection. Transplantation 1998;65:1072–1075.
43. Salcedo M, Vaquero J, Bañares R, et al. Response to steroids in de novo autoimmune hepatitis after liver transplantation. Hepatology 2002;35:349–356.
44. Riva S, Sonzogni A, Bravi M, et al. Late graft dysfunction and autoantibodies after liver transplantation in children: preliminary results of an Italian experience. Liver Transplant 2006;12:573–577.
45. Mieli-Vergani G, Vergani D. De novo autoimmune hepatitis after liver transplantation. J Hepatol 2004;40:3–7.
46. Inui A, Sogo T, Komatsu H, et al. Antibodies against cytokeratin 8/18 in a patient with de novo autoimmune hepatitis after living-donor liver transplantation. Liver Transplant 2005;11:504–507.
47. Hernandez HM, Kovarik P, Whitington PF, et al. Autoimmune hepatitis as a late complication of liver transplantation. J Pediatr Gastroenterol Nutr 2001;32:131–136.
48. Dubel L, Farges O, Sato Y, et al. Development of anti-tissue antibodies in the rat liver transplant model. Transplantation 1998;65:1135–1137.
49. Andries S, Casamayou L, Sempoux C, et al. Posttransplant immune hepatitis in pediatric liver transplant recipients: incidence and maintenance therapy with azathioprine. Transplantation 2001;72:267–272.
50. Richter A, Grabhorn E, Helmke K, et al. Clinical relevance of autoantibodies after pediatric liver transplantation. Clin Transpl 2007;21:427–432.
51. Gupta P, Hart J, Millis JM, et al. De novo hepatitis with autoimmune antibodies and atypical histology: a rare cause of late graft dysfunction after pediatric liver transplantation. Transplantation 2001;71:664–668.
52. Spada M, Bertani A, Sonzogni A, et al. A cause of late graft dysfunction after liver transplantation in children: de-novo autoimmune hepatitis. Transplant Proc 2001;33:1747–1748.
53. Miyagawa-Hayashino A, Haga H, Egawa H, et al. Outcome and risk factors of de novo autoimmune hepatitis in living-donor liver transplantation. Transplantation 2004;78:128–135.
54. Venick RS, McDiarmid SV, Farmer DG, et al. Rejection and steroid dependence: unique risk factors in the development of pediatric posttransplant de novo autoimmune hepatitis. Am J Transplant 2007;7:955–963.
55. Heneghan MA, Portmann BC, Norris SM, et al. Graft dysfunction mimicking autoimmune hepatitis following liver transplantation in adults. Hepatology 2001;34:464–470.
56. Jones DE, James OF, Portmann B, et al. Development of autoimmune hepatitis following liver transplantation for primary biliary cirrhosis. Hepatology 1999;30:53–57.
57. Tan CK, Sian Ho JM. Concurrent de novo autoimmune hepatitis and recurrence of primary biliary cirrhosis post-liver transplantation. Liver Transplant 2001;7:461–465.
58. Berardi S, Lodato F, Gramenzi A, et al. High incidence of allograft dysfunction in liver transplanted patients treated with pegylated-interferon alpha-2b and ribavirin for hepatitis C recurrence: possible de novo autoimmune hepatitis? Gut 2007;56:237–242.
59. Fiel MI, Agarwal K, Stanca C, et al. Posttransplant plasma cell hepatitis (de novo autoimmune hepatitis) is a variant of rejection and may lead to a negative outcome in patients with hepatitis C virus. Liver Transplant 2008;14:861–871.
60. Lodato F, Azzaroli F, Tamè MR, et al. G-CSF in Peg-IFN induced neutropenia in liver transplanted patients with HCV recurrence. World J Gastroenterol 2009;15:5449–5454.
61. Ward SC, Schiano TD, Thung SN, et al. Plasma cell hepatitis in hepatitis C virus patients post-liver transplantation: case-control study showing poor outcome and predictive features in the liver explant. Liver Transplant 2009;15:1826–1833.
62. Kontorinis N, Agarwal K, Elhajj N, et al. Pegylated interferon-induced immune-mediated hepatitis post-liver transplantation. Liver Transplant 2006;12:827–830.
63. Selzner N, Guindi M, Renner EL, et al. Immune-mediated complications of the graft in interferon-treated hepatitis C positive liver transplant recipients. J Hepatol 2010;55:207–217.
64. Albert LJ, Inman RD. Molecular mimicry and autoimmunity. N Engl J Med 1999;341:2068–2074.

65. Bogdanos DP, Choudhuri K, Vergani D. Molecular mimicry and autoimmune liver disease: virtuous intentions, malign consequences. Liver 2001;21:225–232.
66. Sakaguchi S, Sakaguchi N. Role of genetic factors in organ-specific autoimmune diseases induced by manipulating the thymus or T cells, and not self-antigens. Rev Immunogenet 2000; 2:147–153.
67. Gao EK, Lo D, Cheney R, et al. Abnormal differentiation of thymocytes in mice treated with cyclosporin A. Nature 1988;336: 176–179.
68. Bucy RP, Xu XY, Li J, et al. Cyclosporin A-induced autoimmune disease in mice. J Immunol 1993;151:1039–1050.
69. Wu DY, Goldschneider I. Cyclosporin A-induced autologous graft-versus-host disease: a prototypical model of autoimmunity and active (dominant) tolerance coordinately induced by recent thymic emigrants. J Immunol 1999;162:6926–6933.
70. Damoiseaux JG, van Breda Vriesman PJ. Cyclosporin A-induced autoimmunity: the result of defective de novo T-cell development. Folia Biol (Praha) 1998;44:1–9.
71. Shi YF, Sahai BM, Green DR. Cyclosporin A inhibits activation-induced cell death in T-cell hybridomas and thymocytes. Nature 1989;339:625–626.
72. Zeiser R, Nguyen VH, Beilhack A, et al. Inhibition of $CD4^+CD25^+$ regulatory T-cell function by calcineurin-dependent interleukin-2 production. Blood 2006;108:390–399.
73. Thornton AM, Donovan EE, Piccirillo CA, et al. Cutting edge: IL-2 is critically required for the in vitro activation of CD4(+)CD25(+) T cell suppressor function. J Immunol 2004;172:6519–6523.
74. Zheng XX, Kang HG, Zhang D, et al. Effects of cyclosporine on transplant tolerance: the role of IL-2. Am J Transplant 2007;7: 1907–1916.
75. Hess AD, Fischer AC. Immune mechanisms in cyclosporine-induced syngeneic graft-versus-host disease. Transplantation 1989;48: 895–900.
76. Hess AD, Horwitz LR, Laulis MK, et al. Cyclosporine-induced syngeneic graft-vs-host disease: prevention of autoaggression by treatment with monoclonal antibodies to T lymphocyte cell surface determinants and to MHC class II antigens. Clin Immunol Immunopathol 1993;69:341–350.
77. Demirkiran A, Kok A, Kwekkeboom J, et al. Low circulating regulatory T-cell levels after acute rejection in liver transplantation. Liver Transplant 2006;12:277–284.
78. Longhi MS, Ma Y, Bogdanos DP, et al. Impairment of CD4(+) CD25(+) regulatory T-cells in autoimmune liver disease. J Hepatol 2004;41:31–37.
79. Aguilera I, Wichmann I, Sousa JM, et al. Antibodies against glutathione S-transferase T1 (GSTT1) in patients with de novo immune hepatitis following liver transplantation. Clin Exp Immunol 2001;126:535–539.
80. Rodriguez-Mahou M, Salcedo M, Fernandez-Cruz E, et al. Antibodies against glutathione S-transferase T1 (GSTT1) in patients with GSTT1 null genotype as prognostic marker: long-term follow-up after liver transplantation. Transplantation 2007;83:1126–1129.
81. Salcedo M, Rodriguez-Mahou M, Rodriguez-Sainz C, et al. Risk factors for developing de novo autoimmune hepatitis associated with anti-glutathione S-transferase T1 antibodies after liver transplantation. Liver Transplant 2009;15:530–539.
82. Yoshizawa K, Shirakawa H, Ichijo T, et al. De novo autoimmune hepatitis following living-donor liver transplantation for primary biliary cirrhosis. Clin Transpl 2008;22:385–390.
83. Grammatikopoulos T, Mytilinaiou MG, Bogdanos DP, et al. Glutathionine S-transferase theta 1 (Gstt1) mismatch does not account for post liver transplant de novo autoimmune hepatitis. Hepatology 2009;50:635a–636a.

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Conflicts of interest

The authors disclose no conflicts.

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The Impaired Immune Regulation of Autoimmune Hepatitis Is Linked to a Defective Galectin-9/Tim-3 Pathway

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In autoimmune hepatitis (AIH), liver-damaging CD4 T cell responses are associated with defective $CD4^{pos}CD25^{pos}$ regulatory T cells (T-regs). Galectin-9 (Gal9), a β-galactosidase–binding protein expressed by T-regs, is key to their function, inhibiting T helper 1 immune responses by binding T cell immunoglobulin and mucin domain 3 (Tim-3) on CD4 effector cells. We investigated whether impaired immunoregulation in AIH results from reduced expression of Gal9 in T-regs and/or Tim-3 on CD4 effector cells. Circulating $Gal9^{pos}CD4^{pos}CD25^{pos}$ and $Tim\text{-}3^{pos}CD4^{pos}CD25^{neg}$ T cell phenotype was assessed by flow cytometry in 75 AIH patients. To evaluate whether Tim-3 expression renders $CD4^{pos}CD25^{neg}$ T cells amenable to T-reg control, purified $CD4^{pos}CD25^{neg}Tim\text{-}3^{pos}$ ($Tim\text{-}3^{pos}$) and $CD4^{pos}CD25^{neg}Tim\text{-}3^{neg}$ ($Tim\text{-}3^{neg}$) cells were cocultured with T-regs. To determine whether Gal9 expression is essential to function, T-regs were treated with small interfering RNA (siRNA) to repress Gal-9 translation; T-reg suppressor function was assessed by proliferation. In AIH, $Tim\text{-}3^{pos}$ cells within $CD4^{pos}CD25^{neg}$ cells and their $T\text{-}bet^{pos}$ and $RORC^{pos}$ subsets were fewer and contained higher numbers of interferon-γ ($IFN\gamma)^{pos}$ and interleukin (IL)-17^{pos} cells than healthy subjects (HS). In AIH and HS, $Tim\text{-}3^{pos}$ cells proliferated less vigorously and were more susceptible to T-reg control than $Tim\text{-}3^{neg}$ cells. In AIH, $Gal9^{pos}$T-regs were fewer and contained less $FOXP3^{pos}$, $IL\text{-}10^{pos}$, and transforming growth factor β^{pos} and more $IFN\gamma^{pos}$ and IL-17^{pos} cells than HS. siRNA treatment of Gal-9^{pos} T-regs drastically reduced T-reg ability to suppress $CD4^{pos}CD25^{neg}$ and $Tim\text{-}3^{pos}$ cell proliferation in AIH and HS. $Tim\text{-}3^{pos}$ cell percentage correlated inversely with aminotransferase and $CD25^{neg}T\text{-}bet^{pos}$ cell values. *Conclusion*: Reduced levels of Tim-3 on $CD4^{pos}CD25^{neg}$ effector cells and of Gal9 in T-regs contribute to impaired immunoregulation in AIH by rendering effector cells less prone to T-reg control and T-regs less capable of suppressing. (HEPATOLOGY 2012;00:000-000)

Autoimmune hepatitis (AIH) is a progressive inflammatory liver disorder characterized by hypergammaglobulinemia, circulating autoantibodies, and histologically by a florid mononuclear cell infiltration referred to as interface hepatitis.[1,2] CD4 effector lymphocytes are the main orchestrators of liver damage in AIH, their proliferation and proinflammatory cytokine secretion (e.g., interferon-γ [IFNγ]) being correlated with the activity and severity of liver disease.[3] We have provided evidence that in AIH, the extent of autoreactive CD4 T cell effector immune responses is associated with a numerical and functional impairment of $CD4^{pos}CD25^{high}$ regulatory T cells (T-regs),[4-6] a lymphocyte subset central to immunotolerance maintenance.[7,8] Because they are defective, T-regs in AIH are unable to control proliferation and effector cytokine production (i.e., IFNγ, interleukin [IL]-17) by responder CD4 T cells stimulated *in vitro* with polyclonal or antigen-specific stimuli.[9]

Whether impaired immunoregulation in AIH is mainly due to a primary T-reg defect or also to low

Abbreviations: AIH, autoimmune hepatitis; AILD, autoimmune liver disease; ASC, autoimmune hepatitis/sclerosing cholangitis; AST, aspartate aminotransferase; Cy, cytochrome; FITC, fluorescein isothiocyanate; Gal9, galectin-9; HS, healthy subject; IFN-γ, interferon-γ; IgG1, immunoglobulin G1; IL, interleukin; mAb, monoclonal antibody; MOG, myelin-oligodendrocyte-glycoprotein; PBMC, peripheral blood mononuclear cell; PE, phycoerythrin; siRNA, small interfering RNA; TGFβ, transforming growth factor β; Th, T helper; T-regs, regulatory T cells; Tim-3, T cell immunoglobulin and mucin domain 3.

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J_ID: ZHE Customer A_ID: 11-2317.R1 Customer Art: ZHE Date: 2-March-12 Stage: Page: 2

responsiveness of CD4 effector cells to T-reg control is unclear. Murine studies have shown that galectin-9 (Gal9), a member of the galectin family expressed by AQ6 T-regs, inhibits T helper 1 (Th1) effector immune responses after binding to the T cell immunoglobulin and mucin domain 3 (Tim-3), its receptor on CD4 effector cells.[10,11] In the context of experimental autoimmune encephalomyelitis, mice immunized with an immunodominant myelin-oligodendrocyte-glycoprotein (MOG) peptide run a more severe disease course and have a higher mortality rate than mice immunized with MOG peptide but injected with Gal9, where a loss of MOG-specific IFNγ-producing CD4 T cells is observed.[12] On the other hand, the blockade of Tim-3 on Th1 cells through anti–Tim-3 antibody administration accelerates diabetes development in nonobese diabetic AQ7 mice and prevents transplantation tolerance in a model of islet allograft.[13,14] In humans, down-regulation of Tim-3 has been reported in T cell clones established from the cerebrospinal fluid of patients with multiple sclerosis and found to be associated with high levels of IFNγ secretion.[15,16] In hepatitis C–infected patients, overexpression of Tim-3 characterizes CD4 and CD8 T cells and is associated with viral persistence.[17,18] Blockade of Tim-3 leads to restoration of virus-specific immune responses, particularly of hepatocyte-directed cytotoxicity,[18] indicating that immunotherapeutic control of Tim-3 expression may favor viral clearance. All these investigations highlight the importance of the Gal9/Tim-3 interaction in immunoregulation, expression of Gal9 and Tim-3 reflecting the ability of T-regs to suppress and the responsiveness of effector cells to T-reg control.

The aim of the present study is to explore the role of the Gal9/Tim-3 pathway in AIH and particularly to determine whether impaired immunoregulation in this condition is the result of defective T-reg function, low responsiveness of the effectors to T-reg control, or both.

Patients and Methods

Patients and Controls. Seventy-five patients with antinuclear antibody and/or smooth muscle antibody positive AIH (44 female patients) were studied. A liver biopsy performed at the time or close to diagnosis showed histological features of interface hepatitis in all. Eighteen of them had bile duct changes characteristic of sclerosing cholangitis on retrograde cholangiography and were diagnosed as having overlap autoimmune hepatitis/sclerosing cholangitis (ASC) syndrome.[19] When considered together, AIH and ASC are henceforth indicated as autoimmune liver disease (AILD). The median age at the time of the study was 13.8 years (range, 8.2-21.2 years). All patients were studied while on immunosuppressive treatment, since T-regs collected at disease presentation before immunosuppression have been shown to be inefficient at suppressing cell proliferation.[4,20] Fifty-five patients were in remission (i.e., with normal aminotransferase levels), while 20 were studied during an episode of relapse. Treatment consisted of prednisolone (2.5-5 mg daily at remission and 1-2 mg/kg/day at relapse) and azathioprine (1-2 mg/kg/day). Demographic and biochemical data are shown in Table 1. Twenty-six HS T1 (20 female patients; median age, 29.2 years [range, AQ8 22.6-39.4 years]) served as normal controls, the disparity between patient and control age deriving from ethical constraints in obtaining blood from healthy children. To test whether age disparity may account for differences, patients were divided into two subgroups ($\leq$14 years and >14 years). Written consent was obtained from each patient. The study was approved by the Ethical Committee of King's College Hospital, London, UK.

Cell Separation. Peripheral blood mononuclear cells (PBMCs) were obtained as described.[5] Mononuclear cell viability as determined by trypan blue exclusion exceeded 98%.

Flow Cytometry. PBMCs were used either fresh or cryopreserved, the latter being stored in liquid nitrogen until the time of testing. Preliminary experiments in which the cell preparations from the same patients were tested before and after cryopreservation showed no significant difference in viability and behavior in culture when assessed for proliferation and cytokine

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J_ID: ZHE Customer A_ID: 11-2317.R1 Customer Art: ZHE Date: 2-March-12 Stage: Page: 3

Table 1. Patient Demographics and Laboratory Data

AILD	No. of Patients (N = 75)	Sex	Age, Years	AST (Normal: <50 IU/L)	Bilirubin (Normal: <20 μmol/L)	IgG (Normal: 6.5-17 g/L)	Autoantibody Titer (Reciprocal) ANA	SMA	LKM-1
Relapse	20	13 F, 7 M	13.6 (9.3-19.5)	84 (58-761)	24 (5-705)	17.1 (10.3-43.9)	40 (0-160)	20 (0-160)	Negative
Remission	55	31 F, 24 M	13.8 (8.2-21.2)	30 (16-49)*	7 (4-46)**	12.3 (5.7-27.1)***	10 (0-80)	0 (0-80)	Negative

Data are presented as the median (range) unless noted otherwise;
Abbreviations: ANA, anti-nuclear antibody; F, female; LKM-1, liver kidney microsomal antibody type 1; M, male; SMA, smooth muscle antibody.
*$P < 0.001$,
**$P = 0.02$,
***$P = 0.004$ when comparing AST, bilirubin, and IgG levels between AILD at remission and at relapse.

production in the case of effectors and ability to suppress in the case of T-regs. Unfractionated cells were stained with allophycocyanin-cytochrome (Cy)-7–conjugated anti-CD4, fluorescein isothiocyanate (FITC)-conjugated, or phycoerythrin (PE)-Cy-7–conjugated anti-CD25, PE-conjugated anti-CD127 (all from BD Bioscience Discovery Labware, Oxford, UK) and PE- or allophycocyanin-conjugated anti–Tim-3 (R&D Systems, Abingdon, UK) monoclonal antibodies (mAbs). Cells were incubated at 4°C in the dark for 30 minutes, washed with phosphate-buffered saline/1% fetal bovine serum, resuspended, and analyzed by flow cytometry on a Becton Dickinson fluorescent-activated cell sorter (FACSCantoII, Becton Dickinson Immunocytochemistry Systems, San Jose, CA). FACSDiva software was used for analysis. A minimum of 2×10^4 gated events was acquired for each sample.

The percentage of cells positive for FOXP3, T-bet, GATA-3, and RORC, transcription factors of T-regs, Th1, Th2 and Th17 cells, was determined by intracellular staining after cell fixation and permeabilization with Cytofix/Cytoperm (BD Bioscience) and counterstaining with FITC-conjugated anti-FOXP3 (clone PCH101), peridinin chlorophyll protein-Cy5–conjugated anti–T-bet, PE-Cy7–conjugated anti-GATA3, or PE-conjugated anti-RORC mAbs (all from eBioscience, Hatfield, UK). The percentage of Gal9-positive cells was determined after cell incubation with mouse immunoglobulin G1 (IgG1) anti-human Gal9 mAb (MBL, Nagoya, Japan) and with PE-conjugated anti-IgG1 secondary antibody (BD Bioscience).

The percentage of IFNγ, IL-17, IL-10, and transforming growth factor β (TGFβ)-producing cells was assessed before and after exposure to phorbol 12-mystrate 13-acetate (10 ng/mL)/ionomycin (500 ng/mL) (both from Sigma Aldrich, Gillingham, UK), incubation with brefeldin A (10 μg/mL; Sigma-Aldrich) for 5 hours and counterstaining with FITC- or allophycocyanin-conjugated anti–IL-17 (eBioscience), anti-IFNγ (IQ Products, Groningen, The Netherlands), anti–IL-10 (BD Bioscience) and peridinin chlorophyll protein–conjugated anti-TGFβ mAbs (R&D Systems). Flow cytometry was performed as above.

Cell Purification. $CD4^{pos}CD25^{pos}$ (henceforth referred to as T-regs) and $CD4^{pos}CD25^{neg}$ cells were isolated from PBMCs using immunomagnetic beads (Dynal Invitrogen, Oslo, Norway) as described.

$CD4^{pos}CD25^{neg}$ cells were further purified according to the expression of Tim-3. In brief, $CD4^{pos}CD25^{neg}$ cells were incubated with PE-conjugated anti–Tim-3 mAbs for 30 minutes, then with microbeads conjugated to monoclonal anti-PE antibodies (Miltenyi Biotec, Bergisch-Gladbach, Germany) for a further 15 minutes at 4°C. $CD4^{pos}CD25^{neg}Tim\text{-}3^{neg}$ and $CD4^{pos}CD25^{neg}Tim\text{-}3^{pos}$ (henceforth $Tim\text{-}3^{neg}$ and $Tim\text{-}3^{pos}$) populations were purified by negative and positive selection, respectively, using MS columns (Miltenyi Biotec) according to the manufacturer's instructions. $CD4^{pos}CD25^{pos}CD127^{neg}$ cells (henceforth referred to as $CD127^{neg}$ T-regs), or "true" T-regs,[21] were purified from $CD4^{pos}CD25^{pos}$ cells after incubation with PE-conjugated anti-CD127 (BD Bioscience) and anti-PE microbeads (Miltenyi Biotec) (see above).

Proliferation Assay. Once purified, T-regs or $CD127^{neg}$ T-regs were added to autologous $CD4^{pos}CD25^{neg}$, $Tim\text{-}3^{pos}$ and $Tim\text{-}3^{neg}$ cells at a ratio of $1/8^4$. Cells were cultured at 37°C and 5% CO_2 for 5 days in the presence of anti-CD3/anti-CD28 T cell expander (ratio bead/cell: 1/2) (Dynal Invitrogen) and recombinant IL-2 (30 U/mL) (Chiron, Amsterdam, The Netherlands). In parallel, $CD4^{pos}CD25^{neg}$, $Tim\text{-}3^{pos}$, and $Tim\text{-}3^{neg}$ responder cells were cultured on their own under identical conditions and used as controls. All experiments were performed in duplicate. For the last 18 hours, cells were pulsed with 0.25 μCi/well ^{3}H-thymidine and harvested using a multichannel harvester. The amount of incorporated ^{3}H-thymidine was determined using a β-counter (Canberra Packard, Pangbourne, UK). The percentage inhibition was

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J_ID: ZHE Customer A_ID: 11-2317.R1 Customer Art: ZHE Date: 2-March-12 Stage: Page: 4

calculated using the following formula: (1 − cpm in the presence of T-regs or $CD127^{neg}$ T-regs/cpm in the absence of T-regs or $CD127^{neg}$ T-regs).

Neutralization Assay. To investigate whether the susceptibility of the responder cells to T-reg control is related to the release of effector cytokines, $CD4^{pos}CD25^{neg}$, Tim-3^{pos}, and Tim-3^{neg} cells were exposed to anti-IFNγ and anti–IL-17 neutralizing antibodies (10 μg/mL) (R&D Systems) for 12 hours before T-regs were added and during the 5-day coculture period. To assess whether T-reg control over responder cell proliferation relates to IL-10 secretion, anti-human IL-10–neutralizing antibodies (R&D Systems) were added at 10 μg/mL to T-regs cocultured with the effectors.

Gal9 Small Interfering RNA To evaluate whether the expression of Gal9 by T-regs is relevant to their ability to suppress, T-regs and $CD127^{neg}$ T-regs were treated with a set of three Stealth RNAis to block the expression of Gal9. Cells were resuspended in Opti-MEM medium (Invitrogen Life Technologies) at 2-3 $\times 10^6$/mL and transfected using Lipofectamine RNAiMAX reagent (Invitrogen Life Technologies) according to the manufacturer's instructions. Gal9-specific Stealth RNAis were used at a final concentration of 3 nM. A glyceraldehyde 3-phosphate dehydrogenase Stealth RNAi and a negative control Stealth RNAi (Invitrogen Life Technologies) served as positive and negative controls for transfection, respectively. Following overnight incubation at 37°C and 5% CO_2, aliquots of 2.5×10^5 cells were collected to extract RNA and assess Gal9 expression by real-time polymerase chain reaction as described.[22] Following small interfering RNA (siRNA) treatment, cells were cocultured with $CD4^{pos}CD25^{neg}$, Tim-3^{pos}, and Tim-3^{neg} responder cells, whose proliferation was assessed as detailed above. The effect of Gal9 gene knockdown on T-reg suppressor ability was also evaluated after sorting $CD25^{pos}$ cells into $CD25^{high}$ and $CD25^{low}$. $CD25^{pos}$ cells were sorted using a FACSAria (Becton Dickinson Immunocytochemistry Systems). The purity of both $CD25^{low}$ and $CD25^{high}$ cells exceeded 98%.

Statistical Analysis. The normality of variable distribution was assessed by the Kolmogorov-Smirnov goodness of fit test; once the hypothesis of normality was accepted ($P > 0.05$), comparisons were performed by paired and unpaired Student t test as appropriate. Correlation analysis was determined by Pearson's correlation coefficient. Results are expressed as the mean ± SEM. $P < 0.05$ was considered significant; $P \geq 0.05$ and $P \leq 0.15$ were considered to indicate a trend toward significance.

Results

Enumeration and Characterization of $CD4^{pos}CD25^{neg}$Tim-3^{pos} Cells. The percentage of Tim-3^{pos} lymphocytes within $CD4^{pos}CD25^{neg}$ cells was lower in patients (4.2 ± 0.4) than in HS (7.9 ± 0.8; $P < 0.001$) whether they were studied at relapse or during remission (Fig. 1A,B). Tim3^{pos} cells were lower at relapse than during remission (Fig. 1B). Within AILD patients, no differences were observed between AIH and ASC patients and between the two age subgroups. F1

The percentage of Tim-3^{pos} cells within the T-bet^{pos} and the $RORC^{pos}$ effector subsets was lower in AILD (T-bet^{pos}: 5.1 ± 0.7; $RORC^{pos}$: 3.9 ± 0.3) than in health (T-bet^{pos}: 13.6±1.7, $P < 0.001$; $RORC^{pos}$: 8.2 ± 1.1, $P = 0.02$).

Compared with HS (Table 2), the Tim-3^{pos} subset in AILD contained more $RORC^{pos}$, IFNγ^{pos}, and IL-17^{pos} cells and similar numbers of T-bet^{pos}, GATA-3^{pos}, $FOXP3^{pos}$, IL-10^{pos}, and TGFβ^{pos} cells; the Tim-3^{neg} subset in AILD contained more T-bet^{pos}, $RORC^{pos}$, IFNγ^{pos}, and IL-17^{pos} cells, fewer GATA-3^{pos} cells, and similar proportions of $FOXP3^{pos}$, IL-10^{pos}, and TGFβ^{pos} lymphocytes. Compared with the Tim-3^{neg} cell population, Tim-3^{pos} lymphocytes in AILD contained more T-bet^{pos}, $FOXP3^{pos}$, IFNγ^{pos}, IL-17^{pos}, IL-10^{pos}, and TGFβ^{pos} cells, fewer GATA-3^{pos} cells, and similar numbers of $RORC^{pos}$ cells; in HS, Tim-3^{pos} lymphocytes contained more T-bet^{pos}, IFNγ^{pos}, IL-17^{pos}, IL-10^{pos}, and TGFβ^{pos} cells, fewer GATA-3^{pos} cells, and similar numbers of $RORC^{pos}$ and $FOXP3^{pos}$ cells (Table 2). T2

Enumeration and Characterization of $CD4^{pos}CD25^{pos}Gal9^{pos}$ Cells. The percentage of $Gal9^{pos}$ cells within $CD4^{pos}CD25^{pos}$ cells was lower in patients (30.6 ± 3.1) than in HS (49.4 ± 3; $P < 0.001$), this difference being evident also when the $CD25^{low}$ (23.9 ± 2.9 versus 44.7 ± 3.2; $P < 0.001$), $CD25^{med}$ (29.5 ± 3.1 versus 49.9 ± 2.8; $P < 0.001$), and $CD25^{high}$ (35.2 ± 2.1 versus 65.6 ± 3.8; $P < 0.001$) fractions were analyzed separately (Fig. 2). $CD127^{neg}$ T-regs contained the highest percentage of $Gal9^{pos}$ cells. This percentage was lower in AILD (40.9 ± 4.1) than in HS (72.2 ± 4.8; $P < 0.001$). Within AILD, there was no difference between patients with AIH and those with ASC and between the two age subgroups. F2

Compared with HS (Table 3), the $Gal9^{pos}$ T-reg subset in AILD contained more IFNγ^{pos} and IL-17^{pos} cells, fewer $FOXP3^{pos}$, IL-10^{pos}, and TGFβ^{pos} cells and similar numbers of T-bet^{pos}, GATA-3^{pos}, and T3

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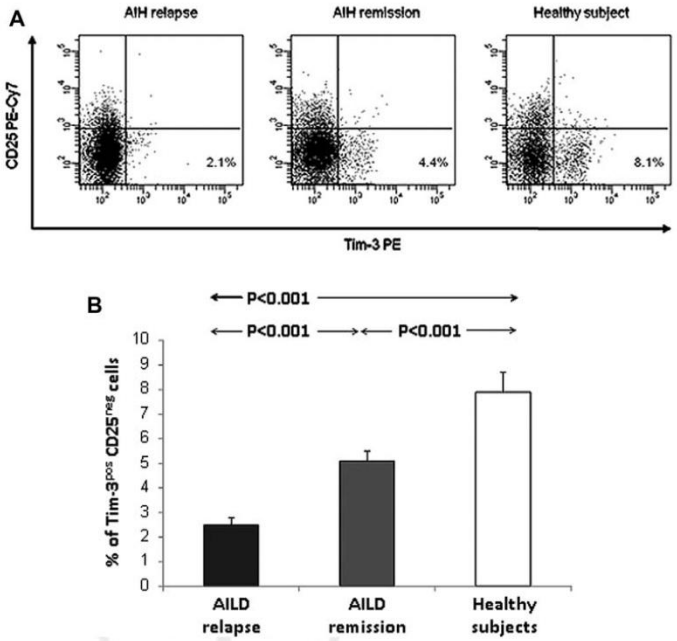

Fig. 1. Percentage of Tim-3^{pos} cells within CD4^{pos}CD25^{neg} T lymphocytes. (A) Percentage of Tim-3^{pos} cells in one representative AIH patient at relapse, in one AIH patient at remission, and in one healthy subject. Dot plots of Tim-3 PE (*x* axis) versus CD25 PE-Cy7. Cells are gated on CD4 lymphocytes. The percentage of Tim-3^{pos} cells is indicated in the lower right quadrant of each dot plot. (B) Percentage of Tim-3^{pos}CD25^{neg} cells in 10 AILD patients during relapse, 30 AILD patients in remission, and 15 HS.

RORC^{pos} cells; the Gal9^{neg} subset in AILD contained more RORC^{pos}, IFNγ^{pos} and IL-17^{pos} cells, fewer FOXP3^{pos} cells, and similar proportions of T-bet^{pos}, GATA3^{pos}, IL-10^{pos}, and TGFβ^{pos} lymphocytes. Compared with the Gal9^{neg} population, Gal9^{pos} T-regs in AILD contained more FOXP3^{pos} and IL-10^{pos} cells, fewer RORC^{pos} and IL-17^{pos} cells, and similar numbers of T-bet^{pos}, GATA-3^{pos}, IFNγ^{pos}, and TGFβ^{pos} cells; in HS, Gal9^{pos} T-regs contained more FOXP3^{pos}, IL-10^{pos}, and TGFβ^{pos} cells, fewer RORC^{pos} cells, and similar numbers of T-bet^{pos}, GATA-3^{pos}, IFNγ^{pos}, and IL-17^{pos} cells.

Table 2. Percentage of T-bet^{pos}, GATA-3^{pos}, RORC^{pos}, FOXP3^{pos}, IFNγ^{pos}, IL-17^{pos}, IL-10^{pos}, and TGFβ^{pos} Cells Within CD4^{pos}CD25^{neg}, Tim-3^{pos}, and Tim-3^{neg} Cells

	Tim-3^{pos}			Tim-3^{neg}				
Cell Type	AILD, %	HS, %	*P**	AILD, %	HS, %	*P*†	*P*‡	*P*§
T-bet^{pos}	91.2 ± 0.8	93.1 ± 0.5	NS	10.1 ± 0.9	6.1 ± 0.9	0.005	<0.001	<0.001
GATA-3^{pos}	0.71 ± 0.23	0.78 ± 0.24	NS	2 ± 0.5	2.8 ± 0.4	0.08	0.02	0.01
RORC^{pos}	7.2 ± 0.9	2.9 ± 0.5	0.03	5.9 ± 0.4	3.5 ± 0.5	0.02	NS	NS
FOXP3^{pos}	2.84 ± 0.85	2.74 ± 0.49	NS	0.5 ± 0.2	1.6 ± 0.8	NS	0.06	NS
IFNγ^{pos}	11.2 ± 0.8	4.2 ± 0.6	<0.001	6.09 ± 0.7	2.4 ± 0.3	0.001	<0.001	<0.001
IL-17^{pos}	7.9 ± 1.2	4.3 ± 0.8	0.04	3.1 ± 0.7	1.5 ± 0.2	0.07	0.013	0.017
IL-10^{pos}	7.1 ± 1	9.9 ± 1.2	NS	5.1 ± 0.7	6.7 ± 1	NS	0.11	0.09
TGFβ^{pos}	6.8 ± 0.4	9.8 ± 1.9	NS	3.9 ± 1.2	4.7 ± 0.4	NS	0.03	0.03

Data are presented as the mean ± SEM and refer to 20 AILD patients and 12 HS.
Abbreviation: NS, not significant.
*Within the Tim-3^{pos} subset between AILD and HS.
†Within the Tim-3^{neg} subset between AILD and HS.
‡Between the Tim-3^{pos} and the Tim-3^{neg} subset in AILD.
§Between the Tim-3^{pos} and the Tim-3^{neg} subset in HS.

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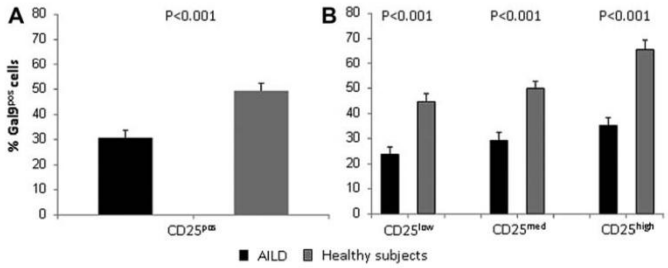

Fig. 2. Percentage of Gal9^{pos} T-regs. Percentage of Gal9^{pos} lymphocytes within unfractionated CD4^{pos}CD25^{pos} cells (A) and within the CD4^{pos}CD25^{low}, CD4^{pos}CD25^{med}, and CD4^{pos}CD25^{high} subsets (B) in patients (black bars, n = 40) and HS (gray bars, n = 20). Gal9^{pos} cells were lower in AILD than in HS both when undivided CD25^{pos} cells and when individual CD25 low, medium, and high cell fractions were analyzed.

Proliferation of CD4^{pos}CD25^{neg}, Tim-3^{pos}, and Tim-3^{neg} Cells and Responsiveness to T-reg Control. The proliferation of unfractionated CD25^{neg} cells was lower than that of Tim-3^{neg} cells (AILD, $P = 0.02$; HS, $P = 0.001$) and higher than that of Tim-3^{pos} cells (AILD, $P = 0.03$; HS, $P = 0.04$) (Fig. 3). Addition of T-regs reduced cell proliferation by 26% (P not significant) in AILD and 53% ($P = 0.007$) in HS when unfractionated CD25^{neg} cells were used as responders; by 23% (P not significant) and 25% (P not significant) when Tim-3^{neg} cells were the responders; and by 47% ($P = 0.03$) and 62% ($P = 0.001$) when the responder cells were Tim-3^{pos} (Fig. 4).

Addition of CD127^{neg} T-regs reduced proliferation of CD25^{neg} cells by 46% in AILD ($P < 0.001$) and 60% in HS ($P < 0.001$) and proliferation of Tim-3^{pos} cells by 56% in AILD ($P = 0.006$) and 69% in HS ($P < 0.001$).

As the percentage of IFNγ-producing and, to a lesser extent, IL-17–producing cells was higher in Tim-3^{pos} than in Tim-3^{neg} cells, we investigated the effect of IFNγ and IL-17 neutralization on the ability of CD25^{neg}, Tim-3^{neg}, and Tim-3^{pos} cells to be regulated by T-regs. Addition of anti-IFNγ–neutralizing antibodies did not change CD25^{neg} and Tim-3^{neg} cell responsiveness to T-reg control (Fig. 4A,B). In contrast, treatment with anti-IFNγ abrogated Tim-3^{pos} responsiveness to T-reg control (Fig. 4C). Exposure to anti-IFNγ–neutralizing antibodies, while leaving unchanged the percentage of Tim-3^{pos} lymphocytes within undivided CD4 cells in both AILD patients and HS, tended to decrease that of Tim-3^{pos} lymphocytes within CD25^{neg} cells in HS after 12-hour (8.5 ± 0.4 versus 6.2 ± 1.2; $P = 0.12$) and 5-day (15.5 ± 1.6 versus 12.2 ± 0.6; $P = 0.1$) culture. Similarly, while no difference in Tim-3 expression on a cell-per-

Table 3. Percentage of T-bet^{pos}, GATA-3^{pos}, RORC^{pos}, FOXP3^{pos}, IFNγ^{pos}, IL-17^{pos}, IL-10^{pos}, and TGFβ^{pos} Cells Within CD4^{pos}CD25^{pos}, Gal9^{pos}, and Gal9^{neg} Cells

	Gal9^{pos}			Gal9^{neg}				
Cell Type	AILD, %	HS, %	P*	AILD, %	HS, %	P†	P‡	P§
T-bet^{pos}	9.5 ± 1.3	9.4 ± 4.4	NS	9.7 ± 1.5	8.1 ± 1.6	NS	NS	NS
GATA-3^{pos}	7.3 ± 1.2	7.4 ± 3.8	NS	5.6 ± 1.6	5.6 ± 1	NS	NS	NS
RORC^{pos}	1.1 ± 0.6	0.9 ± 0.4	NS	8.6 ± 0.9	4.4 ± 0.8	0.04	0.005	0.02
FOXP3^{pos}	14.4 ± 2	42.8 ± 3.1	<0.001	2.6 ± 0.5	16.4 ± 1.5	<0.001	<0.001	<0.001
IFNγ^{pos}	4.4 ± 0.6	2.1 ± 0.3	0.002	5.7 ± 0.7	2.2 ± 0.4	<0.001	NS	NS
IL-17^{pos}	4.1 ± 0.6	1.75 ± 0.2	0.002	7.9 ± 1.2	2.3 ± 0.7	<0.001	0.01	NS
IL-10^{pos}	5.1 ± 0.6	9.1 ± 0.5	<0.001	2.3 ± 0.2	3.3 ± 0.4	NS	0.001	<0.001
TGFβ^{pos}	6.3 ± 0.7	8 ± 0.4	0.04	5.3 ± 0.5	5.6 ± 1.1	NS	NS	0.07

Data are presented as the mean ± SEM and refer to 23 AILD patients and 17 HS.
Abbreviation: NS, not significant.
*Within the Gal9^{pos} subset between AILD and HS.
†Within the Gal9^{neg} subset between AILD and HS.
‡Between the Gal9^{pos} and the Gal9^{neg} subset in AILD.
§Between the Gal9^{pos} and the Gal9^{neg} subset in HS.

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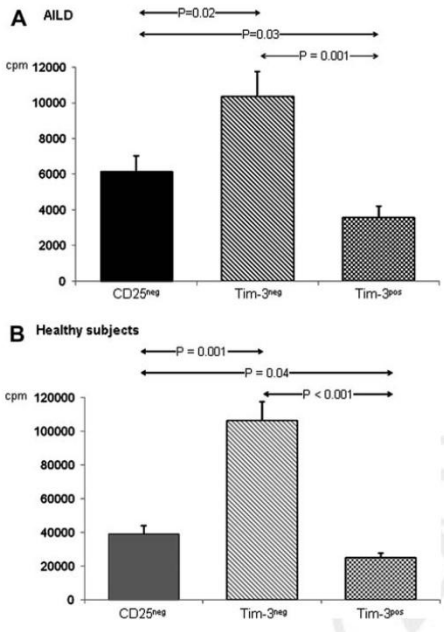

Fig. 3. Proliferation of $CD4^{pos}CD25^{neg}$, $Tim\text{-}3^{neg}$, and $Tim\text{-}3^{pos}$ responder cells. $CD4^{pos}CD25^{neg}$ cells were purified from PBMCs by two-step negative selection using immunomagnetic beads. $Tim\text{-}3^{neg}$ and $Tim\text{-}3^{pos}$ cell fractions were obtained following incubation of $CD25^{neg}$ cells with PE-labeled Tim-3 mAbs and with anti-PE microbeads. Unfractionated $CD25^{neg}$ cells (black bars) and $Tim\text{-}3^{neg}$ (diagonal striped bars) and $Tim\text{-}3^{pos}$ (checkered bars) fractions from (A) AILD patients ($n = 18$) and (B) HS ($n = 9$) were cultured for 5 days in the presence of CD3/CD28 T cell expander and recombinant IL-2. Results are expressed as the mean + SEM.

basis (expressed as mean fluorescence intensity) was noted in CD4 undivided cells in both AILD patients and HS, within $CD25^{neg}$ cells from HS, Tim-3 mean fluorescence intensity tended to decrease after 12-hour (798.5 ± 35.3 versus 678 ± 62.2; $P = 0.14$) and 5-day (978.2 ± 62.6 versus 808.2 ± 86.3; $P = 0.14$) culture. In HS, anti-IFNγ–induced decrease in $Tim\text{-}3^{pos}$ $CD25^{neg}$ cells and in Tim-3 mean fluorescence intensity was abrogated by T-reg addition.

Treatment with anti–IL-17–neutralizing antibodies did not change the responsiveness of $CD25^{neg}$, $Tim\text{-}3^{neg}$, and $Tim\text{-}3^{pos}$ responder cells to T-reg control in both patients and HS. Addition of anti-IFNγ and anti–IL-17–neutralizing antibodies together left unchanged the responsiveness of $CD25^{neg}$ and $Tim\text{-}3^{neg}$ cells, while reduced that of $Tim\text{-}3^{pos}$ cells (Fig. 4A-C).

To explore whether T-reg control over responder cell proliferation is influenced by IL-10 secretion, anti–IL-10–neutralizing antibodies were added to T-regs cocultured with effectors obtained from four AILD patients and four HS. Following treatment, T-reg inhibition of $CD25^{neg}$ cell proliferation did not change in AILD but was decreased from 51% to 34% ($P = 0.07$) in HS. Anti–IL-10–neutralizing antibody treatment resulted in no change in the ability of T-regs to suppress $Tim\text{-}3^{neg}$ cells, but decreased T-reg inhibition over $Tim\text{-}3^{pos}$ cell proliferation from 42% to 36% ($P = 0.06$) in AILD and from 56% to 48% ($P = 0.04$) in HS.

Gal9 Gene Knockdown. Treatment of T-regs with Gal9-RNAi led to a decrease in the expression of *Gal9* gene by 86% in AILD and by 88% in HS.

After addition of Gal9-siRNA–treated T-regs, inhibition of cell proliferation was reduced by 43.5% in AILD ($P < 0.001$) and by 67% in HS ($P < 0.001$) when $CD25^{neg}$ cells were used as responders, by 43% ($P < 0.001$) and 48.7% ($P = 0.038$) when $Tim\text{-}3^{neg}$ cells were the responders, and by 70.4% and 73.5% when the responders were $Tim\text{-}3^{pos}$ lymphocytes.

After treatment with Gal9-RNAi, $CD127^{neg}$ T-reg suppressor function was reduced by 54.4% ($P < 0.001$) in AILD and 66.7% ($P < 0.001$) in HS when responders were $CD25^{neg}$ cells; by 36.4% (P = NS) in AILD and 51.62% ($P = 0.06$) in HS when responders were $Tim\text{-}3^{neg}$ cells; and by 75% ($P = 0.001$) in AILD and 71% ($P < 0.001$) in HS when responders were $Tim\text{-}3^{pos}$ cells.

In two HS where higher numbers of cells were available, the effect of Gal9 gene knockdown on T-reg suppression was also evaluated after sorting T-regs according to their CD25 expression. $CD25^{high}$ T-reg suppressor function was markedly reduced by Gal9 siRNA treatment: inhibition of $CD25^{neg}$ cell proliferation was 57% following addition of untreated $CD25^{high}$ T-regs and 14% after addition of Gal9 siRNA-treated $CD25^{high}$ T-regs. No difference in the ability of $CD25^{low}$ T-regs to suppress was noted before and after Gal9 siRNA treatment (25% versus 27%).

Correlations. The percentage of $Tim\text{-}3^{pos}$cells was inversely correlated with the levels of aspartate aminotransferase (AST) ($r = -0.47$; $P = 0.002$). The percentage of $CD25^{neg}T\text{-}bet^{pos}$ cells was positively correlated with AST levels ($r = 0.69$; $P < 0.01$) and negatively with that of $Tim\text{-}3^{pos}$ cells ($r = -0.91$; $P < 0.001$).

The percentage of $Gal9^{pos}$ T-regs was inversely correlated with the titer of IgG ($r = -0.39$, $P < 0.02$), smooth muscle antibody ($r = -0.32$, $P = 0.05$), and,

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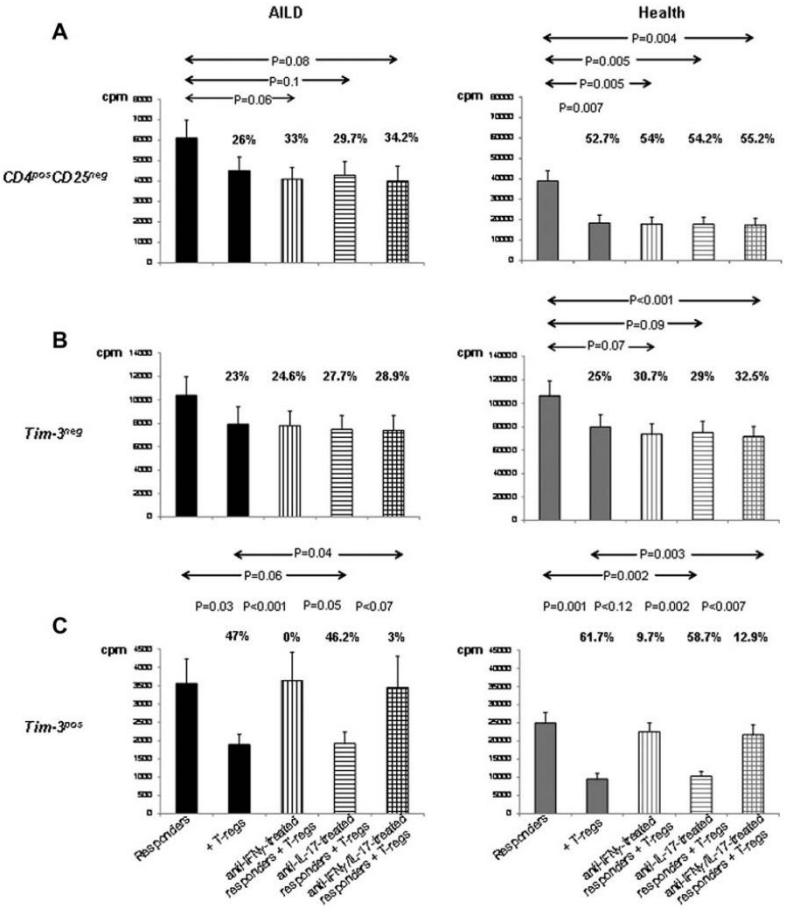

Fig. 4. Suppression of $CD4^{pos}CD25^{neg}$, Tim-3^{neg}, and Tim-3^{pos} responder cell proliferation by T-regs. T-regs purified from AILD patients (left plots, n = 18) and HS (right plots, n = 9) were added to (A) $CD4^{pos}CD25^{neg}$, (B) Tim-3^{neg}, or (C) Tim-3^{pos} responder cells, either untreated or treated with anti-IFNγ (vertically striped bars), anti–IL-17 (horizontally striped bars), or anti-IFNγ + anti-IL-17 (thatched bars) neutralizing antibodies. After 5-day coculture, responder cell proliferation was assessed by ^{3}H-thymidine incorporation. Bars represent the mean + SEM proliferation of untreated or neutralizing antibody–treated responder cells before and after T-reg addition. Percentage inhibition of responder cell proliferation following T-reg addition is indicated.

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to a lesser extent, antinuclear antibody ($r = -0.27$, $P < 0.1$).

Discussion

This study shows that in AILD numerical and functional T-reg impairment is accompanied by low susceptibility of responder cells to the control exerted by T-regs, indicating that there is a defect both at the regulatory and effector cell levels. Thus, the present results, besides confirming our previous observations on reduced number and suppressor function of T-regs in AILD, provide evidence that low expression of Gal9 could be one of the mechanisms responsible for T-reg impairment, as highlighted by gene knockdown experiments where T-regs treated with a set of Gal9-specific

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Stealth RNAis were less effective at controlling the proliferation of responder cells, especially in the case of $CD25^{high}$ T-regs.

Neutralization experiments showing reduced T-reg suppression following treatment with anti–IL-10–neutralizing antibodies indicate that $Gal9^{pos}$ T-regs partly act through IL-10 production/secretion. This finding suggests that $Gal9^{pos}$ T-regs, in addition to regulating effectors via induction of their apoptosis,[23] can deliver suppression via IL-10 secretion. That $Gal9^{pos}$ T-regs exert a control over disease activity was supported by the observation of an inverse correlation between $Gal9^{pos}$ T-regs and IgG levels and titers of autoantibodies, which are the serological markers of the disease. These results echo a murine study wherein the context of autoimmune encephalomyelitis Gal9 was shown to have a direct effect on disease severity, as mice immunized with a MOG immunodominant peptide and injected *in vivo* with Gal9 had a less severe disease course and a lower mortality rate than mice that were not injected.[12]

In AILD, decreased percentage of $Gal9^{pos}$ T-regs is mirrored by a down-modulation of Tim-3 on the surface of $CD4^{pos}CD25^{neg}$ cells. Whether down-regulation of Tim-3 on effectors is the result of low $Gal9^{pos}$ T-reg number or a defective Th1 cell maturation/differentiation process is unclear. Our data demonstrating low percentage of Tim-3 on $T\text{-}bet^{pos}$ cells support the second hypothesis and suggest that in AILD, Th1 cells arrest at a T-bet positive status having acquired effector properties, without differentiating into the T-bet/Tim-3 double positive status, which characterizes terminally differentiated effector cells that are susceptible to T-reg control. This is in line with the report of Anderson et al.,[24] who documented that $T\text{-}bet^{-/-}$ mice have defective Tim-3 expression on CD4 T cells and that T-bet binds directly to the Tim-3 promoter, regulating its expression. Future studies in AILD should also measure soluble Tim-3, as its role is controversial: while administration of soluble Tim-3-Ig fusion protein augments Th1 immune responses in SJL/J mice immunized with a myelin proteolipid protein peptide,[14] the overexpression of soluble Tim-3 by in vivo delivery of plasmid DNA in C57BL/6 mice inoculated with melanoma cells results in the inhibition of Th1 cytokines and impaired T cell antitumor response.[25]

That Tim-3 renders effectors responsive to T-reg control was clearly shown in the current study by a series of experiments in which the $Tim\text{-}3^{neg}$ and the $Tim\text{-}3^{pos}$ cell fractions were tested for their ability to be regulated by T-regs. The $Tim\text{-}3^{pos}$ fraction was the most amenable to T-reg control, especially when $CD127^{neg}$ T-regs were used as suppressors; responsiveness of $Tim\text{-}3^{pos}$ cells, however, was more evident in health than in AILD, indicating that in this condition, immunoregulation impairment includes low responder cell susceptibility to T-reg suppression in addition to a defective T-reg function. It is therefore plausible that, as supported by our data, in AILD $T\text{-}bet^{pos}/Tim\text{-}3^{neg}$ cells prevail over the $T\text{-}bet^{pos}/Tim\text{-}3^{pos}$ cells, accounting for the high number of poorly controllable effectors that contribute to the unfolding of the liver damage. That Tim-3 down-modulation is associated with disease activity was confirmed by the strong negative correlation between percentage of $Tim\text{-}3^{pos}$ effectors and AST levels.

Further phenotypic and functional analyses performed in the current study indicate that regulation of $Tim\text{-}3^{pos}$ cells by T-regs depends on IFNγ production, as blockade of IFNγ results in reduced Tim-3 expression of $CD4^{pos}CD25^{neg}$ cells and lower susceptibility of $Tim\text{-}3^{pos}$ cells to T-reg control. These data suggest that a proinflammatory signature is necessary for the effectors in order to be "seen" and consequently regulated by T-regs via the Gal9/Tim-3 pathway.

The higher percentage of IL-17–producing and $RORC^{pos}$ cells within the $Tim\text{-}3^{pos}$ fraction shows that Tim-3 is expressed also on other types of effectors, i.e. Th17 cells, a subset involved in the autoimmune liver damage.[1] Tim-3 expression by Th17 cells is likely to contribute to their ability to be controlled by T-regs, as reported by Chen et al.[26] in mice and Hastings et al.[27] in humans. Relevant to our findings, in the collagen-induced arthritis model, treatment with Gal9 blocks Th17 cell induction and IL-17 messenger RNA expression even when CD4-naïve cells are exposed to TGFβ and IL-6, cytokines central to Th17 development in mice.[28]

The information obtained in the present study is crucial for devising T-reg–based immunotherapy for the possible treatment/cure of AILD. In view of the reported direct role of Gal9 in T-reg induction and differentiation in a murine setting,[28] further studies should explore whether in the context of AILD T-reg suppression could be restored by transfecting T-regs with human Gal9 complementary DNA or, alternatively, by culturing them in the presence of Gal9. In this context it, is of interest that culture of PBMCs in the presence of Gal9 results in the expansion of $CD4^{pos}CD25^{pos}FOXP3^{pos}CD127^{low}$ T-regs both in hepatitis C–infected patients and healthy subjects.[29]

In conclusion, our data show that defective immunoregulation is due not only to T-reg inability to suppress, but also to a low susceptibility of effector cells

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to T-reg control due to decreased expression of Tim-3. Because Tim-3 expression is reduced when the disease is active, adoptive transfer of autologous T-regs may be more efficacious once inflammation is dampened by immunosuppression to render effectors more amenable to T-reg inhibition.

References

1. Longhi MS, Ma Y, Mieli-Vergani G, Vergani D. Aetiopathogenesis of autoimmune hepatitis. J Autoimmun 2010;34:7-14.
2. Gregorio GV, Portmann B, Reid F, Donaldson PT, Doherty DG, McCartney M, et al. Autoimmune hepatitis in childhood: a 20-year experience. HEPATOLOGY 1997;25:541-547.
3. Ma Y, Bogdanos DP, Hussain MJ, Underhill J, Bansal S, Longhi MS, et al. Polyclonal T-cell responses to cytochrome P450IID6 are associated with disease activity in autoimmune hepatitis type 2. Gastroenterology 2006;130:868-882.
4. Longhi MS, Ma Y, Bogdanos DP, Cheeseman P, Mieli-Vergani G, Vergani D. Impairment of CD4(+)CD25(+) regulatory T-cells in autoimmune liver disease. J Hepatol 2004;41:31-37.
5. Longhi MS, Hussain MJ, Mitry RR, Arora SK, Mieli-Vergani G, Vergani D, et al. Functional study of CD4+CD25+ regulatory T cells in health and autoimmune hepatitis. J Immunol 2006;176:4484-4491.
6. Ferri S, Longhi MS, De Molo C, Lalanne C, Muratori P, Granito A, et al. A multifaceted imbalance of T cells with regulatory function characterizes type 1 autoimmune hepatitis. HEPATOLOGY 2010;52: 999-1007.
7. Sakaguchi S. Naturally arising CD4+ regulatory T cells for immunologic self-tolerance and negative control of immune responses. Annu Rev Immunol 2004;22:531-562.
8. Sakaguchi S, Yamaguchi T, Nomura T, Ono M. Regulatory T cells and immune tolerance. Cell 2008;133:775-787.
9. Longhi MS, Hussain MJ, Kwok WW, Mieli-Vergani G, Ma Y, Vergani D. Autoantigen-specific regulatory T cells, a potential tool for immune-tolerance reconstitution in type-2 autoimmune hepatitis. HEPATOLOGY 2011;53:536-547.
10. Anderson AC, Anderson DE. TIM-3 in autoimmunity. Curr Opin Immunol 2006;18:665-669.
11. Rodriguez-Manzanet R, DeKruyff R, Kuchroo VK, Umetsu DT. The costimulatory role of TIM molecules. Immunol Rev 2009;229: 259-270.
12. Zhu C, Anderson AC, Schubart A, Xiong H, Imitola J, Khoury SJ, et al. The Tim-3 ligand galectin-9 negatively regulates T helper type 1 immunity. Nat Immunol 2005;6:1245-1252.
13. Sanchez-Fueyo A, Tian J, Picarella D, Domenig C, Zheng XX, Sabatos CA, et al. Tim-3 inhibits T helper type 1-mediated auto- and alloimmune responses and promotes immunological tolerance. Nat Immunol 2003;4:1093-1101.
14. Sabatos CA, Chakravarti S, Cha E, Schubart A, Sanchez-Fueyo A, Zheng XX, et al. Interaction of Tim-3 and Tim-3 ligand regulates T helper type 1 responses and induction of peripheral tolerance. Nat Immunol 2003;4:1102-1110.
15. Koguchi K, Anderson DE, Yang L, O'Connor KC, Kuchroo VK, Hafler DA. Dysregulated T cell expression of TIM3 in multiple sclerosis. J Exp Med 2006;203:1413-1418.
16. Yang L, Anderson DE, Kuchroo J, Hafler DA. Lack of TIM-3 Immunoregulation in multiple sclerosis. J Immunol 2008;180:4409-4414.
17. Golden-Mason L, Palmer BE, Kassam N, Townshend-Bulson L, Livingston S, McMahon BJ, et al. Negative immune regulator Tim-3 is overexpressed on T cells in hepatitis C virus infection and its blockade rescues dysfunctional CD4+ and CD8+ T cells. J Virol 2009;83: 9122-9130.
18. McMahan RH, Golden-Mason L, Nishimura MI, McMahon BJ, Kemper M, Allen TM, et al. Tim-3 expression on PD-1+ HCV-specific human CTLs is associated with viral persistence, and its blockade restores hepatocyte-directed in vitro cytotoxicity. J Clin Invest 2010; 120:4546-4557.
19. Gregorio GV, Portmann B, Karani J, Harrison P, Donaldson PT, Vergani D, et al. Autoimmune hepatitis/sclerosing cholangitis overlap syndrome in childhood: a 16-year prospective study. HEPATOLOGY 2001;33: 544-553.
20. Longhi MS, Ma Y, Mitry RR, Bogdanos DP, Heneghan M, Cheeseman P, et al. Effect of CD4+ CD25+ regulatory T-cells on CD8 T-cell function in patients with autoimmune hepatitis. J Autoimmun 2005; 25:63-71.
21. Longhi MS, Mitry RR, Samyn M, Scalori A, Hussain MJ, Quaglia A, et al. Vigorous activation of monocytes in juvenile autoimmune liver disease escapes the control of regulatory T-cells. HEPATOLOGY 2009;50: 130-142.
22. Longhi MS, Meda F, Wang P, Samyn M, Mieli-Vergani G, Vergani D, et al. Expansion and de novo generation of potentially therapeutic regulatory T cells in patients with autoimmune hepatitis. HEPATOLOGY 2008;47:581-591.
23. Kashio Y, Nakamura K, Abedin MJ, Seki M, Nishi N, Yoshida N, et al. Galectin-9 induces apoptosis through the calcium-calpain-caspase-1 pathway. J Immunol 2003;170:3631-3636.
24. Anderson AC, Lord GM, Dardalhon V, Lee DH, Sabatos-Peyton CA, Glimcher LH, et al. T-bet, a Th1 transcription factor regulates the expression of Tim-3. Eur J Immunol 2010;40:859-866.
25. Geng H, Zhang GM, Li D, Zhang H, Yuan Y, Zhu HG, et al. Soluble form of T cell Ig mucin 3 is an inhibitory molecule in T cell-mediated immune response. J Immunol 2006;176:1411-1420.
26. Chen Y, Langrish CL, McKenzie B, Joyce-Shaikh B, Stumhofer JS, McClanahan T, et al. Anti–IL-23 therapy inhibits multiple inflammatory pathways and ameliorates autoimmune encephalomyelitis. J Clin Invest 2006;116:1317-1326.
27. Hastings WD, Anderson DE, Kassam N, Koguchi K, Greenfield EA, Kent SC, et al. TIM-3 is expressed on activated human CD4+ T cells and regulates Th1 and Th17 cytokines. Eur J Immunol 2009;39: 2492-2501.
28. Seki M, Oomizu S, Sakata KM, Sakata A, Arikawa T, Watanabe K, et al. Galectin-9 suppresses the generation of Th17, promotes the induction of regulatory T cells, and regulates experimental autoimmune arthritis. Clin Immunol 2008;127:78-88.
29. Mengshol JA, Golden-Mason L, Arikawa T, Smith M, Niki T, McWilliams R, et al. A crucial role for Kupffer cell-derived galectin-9 in regulation of T cell immunity in hepatitis C infection. PLoS One 2010;5: e9504.

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Inhibition of Interleukin-17 Promotes Differentiation of CD25$^-$ Cells Into Stable T Regulatory Cells in Patients With Autoimmune Hepatitis

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BACKGROUND & AIMS: Patients with autoimmune hepatitis (AIH) have reduced numbers and function of CD4$^+$CD25^{high}FOXP3$^+$ T regulatory cells (Tregs). Tregs can be generated from CD25$^-$ (ngTreg) cells, which suppress the immune response less efficiently than Tregs. We investigated whether their differentiation into T-helper (Th)17 cells, an effector subset that has the same CD4$^+$ progenitors as Tregs, accounts for the reduced suppressive functions of ngTregs. We investigated whether blocking interleukin (IL)-17 increased the immunosuppressive activity of Tregs. **METHODS:** ngTregs were generated from 36 patients with AIH and 23 healthy subjects (controls). During Treg differentiation, expression of IL-17 was inhibited by physical removal of IL-17–secreting cells, exposure to recombinant transforming growth factor β or neutralizing antibodies against IL-6 and IL-1β (to promote differentiation of ngTregs vs Th17 cells), small inhibitory RNAs specific for the Th17 transcription factor RORC, or a combination of all these approaches. **RESULTS:** ngTregs from patients with AIH contained greater proportions of IL-17$^+$ and RORC$^+$ cells than Tregs from controls. All approaches to inhibit IL-17 increased expression of FOXP3 by ngTregs and their suppressive functions. Inhibition of IL-17 led to development of ngTregs that were phenotypically stable and did not acquire proinflammatory properties after exposure to IL-6 and IL-1β. **CONCLUSIONS: Blocking Th17 allows ngTregs to differentiate into functionally stable immune inhibitory cells; this approach might be developed for therapy of patients with AIH.**

Keywords: Immunotherapy; Liver Inflammation; Immune Regulation; T-Cell Development.

BASIC AND TRANSLATIONAL LIVER

Experimental and clinical evidence indicates that failure of immunoregulation is key to development of autoimmune disease. CD4$^+$CD25^{high}FOXP3$^+$ T regulatory cells (Tregs) are major players in maintaining immune homeostasis; defective Treg numbers and function result in autoimmune disorders in mice and humans.[1–5] Numerical and functional Treg impairment is documented in autoimmune hepatitis (AIH),[6–8] an inflammatory condition characterized by hypergammaglobulinemia, circulating autoantibodies, and interface hepatitis.[9,10] Treg impairment in AIH varies with disease stage, appearing worse at presentation than during remission, showing functional restoration potential.[6,7]

We reported that following exposure to anti-CD3/anti-CD28 and high interleukin (IL)-2 concentrations, Tregs derived from CD4$^+$CD25^{high} cells expand in both healthy subjects (HS) and patients with AIH.[11] Tregs expressing higher levels of FOXP3 and suppressing more efficiently than freshly isolated Tregs[11] can be expanded, but in limited numbers, hampering their immunotherapeutic use. To overcome this limitation, using a similar experimental approach, we generated Tregs from CD4$^+$CD25$^-$ cells, an effector subset also containing lymphocytes with regulatory function.[12] Differently from Tregs obtained from conventional CD4$^+$CD25^{high} cells, newly generated Tregs (ngTregs) have greater ability to expand and are more resistant to apoptosis,[11] features important for therapeutic application. However, despite their Treg phenotype (CD25^{high}, CD127^{low}, and FOXP3$^+$), ngTregs suppress less efficiently than Tregs expanded from CD4$^+$CD25^{high} cells.[11] During differentiation, ngTregs may display effector cell features (ie, secretion/production of proinflammatory cytokines), offsetting their suppressive function.[13] This may derive from incomplete transition to a terminal regulatory status of a proportion of cells producing IL-17.[14] IL-17–producing CD4 effector T-cells (Th17 cells) originate from the same progenitors as Tregs in the presence of IL-6, IL-21, and transforming growth factor (TGF)-β in mice and IL-6, IL-1β, IL-23, and TGF-β in humans.[15–20]

The aims of this study were to assess whether the reduced suppressor function of ngTregs derives from their ability to produce IL-17 and whether control of those ngTregs producing IL-17 has a favorable effect on ngTreg function.

Patients and Methods

Patients and Controls

Thirty-six patients with anti-nuclear antibody (ANA)-positive and/or anti–smooth muscle antibody (SMA)-positive

Abbreviations used in this paper: AIH, autoimmune hepatitis; ANA, anti-nuclear antibody; HS, healthy subjects; IFN, interferon; IL, interleukin; MFI, mean fluorescence intensity; ngTreg, newly generated T regulatory cell; PBMC, peripheral blood mononuclear cell; rIL-2, recombinant interleukin-2; rTGF-β, recombinant transforming growth factor β; siRNA, small interfering RNA; SMA, anti–smooth muscle antibody; TGF, transforming growth factor; Th, T-helper; Treg, T regulatory cell.

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AIH (AIH type 1; 23 female; median age, 13.9 years; range, 8.6–17.9 years) were studied, all having histologic evidence of interface hepatitis at diagnosis. Patients were studied during immunosuppressive treatment, because pretreatment Tregs are inefficient at suppressing effector functions of their targets.[7] Thirty patients were in remission (normal aspartate aminotransferase levels), four were in relapse, and 2 had recently started immunosuppressive treatment (aspartate aminotransferase levels, 59 and 69 IU/L; normal value, <50 IU/L). At the time of study, median aspartate aminotransferase level was 30 IU/L (range, 18–79 IU/L), bilirubin level was 8 μmol/L (range, 4–29 μmol/L; normal value, <20 μmol/L), immunoglobulin G level was 13.3 g/L (range, 6.6–28.6 g/L; normal value, 6.5–17 g/L), ANA titer was 1/40 (range, 1/10–1/320), and SMA titer was 1/160 (range, 1/20–1/2560). Patients were receiving prednisolone (2.5–5 mg daily) and azathioprine (1–2 mg/kg/day). Controls were 23 HS (19 female; median age, 31 years; range, 25–41 years), recruited from hospital staff, because for ethical reasons blood could not be obtained from healthy children. To test whether age disparity may account for differences, patients were divided into 2 subgroups (≤14 and >14 years old). Informed consent was obtained from each subject. The study was approved by the local ethics committee.

Cell Separation

Peripheral blood mononuclear cells (PBMCs) were obtained as previously described.[6] Cell viability, determined by Trypan blue exclusion, exceeded 98%.

Characterization of Cytokine Profile

The percentage of cytokine-producing ngTregs was determined by intracellular staining.[6,7] Cells were stained with fluorescein isothiocyanate– or APC-Cy7–conjugated anti-CD4, APC-conjugated or PE-Cy7–conjugated anti-CD25 monoclonal antibodies (BD Bioscience, Oxford, England), fluorescein isothiocyanate– or PE-conjugated anti–IL-17A (eBioscience, San Diego, CA), PE-conjugated monoclonal antibodies to interferon (IFN)-γ (IQ Products, Groningen, The Netherlands), IL-10 (BD Bioscience), IL-1β and IL-6 (eBioscience), and PerCP-conjugated anti-human LAP (TGF-β1) (R&D Systems, Abingdon, England). Flow cytometry was performed on a BD FACSCanto II (Immunocytochemistry Systems, BD Bioscience), acquiring a minimum of 1×10^4 gated events/sample; FACSDiva software was used for analysis.

Cell Purification

$CD4^+CD25^-$ and $CD4^+CD25^{high}$ cells were purified from PBMCs using immunomagnetic beads (Dynal Invitrogen, Oslo, Norway).[8] Their purity exceeded 90% and 95%, respectively.

Th17 Depletion

Freshly isolated $CD4^+CD25^-$ cells were cultured at 5×10^5 cells/well with anti-CD3/anti-CD28 T-cell expander (1 bead/cell) (Dynal Invitrogen), and recombinant IL-2 (rIL-2) (Eurocetus, Amsterdam, The Netherlands) was added at 300 U/mL the first week and each time the exhausted medium was replaced. Two-week expanded $CD4^+CD25^-$ cells were depleted of IL-17–secreting cells (Supplementary Figure 1) according to Streeck et al[21] (see Supplementary Materials and Methods). Following depletion, the frequency of IL-17–secreting cells within ngTregs was always <0.5%. IL-17–depleted cells were cultured for 3 additional weeks with 300 U/mL rIL-2. The frequency of cells positive for IL-17, RORC, a Th17 transcription factor,[22] and FOXP3 was monitored weekly. After a 3-week culture, ngTregs were purified from expanded $CD4^+CD25^-$ cells by CD4-negative selection followed by CD25-positive selection (Dynal Invitrogen)[8] (purity >95%) and their ability to suppress was assessed in a proliferation assay (see the following text). The frequency of $IL\text{-}17^+$, $RORC^+$, and $FOXP3^+$ cells during the 3-week culture, as well as the suppressor function of ngTregs isolated at the end of the third week, were also determined after stimulating Th17-depleted ngTregs with IL-6 (50 ng/mL) and IL-1β (10 ng/mL).

Phenotype and ability to suppress ngTregs obtained from $CD4^+CD25^-$ cells after treatment with neutralizing antibodies and/or cytokines and *RORC* gene knockdown were also evaluated (see the following text).

Characterization of ngTregs and Th17 Cell Phenotype

The phenotype of ngTregs was determined by flow cytometry using monoclonal antibodies to the surface markers CD25, CD62L, CD45RO, and CD127 (eBioscience) and to the intracellular molecules Granzyme B (BD Bioscience), a cytotoxic enzyme linked to Treg immunosuppressive function,[23] FOXP3 (clone PCH101; eBioscience), and CTLA-4 (BD Bioscience). Frequencies of IFN-γ, IL-10, IL-17A, IL-6, and IL-1β–producing ngTregs were determined as described previously. Expression of CD196 (CCR6) and IL-23 receptor, markers of Th17 cells,[24,25] and RORC was assessed in IL-17–producing CD4 cells using PerCP-conjugated anti-human CCR6 (BD Bioscience), anti-human IL-23 receptor (R&D Systems), and PE-conjugated anti-human RORC (eBioscience) monoclonal antibodies. Cells were analyzed by flow cytometry after gating on CD4 lymphocytes.

Inhibition of Proliferation

Following purification, ngTregs were added at a 1:8 ratio[6,7] to autologous $CD4^+CD25^-$ cells ($5–10 \times 10^4$/well) after 48-hour resting in T-cell expander- and IL-2–free medium. Experiments were performed in duplicate. After a 5-day culture, cells were pulsed with 0.25 μCi/well ^{3}H-thymidine and harvested 18 hours later. Incorporated thymidine was measured by a beta counter (Canberra Packard Ltd, Pangbourne, England). Percentage inhibition was calculated using the following formula: 1 – (Counts per Minute in the Presence of ngTregs/Counts per Minute in the Absence of ngTregs) × 100. Transwell assays were performed to determine whether ngTregs suppress through a direct cell-to-cell contact mechanism (see Supplementary Materials and Methods).

De Novo Generation of Tregs in the Absence or Presence of Neutralizing Antibodies and/or Cytokines

In preliminary experiments to determine the best maneuvers for inhibiting Th17 cell differentiation and boosting Treg function, exposure to rapamycin, retinoic acid, IL-17 neutralizing antibodies, and RORC-specific small interfering RNA (siRNA) were tested. Because the former 2 procedures proved ineffective (see Supplementary Materials and Methods), the latter were chosen.

$CD4^+CD25^-$ cells were cultured in the presence of T-cell expander and rIL-2 with or without recombinant TGF-β (5 ng/mL) and antibodies to IL-17A, IL-6, IL-1β, and IL-23 (R&D Systems), cytokines important for the development and differentiation of human Th17 cells, used at 10 μg/mL either individually or in combination. Following 4-week culture, ngTregs were purified (Supplementary Figure 1). True Treg (ie,

$CD4^+CD25^{high}CD127^-$) T cells were obtained from ngTregs as previously described.[26] Their purity exceeded 90%. Exposure to all neutralizing antibodies/cytokines tested did not affect cell viability throughout the culture period.

RORC *Gene Knockdown*

$CD4^+CD25^-$ cells expanded for 2 and 3 weeks were treated with a set of 3 Stealth RNAi siRNAs (Invitrogen Life Technologies) that block the expression of the Th17 transcription factor RORC. For transfection procedure, see Supplementary Materials and Methods. Transfections were performed at the end of the second and third week of culture (Supplementary Figure 1). After the first round of transfection, cells were washed and resuspended in culture medium with 300 U/mL rIL-2. Following the second transfection round, cells were washed and resuspended in culture medium before $CD4^+CD25^{high}$ cell purification. Following overnight incubation at 37°C and 5% CO_2, aliquots of 2.5×10^5 cells were collected to extract RNA and assess *RORC* gene expression by real-time polymerase chain reaction. Phenotype and suppressor function of RORC siRNA-treated cells were assessed as previously described. No significant changes in ngTreg viability were noted after transfection.

NgTreg phenotype and function were also assessed after combining the 3 Th17 inhibitory strategies. Briefly, $CD4^+CD25^-$ cells, expanded for 2 weeks with anti-CD3/anti-CD28 T-cell expander and rIL-2, were treated with anti–IL-6, anti–IL-1β, and recombinant Transforming Growth Factor-β (rTGF-β), immunomagnetically depleted of IL-17–secreting cells, and transfected (2 rounds) with RORC siRNAs. After the second transfection, ngTregs were purified and their phenotype, cytokine production, and suppressor ability evaluated.

Quantification of RORC *Gene Expression*

Expression of *RORC* gene was evaluated in freshly purified $CD4^+CD25^-$ cells and in RORC-specific siRNA untreated or treated ngTregs. RNA extraction and retrotranscription into complementary DNA were performed as described.[11] *RORC* gene transcripts were quantified by real-time polymerase chain reaction (see Supplementary Materials and Methods).

Statistical Analysis

Paired and unpaired Student *t* test was used for comparing normally distributed data; Wilcoxon rank sum test or Mann–Whitney test was used for non-normally distributed data. Results are expressed as mean ± SEM. $P < .05$ was considered significant.

Results

Frequency of IL-17–Producing Lymphocytes and Levels of RORC *Gene Transcripts*

The frequency of IL-17–producing $CD4^+CD25^-$ cells, freshly isolated from PBMCs, increased markedly in $CD4^+CD25^-$ cells purified after 4-week expansion of $CD25^{neg}$ cells ($P < .001$) and was higher in patients than in HS at both time points (Figure 1*A*). The frequency of IL-17–producing $CD4^+CD25^{high}$ cells isolated from 4-week expanded $CD25^-$ cells was higher than in freshly isolated $CD4^+CD25^{high}$ cells in both groups ($P < .001$ for both) (Figure 1*B*). In AIH, no difference in the frequency of IL-17–producing $CD25^-$ cells and IL-17–producing $CD25^{high}$ cells or in the ability to generate ngTregs was observed between the 2 age subgroups. Figure 1*C* shows the frequency of IL-17–producing $CD25^-$ and $CD25^{high}$ cells freshly isolated from PBMCs or after expansion of $CD25^-$ cells in one patient with AIH and one HS.

Among freshly isolated $CD4^+CD25^-$ cells, besides IL-17, we identified IL-1β– and IL-6–producing cells, with their frequency higher in AIH (IL-1β, 9.6 ± 1.25; IL-6, 5.8 ± 0.9) than in HS (IL-1β, 6.9 ± 1.6 [$P = .078$]; IL-6, 3.7 ± 0.5 [$P = .039$]).

Akin to the frequency of IL-17 cells, the levels of *RORC* gene transcripts in freshly isolated $CD4^+CD25^-$ cells increased in $CD25^-$ cells purified after 4-week expansion, but this increase did not reach statistical significance. Levels of *RORC* gene transcripts were significantly higher in patients than in HS at both time points (Figure 2*A*). Levels of *RORC* gene transcripts in $CD4^+CD25^{high}$ cells isolated from 4-week expanded $CD25^-$ cells were higher than in freshly isolated $CD4^+CD25^{high}$ cells in both groups (AIH, $P = .01$; HS, $P = .028$) (Figure 2*B*).

In addition to being $RORC^+$, IL-17–producing cells were $CCR6^+$, IL-23 receptor $^+$, and IFN-γ^- (Supplementary Figure 2).

To remove IL-17–producing cells and/or to condition the Treg maturation toward a fully differentiated Treg profile (see the following text), $CD25^-$ cells were subjected during expansion to a variety of strategies, applied individually or in combination.

Th17 Depletion

Depletion of IL-17–secreting cells from 2-week stimulated $CD4^+CD25^-$ cells (Supplementary Figure 1) was performed in 9 patients with AIH and 6 HS. The frequency of IL-17–producing cells and that of $RORC^+$ cells within the Th17-depleted cell fraction was negligible, remaining so over the 3 additional weeks of culture (Supplementary Figure 3*A* and *B*), while the frequency of $FOXP3^+$ cells increased throughout this period (Supplementary Figure 3*C*). As previously reported,[11] FOXP3 mean fluorescence intensity (MFI) peaked at week 2 and decreased by the end of week 3, although it remained higher than at the end of week 1 of expansion (Supplementary Figure 3*C*). ngTregs, purified from the Th17-depleted cell fraction and challenged with IL-6 and IL-1β, cytokines key to Th17 cell development, remained IL-17^- and $RORC^-$ while expressing high levels of FOXP3 for the whole period of culture (Supplementary Figure 3*A*–*C*).

Suppressor function. In AIH, ngTregs purified from the Th17-depleted cell fraction reduced the mean $CD25^-$ target cell proliferation by 44.2%, an inhibition rate higher than that obtained with ngTregs isolated from nondepleted cells (32%). In contrast, in HS no difference was observed whether Th17-depleted (34.8%) or nondepleted (38%) ngTregs (Table 1) were used. Challenging Th17-depleted ngTregs with IL-6 and IL-1β did not affect their suppressor ability (Table 1).

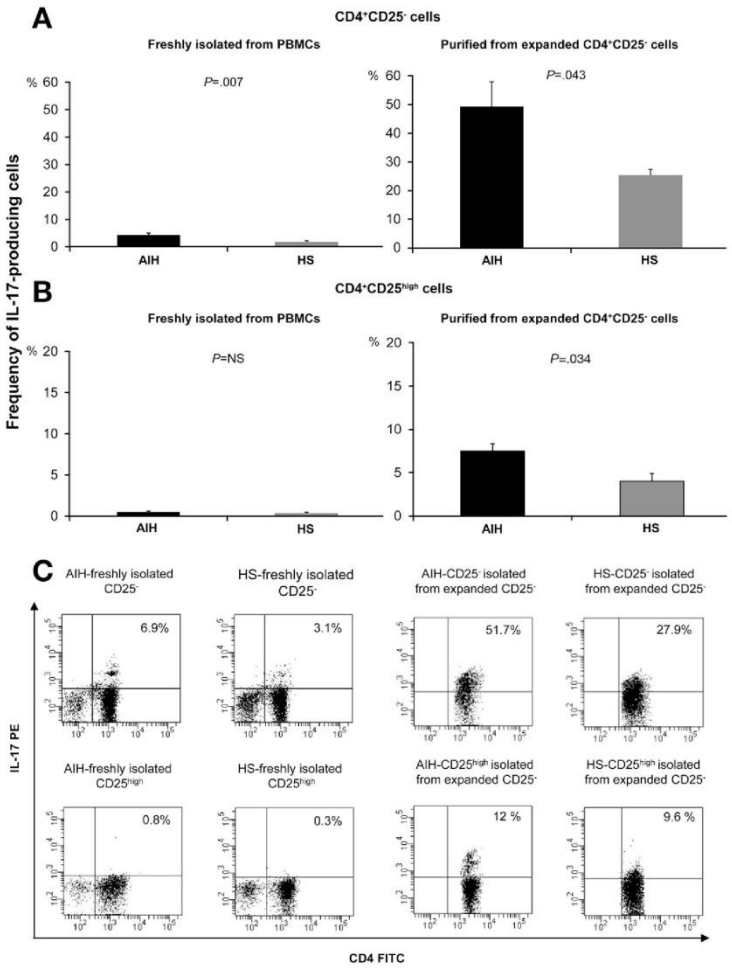

Figure 1. Frequency of IL-17–producing lymphocytes. Frequency of IL-17–producing lymphocytes was determined by intracellular cytokine staining. (*A* and *B*) Mean (+SEM) frequency of (*A*) IL-17–producing $CD4^+CD25^-$ and (*B*) $CD4^+CD25^{high}$ cells isolated from PBMCs (*left*) and from expanded $CD4^+CD25^-$ cells (*right*) in patients with AIH (*black bars*) and HS (*gray bars*). (*C*) Dot plots showing the frequency of IL-17–producing $CD25^-$ or $CD25^{high}$ cells purified from freshly isolated PBMCs or from expanded $CD25^-$ cells from a representative patient with AIH and from a representative HS.

Th17 Inhibition: Effect of rTGF-β and Anti–IL-6/Anti–IL-1β Neutralizing Antibodies

The cytokine/neutralizing antibody combination was most effective at reducing the number of IL-17–producing cells within $CD25^-$ cells and ngTregs, and at conditioning the maturation of the latter into fully differentiated Tregs was established in 5 patients and 5 HS. In these studies, antibodies neutralizing IL-17, IL-6, IL-1β, and IL-23 were used either alone or in combination in the absence or presence of rTGF-β. The most effective inhibitory cocktail was found to comprise anti–IL-6, anti–IL-1β, and rTGF-β (Figure 3). This cocktail was then tested on 20 patients with AIH and 17 HS.

Following 4-week expansion in the absence or presence of the cocktail, ngTregs were isolated from expanded $CD4^+CD25^-$ cells (Supplementary Figure 1) and their phenotype and ability to suppress were assessed.

Phenotype. Compared with freshly isolated $CD4^+CD25^{high}$ Tregs and akin to our previous findings,[11] ngTregs from both patients with AIH and HS were $CD25^{high}$, $CD45RO^{high}$, and $CD62L^{high}$ but contained higher numbers of Granzyme B^+ cells (78.3 ± 5.8 vs 3.2 ± 1.2, $P < .001$; data from 2 patients with AIH and 3 HS). Akin to freshly isolated $CD4^+CD25^{high}$ cells,[8] ngTregs suppressed via a cell-to-cell contact mechanism as shown

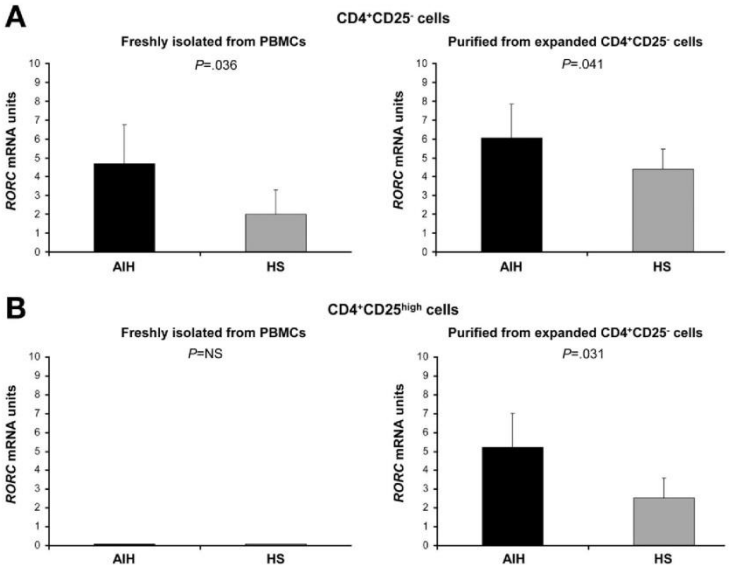

Figure 2. *RORC* gene expression. RORC gene expression was evaluated by real-time polymerase chain reaction. The panels show mean (+SEM) RORC messenger RNA units of (*A*) $CD4^+$ $CD25^-$ and (*B*) $CD4^+CD25^{high}$ cells isolated from PBMCs (*left*) or from expanded $CD4^+CD25^-$ cells (*right*) in patients with AIH (*black bars*) and HS (*gray bars*).

in Transwell experiments (see Supplementary Materials and Methods).

The frequency of $CD25^{high/+}$, $FOXP3^+$, and $CTLA\text{-}4^+$ cells was similar in ngTregs cultured with or without the inhibitory cocktail both in AIH and in HS. In contrast, CD25 and FOXP3 expression in individual cells, detected as MFI, was lower in both groups of subjects among untreated ngTregs than among those cocktail treated (Table 2). CTLA-4 MFI tended to be lower in untreated than in cocktail-treated ngTregs from both AIH and HS

BASIC AND TRANSLATIONAL LIVER

Table 1. $CD4^+CD25^-$ Cell Proliferation in the Presence of Untreated ngTregs or of ngTregs After Th17 Depletion, Th17 Inhibition, or RORC Knockdown

Experimental condition	Patients with AIH	HS
Th17 depletion		
$CD25^-$	A: 99,796 ± 18,859	a: 101,429 ± 18,305
$CD25^-$ + untreated ngTregs	B: 67,679 ± 12,567	b: 63,655 ± 10,642
$CD25^-$ + Th17-depleted ngTregs	C: 55,686 ± 10,340	c: 66,131 ± 11,935
$CD25^-$ + Th17-depleted ngTregs + challenge (exposure to IL-6 + IL-1β)	D: 64,508 ± 11,978	d: 55,988 ± 10,104
	A vs B: P = .017; A vs C: P = .03 A vs D: P = .01; B vs C: P = NS B vs D: P = NS; C vs D: P = NS	a vs b: P = .001; a vs c: P = .002 a vs d: P = .047; b vs c: P = NS b vs d: P = NS; c vs d: P = NS
Th17 inhibition		
$CD25^-$	E: 111,034 ± 12,149	e: 116,286 ± 26,228
$CD25^-$ + untreated ngTregs	F: 78,279 ± 8565	f: 68,027 ± 15,343
$CD25^-$ + Th17-inhibited ngTregs	G: 58,626 ± 6414	g: 70,818 ± 15,973
	E vs F: P = .007; E vs G: P = .002 F vs G: P = .08	e vs f: P = .005; e vs g: P = .03 f vs g: P = NS
RORC knockdown		
$CD25^-$	H: 88,099 ± 10,980	h: 107,545 ± 17,949
$CD25^-$ + untreated ngTregs	I: 63,431 ± 7905	i: 66,137 ± 7514
$CD25^-$ + RORC knocked down ngTregs	J: 52,683 ± 6566	j: 49,900 ± 8328
	H vs I: P = .001; H vs J: P = .014 I vs J: P = .02	h vs i: P = .005; h vs j: P = .013 i vs j: P = .1

NOTE. Results expressed as counts per minute (mean ± SEM).
NS, not significant.

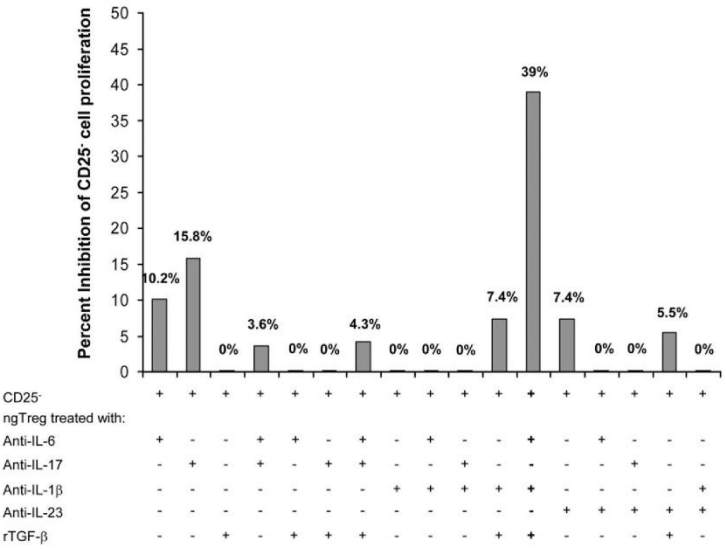

Figure 3. Inhibition of $CD25^-$ cell proliferation by Tregs generated under different neutralizing antibody/cytokine combinations. The columns represent the percentage inhibition of $CD25^-$ target cell proliferation after addition of Tregs generated in the presence of different neutralizing antibody/cytokine combinations. Percentages of inhibition are indicated at the top of each column. The percentage of inhibition obtained in the presence of Tregs treated with anti–IL-6, anti–IL-1β neutralizing antibodies, and rTGF-β was higher than that obtained in the presence of Tregs generated under different antibody/cytokine combinations ($P < .05$ for all). ngTreg/$CD25^-$ cell ratio is 1/8.

(Table 2). FOXP3 and CTLA-4 MFI in cocktail-treated ngTregs tended to be lower in AIH than in health ($P = .06$ and $P = .12$, respectively). Exposure to the inhibitory cocktail induced a decrease in the percentage of IFN-γ and IL-17–producing cells among ngTregs in both patients with AIH and HS (Table 2), with this decrease more marked in AIH (IFN-γ, 3-fold; IL-17, 4.3-fold) than in health (IFN-γ, 1.7-fold; IL-17, 2.2-fold), but left unchanged the frequency of IL-10–producing cells in both groups (Table 2). The frequency of IL-10–producing cells within untreated and cocktail-treated ngTregs was lower in patients than in controls ($P = .009$ and $P = .1$ respectively).

Suppressor function. In AIH, treatment with anti–IL-6, anti–IL-1β, and rTGF-β enhanced ngTreg suppressor function, with the proliferation of $CD25^-$ target cells decreased by 29.5% after addition of untreated and by 47.2% after addition of cocktail-treated ngTregs (Table 1). In contrast, in HS, treatment with the inhibitory cocktail did not affect the ability of ngTregs to suppress, with the proliferation of $CD25^-$ target cells reduced by 41.5% after addition of untreated and by 39.1% after addition of treated ngTregs.

Enrichment of $CD127^-$ ngTregs. In 6 patients with AIH and 6 HS (including 2 patients and 3 controls whose cocktail-treated ngTregs had been tested for phenotype and function) in whom sufficient numbers of ngTregs were obtained, further purification of Tregs on the basis of CD127 expression was performed.[26] No difference in the frequency of $CD25^{high/+}$, $FOXP3^+$, and $CTLA\text{-}4^+$ ngTregs was observed before and after CD127 depletion among cocktail-treated and untreated ngTregs obtained from either patients with AIH or HS. Among patients with AIH, depletion of $CD127^+$ cells from ngTregs obtained from untreated $CD4^+CD25^-$ cells led to an increase of CD25, FOXP3, and CTLA-4 MFI; an increase of CTLA-4 MFI was observed also among CD127-depleted ngTregs obtained from cocktail-treated $CD4^+$ $CD25^-$ cells (Figure 4*A*). Among HS, depletion of $CD127^+$ cells from ngTregs obtained from untreated $CD4^+CD25^-$ cells led to an increase of FOXP3 and CTLA-4 MFI; a marked increase of CTLA-4 MFI was observed in CD127-depleted ngTregs obtained from cocktail-treated $CD4^+CD25^-$ cells (Figure 4*A*). CTLA-4 MFI in cocktail-treated ngTregs after CD127 depletion was lower in AIH than in health ($P = .04$). Whether obtained in the absence or presence of cocktail, $CD127^-$ ngTregs had lower frequencies of IFN-γ–, IL-17–, and IL-10–producing cells than CD127 nondepleted ngTregs in both AIH and health (Figure 4*B*). The frequency of IL-10–producing cells within CD127-depleted ngTregs tended to be lower in patients than in controls both in the absence and presence of cocktail treatment ($P = .1$ and $P = .08$, respectively). In 2 patients with AIH and 3 HS, in whom the effect of $CD127^-$ enrichment on TGF-β production by ngTregs was also tested, a decrease in IL-10–producing ngTregs was paralleled by an increase in the frequency of ngTregs producing TGF-β (5.7 ± 2.3 vs 33.9 ± 16.8, $P = .1$).

CD127-depleted ngTregs inhibited target cell proliferation by 47.2% in AIH and 39.1% in health in the absence of treatment and by 58.7% and 51.2% following cocktail treatment (Figure 4*C*).

Table 2. Phenotype of Untreated and Treated ngTregs

	Untreated ngTregs	Treated ngTregs	*P* value
Patients with AIH			
Th17 inhibition			
CD25 (MFI)	75.5 ± 9.2	106.8 ± 11.3	.039
FOXP3 (MFI)	56.6 ± 7.5	76.9 ± 10.6	.079
CTLA-4 (MFI)	79.1 ± 7.4	101.4 ± 8.9	NS
IFN-γ$^+$ (%)	3.9 ± 1.2	1.3 ± 0.6	.02
IL-17$^+$ (%)	14.2 ± 4.7	3.3 ± 1.2	.043
IL-10$^+$ (%)	1.23 ± 0.4	1.4 ± 0.8	NS
RORC knockdown			
CD25 (MFI)	81.1 ± 18.5	112 ± 25.6	.014
FOXP3 (MFI)	51.7 ± 2.5	67.6 ± 4.6	.088
CTLA-4 (MFI)	106.6 ± 10	105.4 ± 9.9	NS
IFN-γ$^+$ (%)	3.2 ± 10.7	1.6 ± 0.3	.086
IL-17$^+$ (%)	10.1 ± 2.2	3.9 ± 0.8	.026
IL-10$^+$ (%)	1.4 ± 0.4	0.6 ± 0.2	NS
HS			
Th17 inhibition			
CD25 (MFI)	66.6 ± 14.3	101.4 ± 8.9	.015
FOXP3 (MFI)	59.4 ± 4.1	121.4 ± 16.7	.002
CTLA-4 (MFI)	98.9 ± 14.7	174.1 ± 25.6	.09
IFN-γ$^+$ (%)	4.1 ± 0.7	2.4 ± 0.7	.024
IL-17$^+$ (%)	9.3 ± 2.9	4.3 ± 1.6	.043
IL-10$^+$ (%)	5.4 ± 1.2	5.8 ± 2.8	NS
RORC knockdown			
CD25 (MFI)	63.3 ± 7.2	115.8 ± 13.1	.002
FOXP3 (MFI)	52.9 ± 3.5	61.9 ± 4.1	.036
CTLA-4 (MFI)	104.9 ± 10.7	126.9 ± 12.9	.044
IFN-γ$^+$ (%)	5.1 ± 1	2.6 ± 0.5	.088
IL-17$^+$ (%)	7.3 ± 1.5	2.8 ± 0.6	.01
IL-10$^+$ (%)	3.8 ± 0.9	8 ± 1.8	.07

NOTE. Treated ngTregs indicates ngTregs obtained after Th17 inhibition or RORC knockdown. Results are shown as mean ± SEM. *P* value comparing CD25, FOXP3, and CTLA-4 MFI and the frequency of IFN-γ$^+$, IL-17$^+$, and IL-10$^+$ cells between untreated and treated ngTregs.
NS, not significant.

BASIC AND TRANSLATIONAL LIVER

Th17 Inhibition Through RORC Knockdown

Treatment of expanded CD4$^+$CD25$^-$ cells with RORC-specific siRNAs, performed in 10 patients with AIH and 6 HS, led to a decrease in the expression of *RORC* gene by 86.6% in AIH and by 86.9% in HS. A decrease in *RORC* gene expression following siRNA treatment was paralleled by a decrease in the frequency of RORC$^+$ ngTregs.

Phenotype. The frequency of CD25^{high} cells was lower in untreated than in RORC siRNA-treated ngTregs in patients (P = .07) and HS (P = .086), whereas no difference in the frequency of FOXP3$^+$ and CTLA-4$^+$ cells from both groups was noted before and after treatment. CD25, FOXP3, and CTLA-4 MFI was higher in treated than in untreated cells from both AIH and HS; in the case of CTLA-4, this difference reached statistical significance in health only (Table 2). FOXP3 and CTLA-4 MFI of RORC siRNA-treated ngTregs was lower in AIH than in health (P = .13 and P = .03, respectively). Treatment with RORC siRNA reduced the frequency of IFN-γ– and IL-17–producing ngTregs and increased that of Tregs producing IL-10 in HS only (Table 2). The frequency of RORC siRNA-treated IL-10–producing ngTregs was lower in patients with AIH than in HS (P = .001).

Suppressor function. After addition of siRNA-treated ngTregs, the mean CD25$^-$ target cell proliferation was reduced by 40.2% in patients with AIH and by 53.6% in HS, with these inhibition values higher than after addition of untreated ngTregs (28% in AIH [P = .007] and 38.5% [P = NS] in HS) (Table 1).

No differences in the phenotype and ability to suppress ngTregs obtained after Th17 depletion, inhibition, or RORC knockdown were noted between the 2 AIH age subgroups.

Th17 Inhibition Following Combined Treatment With Neutralizing Antibody/rTGF-β, RORC Knockdown, and IL-17–Secreting Cell Removal

In both patients with AIH and HS, a decrease in IL-17– and IFN-γ–producing cells was more marked when ngTregs were subjected to all the previously described anti–IL-17 maneuvers in combination (IL-17: 75.8% in AIH, 64% in HS; IFN-γ: 82.2% in AIH, 84.3% in HS) than when they were submitted to individual anti–IL-17 strategies (average for IL-17: 69.1% in AIH, 58% in HS; for IFN-γ: 57.5% in AIH, 45.3% in HS), while the frequencies of CD25, FOXP3, and CTLA-4 cell positivity and MFI were similar between combined and individual approaches. There was also no difference in the frequency of IL-10–producing ngTregs after combined or individual treatment.

CD25$^-$ target cell proliferation was reduced by an average of 43.8% in AIH and 42.5% in health when ngTregs were treated with individual approaches and by 63.2% and by 82% when the three strategies were combined. Figure 5 shows the decrease in CD25$^-$ cell proliferation after addition of ngTregs treated with individual or combined approaches in one patient with AIH and in one HS.

Discussion

Adoptive transfer of Tregs is a promising mode of treatment for autoimmune diseases,[27–29] particularly when the target antigen is known; in AIH type 2 Tregs specific for cytochrome P450IID6, the main autoantigen in this condition, grow readily in culture and are highly efficient in suppressing effectors of damage in low numbers.[30] For autoimmune diseases where the target antigen is less well characterized, such as AIH type 1, immunoregulatory treatment would depend on the possibility of generating large numbers of non–antigen-specific Tregs from the circulating CD4$^+$CD25^{high}FOXP3$^+$ T cells, which are difficult to expand and prone to apoptosis, or from CD4$^+$CD25$^-$ cells with regulatory potential that expand much more efficiently and are more resistant to apoptosis.

The present study, however, shows that in AIH and health, a high proportion of newly generated Tregs, particularly in patients, bear the phenotypic and functional signature of Th17 cells despite expressing classic Treg markers, raising questions on their potential immune

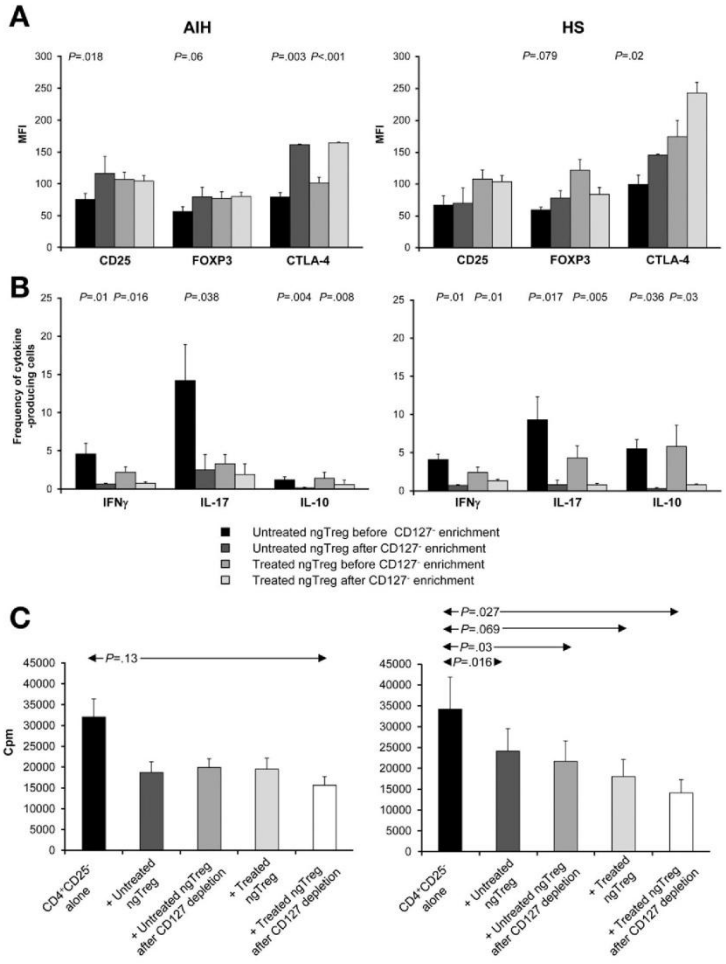

Figure 4. Effect of anti–IL-6, anti–IL-1β neutralizing antibodies, and rTGF-β on phenotype and suppressive function of CD127 depleted ngTregs obtained from $CD4^+CD25^-$ cells. (*A*) Mean (+SEM) CD25, FOXP3, and CTLA-4 MFI and (*B*) mean (+SEM) frequency of IFN-γ–, IL-17–, and IL-10–producing ngTregs before and after CD127 depletion in patients with AIH (*left*) and HS (*right*). *Black and dark gray bars* refer to ngTregs obtained from $CD4^+CD25^-$ cells in the absence and *light gray bars* in the presence of the inhibitory cocktail. (*C*) Mean (+SEM) counts per minute of $CD25^-$ cells from patients with AIH (*left*) and HS (*right*) cultured either alone or with untreated ngTregs; untreated ngTregs after CD127 depletion; ngTregs obtained from $CD25^-$ cells treated with anti–IL-6, anti–IL-1β, and rTGF-β; and ngTregs obtained from $CD25^-$ cells treated with anti–IL-6, anti–IL-1β, and rTGF-β after CD127 depletion. ngTreg/$CD25^-$ cell ratio is 1/8.

therapeutic use; importantly, however, it also shows that this limitation can be overcome by conditioning the differentiation process of the IL-17–producing ngTregs toward a fully polarized Treg phenotype with consequent strengthening of their suppressive function.

We show that exposure of $CD25^-$ cells to a Treg generating polyclonal T-cell stimulus, consisting of anti-CD3/anti-CD28 T-cell expander in the presence of high concentrations of IL-2, leads to the generation of new Tregs that express high levels of CD25, FOXP3, and CTLA-4. We also show that ngTregs from patients with AIH mediate suppression by direct cell contact, akin to what we previously observed for freshly isolated Tregs.[8] Such direct cell contact effect may be mediated at least in part by Granzyme B, which is highly expressed by ngTregs from both HS, as previously reported,[31] and from patients with AIH, as shown in this study.

A proportion of the newly generated Tregs, however, produces IL-17, with the frequency of IL-17–producing cells higher among ngTregs than in pre-expansion freshly isolated $CD25^-$ or $CD25^{high}$ cells. Up-regulation of *RORC* by $FOXP3^+$ ngTregs upon polyclonal expansion (Figure 2*B*) indicates that these cells exhibit both regulatory and effector features at the transcriptional level.

To assess how regulatory T-cell function is influenced by the presence of Th17 ngTregs and affected by their removal/control, we have used different approaches, either alone or in combination.

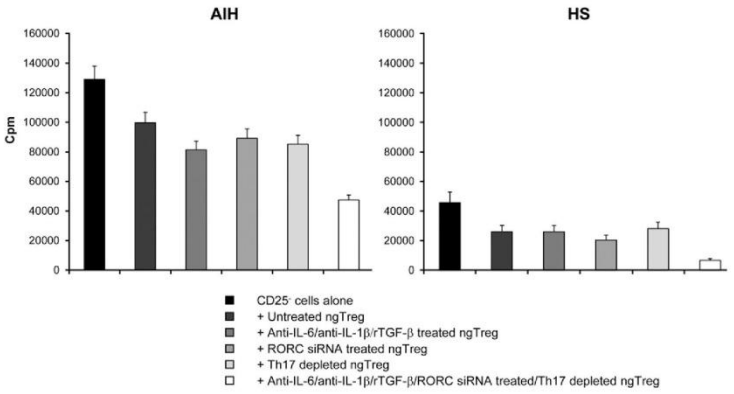

Figure 5. Inhibition of $CD25^-$ cell proliferation following addition of ngTregs treated with separate or combined anti–IL-17 maneuvers. Mean (+SEM) counts per minute of $CD25^-$ target cells cultured alone; with untreated ngTregs; with ngTregs after neutralizing antibody/cytokine treatment, siRNA treatment, Th17 depletion; or after treatment with the 3 maneuvers combined in patients with AIH (*left*) and in HS (*right*). ngTreg/$CD25^-$ cell ratio is 1/8.

The physical removal of IL-17 ngTregs using immunomagnetic depletion led to a significant enhancement of ngTreg suppressor function. Of relevance to the therapeutic role of these cells, during the 3-week expansion after Th17 depletion, the phenotype of $CD25^{high}$ Tregs remained stable, with no reappearance of IL-17 and RORC positivity, suggesting that elimination of IL-17 orients $CD25^{high}$ cells toward a fully differentiated Treg phenotype. Moreover, challenge with IL-6 and IL-1β, cytokines promoting Th17 cell development, did not drive IL-17–depleted ngTregs toward an effector phenotype, because they remained negative for RORC and IL-17 and continued to express high levels of FOXP3 with preserved suppressor function.

A second approach to counteract the Th17 influence and promote a polarized Treg status was to generate ngTregs in an environment that precludes Th17 cell development. To achieve this, besides using antibodies neutralizing IL-6 and IL-1β, we treated $CD25^-$ cells with recombinant TGF-β to boost their regulatory function. We observed a marked reduction in the frequency of IL-17–producing cells and increased ngTreg FOXP3/CTLA-4 expression and suppressive function. This neutralizing maneuver also reduced the frequency of IFN-γ–producing ngTregs without affecting the Th2 lineage, akin to the findings of Burgler et al following siRNA-mediated *RORC2* knockdown in human CD4 naïve cells, interpreted as an indirect effect of the increase in FOXP3 expression.[32] The results of this IL-17 inhibitory approach are of particular relevance in AIH, because IL-6 and IL-1β as well as IFN-γ are produced abundantly during the active phases of the disease, and suggest that therapeutic adoptive transfer of Tregs will require preliminary control of the inflammatory milieu,[33] which might promote emergence of Th17 cells.

The third approach, based on the selection within ngTregs of $CD127^{-/low}$ cells (ie, cells with the greatest ability to suppress) enabled us to obtain highly polarized Tregs characterized by enhanced suppressor function, marked elevation in CTLA-4 expression, a molecule whose immune regulatory role has been shown in the context of rheumatoid arthritis,[34] and reduction in IL-17– and IFN-γ–producing ngTregs. This approach also resulted in ngTregs containing a lower frequency of IL-10–producing cells and increased numbers of lymphocytes producing TGF-β, a cytokine linked to Treg suppression.[8]

An alternative approach was to treat $CD25^-$ cells with RORC-specific siRNA to knock down the translation of *RORC*, thus interfering with the Th17 transcriptional program. Akin to what was observed after milieu conditioning with the anti–IL-17 cytokine/antibody cocktail, *RORC* knockdown not only augmented the suppressor function of ngTregs and enhanced their FOXP3 and CTLA-4 expression, but also reduced their expression of proinflammatory cytokines.

Lastly, when we combined all anti-Th17 approaches, we observed an additive effect with a greater decrease in IL-17– and IFN-γ–producing ngTregs alongside a substantial enhancement of ngTreg suppressive ability, indicating that fully polarized and functionally stable Tregs can be obtained from $CD4^+CD25^-$ T cells by combining the removal of IL-17–producing Tregs, the conditioning of the milieu, and the repression of *RORC*. That conditioning the inflammatory milieu is a prerequisite for Treg adoptive transfer is indicated by the improvement in Treg function and number during drug-induced remission in AIH[6,7] and by the enhancement of Treg function when tissue inflammation is pre-emptively controlled in autoimmune encephalomyelitis.[33]

In conclusion, the information obtained in the present study is an important step toward the establishment of new therapeutic maneuvers in autoimmune disease. Although further investigation is needed to confirm the effectiveness, stability, and half-life of autologous ngTregs under good manufacturing practice procedures, we show that differentiation of $CD25^-$ cells into stable Tregs can be achieved through abrogation of Th17 cell development. This is of particular relevance for immu-

notherapeutic purposes aiming at reestablishing immune tolerance through Treg infusion, especially in those conditions, such as AIH type 1, in which antigen specificity is not well defined.

Supplementary Material

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at www.gastrojournal.org, and at http://dx.doi.org/10.1053/j.gastro.2012.02.041.

References

1. Sakaguchi S, Yamaguchi T, Nomura T, et al. Regulatory T cells and immune tolerance. Cell 2008;133:775–787.
2. Shevach EM, DiPaolo RA, Andersson J, et al. The lifestyle of naturally occurring CD4+ CD25+ Foxp3+ regulatory T cells. Immunol Rev 2006;212:60–73.
3. Lindley S, Dayan CM, Bishop A, et al. Defective suppressor function in CD4(+)CD25(+) T-cells from patients with type 1 diabetes. Diabetes 2005;54:92–99.
4. Viglietta V, Baecher-Allan C, Weiner HL, et al. Loss of functional suppression by CD4+CD25+ regulatory T cells in patients with multiple sclerosis. J Exp Med 2004;199:971–979.
5. Ehrenstein MR, Evans JG, Singh A, et al. Compromised function of regulatory T cells in rheumatoid arthritis and reversal by anti-TNFalpha therapy. J Exp Med 2004;200:277–285.
6. Longhi MS, Ma Y, Bogdanos DP, et al. Impairment of CD4(+)CD25(+) regulatory T-cells in autoimmune liver disease. J Hepatol 2004;41:31–37.
7. Longhi MS, Ma Y, Mitry RR, et al. Effect of CD4+ CD25+ regulatory T-cells on CD8 T-cell function in patients with autoimmune hepatitis. J Autoimmun 2005;25:63–71.
8. Longhi MS, Hussain MJ, Mitry RR, et al. Functional study of CD4+CD25+ regulatory T cells in health and autoimmune hepatitis. J Immunol 2006;176:4484–4491.
9. Longhi MS, Ma Y, Mieli-Vergani G, et al. Aetiopathogenesis of autoimmune hepatitis. J Autoimmun 2010;34:7–14.
10. Gregorio GV, Portmann B, Reid F, et al. Autoimmune hepatitis in childhood: a 20-year experience. Hepatology 1997;25:541–547.
11. Longhi MS, Meda F, Wang P, et al. Expansion and de novo generation of potentially therapeutic regulatory T cells in patients with autoimmune hepatitis. Hepatology 2008;47:581–591.
12. Walker MR, Kasprowicz DJ, Gersuk VH, et al. Induction of FoxP3 and acquisition of T regulatory activity by stimulated human CD4+CD25- T cells. J Clin Invest 2003;112:1437–1443.
13. Tartar DM, VanMorlan AM, Wan X, et al. FoxP3+RORgammat+ T helper intermediates display suppressive function against autoimmune diabetes. J Immunol 2010;184:3377–3385.
14. Beriou G, Costantino CM, Ashley CW, et al. IL-17-producing human peripheral regulatory T cells retain suppressive function. Blood 2009;113:4240–4249.
15. Bettelli E, Carrier Y, Gao W, et al. Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells. Nature 2006;441:235–238.
16. Korn T, Bettelli E, Oukka M, et al. IL-17 and Th17 cells. Annu Rev Immunol 2009;27:485–517.
17. Miossec P, Korn T, Kuchroo VK. Interleukin-17 and type 17 helper T cells. N Engl J Med 2009;361:888–898.
18. Korn T, Bettelli E, Gao W, et al. IL-21 initiates an alternative pathway to induce proinflammatory T(H)17 cells. Nature 2007;448:484–487.
19. Acosta-Rodriguez EV, Napolitani G, Lanzavecchia A, et al. Interleukins 1beta and 6 but not transforming growth factor-beta are essential for the differentiation of interleukin 17-producing human T helper cells. Nat Immunol 2007;8:942–949.
20. Manel N, Unutmaz D, Littman DR. The differentiation of human T(H)-17 cells requires transforming growth factor-beta and induction of the nuclear receptor RORgammat. Nat Immunol 2008;9:641–649.
21. Streeck H, Cohen KW, Jolin JS, et al. Rapid ex vivo isolation and long-term culture of human Th17 cells. J Immunol Methods 2008;333:115–125.
22. Ivanov II, McKenzie BS, Zhou L, et al. The orphan nuclear receptor RORgammat directs the differentiation program of proinflammatory IL-17+ T helper cells. Cell 2006;126:1121–1133.
23. Gondek DC, Lu LF, Quezada SA, et al. Cutting edge: contact-mediated suppression by CD4+CD25+ regulatory cells involves a granzyme B-dependent, perforin-independent mechanism. J Immunol 2005;174:1783–1786.
24. Singh SP, Zhang HH, Foley JF, et al. Human T cells that are able to produce IL-17 express the chemokine receptor CCR6. J Immunol 2008;180:214–221.
25. Awasthi A, Riol-Blanco L, Jager A, et al. Cutting edge: IL-23 receptor gfp reporter mice reveal distinct populations of IL-17-producing cells. J Immunol 2009;182:5904–5908.
26. Longhi MS, Mitry RR, Samyn M, et al. Vigorous activation of monocytes in juvenile autoimmune liver disease escapes the control of regulatory T-cells. Hepatology 2009;50:130–142.
27. Roncarolo MG, Battaglia M. Regulatory T-cell immunotherapy for tolerance to self antigens and alloantigens in humans. Nat Rev Immunol 2007;7:585–598.
28. Riley JL, June CH, Blazar BR. Human T regulatory cell therapy: take a billion or so and call me in the morning. Immunity 2009;30:656–665.
29. Brusko TM, Putnam AL, Bluestone JA. Human regulatory T cells: role in autoimmune disease and therapeutic opportunities. Immunol Rev 2008;223:371–390.
30. Longhi MS, Hussain MJ, Kwok WW, et al. Autoantigen-specific regulatory T cells, a potential tool for immune-tolerance reconstitution in type-2 autoimmune hepatitis. Hepatology 2011;53:536–547.
31. Efimova OV, Kelley TW. Induction of granzyme B expression in T-cell receptor/CD28-stimulated human regulatory T cells is suppressed by inhibitors of the PI3K-mTOR pathway. BMC Immunol 2009;10:59.
32. Burgler S, Mantel PY, Bassin C, et al. RORC2 is involved in T cell polarization through interaction with the FOXP3 promoter. J Immunol 2010;184:6161–6169.
33. Korn T, Reddy J, Gao W, et al. Myelin-specific regulatory T cells accumulate in the CNS but fail to control autoimmune inflammation. Nat Med 2007;13:423–431.
34. Flores-Borja F, Jury EC, Mauri C, et al. Defects in CTLA-4 are associated with abnormal regulatory T cell function in rheumatoid arthritis. Proc Natl Acad Sci U S A 2008;105:19396–19401.

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Acknowledgments

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Conflicts of interest

The authors disclose no conflicts.

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Supplementary Materials and Methods

Th17 Depletion

A total of 3×10^6 expanded $CD4^+CD25^-$ cells were treated with 20 μL capture complex, consisting of biotin-labeled anti-CD45 (Caltag-Medsystems, Buckingham, England) and anti–IL-17 (eBioscience) antibodies and avidin (Sigma-Aldrich, Dorset, England). Following incubation on ice for 15 minutes, 9 mL RPMI 10% fetal calf serum was added to the cell suspension. After rotation at 37°C for 90 minutes, cells were stained with PE-conjugated anti–IL-17 monoclonal antibodies (eBioscience). After 20-minute incubation and washing, anti-PE microbeads were added and IL-17–positive and –negative cell fractions were purified using MS columns (Miltenyi Biotec, Bergisch-Gladbach, Germany).

Transwell Experiments

Transwell experiments were performed as described previously.[1] Briefly, ngTregs were added to autologous $CD4^+CD25^-$ cells seeded at 1×10^5/well in the lower chamber of a 24-well plate. ngTregs were either cultured in the lower chambers directly in contact with the target cells or in the upper chambers separated from the target cells by a 0.4-μm pore membrane (BD Biosciences Discovery Labware, Oxford, England). Control cultures using $CD4^+CD25^-$ cells on their own were performed under identical conditions. Experiments were performed in duplicate. Cells were cultured for 5 days in the presence of anti-CD3/anti-CD28 T-cell expander (ratio bead/cell, 1:2) and rIL-2. On the last day of culture, cells from the lower chambers were collected and transferred after centrifugation in 96-well plates and their proliferative response was tested by ^{3}H-thymidine incorporation.

De Novo Generation of Tregs in the Presence of Rapamycin or Retinoic Acid

Freshly isolated $CD4^+CD25^-$ cells from 3 patients with AIH and 2 HS were cultured at 5×10^5 cells/well in the presence of T-cell expander (1 bead/cell) (Dynal Invitrogen), rIL-2 (300 U/mL) (Eurocetus), and either rapamycin[2] (10 nmol/L) or retinoic acid[3,4] (10 nmol/L) (both from Sigma-Aldrich, Gillingham, United Kingdom). These concentrations were determined following titration experiments, where concentrations of 1, 10, and 100 nmol/L for rapamycin and 5, 10, and 20 nmol/L for retinoic acid were tested. Concentrations of 10 nmol/L for rapamycin and 10 nmol/L for retinoic acid were chosen because they were associated with the lowest ngTreg CD127 expression and the highest FOXP3 expression. Cells were cultured for 4 weeks; at the end of the culture period, $CD4^+CD25^{high}$ cells were purified using immunomagnetic beads and their phenotype and suppression function evaluated.

RORC *Gene Knockdown*

Transfection procedure. Expanded $CD4^+CD25^-$ cells were resuspended in Opti-MEM medium (Invitrogen Life Technologies) at $2–3 \times 10^6$/mL and then transfected using 8 μL Lipofectamine RNAiMax (Invitrogen Life Technologies). RORC-specific siRNA were used at a final concentration of 3 nmol/L. RORC siRNA working concentration and Lipofectamine RNAiMax volume were determined after performing a series of preliminary experiments in which Stealth RNAi concentrations ranging from 0.75 to 24 nmol/L and Lipofectamine RNAiMax volumes ranging from 1 to 16 μL/mL of total cell suspension were tested. A RORC siRNA concentration of 3 nmol/L and a Lipofectamine RNAiMax volume of 8 μL proved to be most effective at inducing gene knockdown with minimal toxicity to the cells. A GAPDH-specific Stealth RNAi siRNAs and a negative control Stealth RNAi siRNAs (Invitrogen Life Technologies) served as controls.

Quantification of RORC *Gene Expression*

RORC gene transcripts were quantified using gene-specific probes and TaqMan Master Mix (Applied Biosystems, Warrington, England). Complementary DNA was used at 4 μg/mL. Polymerase chain reaction amplification conditions were as described.[5] Samples were run in triplicate using a real-time polymerase chain reaction thermocycler (ABI Prism Sequence Detection Systems, Applied Biosystems, Foster City, CA), and results were analyzed by matched software. Relative expression of *RORC* was determined by normalization to *GAPDH* and, after *RORC* gene knockdown, by normalization to ribosomal RNA (kindly donated by Dr R. R. Mitry).

Supplementary Results

Transwell Assay

The mean $CD25^-$ cell count per minute decreased by 39.5% in HS and 33.5% in patients with AIH when $CD25^-$ cells were cultured in direct contact with ngTregs (Supplementary Figure 4). No inhibition of $CD25^-$ cell proliferation was observed when ngTregs were kept separated by a Transwell membrane (Supplementary Figure 4).

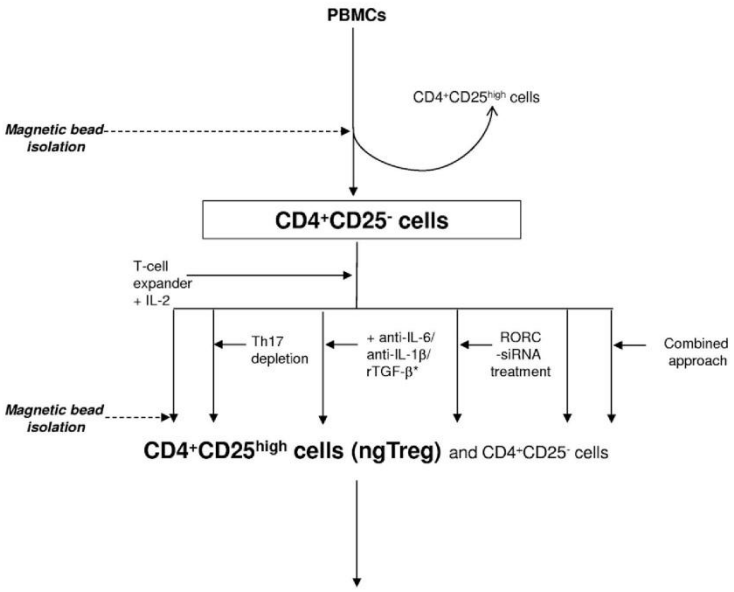
De Novo Treg Generation Following Exposure to Rapamycin or Retinoic Acid

Exposure to rapamycin, while reducing the frequency of IL-17–producing ngTregs by 54%, did not have any effect on the number of ngTregs producing IFN-γ and on the ability of ngTregs to suppress. Reduction in IL-17–producing ngTregs obtained in the presence of rapamycin was either similar or lower than that obtained by treating ngTregs with neutralizing antibodies/cytokine cocktail (76.8% in AIH and 54% in HS) or RORC-specific siRNA (61.5% in AIH and 62.1% in HS). Exposure to retinoic acid resulted in no inhibition of IL-17– and

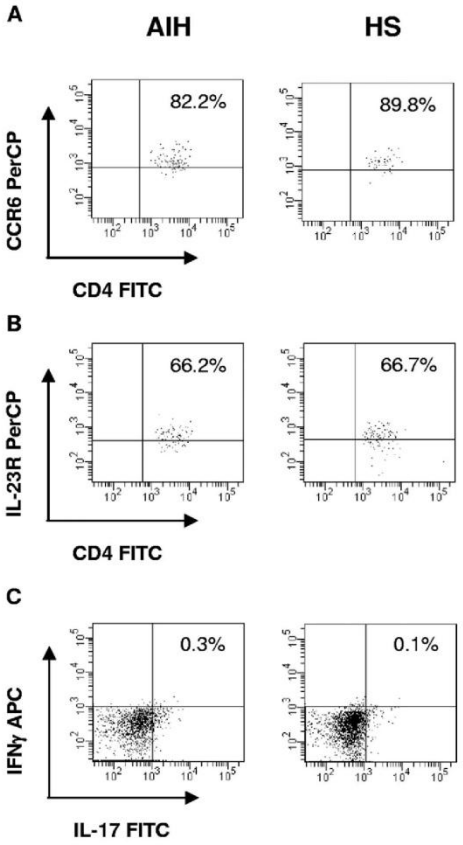
IFN-γ–producing ngTregs and in no suppressive function.

Supplementary References

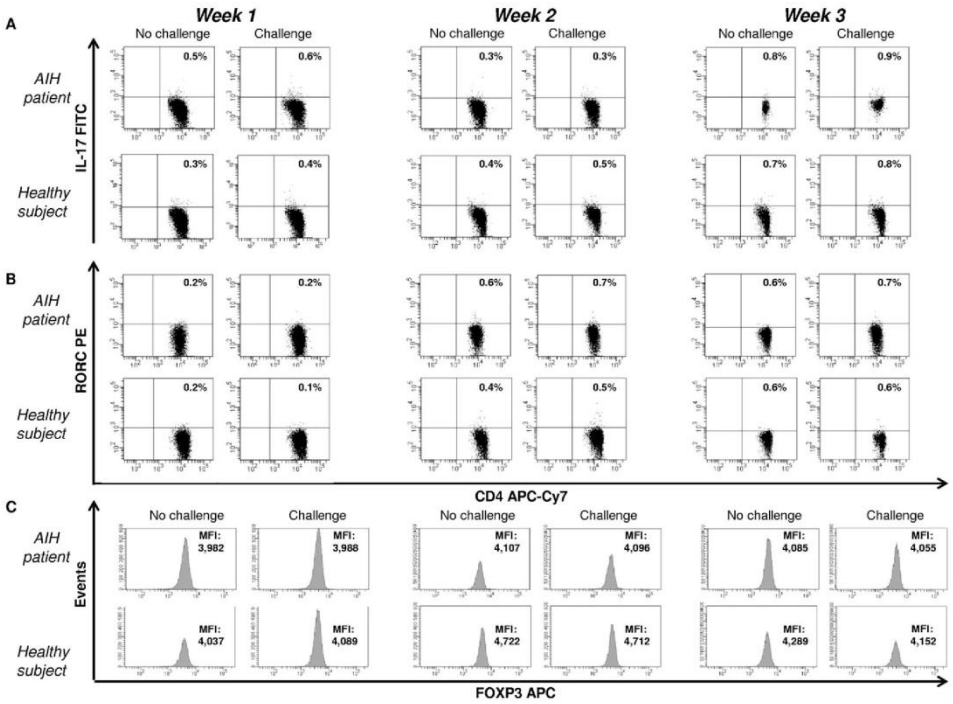
1. Longhi MS, Hussain MJ, Mitry RR, et al. Functional study of CD4+CD25+ regulatory T cells in health and autoimmune hepatitis. J Immunol 2006;176:4484–4491.
2. Strauss L, Whiteside TL, Knights A, et al. Selective survival of naturally occurring human CD4+CD25+Foxp3+ regulatory T cells cultured with rapamycin. J Immunol 2007;178:320–329.
3. Elias KM, Laurence A, Davidson TS, et al. Retinoic acid inhibits Th17 polarization and enhances FoxP3 expression through a Stat-3/Stat-5 independent signaling pathway. Blood 2008;111:1013–1020.
4. Wang J, Huizinga TW, Toes RE. De novo generation and enhanced suppression of human CD4+CD25+ regulatory T cells by retinoic acid. J Immunol 2009;183:4119–4126.
5. Longhi MS, Meda F, Wang P, et al. Expansion and de novo generation of potentially therapeutic regulatory T cells in patients with autoimmune hepatitis. Hepatology 2008;47:581–591.



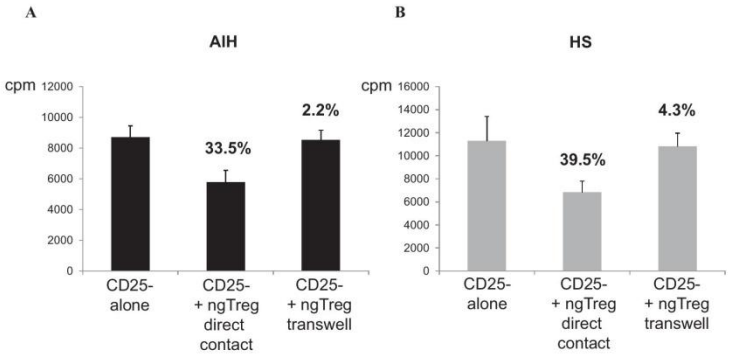
Supplementary Figure 1. Production of newly generated Tregs. To newly generate $CD4^+CD25^{high}$ T cells (ngTregs), immunomagnetic beads were used to separate $CD4^+CD25^-$ cells from naturally occurring $CD4^+CD25^{high}$ T cells within PBMCs. Purified $CD4^+CD25^-$ cells were then exposed to anti-CD3/anti-CD28 T-cell expander/high-dose IL-2 and cultured for 4 weeks. At the end of culture, newly generated $CD4^+CD25^{high}$ cells were immunomagnetically isolated and tested directly or after exposure to one of the following 4 treatments: (1) Th17 depletion (at the end of the second week of culture), (2) treatment with anti–IL-6/anti–IL-1β/rTGF-β (added at the same time of T-cell expander and IL-2), (3) transfection with RORC siRNA (at the end of the second and third week of culture), and (4) a combination of strategies 1–3. Purified ngTregs were then tested for their phenotype, cytokine production, and suppressor function.



Supplementary Figure 2. Phenotype of Th17 cells. Dot plots showing the frequency of CD4 cells positive for (*A*) CCR6, (*B*) IL-23R, and (*C*) IFN-γ within IL-17–producing CD25$^-$ cells isolated from one representative patient with AIH (*left*) and one representative HS (*right*). Cells in *A* and *B* are gated on IL-17$^+$ lymphocytes; cells in *C* are gated on CD4 lymphocytes.



Supplementary Figure 3. Phenotype of Th17-depleted ngTregs. Results obtained in a representative patient with AIH and a representative HS are shown. (*A* and *B*) Frequency of (*A*) IL-17–producing and (*B*) RORC$^+$ cells within Th17-depleted ngTregs (purified from 2-week CD4$^+$CD25$^-$ cells stimulated with anti-CD3/anti-CD28 T-cell expander at 1 bead per cell and rIL-2 at 300 U/mL) during a 3-week culture period in the absence or presence of proinflammatory challenge with IL-6 and IL-1β, applied simultaneously. (*C*) MFI of FOXP3 in Th17-depleted ngTregs during the same culture period in the absence or presence of the challenge.



Supplementary Figure 4. Transwell experiments. ngTregs from 2 HS and 3 patients with AIH were added to autologous $CD4^+CD25^-$ cells seeded in the lower chamber of a 24-well plate. ngTregs were added either directly or separated by a Transwell semipermeable membrane. $CD25^-$ cell proliferation was tested by thymidine incorporation. The plots show the proliferation of $CD25^-$ cells in (*A*) patients with AIH and (*B*) HS. Columns in each plot represent the mean (+SEM) counts per minute of $CD25^-$ cells cultured on their own and after addition of ngTregs in the presence or absence of Transwell membrane.

Key Paper Evaluation

EXPERT REVIEWS

Effect of ethnicity on the clinical presentation and outcome of autoimmune hepatitis

Expert Rev. Gastroenterol. Hepatol. 6(3), 267–269 (2012)

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Evaluation of: Wong RJ, Gish R, Frederick T, Bzowej N, Frenette C. The impact of race/ethnicity on the clinical epidemiology of autoimmune hepatitis. *J. Clin. Gastroenterol.* 46(2), 155–161 (2012).

Autoimmune hepatitis (AIH) is a progressive inflammatory disorder affecting children and adults of both sexes. Although AIH is known to occur in all geographical areas, racial differences have not been studied systematically. The paper by Wong *et al.* reports a retrospective study analyzing AIH epidemiology among ethnically different populations referred to a tertiary-care hospital. A total of 183 patients were included and divided according to their ethnicity into Hispanic, Asian and Caucasian groups. Age at diagnosis and sex distribution was similar in all three groups. Compared with Caucasians, Asian patients had a higher international normalized ratio, whereas, in addition to higher international normalized ratio, Hispanics also showed lower levels of albumin and platelets, as well as higher prevalence of biopsy-proven cirrhosis at presentation. Despite this, Kaplan–Meier analysis showed that Hispanics had the best survival outcomes, followed by Caucasians. Asians had the poorest survival outcome.

Keywords: Asians • autoimmune hepatitis • Caucasians • clinical outcome • epidemiology • Hispanics

Methods & results

The authors of the evaluated article investigated the clinical presentation and outcomes of type 1 autoimmune hepatitis (AIH) in different US residents of different ethnicity/race who were referred to a tertiary-care hospital. Clinical, biochemical, immunological and histological data at presentation of patients diagnosed with AIH from 1999 to 2009 were retrospectively reviewed. The authors then performed Kaplan–Meier analysis to estimate survival among different race groups. Primary outcome measures included time to death or liver transplantation (LT). Development of hepatocellular carcinoma was used as a secondary measure of outcome.

Of the 322 patients screened, 183 fulfilled the criteria for either definite or probable AIH according to the revised International Autoimmune Hepatitis Group (IAIHG) scoring system and were thus included in the study; 61 patients had histological evidence of primary biliary cirrhosis (PBC) and were diagnosed as having PBC-AIH variant syndrome. Patients were divided according to their ethnic background into three different groups: Caucasians (n = 146; 79.8%), Hispanics (n = 20; 10.9%) and Asians (n = 17; 9.3%). African – Americans, who accounted for only two AIH diagnoses, were excluded from the study.

The mean age at diagnosis was 49.3 years (range: 36.7–61.9 years); 80.3% of patients were women. Age and female/male ratio did not differ among the different groups studied. The presence of PBC-AIH variants was considerably less frequent in Asians (n = 3; 17.6%) than in Hispanics (n = 7; 35%) and Caucasians (n = 53; 36.3%). At presentation, the model for end-stage liver disease (MELD) score did not differ among the different races. However, Asians and Hispanics presented with a higher INR compared with Caucasians. Hispanics also had lower levels of albumin and platelets at presentation. Consistent with this, when liver biopsies

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were analyzed Hispanics showed the highest frequency of cirrhosis (n = 11; 55%) compared with Caucasians (n = 44; 30%) and Asians (n = 5; 29%).

The authors have then investigated the outcome of AIH during a mean follow-up time of 69.0 months (range: 24.9–113.1 months). Progression to LT was high within all groups (Caucasians: n = 27, 18.5%; Hispanics: n = 5, 25%; Asians: n = 4, 23.5%) but was not statistically different between the different groups. In terms of survival of those patients who were not transplanted, mortality was significantly higher in Asians (n = 5; 29.4%) compared with Hispanics (n = 1; 5%) and Caucasians (n = 11; 7.5%). Similarly, Asians showed the highest incidence of hepatocellular carcinoma (n = 3; 17.6%), followed by Hispanics (n = 2; 10%) and Caucasians (n = 4; 2.7%). A Kaplan–Meier analysis showed that the probability of a poor outcome was significantly higher in Asians followed by Caucasians, whereas Hispanics demonstrated the best survival outcomes.

Discussion & expert commentary

AIH is an inflammatory liver disorder that can progress to cirrhosis and liver failure despite immunosuppressive treatment. It is characterized by hypergammaglobulinemia, interface hepatitis and seropositivity for autoantibodies: antinuclear (ANA) and antismooth muscle (SMA) antibodies, defining AIH type 1 (AIH-1), and liver–kidney microsomal antibody type 1 (LKM-1) and anticytosol antibody type 1 (LC-1), defining AIH type 2 (AIH-2) [1].

The etiology of AIH is unknown, although both genetic and environmental factors are involved [2]. In spite of its global distribution, AIH prevalence and behavior is known to vary according to ethnicity [1].

The results of this study [3], showing the impact of race on the presentation and outcomes of AIH-1, represent a further important step for the comprehension of this heterogeneous disease. While similar studies conducted on the US population showed that black patients have a more aggressive clinical course than non-blacks [4,5], and that Alaskan natives exhibited a higher frequency of acute icteric disease than their non-native counterparts [6], this is the first study to demonstrate a more severe presentation and a worse outcome in US AIH patients from Hispanic and Asian background.

Hispanic patients had more aggressive presentation both biochemically and histologically in comparison with non-Hispanics. At the initial presentation, they were almost two-times more likely to have cirrhosis than Caucasians and Asians. Interestingly, however, Hispanic AIH patients required LT at the same rate as other ethnic groups and showed the best survival outcome. The impact of the presence of cirrhosis at presentation on the natural history of AIH has been evaluated by other studies, the majority of them showing that cirrhosis correlates with a poorer outcome [5,7–9]. However, in line with the evaluated article, a large study by Roberts *et al*. has shown that the presence of histological cirrhosis at diagnosis did not diminish long-term survival expectations [10]. In view of both studies, it should be emphasized that AIH responds to immunosuppressive treatment, whatever the degree of liver impairment.

Asian AIH patients, on the other hand, demonstrated a higher rate of mortality and thus displayed the worst survival curve. Three possible explanations for this negative outcome may be proposed. First, as this was a hospital-based study, and was consequently limited by tertiary referral bias, it may have resulted in an overestimation in severity, with an underestimation of incidence and prevalence. This bias, however, would have affected all groups studied equally and thus is unlikely to account for the worst outcome reported in Asians. Second, socioeconomic status, healthcare access and thus quality of care are factors that must always be considered when assessing disease manifestations within ethnic groups [1]. However, since Hispanics were the group presenting with more advanced disease, it seems unlikely that Asians were seeking medical attention for longer than other groups. Third, it is conceivable that genetic variations may have accounted for the different natural histories of the disease observed among the diverse ethnic groups. AIH is a 'complex trait' disease; in other words, it does not follow any Mendelian pattern of inheritance. Its exact mode of inheritance is unknown, although like other human complex trait disorders it involves one or more genes that, acting alone or in concert, and interacting with environmental factors, increase or reduce the risk of the trait [11]. Previous studies have clearly shown that genetic factors influence AIH occurrence, clinical expression and response to corticosteroid therapy [12,13]. In this regard, the strongest genetic associations relate to genes located within the major histocompatibility complex (MHC) – the human leukocyte antigen (HLA) region – on the short arm of chromosome 6, particularly those encoding *DRB1* alleles [14]. In Europeans and white North Americans, the alleles conferring susceptibility to AIH-1 are *DRB1*0301* and *DRB1*0401*, which encode the HLA DR3 and DR4 antigens, respectively [15,16]. A later study, comparing US and Brazilian AIH populations, has indicated that possession of the HLA *DRB1*1301* allele predisposes to pediatric AIH-1 in the Brazilian population [17], thus suggesting that the genetic predisposition varies among different populations. The current study, however, provides no information about HLA class II genotypes among different AIH ethnic patients, therefore constituting one key limitation in interpreting the data, and according to us this should be addressed in future studies.

In summary, the results of this study show that the clinical course of AIH-1 is different in patients of diverse ethnic backgrounds. It is likely that a combination of different factors may account for this observation, including time of diagnosis, treatment regimen and dosage, and importantly, genetic disparities between different groups.

Five-year view

The evaluated article is a step forward in the characterization of AIH among different ethnic/racial groups. The authors show that presentation and outcome of AIH vary between

races. The reasons for this disparity should be addressed in the future, particularly to understand whether patients belonging to particular ethnic groups have a more advanced disease at presentation, a poor initial response to immunosuppressive therapy and/or a specific genetic predisposition. Further studies also need to focus on the development of different treatment protocols, particularly for Asian patients who displayed the worst outcome.

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Key issues

- Clinical presentation and outcomes in autoimmune hepatitis (AIH) are determined, at least in part, by ethnicity/race.
- Compared with Caucasians, Hispanic patients with AIH appear to present with higher rates of cirrhosis, whereas Asians show a worse overall survival.
- Future studies are needed to establish the immunogenetic influence on the development, clinical course and response to therapy of AIH in ethnically different patients.

References

1 Manns MP, Czaja AJ, Gorham JD *et al.*; American Association for the Study of Liver Diseases. Diagnosis and management of autoimmune hepatitis. *Hepatology* 51(6), 2193–2213 (2010).

2 Liberal R, Longhi MS, Mieli-Vergani G, Vergani D. Pathogenesis of autoimmune hepatitis. *Best Pract. Res. Clin. Gastroenterol.* 25(6), 653–664 (2011).

3 Wong RJ, Gish R, Frederick T, Bzowej N, Frenette C. The impact of race/ethnicity on the clinical epidemiology of autoimmune hepatitis. *J. Clin. Gastroenterol.* 46(2), 155–161 (2012).

4 Lim KN, Casanova RL, Boyer TD, Bruno CJ. Autoimmune hepatitis in African Americans: presenting features and response to therapy. *Am. J. Gastroenterol.* 96(12), 3390–3394 (2001).

5 Verma S, Torbenson M, Thuluvath PJ. The impact of ethnicity on the natural history of autoimmune hepatitis. *Hepatology* 46(6), 1828–1835 (2007).

6 Hurlburt KJ, McMahon BJ, Deubner H, Hsu-Trawinski B, Williams JL, Kowdley KV. Prevalence of autoimmune liver disease in Alaska Natives. *Am. J. Gastroenterol.* 97(9), 2402–2407 (2002).

7 Verma S, Gunuwan B, Mendler M, Govindrajan S, Redeker A. Factors predicting relapse and poor outcome in type I autoimmune hepatitis: role of cirrhosis development, patterns of transaminases during remission and plasma cell activity in the liver biopsy. *Am. J. Gastroenterol.* 99(8), 1510–1516 (2004).

8 Verma S, Redeker A. In type 1 autoimmune hepatitis, is cirrhosis at presentation or follow-up associated with a poorer outcome? *Hepatology* 42(5), 1237; author reply 1237–author reply 1238 (2005).

9 Feld JJ, Dinh H, Arenovich T, Marcus VA, Wanless IR, Heathcote EJ. Autoimmune hepatitis: effect of symptoms and cirrhosis on natural history and outcome. *Hepatology* 42(1), 53–62 (2005).

10 Roberts SK, Therneau TM, Czaja AJ. Prognosis of histological cirrhosis in type 1 autoimmune hepatitis. *Gastroenterology* 110(3), 848–857 (1996).

11 Donaldson PT. Genetics of liver disease: immunogenetics and disease pathogenesis. *Gut* 53(4), 599–608 (2004).

12 Czaja AJ, Carpenter HA, Santrach PJ, Moore SB. Significance of HLA DR4 in type 1 autoimmune hepatitis. *Gastroenterology* 105(5), 1502–1507 (1993).

13 Czaja AJ, Strettell MD, Thomson LJ *et al.* Associations between alleles of the major histocompatibility complex and type 1 autoimmune hepatitis. *Hepatology* 25(2), 317–323 (1997).

14 Donaldson PT. Genetics of autoimmune and viral liver diseases; understanding the issues. *J. Hepatol.* 41(2), 327–332 (2004).

15 Donaldson PT, Doherty DG, Hayllar KM, McFarlane IG, Johnson PJ, Williams R. Susceptibility to autoimmune chronic active hepatitis: human leukocyte antigens DR4 and A1-B8-DR3 are independent risk factors. *Hepatology* 13(4), 701–706 (1991).

16 Strettell MD, Donaldson PT, Thomson LJ *et al.* Allelic basis for HLA-encoded susceptibility to type 1 autoimmune hepatitis. *Gastroenterology* 112(6), 2028–2035 (1997).

17 Czaja AJ, Souto EO, Bittencourt PL *et al.* Clinical distinctions and pathogenic implications of type 1 autoimmune hepatitis in Brazil and the United States. *J. Hepatol.* 37(3), 302–308 (2002).

APPENDIX III

Oral presentations

1. **R. Liberal**, Y. Ma, G. Mieli-Vergani, D. Vergani, M.S. Longhi. “Dysregulation of GAL-9/TIM3 pathway mirrors T-reg impairment in autoimmune hepatitis", presented at the 7th International Congress on Autoimmunity, Ljubljana, Slovenia, 2010, and at the 14th International Congress of Immunology, Kobe, Japan, 2010
2. **R. Liberal**, Y. Ma, G Mieli-Vergani, D. Vergani, M.S. Longhi. "Tim3 down-regulation renders CD4 effector cells less susceptible to T-reg control in patients with autoimmune hepatitis", presented at Annual Meeting of the British Association for the Study of the Liver, Edinburgh, UK, 2010
3. **R. Liberal**. "Reduced expression of TIM3 renders CD4 cells less amenable to immune-regulation in autoimmune hepatitis", presented at the 7th International Postgraduate Conference, Hradec Kralove, Czech Republic, 2010
4. **R. Liberal**. “Role of TIM-3/galectin-9 pathway in autoimmune hepatitis”, Academic Seminar Programme, King´s College London, London, UK, 2011
5. **R. Liberal**, J. Gandara, S. Ferreira, V. Lopes, G. Mieli-Vergani, D. Vergani, J. Daniel, H.P. Miranda. “De-novo autoimmune hepatitis following liver transplantation for familial amyloidotic polyneuropathy”, presented at VIIth International Symposium on Familial Amyloidotic Polyneuropathy, Kumamoto, Japan, 2011.
6. **R. Liberal**, C.R. Grant, B. Holder, G. Mieli-Vergani, D. Vergani, M.S. Longhi. “Phenotypic and functional signature of $CD4^{pos}CD25^{high}CD127^{low}$ regulatory T cells in autoimmune hepatitis”, presented at 8^{th} International Congress on Autoimmunity, Granada, Spain, 2012

7. **R. Liberal**, C.R. Grant, B. Holder, G. Mieli-Vergani, D. Vergani, M.S. Longhi. "Reduced expression of inhibitory molecules characterises CD4 and CD8 effector cells in autoimmune hepatitis", presented at 8th International Congress on Autoimmunity, Granada, Spain, 2012
8. **R. Liberal**, C.R. Grant, B. Holder, G. Mieli-Vergani, D. Vergani, M.S. Longhi. "The T-cell effector phenotype profile in autoimmune sclerosing cholangitis differs from that of autoimmune hepatitis", presented at 8th International Congress on Autoimmunity, Granada, Spain, 2012

Poster presentations

1. **R. Liberal**, Y. Ma, G. Mieli-Vergani, D. Vergani, M.S. Longhi. “Dysregulation of GAL-9/TIM-3 pathway mirrors T-reg impairment in autoimmune hepatitis", presented at the 45th Annual Meeting of the European Association for the Study of the Liver, Vienna, Austria, 2010
2. **R. Liberal**, Y. Ma, G Mieli-Vergani, D. Vergani, M.S. Longhi. "Tim3 down-regulation renders CD4 effector cells less susceptible to T-reg control in patients with autoimmune hepatitis", presented at the 61st Liver Meeting of the American Association for the Study of the Liver, Boston, USA, 2010
3. **R. Liberal**, Y. Ma, G. Mieli-Vergani, D. Vergani, M.S. Longhi. “Reduced galectin-9 expression is associated with defective T-reg control of effector cells in patients with autoimmune hepatitis”, presented at the 46th Annual Meeting of the European Association for the Study of the Liver, Berlin, Germany, 2011
4. M.S. Longhi, **R. Liberal**, G. Mieli-Vergani, Y. Ma, D. Vergani. “Inhibition of Th17-cell development enhances the suppressive function of newly generated T-regs in patients with autoimmune hepatitis”, presented at the 46th Annual Meeting of the European Association for the Study of the Liver, Berlin, Germany, 2011
5. **R. Liberal**, J. Gandara, S. Ferreira, V. Lopes, G. M.S. Longhi, G. Mieli-Vergani, D. Vergani, J. Daniel, H.P. Miranda. De-novo autoimmune hepatitis following liver transplantation for familial amyloidotic polyneuropathy, presented at the 62nd Liver Meeting of the American Association for the Study of the Liver, San Francisco, USA, 2011

6. **R. Liberal**, C.R. Grant, B. Holder, G. Mieli-Vergani, D. Vergani, M.S. Longhi. "Reduced expression of Tim-3 renders Th1 and Th17 effector cells less amenable to T-reg control in autoimmune hepatitis", presented at the 62nd Liver Meeting of the American Association for the Study of the Liver, San Francisco, USA, 2011, and at British Society for Immunology Congress, Liverpool, UK, 2011
7. **R. Liberal**, C.R. Grant, B. Holder, G. Mieli-Vergani, D. Vergani, M.S. Longhi. "Defective T-regulatory function in autoimmune hepatitis may partially derive from a pro-inflammatory skewing of $Gal9^{+}$ T-regs", presented at the 62nd Liver Meeting of the American Association for the Study of the Liver, San Francisco, USA, 2011, and at British Society for Immunology Congress, Liverpool, UK, 2011
8. C.R. Grant, **R. Liberal**, B. Holder, Y. Ma, G. Mieli-Vergani, D. Vergani, M.S. Longhi. "Phenotypic and functional stability of regulatory T cells in autoimmune hepatitis", presented at British Society for Immunology Congress, Liverpool, UK, 2011
9. **R. Liberal**, J. Gandara, S. Ferreira, V. Lopes, G. M.S. Longhi, G. Mieli-Vergani, D. Vergani, J. Daniel, H.P. Miranda. "Incidence, clinical presentation and outcome of de-novo autoimmune hepatitis after liver transplantation for familial amyloidotic polyneuropathy", presented at European Association for the Study of the Liver Special Conference on Liver Transplantation, Lisbon, Portugal, 2011
10. C.R. Grant, **R. Liberal**, B. Holder, Y. Ma, G. Mieli-Vergani, D. Vergani, M.S. Longhi. "Low CD39 expression marks severe regulatory T-reg impairment in

patients with autoimmune sclerosing cholangitis", presented at the 47th Annual Meeting of the European Association for the Study of the Liver, Barcelona, Spain, 2012

11. **R. Liberal**, C.R. Grant, B. Holder, G. Mieli-Vergani, D. Vergani, M.S. Longhi. "Phenotypic and functional signature of $CD4^{pos}CD25^{high}CD127^{low}$ regulatory T cells in autoimmune hepatitis", presented at the 47th Annual Meeting of the European Association for the Study of the Liver, Barcelona, Spain, 2012
12. **R. Liberal**, C.R. Grant, B. Holder, G. Mieli-Vergani, D. Vergani, M.S. Longhi. "Reduced expression of inhibitory molecules characterises CD4 and CD8 effector cells in autoimmune hepatitis", presented at the 47th Annual Meeting of the European Association for the Study of the Liver, Barcelona, Spain, 2012
13. **R. Liberal**, C.R. Grant, B. Holder, G. Mieli-Vergani, D. Vergani, M.S. Longhi. "The T-cell effector phenotype profile in autoimmune sclerosing cholangitis differs from that of autoimmune hepatitis", presented at the 47th Annual Meeting of the European Association for the Study of the Liver, Barcelona, Spain, 2012
14. B.S. Holder, C.R.Grant, **R. Liberal,** Y. Ma, G. Mieli-Vergani, D. Vergani, M.S Longhi. "Cytochrome P450IID6-specific T-regs generated from patients with autoimmune hepatitis type-2 are phenotypically stable upon expansion and pro-inflammatory challenge", presented at the 47th Annual Meeting of the European Association for the Study of the Liver, Barcelona, Spain, 2012