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UNIVERSITY OF GLASGOW

THE ROLE OF TH17 CELLS IN A MODEL OF RHEUMATOID ARTHRITIS

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ABSTRACT

Introduction: While many studies on rheumatoid arthritis have focused on the active phase of the disease, the events that lead to the development of autoimmunity remain poorly defined. We have developed a model of breach of self tolerance, where a Th1 response to irrelevant antigen (OVA) results in arthropathy associated with spontaneous induction of autoreactive T and B cell responses, which allows the investigation of the immnologival events that lead to the development of autoreactivity. Employing this model the role of Th17 cells, a a subset of IL-17 producing $CD4^+$ T important in autoimmunity, was investigated in the development of autoimmunity. In addition, the relative ability of Th1 and Th17 polarised populations in supporting B cell responses was analysed. Finally, in this thesis the role of sterile damage regulation in the development of autoimmunity was assesed, by investigating the role of Siglec-G, a molecule involved in DAMP-signalling regulation, in this process.

Results: Transfer of OVA specific Th17 cells induced similar levels of inflammation as Th1 cells, and could induce a breach of self tolerance as demonstrated by CII specific T and B cell responses. While the CII specific T cells in the Th1 recipients produced IFNγ and not IL-17, surprisingly the CII T cell responses in the Th17 recipients were predominantly IFNγ producers. Whereas the transferred OVA specific Th1 population retained its phenotype, the transferred Th17 population displayed significantly reduced IL-17 production. However, cells polarised under Th17 conditions expanded in a higher degree and persisted for longer time in response to immunisation. This resulted in a higher ability of Th17 polarised population in supporting B cell responses. Finally in this thesis, preliminaty data for a role of Siglec-G in the development of autoimmunity were presented, as Siglec-G deficient mice were protected from the development of autoreactive B cell responses.

Conclusion: The results of this thesis suggest that the developing autoimmuniy in both Th1 and Th17 models is mediated by Th1 cells. These studies highlight the plasticity of transferred cell populations in vivo, and support the use of blocking and fate-mapping studies to definitively address how auto-reactive responses develop.

Authors declaration

"I declare that, except where explicit reference is made to the contributionof others,that this dissertation is the result of my own work and has not been submitted forany other degree atthe UniversityofGlasgow orany other institution."

Signature:

Printed Name: AgapitosPatakas

Abbreviations

Anti-Citrullinated Protein/Peptide Antibodies	ACPA
Antigen	Ag
Aryl-hydrocarbon Receptor	AhR
Antigen Induced Arthritis	AIA
Activation-Induced Cell Death	AICD
Anti-Nuclear Antibodies	ANA
ANalysis Of VAriance	ANOVA
Antigen Presenting Cells	APCs
A Proliferation Inducing Ligand	APRIL
Arginine	Arg
Bcl-2-associated X protein	Bax
B cell lymphoma-2	Bcl-2
B cell Receptor	BcR
Immunoglobulin Binding Protein	BiP
B Lympocyte stimulator	Blys
Bovine Serum Albumin	BSA
Collagen Antibody-Induced Arthritis	CAIA
Cluster of differentiation	CD
conventional DCs	cDCs
Complete Freund's Adjuvant	CFA
Collagen-Induced Arthritis	CIA
Collagen Type II	CII
Cytotoxic T-Lymphocyte Antigen	CTLA
Curdlan	CUR
C	Cysteine
Decay accelerating Factor	DAF
Damage-Associated Molecular Patterns	DAMPs
Dendritic Cells	DCs
draining Lymph Nodes	dLNs
Disease modifying anti-rheumatic drugs	DMARDs
Deoxyribonucleic acid	DNA
Experimental Autoimmune Engephalomyelitis	EAE
Ethylenediaminetetraacetic acid	EDTA
5-ethynyl-2´-deoxyuridine	EDU
Enhanced Green Fluorescent Protein	EGFP
Enzyme-Linked Immunosorbent Assay	ELISA
Fluorescence-activated cell sorting	FACS
Fc Receptor	FcR
Fetal Calf Serum	FCS
Follicular Dendritic Cells	FDC

Fibroblast Growth Factor	FGF
6-Formyl-Indolo[3,2-b]CarbaZole	FICZ
Fluorescein isothiocyanate	FITC
Forkhead box P3	FoxP3
Germinal Centre	GC
Granulocyte Colony Stimulating Factor	G-CSF
Green Fluorescent Protein	GFP
Glucocorticoid-Induced Tumor necrosis factor Receptor family−related	GITR
Granulocyte Monocyte Colony Stimulating Factor	GM-CSF
Glucose-6-Phospate Isomerase	GPI
Gradient Reflective Index leNses	GRIN
Genome Wide Association Studies	GWAS
Granzyme B	GZMB
Heamatoxylin and Eosin	H&E
Heat aggregated Ovalbumin	HAO
Human Cartilage antigen glycoprotein-39	HCgp-39
Hen Egg Lysozyme	HEL
Human Leukocyte Antigen	HLA
H2.0-like homeobox protein	HLX
High Motility Group Protein B	HMGB
Horse-Radish-Peroxidase	HRP
Heat shock protein	Hsp
intra-venous	i.v.
IntraCellular Adhension Molecule	ICAM
Inducible Costimulator	ICOS
Interferon	IFN
Immunoglobulin	Ig
IFNγ-Inducing Factor	IGIF
Interleukin	IL
Iscove's Modified Dulbecco's Media	IMDM
Immune dysregulation,Polyendocrinopathy, Enteropathy, X-linked syndrome	IPEX
Interferon Regulatory Factor	IRF
Immunoreceptor Tyrosine-based Inhibitory Motifs	ITIM
Inducible T cell kinase	Itk
Kilo Dalton	Kda
Knock Out	KO
Lymphocytic ChorioMeningitis Virus	LCMV
L	Ligand
Lymph Node	LN
Laser Scanning Microscope	LSM
Lympoid Tissue inducer	Lti

Lymphotoxin-Betta	Lt-β
Medium	M
Magnetic-activated Cell Sorting	MACS
Monocyte Chemotactic Protein 1	MCP-1
Major Histocompatibility Complex class II	MHCII
mesenteric Lymph Nodes	mLNs
Matrix MetalloProteinase	MMP
Multi-Photon Laser Scanning Microscopy	MPLSM
Magnetic Resonance Imaging	MRI
Multiple Sclerosis	MS
Nuclear Factor of Activated T cells	NFAT
Nuclear Factor kappa B	NFκB
Natural Killer	NK
Non-Obese-Diabetic	NOD
Nucleotide Oligomerization Domain	NOD
Osteoarthritis	OA
Optimal Cutting Temperature	OCT
Optical Density	OD
Osteclastogenesis Differentiation Factor	ODF
Osteoprotegerin	OPG
Ovalbumin	OVA
Peptidylarginine Diaminase	PAD
Pathogen-Associated Molecular Patterns	PAMPs
Phosphate buffered saline	PBS
Programmed Death-1	PD-1
plasmacytoid DCs	pDCs
Positron Emmision Tomography	PET
ParaFormAldehyde	PFA
Prostogladin E	PGE
Propidium Iodine	PI
PhosphoInositide-3 Kinase	PI3K
Phospolipase C	PLC
Phorbol-12-Myristat-13-Acetate	PMA
Pattern Recognition Receptor	PRR
Porcine reproductive and respiratory syndrome virus	PRSV
PhospatidylSerine	PS
Protein Tyrosine Phosphatase Non-receptor type 22	PTN22
Rheumatoid Arthritis	RA
Receptor antagonist	Ra
Receptor Activator of NFκB	RANK
Receptor Activator of NFκB Ligand	RANKL
Red Blood Cell	RBC
R	Receptor

Rheumatoid Factor	RF
Retinoic Orphan Receptor	ROR
Roswell Park Memorial Institute	RPMI
subcutaneous	s.c.
Severe Compined Immunodeficiency	SCID
Standard Deviation	SD
Shared Epitope	SE
Standard Error of Mean	SEM
Sialic acid binding Ig-like lectin	Siglec
Systemic Lupus Erythematosus	SLE
Single Nucleotide Polymorphism	SNP
Suppressor Of Cytokine Signalling	SOCS
Single photon emission computed tomography	SPECT
Specific Pathogen Free	SPF
Signal Transducer and Activator of Trascription	STAT
T-box expressed in T cells	Tbet
T cell Receptor	TcR
T Follicular Helper	TFH
Transgenic	Tg
Transforming Growth Factor	TGF
T helper	Th
Toll-Like Receptor	TLR
3,3', 5,5' tetramethyl benzidine	TMB
Tumour Necrosis Factor, Alpha-Induced Protein	TNFAIP
Tumour Necrosis Factor, alpha	TNFα
Trinitrophenol	TNP
Tris/Base NaCl Tween 20	TNT
T Regulatory	T_{REG}
Tryptophan	Trp
Vascular Cell Adhension Molecule	VCAM
Vascular Endothelial Growth Factor	VEGF
Zeta-chain-Associated Protein kinase 70	ZAP70

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Chapter 1: Introduction

1.1 Aims

Rheumatoid arthritis (RA) is a complex systemic autoimmune disease that predominantly targets synovial joints, especially the small joints of the hands and feet, and is characterized by joint destruction and chronic disability(1). Its occurrence is about 1%(1) of the population and presents a significant economical burden to the health system and society (estimated €45.1 billions in Europe)(2).

RA pathology can be subdivided in three stages: autoimmunity, inflammation and bone destruction(3). Susceptible individuals, under the influence of various environmental factors, develop an underlying autoimmunity that manifests as autoantibodies, such as rheumatoid factor (RF) and anti-citrullinated protein/peptide antibodies (ACPA)(3). This stage can precede the clinical manifestation of the disease by as much as ten years(4) and is relatively understudied compared to the active phase of the disease (Fig 1.1). This is due to the fact that only tissue from active arthritis patients is available, and most animal models resemble the articular phase of the disease. Recently our group has developed a model of early RA based upon the adoptive transfer of T helper (Th)1 polarised T-cell receptor (TcR) transgenic (Tg) T cells specific for ovalbumin (OVA)(5). The advantage of this model is that the precise development, migration, antigen (Ag) specificity and the contribution of the T cell phenotype to the pathology can be monitored and regulated. More importantly, this model is characterized by development of autoreactivity in the form of B and T cell responses against collagen type II (CII)(5-7), as these mice were never immunised with this protein. It therefore provides a useful tool to investigate the early immunological mechanisms that lead to autoimmunity in the context of arthritis.

Th17 cells are the latest addition in the effector Th cell repertoire and are characterised by the production of interleukin (IL)-17. Their discovery in models such as experimental autoimmune encephalomyelitis (EAE) and collagen-induced arthritis (CIA)(8;9) marked a shift from the traditional view that autoimmune diseases are Th1-mediated conditions. Even though some data suggest a role for IL-

17 and Th17 cells in inflammation and bone destruction that characterizes RA(10-13) their role in the early immunological events that lead to breach of self tolerance is unknown. Moreover, the role of these cells in crucial aspects of the adaptive immune, namely their ability to support T-cell dependent B cell responses is relatively understudied.

Employing the aforementioned model of breach of self tolerance in the context of arthritis and other adoptive transfer models the following aims will be pursued:

- Develop a robust and reproducible Th17 polarisation protocol that will be employed through out this thesis
- Investigate the role of Th17 versus Th1 T cells in the immunological events that lead to the breach of self tolerance in experimental arthritis
- Investigate the role of Th17 effector cells in supporting T-cell dependent B cell responses

In the last part of this thesis, the role of sialic acid binding Ig-like lectins (Siglec)-G, a sialic acid binding lectin, that has been proposed to play a part in mechanisms that discriminate between damage and pathogen associated derived signals, which might underlie the breach of self tolerance, will also be investigated.
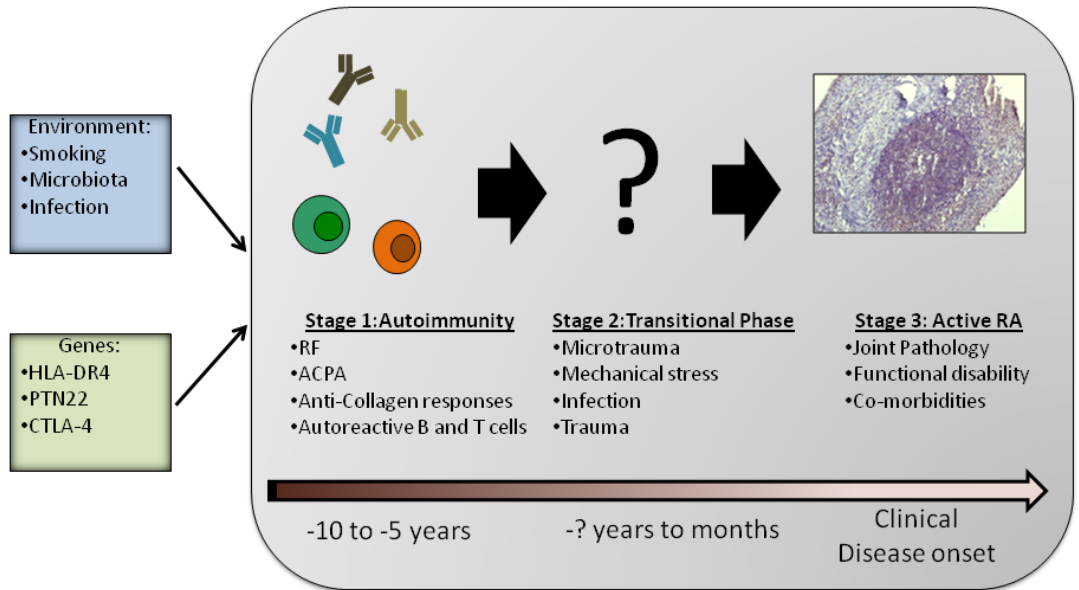
Fig 1.1 Pathogenesis of RA

The pathogenesis of RA can be grossly subdivided into 3 stages. Under the influence of various environmental factors (e.g. smoking, microorganisms) individuals that carry various disease-associated genetic traits (e.g *HLA-DR4, PTN22*) may develop autoimmunity in the form of autoantibodies (Rheumatoid Factor, Anti-Citrullinated Protein/Peptide Antibodies), some-times years before clinical signs of the disease. The mechanisms of transition to the clinical phase of the disease are still poorly defined but biomechanical events and trauma might be involved. Initiation of the disease is characterized by systemic inflammation that leads to joint destruction and co-morbidity.

1.2 Pathology of Rheumatoid arthritis: a brief overview

RA is a systemic inflammatory disease of unknown aetiology. Clinically it manifests as a symmetric polyarthritis associated with swelling and pain in multiple joints, often initiated from the joins of the hand, wrist and feet(14). RA can also affect other organs which result in conditions such as vasculitis, pleuritis and pericarditis(15). In the next section a brief overview of RA pathology will be presented, starting from the preclinical stage of the disease and expanding to the joint pathology and associated co-morbidities. Also a brief description of the healthy synovial membrane will be presented to contextualise the changes developing in an RA joint.

1.2.1 Normal Synovium

The synovial membrane is a connective tissue layer that covers the inner surface of the joint, tendon, sheaths and bursae(16). It has two main layers, the synovial lining and the synovial sublining(16). The synovial lining is composed by two major types of synovial cells which morphologically, phenotypically and functionally can be subdivided to'macrophage-like' (type A) and 'fibroblast-like' (type B) synoviocytes(17;18) (Fig 1.2a). The type A cells express various macrophage markers such as CD68, Fcγ receptors, CD14 and CD45 (common leukocyte antigen) and major histocompatibility complex class II (MHCII)(19). The type B synoviocytes on the other hand express rather specifically vascular cell adhesion molecule-I (VCAM-I) and decay accelerating factor (DAF)(16). They differ by other type of fibroblasts by the expression of α6β1 intergrin, which binds to the basement membrane component laminin(16;20). The synovial fibroblasts are the primary stromal cells of the joints and are responsible for the production of collagen I, III, IV an V and other connective tissue components that support the joint, such as fibronectin, laminin, chondroitin and heparan sulphate(16). Furthermore they produce and secrete hyaluronic acid into the joint cavity providing lubrication to its components(21;22). The synovial sublining consist of soft, loose connective tissue based on a network of elastic fibres and different

collagens, such as collagen I, III, IV, V and VI, fibronectin, laminin and proteoclycans(23). The synovial fluid is a plasma dialysate formed by diffusion through the synovial lining and sublining(16). It is acellular under physiological condition and its supplementation with hyaluronan accounts for its viscosity(16).

1.2.2 Preclinical RA

The relatively superficial question "When does RA start?" is still challenging researchers and clinicians. It has been demonstrated in various studies that both RF and ACPA are present in patients' sera years before the development of clinical disease(4;24-26). The first study demonstrating RF preceding clinical signs of RA took place in Finland 20 years which reported that two thirds of the patients investigated had developed RF four year before disease onset(24). A more recent study in Sweden employed blood samples from 83 donors that subsequently developed RA(26). This study revealed that the prevalence for ACPA was 33.7%, 16.9% IgG-RF, 19.3% IgM-RF, and 33.7% IgA-RF, which was significantly higher than healthy controls (26). Another study has demonstrated the presence of IgM-RF and ACPA in almost half of the patient investigated, at a median of 4.5 years before disease onset(4). More importantly it demonstrated that the autoantibody titres increased as the onset of disease approached(4). The presence of these autoantibodies and especially the fact that they are class switched suggests an active adaptive immune response against a various autoantigens, which is initiated years before the clinical signs of the disease. There are studies in animal models demonstrating a role for autoantibodies in the development of the articular phase of RA(27-29), however why and how this antibodies develop in humans is still unclear. Understanding the events surrounding the breach of self tolerance associated with RA could therefore reveal markers associated with the onset of preclinical disease and signal a window of early intervention that would prevent the initiation of the cascade of events leading to symptomatic disease.

1.2.3 Inflamed synovium

In RA the phenotype of the synovium is altered and it develops into a thickened, invasive growing tissue that eventually destroys the joint (Fig 1.2b). The acquisition of an activated phenotype by the synovium is a chronic process that develops in several not well defined stages(16). Synovial thickening is one of the main characteristics of RA and can result in a depth of up to eight cells(30). The synovial hyperplasia is probably a combined event of local proliferation of the synovial lining and influx of inflammatory cells(16). Macrophages constitute one of the major determinants of synovial thickening and there are studies that suggest that they account for 80% of the synovial infiltrate, especially in the area adjacent to the joint cartilage (31;32). In addition to hyperplasia, altered function of cells such as fibroblasts is a hallmark of RA(16;33). Activated fibroblasts exhibit many features of transformed cell lines, such as increased cell adhesion molecule expression, proliferation, resistance to apoptosis, oncogene expression and cytokine production(33;34). Inflamed synovial sublining is characterized by pronounced infiltrates of T cells, B cells, natural killer (NK) cells, dendritic cells and mast cells(33;35-38). Lymphocyte aggregates are observed in 50-60% of the RA patients(33). These aggregates can be surrounded by plasma cells, whereas macrophages can infiltrate them(33). Neutrophils are mainly found in the synovial fluid even though they can also be found in the synovial-cartiladge junction(39;40). The role of some of these cells in pathology and joint destruction will be discussed later in this chapter. The thickened synovium has increased requirement for oxygen and nutrients that can only be provided by the generation of new blood vessels. The local hypoxia is a strong stimulus for angiogenesis, whereas the required pro-angiogenic factors, such as Fibroblast Growth Factor (FGF) and Vascular Endothelial Growth Factor (VEGF), are produced by macrophages, synovial fibroblasts and other cells of the synovial infiltrate, such as neutrophils(41-43). Conversely, inhibition of angiogenesis has been reported to inhibit the development of CIA(44).
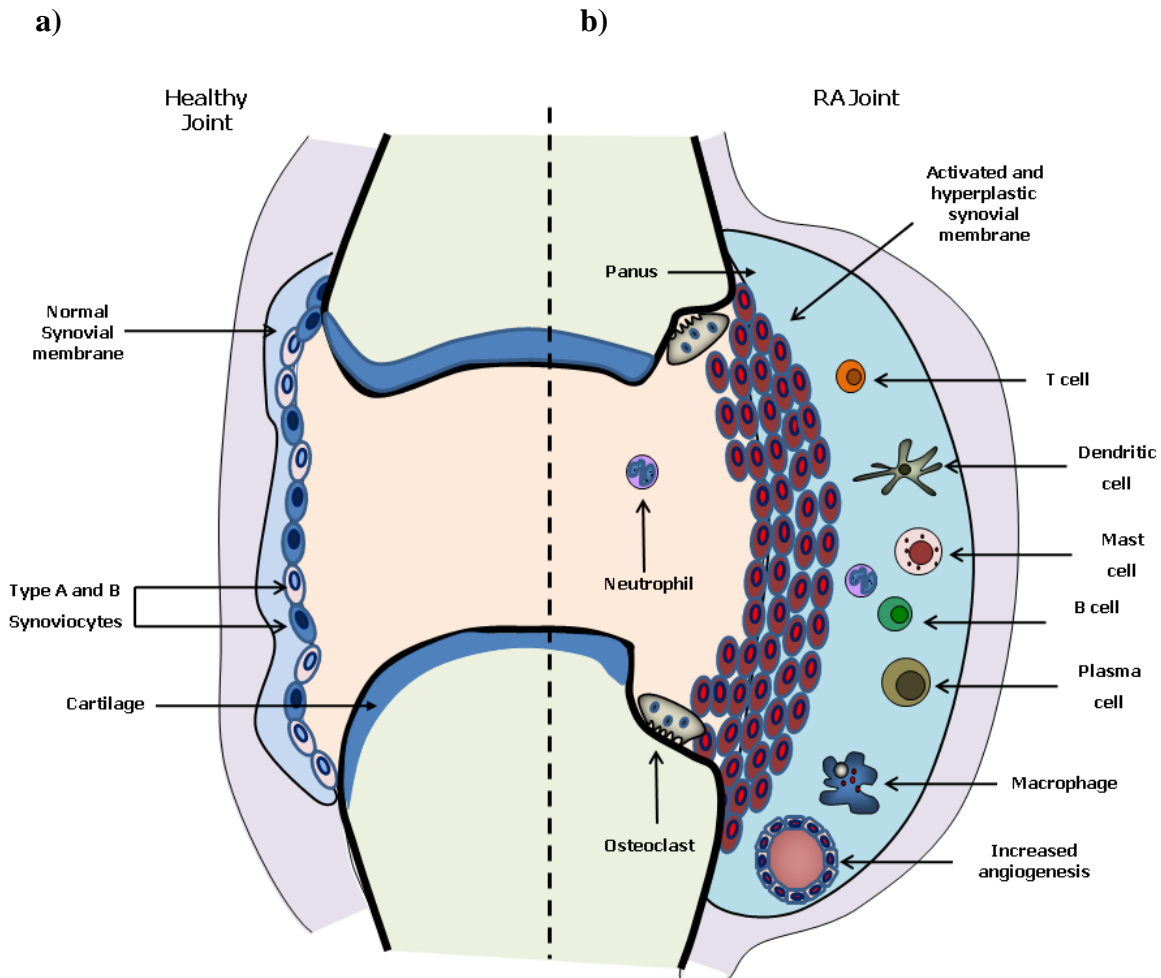
Fig 1.2: Healthy vs. RA synovial membrane

a) The synovium is a relatively acellular structure comprising by a thin layer of macrophage-like (type A) and fibroblast-like (type B) synoviocytes. b) The synovial membrane in RA patients is activated and hyperplastic as the synoviocytes proliferate locally. At the same time, various immune cells are recruited to the inflamed site. The inflamed synovial membrane will gradually invade the joint. The increased cellularity of the RA synovium requires adequate oxygenation which is supported by angiogenesis. The production of cytokines, chemical mediators and degrading enzymes destroys the cartilage and deregulates bone metabolism, which eventually leads to joint destruction.

1.2.4 Extra-articular manifestations of RA

RA is a systemic inflammatory disease, which in addition to the peripheral polyarthritis, can involve other organs and tissues. These manifestations could be either extra-articular symptoms or complications of the disease, however there is no agreed classification for them(45). The incidence and frequency for extra-articular RA varies between studies, but the most common are nodules which are present to up to 30% of cases(45). The most important extra-articular features and complications of RA are summarized in table 1.

Extra-articular manifestations have been thought to be more frequent in severe cases of RA(46). In addition, these manifestations seem to be more often in men and in rheumatoid factor (RF) positive(47) and/or anti-nuclear antibody (ANA) patients(48). Extra-articular features that do not respond to treatment clearly can have an adverse effect in the course of RA. These symptoms are not common and include systemic and ocular vasculitis, Felty's syndrome, interstitial pulmonary fibrosis, neuromyopathies, amyloid and cryoglobulins(45).

There is conciderable evidence linking cardiovascular disease and RA. RA is an independent risk factor for ischemic heart disease(45). More recently it has been shown that congestive heart failure, more than ischemic heart disease, appears to contribute to the overall RA mortality and this is through increased incidence of this condition in RA compared to the general population(49). RA patients have twice the risk of developing congestive heart disease compared to the non-RA population(50) and cardiovascular-disease associated death has been linked with markers of systemic inflammation(51). Studies have shown that the increased incidence of cardiovascular disease or the presence of atherosclerosis is not explained by traditional cardiovascular risk factors (smoking, lipid levels etc), suggesting a role for RA in the development of the disease(52;53).

From the above it is obvious that the extra-articular manifestations of RA have great effect in the life quality and expectancy of RA patients.

Table 1[i]: Extra-articular manifestations of RA

Extra-articular RA	Complication of RA
• Nodules • Raynaud's phenomenon • Secondary Sjogren's syndrome • Interstitial lung disease-pulmonary fibrosis • Pericarditis • Pleuritis • Felty's syndrome • Polyneuropathy, mononeuropathy, mononeuritis multiplex • Myopathy, polymyositis • Episcleritis, Scleritis • Glomerulonephritis • Systemic vasculitis • Benign cutaneous and nail-fold vasculitis • Lymphadenopathy • Weight loss, cachexia, malaise, fatigue, fever • Amyloid	• Cervical myopathy • Chronic leg ulcers • Normochromic normocytic anaemia • Osteoporotic fructure • Carpal tunnel syndrome • Lymphoedema • Hyperviscocity, cryoglobulins • Ischaemic heart disease • Non-Hodgkin's lymphoma • Infections

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[i] Table adapted from reference (45)

1.3 Genes and environment in the induction of breach of self tolerance

As noted above, RA is a multi-factorial condition where genetic, environmental factors and deregulated immune responses have a defining effect on the induction, magnitude and rate of progression of the disease(3). The result of this complexity is that the clinical picture is highly heterogeneous with different subsets of RA being manifested in patients(54;55). However, how these factors promote breach of self tolerance and progression of pathology is ill defined.

1.3.1 Genes

The genetic basis of RA is extremely complex. RA does not aggregate with high prevalence in families and concordance rates in identical twins are relatively low (12-15%)(56). However, the prevalence of the disease between first degree relatives is considerably higher than the general population(56). The description of the Human Leukocyte Antigen (HLA) association with RA is the strongest evidence for a genetic basis of the disease(56;57). Most patients with rheumatoid arthritis express particular HLA-DR alleles like HLA-DRB1*0401, *0404, *0405, *0408, *0101, *0102, *1001 and *1402(58). RA associated HLA-DR alleles share a highly conserved amino acid motif (^{70}QRRAA^{74}, ^{70}RRRAA^{74} or ^{70}QKRAA^{74}) expressed in the third hypervariable region of their DRB1 chain, termed the shared epitope (SE)(58). In different ethnic groups the involved allele varies considerably, for example *0401 and *0404 for Caucasians and *0405 in Japanese(59).

Apart from MHC, the best established locus of susceptibility for RA is protein tyrosine phosphatase non-receptor type 22 (PTPN22) which encodes Lyp, a tyrosine phosphatase expressed by T lymphocytes and regulates TcR transduction(60). The minor allele of a single nucleotide polymorphism (SNP) in PTPN22 has been linked to conditions such as type-I diabetes and RA(61-64). This SNP results in a in an amino acid substitution of arginine (Arg620) for tryptophan (Trp620) in a proline rich motif of the non catalytic C terminal of Lyp(62). It has been reported that the Trp620 allele is a gain of function mutation(65). T

lymphocytes from patients that carried this allele produced reduced amounts of IL-2 in response to TcR stimulation, compared to T cells that did not carry the mutation, whereas induced expression of the mutant *PTN22* transferred this hyporesponsiveness in primary T cells and the Jurkat T cell line(65). It has been suggested that the increased efficacy of the mutant *PTN22* in inhibiting TcR signalling may lead to a defective thymic negative selection, conferring predisposition to autoimmunity(65). This hypothesis is re-enforced by animal data, where deregulated thymic selection results in autoimmune arthritis(66).

Genome wide association studies (GWAS) have revolutionized the study of human disease genetics. These studies represent a powerful tool for the identification of genes involved in common human diseases. A GWAS was undertaken by The Wellcome Trust Case Control Consortium(57) confirmed the association of HLA-DR1 and PTPN22 with RA. Other candidate genes associated with RA that this study revealed are CTLA-4 (only nominal significance), the α and β chain of the IL-2 receptor(IL-2RA and IL-2RB), genes of the TNF pathway (TNFAIP2 (tumour necrosis factor, alpha-induced protein)) and in the regulation of T-cell function (GZMB (granzyme B))(57).

1.3.2 Environment

The amount of data concerning the environmental factors that contribute to the development of the disease is surprisingly scarce. Smoking is the environmental factor most strongly linked to an increased risk of developing RA(67-71). A link has been demonstrated between the HLA-DRB1 shared epitope, citrullination and smoking(72;73). Antibodies to antigens modified by citrullination through deimination of arginine to citrulline are present in about two-thirds of RA patients but are rare in other inflammatory conditions(73). It has been demonstrated that smoking increases protein deimination, which in the presence of the SE, leads to increased risk of developing ACPA positive RA(73).   This lead to the suggestion that smoking triggers citrullination in lungs through activation of peptidylarginine deiminase (PAD)(73), activation of the local antigen presenting cells (APCs) that

enables efficient antigen presentation of the post-translationally modified peptides for which the immune system has not developed tolerance(68;74). In addition, it has been reported that the conversion of arginine to citrulline at the peptide side-chain position interacting with the shared epitope significantly increases peptide-MHC affinity, which could lead to an immune response at individuals carrying the susceptible HLA-DRB1 alleles(75).

Recently great attention has been given to the immuno-modulatory role of mucosal microbiota(76;77). In has been proposed that the normal intestinal flora may protect against the development of inflammatory diseases(78-80). Mice deficient in a G-protein coupled receptor that recognises products of the metabolism of fiber by gut microbes developed exacerbated arthritis in the KxB/N serum-induced arthritis model, which suggests that commensal bacteria might be required for regulation of the immune response(78). Other reports suggests that components of the microbiota drive arthritis development(81;82). A prime example of a possible link of mucosal micro-organisms and RA pathogenesis is *Porphyromonas (P.) gingivalis,* a pathogen linked to periodontal disease development(83). This bacterial species has been linked to the development of immunity against citrullinated proteins due to its ability to produce citrullinated epitopes and its presence in an environment that highly analogous to RA, characterized by bone erosion and chronic inflammation(84;85).

Table 2 lists some of the main environmental factors that have been reported to affect RA pathology:

Table 2: Environmental factors and RA

Environmental factor	Effect	References
Smoking	Increased risk, dependent on magnitude and length of habit, association with anti-CCP antibodies	(67-69;72-74;86)
Alcohol	May decrease risk, lower risk for anti-CCP positive RA	(86;87)
High Birth Weight	Increased Risk	(88)
Oral contraceptives	Lowers the risk of RF positivity	(90)
Breast feeding	Reduced risk	(91)
Socioeconomic status	Inverse association between socioeconomic status, measured by occupational class and education and RA	(92)
Geography	Location of birth and current residence is associated with differential risk of RA	(93)
Microbiota	Intestinal flora could be protective, P. gingivalis promotes disease	(78;81)

1.4 Localisation

The major clinical sign of RA is joint pathology, which manifests as a symmetric polyarthritis with associated swelling and pain in multiple joints, often initially in the joints of the hand, wrist and feet(14). This is recapitulated in many animal models; however, why the systemic autoimmunity that characterizes the preclinical phase of the disease eventually targets the joints is still unknown. Interestingly, studies from our group using an adoptive transfer model of arthropathy reveal early involvement of the articular environment was prerequisite for development of autoreactive responses as immunisation in other sites did not lead to autoimmune arthritis (RA Benson, unpublished data). Reasons related to the environment and function of the joint, namely biomechanical stress, hypoxia, and trauma could potentially explain its preferential involvement in RA. Joint overuse and misuse in conjunction with trauma have been linked to the development of osteoarthritis(94), however their role in RA development is not clear. Interestingly, a case control study links physical trauma with RA onset(95), whereas in experimental arthritis development of the disease was associated with joint microbleeding(96). We could speculate that local microtrauma or infection leads to inflammation, damage, antigen release and activation of resident dendritic cells (DCs), which in genetically susceptible and environmentally conditioned individuals, target the autoimmune response to the joint. In experimental arthritis hypoxia-induced cell death was linked to the release of damage associated molecular patterns (DAMPs), such as HMGB1, that perpetuated the inflammatory response(97). This potentially suggests that regulation of sterile trauma could be important in RA development. It is now accepted that the immune system recognises both DAMPs and pathogen associated molecular patterns (PAMPs), through pattern recognition receptors (PRRs), such as Toll-like receptors (TLRs)(98-100). Less clear is how the immune system discriminates between DAMP and PAMP derived signals. Indeed injury and cell death is followed by the release of DAMPs and probably self antigens, however this does not usually lead to autoimmunity. A good example is cancer radiotherapy, where dying cells release HMGB1, which activates DCs through TLR4 initiating

anti-cancer T cell immunity. Despite the fact that the dying cancer cells will contain autoantigens this does not lead to a generalised autoimmune responses(101). This is established in a significant degree by central and peripheral tolerance mechanisms. Recent studies however, suggest that another possible mechanism is through attenuating the signals initiated by DAMPs(102). It was demonstrated that CD24, also known as heat-stable antigen, can associate with various DAMPs, such as HMGB1, heat-shock protein (Hsp)-70 and Hsp-90(103). Interestingly, CD24 deficient mice were more susceptible to liver injury induced by acetaminophen, a phenomenon mediated by HMGB1, which suggest that this molecule is part of a DAMP regulatory mechanism. As CD24 is a glycosyl-phosphatidyl-inositol anchored molecule(104) it does not have a intracellular signalling domain(105). On the other hand, CD24 and HMGB1 form a complex with Siglec-G(106). This is a member of the Siglec family of Ig-like type I transmembrane proteins, which recognise sialic acids(107). There are 13 siglecs in humans and 8 in mice, and all of them, apart from sialoadhesin and Siglec-H, possess immunoreceptor tyrosine-based inhibitory motifs (ITIM) in their intracellular domains(107). Thus Siglec-G has the potential to initiate regulatory signals by recruiting phosphatases such as SHP-1. Indeed, Siglec-G deficient mice phenocopy the CD24 deficient mice in their lethal acetaminophen response(108). In addition, Siglec-G represses DAMP-mediated NFκB activation, but more importantly does not regulate the inflammatory signals initiated by PAMPs, such as LPS and polyI:C(109). These data suggest that the CD24/Siglec-G complex is a regulatory mechanism that facilitates discrimination between pathogen and damage-derived signals. It is intriguing to speculate that potential deficiencies in regulation of sterile damage initiated by mechanical loading or microtrauma might lead to the articular localisation of RA. Unfortunately, there are no experimental data to support this.

1.5 Mechanisms of disease progression: cells and cytokines

The development of the inflammatory conditions that is RA, involves many different cell types and a complex cytokine networks. An overview of the cellular protagonist and the cytokine networks involved in RA development will be presented in the following sections, focusing mainly on cells of the adaptive immune response.

1.5.1 The role of T cells in RA

A critical role for adaptive immunity in the pathogenesis of RA is supported by the presence of activated T cells in the synovial lesion, by long established association with *HLADRB1* and by recent genome wide scanning studies implicating *ptpn22, cd40, ctla4* and *cd28(57;63;64;110).* Various studies have reported the presence of T cells in the synovial membrane(111;112). Interestingly these studies positioned these cells in association with APCs (either DCs or B cells), which could suggest an active adaptive immune response in the synovial membrane(111;112). T cells from synovial fluids and membrane are mainly highly differentiated activated memory $CD4^+$ $CD45RO^+$ $CD45RB^{dull}$cells(113-115). Interestingly, the synovial membrane environment protects these cells from apoptosis even though they express apoptosis susceptibility markers (Bcl-2^{low}, Bax^{high}, Fas^{high})(116). Other studies have reported that RA patients are characterized by a T cell phenotype ($CD4^+$ $CD28^-$) that has both effector and memory T cell features(117). In adoptive transfer of these cells into SCID mice grafted with human synovial membrane these cells expressed CCR7, CCR5 and CXCR4 and homed at the synovium, but preferentially homed at the lymph node upon activation with CCL5 or CXCL12(117). Upon treatment with IL-12 or TcR activation these cells homed to the synovial membrane via upregulation of CCR5(117). The T cells of RA patients, even the ones that develop the disease at early age, exhibits features of increased ageing as demonstrated by telomere shortening(118). This is observed in both naïve and antigen experienced cells suggesting that is not an antigen driven event, but potentially due to reduced

production of new thymic emigrants and homeostatic proliferation of the T cell repertoire(118).

Numerous animal models further confirm the involvement of T cells in the development of inflammatory arthritis. An intrinsic defect in the TcR signaling (a spontaneous point mutations that alters the encoding of an SH-2 domain of ZAP70) can lead to T-cell dependent arthritis in mice(66). It was reported that altered signal transduction from the TcR through the aberrant ZAP70 defects, changes the threshold of thymic T cell selection, leading to the positive selection of otherwise negatively selected autoimmune T cells(66). Another model where the central role of T cell in the development of arthritis is demonstrated is the IL-1 receptor antagonist (IL-1Ra) deficient mouse model(63). IL-1Ra deficient mice develop spontaneous autoimmune arthritis, only in the presence of T cells, which produce cytokines, such as IL-17 and TNF, important for the development of the disease(119). Furthermore, when the KRN/C57BL/6 TcR transgenic mice were crossed with the Non-Obese Diabetic (NOD) mice (KxB/N) the off-spring developed arthritis, due to the development of T-cell dependent B cell responses against glucose-6-phosphate isomerase (GPI), a glycolytic enzyme that is ubiquitously expressed(27;120).

Various T-cell directed therapies have been developed with the most promising being abatacept, a fully human recombinant fusion protein of the extracellular domain of the endogenous inhibitory molecule cytotoxic T-lymphocyte antigen 4 (CTLA4) and the Fc domain of human $IgG_{1(121)}$. The efficacy of abatacept has been demonstrated in phase II studies in RA patients either nonresponsive to adequate doses of methotrexate or in combination with methotrexate. Treatment responses were sustained to 3 years(122).

Even though there seems to be an important role for T cells in RA it is not clear yet what antigen they recognise. Various molecules, highly expressed in the joint, have been proposed, such as CII, the cartilage protein HCgp-39 and proteoglycans (e.g.

aggrecan), mainly based on their ability to promote arthritis in animal models(123-125). Antigens such as Hsp and immunoglobulin binding protein (BiP) have also been proposed(126;127). The presence of SE in RA patients and its association with anti-citrullinated protein immune responses could suggest that T cells in RA patients recognise citrullinated epitopes(68;72;73). Indeed in the HLA-DRB1*0401 transgenic mice the conversion of arginine to citrulline at the side chain where peptides interact with the SE, leads to increased peptide-MHC affinity and activation of CD4$^+$ cells(128). However there is little evidence directly relating T cells specific for these antigens to the pathogenesis of RA and it is most probable that in different patient groups, different antigenic epitopes will be responsible for the pathology.

1.5.2 The roles of B cells in RA

The important role of B cells in RA pathology can be summarized by the following evidence:

- The presence of autoantibodies in patients with RA
- Presence of B cells in RA synovial membrane
- B cell activation and germinal centre formation in ectopical germinal centers in RA synovial membranes
- The effectiveness of B cell depletion as a treatment of RA

The first evidence for a role of B cells in RA were based on the plethora of auto-antibodies that characterize this condition. Given the easier accessibility of peripheral blood the first feature of B cell contribution that was reported is RF, an autoantibody against the Fc portion of human IgG(129;130). RF is found at about 80% of RA patients but it is not very specific and it is found in other autoimmune conditions and even healthy individual (130). However, there are differences between RF in health and disease. In healthy individuals RF is an IgM produced by B1 cells as "natural" antibody and has low affinity and polyreactivity(131). On the other hand RF in RA patients undergoes class switching as a consequence of help

that the B cells are receiving from T cells(131;132), a phenomenon also observed for ACPA(133).

B cells are a significant but not constant population in RA synovium(132). B cell infiltration is not so prominent in samples of synovium tissue that are lacking a defined level of organization of immune cells, whereas it is more significant in samples that are characterized by large and well organized mononuclear aggregates(134). Importantly as the lymphocyte number increase a defined lymphoid tissue organization appears with defined T and B cell compartmentalization, vascular apparatus and follicular dendritic cell (FDC) network(134). In synovial membrane, B cells are the major source of lymphotoxin-B (LT-β), a cytokine important in normal lymphoid organogenesis(135) suggesting a significant role of B cells in ectopical lymphoid tissue organization. A defined lymphoid architecture in the synovial tissue, with B cells in close interaction with T cells, potentially provides the appropriate microenvironment for B cell activation(136). Different pattern of synovites have been shown to correlate with different markers of B cell activation(137). Tissues containing germinal centers have the highest levels of IgG transcription, compared to samples with diffuse synovitis(137). In addition, increased B cell activation markers such as Blys and APRIL, have been reported in RA synovial membrane(137).

B cells can also act as efficient APCs in antigen specific manner to stimulate T cells and to allow optimal CD4$^+$ T cell memory(138-141). RF$^+$ B cells can take up antigen-IgG immune complexes via their membrane Ig receptors, which are IgG specific. B cells then can process and present peptides from the antigen and provide T cell activation and help, which could lead to responses against self antigens(142). Indeed, a study employing human synovial transplantation into SCID mice demonstrated that the presence of B cells is required for adequate local activation of T cells(143). Furthermore, in the KxB/N model, development of arthritis is based on the cognate recognition of GPI by B and T cells, emphasising the importance of B-T cell communication in the disease establishment(27).

The importance of B cells in RA pathology is depicted by the effectiveness of B cell depletion therapies(144). Rituximab is B-cell-cytolytic chimeric IgG1 CD20-

specific monoclonal antibody, which can potently kill B cells from the pre-B-cell stage to the pre-plasma-cell stage(145). Several B cell depletion agents are now under investigation with a major goal the re-establishment of some form of immunological tolerance.

1.5.3 Innate immune cells in RA: Macrophages and DCs

The myeloid lineage gives rise to several cell types involved in the disease, such as monocytes/macrophages, subtypes of DCs and osteoclasts.

Monocytes/macrophages: The predominance of macrophage derived cytokines in the synovial compartments(146-149) signifies the importance of this cell type in the pathology of RA. In the normal synovial membrane, macrophages predominate in the lining layer, where they scavenge debris from articular structures, and eliminate all the microorganisms entering via the blood or upon trauma(1). In the inflamed synovial membrane activated macrophages is one of the most abundant cell types(1) and the degree of macrophage infiltration directly correlates with clinical status and progression of joint damage(150;151). At the tissue level, pre-activated monocytes infiltrate the synovial membrane, mature into macrophages, which get activated and interact with other synovial cells(152). Activated macrophages confer in the progression of the pathology through

- Production of pro-inflammatory cytokines such as TNF-α(146), IL-1(147), IL-6(149) and GM-CSF(148).

- Production of chemoattractants and chemokines such IL-8(153), MCP-1(154) and MIP-1α(155).

- The overexpression of metalloproteinases, such as MMP9(156) and MMP12(157), which confer to tissue distruction.

- Antigen presentation, even though the relative importance of this cell type compared to other APCs such as dendritic cells and B cells in this function is not clear[1;152].

Dendritic cells: DCs comprise a complicated population of heterogeneous APCs that are critical for the initiation of the adaptive immune response and the

maintenance of both central and peripheral tolerance(158). DCs both in human and mouse can be divided into subsets according to tissue distribution, function and phenotype(159). DCs have been identified in RA synovial fluid and synovial tissue by several groups but their origin, function and potential role in the pathogenesis of the disease are not fully understood(160). A gross subdivision can be made between conventional and plasmacytoid DCs (cDCs and pDCs respectively)(159). Contrary to other APCs, DCs can prime naïve T cells for helper and cytotoxic functions, are essential for the generation of primary antibody responses, and are powerful enhancers of natural killer cells(161). DCs are likely to contribute in several ways in the pathogenesis of RA. Firstly, DCs could prime auto-immune responses by presenting self-antigens. Our group has demonstrated that the presentation of collagen derived peptides by mature bone marrow derived DCs is sufficient to induce arthritis in DBA/1 mice(162). More importantly, in a model of pre-clinical arthritis it was demonstrated that conventional DCs are the cells that orchestrate the initial breach of self tolerance(7). Secondly, DCs could infiltrate the synovial tissue and fluid where they could take up and present antigen locally, perpetuating the disease, however there are no direct evidence to support this(163). Furthermore, DCs, alongside with other immune cells and synoviocytes produce inflammatory mediators that drive the RA pathology(161).

DCs are critical for peripheral and central tolerance. Both cDCs and pDCs have been suggested to have tolerogenic abilities in different environmental settings(164-167). Our group has demonstrated using a model of breach of self tolerance in the context of arthritis, that pDCs can function to limit self reactivity and the consequent pathology(168). To further support a regulatory role for pDCs, identified a tolerogenic CCR9^{+} pDC population has been identified, which can suppress acute host versus graft disease(167). Even though there is an incomplete understanding of how DCs are involved in RA pathology, DCs therapies are currently developed with some success in murine models(169-171), whereas clinical trials have been initiated in UK (http://news.bbc.co.uk/1/hi/health/7560535.stm) and Australia (http://www.uq.edu.au/news/?article=13128).

1.5.4 Non-Immune cells

Osteoclasts: Osteoclasts are multinucleated cells of hemopoeitic origin, they are the primary bone resorbing cells and are essential for the remodeling of bone throughout life(172). These giant cells are a fusion product of up to 20 single cells(173). There are only a few clinical conditions that induce the local formation of osteoclasts and one of them is RA(173). Synovial inflammatory tissue is the source of osteoclasts(173;174). The synovial membrane contains many monocytes/macrophages that could undergo osteoclast differentiation upon contact with the appropriate signals. Two cells are considered very important in providing differentiation signals for the monocytes to become osteoclasts, fibroblasts and T cells(175;176). Fibroblasts express receptor activator of nucleor factor (NF)κB ligand (RANKL), which is a major driver of osteoclast formation(175). T lymphocytes, apart from RANKL, express cytokines such as IL-17 that support osteoclast formation(176). Other cytokines present in the synovial environment such as TNF, IL-1 and IL-6 also are important in RANKL upregulation and thus possibly on osteoclast formation(173). From animal models of arthritis it is evident that osteoclast formation is an early and rapid event of the pathology(177), which eventually leads to the destruction of the joint.

Synovial Fibroblasts: Synovial fibroblasts, together with synovial macrophages, are one of the two main cells compromising the synovial membrane(178). RA synovial fibroblasts are now considered active drivers of RA pathology(179). The physiological function of these cells is to provide the joint cavity and cartilage with plasma proteins and lubricating molecules such as hyaluronic acid(178). Human synovial fibroblasts contribute to disease pathology through the production of inflammatory mediators and chemokines, such as VEGF, IL-15, interferon-β (IFNβ), IL-8, CXCL2, CCL8, CCL5, CXCL10(180-183) and damage promoting enzymes, notably cathepsins and MMPs(184-186). It should be noted that RA synovial fibroblasts differ considerably from fibroblasts from healthy joints. RA

fibroblasts have an activated phenotype which is characterised by morphological differences, long term growth, reduced apoptosis and an altered response to various stimuli(178;187). Indeed a recent study demonstrated that RA fibroblasts were able to spread the pathology by invading unaffected joints, an ability lacking from non-RA fibroblasts(188). Various mechanisms could be involved in the development of this phenotype, amongst them cytokines and growth factors(FGF, IL-17, IL-18, TNF and IL-1)(178;189-192), articular hypoxia of the rheumatoid joint that activates the production of pro-angiogenic and pro-inflammatory factors(193) and expression of proto-oncogenes and tumour supressors molecules(194).

1.5.5 Cytokine networks in RA

Numerous cytokines have been involved in the pathology of RA, amongst them members of the IL-1, IL-12, and TNF superfamilies. More importantly, neutralising antibodies against cytokines is an established therapy for RA, with TNF blocking as the most characteristic example.

IL-1 Superfamily, IL-1, IL-18 and IL-33: IL-1, IL-18 and IL-33 are related by means of origin, receptor structure and signaling pathways(195). The IL-1 family includes IL-1α, IL-1β and IL-1 receptor antagonist (Ra), the IL-18 family includes IL-18 and IL-18 binding protein and the IL-33 family includes IL-33 only(195). The major extracellular forms for these cytokines are IL-1β, IL-18 and IL-33 and are all stored as inactive precursors in cells(195). They are activated by the enzyme caspase-1 to the active form which is released from the cell(195).

IL-1α and IL-1β are produced by various cells such as monocytes, macrophages, neutrophils and hepatocytes(195). They activate cells through IL-1RI(195). IL-1Ra is related to IL-1α and IL-1β but has undergone mutations that renders it capable of binding avidly to the receptor but fails to signal through it, thus acting as specific inhibitor of IL-1(196). All the members of the IL-1 family have been found in abundance in synovial membrane(197;198). There are various studies that report the production of matrix metalloproteinases (MMPs) and prostogladin E_2 by IL-1 and TNFα(192;199) which are very important in tissue degradation and perpetuation of the inflammation. The involvement of IL-1 in the pathology of RA has been shown in many models of experimental arthritis. IL-1 is present in the inflamed synovium of mice with CIA(200), whereas intra-articular delivery of IL-1 into rabbit and rat joints resulted in arthritic manifestation similar to RA(201;202). Furthermore, IL-1Ra-deficient mice develop spontaneous arthritis in an IL-17 dependent manner(203). In addition the significant role of IL-1 in articular damage and bone erosion was demonstrated when human TNF-α overexpressing mice that develop spontaneous arthritis were crossed with IL-1 deficient mice(204). These mice even though they developed synovial inflammation they had significantly

reduced bone erosion and osteoclast formation(204). Due to the accumulating evidence of its importance in RA pathology, methods for blocking its action were investigated. However, blocking its activity using another member of the IL-1 family, the IL-1Ra (anakinra) has failed to produce adequate therapeutic value compared to other biologics(205).

IL-18 was discovered in 1989 and was described as IFNγ inducing factor (IGIF)(206). It is produced as an inactive 24KDa precursor that is cleaved by caspase-1 to its active form(207) and was found to have powerful Th1 promoting activities(208;209). It is produced by various cell types amongst them, macrophages, articular chondrocytes, synoviocytes and osteoblasts(207;210;211). In the context of RA it is considered to be produced by macrophages or DCs and leads synovial T cells to produce IFNγ(212). In the CIA model, injection of IL-18 increased the bone erosion and inflammation(212), whereas its blocking, either with IL-18 binding protein or anti-IL-18 antibodies, reduced the severity of arthritis(213). In RA patients but not OA patients IL-18 has been described to be present in serum and synovial fluid (209;212). IL-18 induces the production of GM-CSF, nitric oxide, TNFα, IL-6 and IFNγ from RA synovial cell cultures, suggesting an important role in the propagation of the disease(209;212).

IL-33 is an IL-1-like cytokine with functional and structural similarities with other members of the IL-1 family(214), which mediates its actions through the IL-33R, also known as ST-2. IL-33 expression has been detected on synovial fibroblasts from RA patients(215). Furthermore, it has been demonstrated that IL-33 exacerbates CIA, whereas IL-33R deficient mice or mice that were administrated with sST2 (an natural antagonist of IL-33) exhibit reduced disease(215).

TNF superfamily: The most important member of this family is TNFα. It forms a membrane bound homodimer cleaved by TNFα-converting enzyme to generate a 17KDa secreted form(216). Two TNF receptors (TNFR) have been described; TNFR1 (p55) and TNFR2(p75)(216).   TNF-blockade is one of the most effective

therapies developed for RA. The introduction of anti-TNF therapy was based on the expression of TNF and its receptors in RA synovial tissue, in vitro studies using RA synovial tissue(217;218) and on animal models of autoimmunity(219).

Three TNF-blocking agents have been introduced in the market since 1998: 1) Infliximab (Remicade), a chimeric anti-TNFα IgG1 antibody; 2) Etanercept (Enbrel), a human dimeric TNF receptor type II-IgG1 fusion protein (TNFR-Fc); 3) Adalimumab (Humira), a human anti-TNF-α IgG_1 antibody genetically engineered through phage display technology(220).

Even though anti-TNF blocking is widely used the mode of action of this therapy is not fully elucidated. Various mechanisms have been proposed for its action. Amongst them, the inhibition of the cytokine cascade initiated by TNF, sequestration of TNF by binding, altered leukocyte recruitment and endothelial activation, reduction of angiogenesis, and generation of regulatory T cells (T_{REG})(216;221-223). The biggest disadvantages of TNF-blocking therapies are the partial response or no-response to the therapy and the susceptibility of the patients to infections like tuberculosis(224).

Another important member of the TNF superfamily that is important for RA development is RANKL. It is a type 2 transmembrane cytokine that is expressed by bone and lymphoid tissue(225). Its critical role is in the differentiation and activation of osteoclasts. RANKL assemples into functional trimers, bind to its receptor, RANK, and induces the differentiation of osteoclasts from their precursor cells(225). It also promotes the bone resorbing activity of osteoclasts and prolongs their survival(225). The other important regulatory component of this system is osteoprotegerin (OPG), a soluble decoy receptor for RANKL, which inhibits RANKL activity by preventing its binding to RANK(225). It is believed that the RANKL/RANK/OPG is critical for bone destruction in RA. RANKL is highly expressed in synovial tissue of RA patients(226;227) and is mainly produced by synovial fibroblasts and T lymphocytes(226;228). Cytokines, such as IL-17, and TLRs (TLR2 and TLR4) have been involved in the production of RANKL by synovial fibroblasts(11;226). A fully human RANKL blocking monoclonal

antibody (denosumab) is in phase II clinical trials for RA with promising results concerning the limitation of bone erosion(229).

IL-6: IL-6 is a pleiotropic cytokine produced by various cell types such as macrophages, fibroblasts, endothelial cells, B and T cells(230). Its action is mediated through the heterodimeric receptor composed by the gp130 and IL-6R subunits(230). Pro-inflammatory cytokines, like IL-1 and TNFα, immune complexes and oxidative stress induce the production of IL-6, mainly though the activation of the NFκB pathway(230).

IL-6 is present in the serum and synovial fluid of patients with RA and its presence correlates with disease activity(230;231). It is involved in the activation of autoreactive T-cells and the production of rheumatoid factor(232). Acting on hepatocytes it induces the production of acute phase proteins(233) and in the presence of the soluble IL-6R it activates the osteoclast precursor cells to differentiate to functional osteoclasts(234).

IL-6 is considered to be lower from TNF in the cytokine hierarchy because TNF-blocking reduces the levels of IL-6(235). However, the fact that IL-6 deficient mice are protected from CIA and IL-6 blocking through targeting either the cytokine or the receptor ameliorates CIA suggest an independent role for IL-6 in RA(230;236;237).

IL-6 blocking is the subject of intense clinical trial activity and a humanized anti-IL6R antibody has bedeveloped with promising results. Tocilizumab, a humanized monoclonal antibody against the IL-6R, is on phase III clinical trials and has been shown to suppress disease activity in patients that are resistant to disease modifying anti-rheumatic drugs (DMARDs)(238).

IL-15: IL-15 is a pleiotropic cytokine involved in lymphoid homeostasis and inflammation(230). It exists in two isoforms one expressed on the membrane or secreted and one that has a cytosolic and nuclear distribution(230). Its action is mediated through a heteromeric receptor composed by IL-15Rα, IL-2/15Rβ, which is shared by IL-2 and a common γc subunit that is shared by many cytokines(230).

It is expressed by immune cells such as macrophages and dendritic cells and non-immune cells like fibroblasts and epithelial cells(230). IL-15 protein and mRNA levels are increased in RA patients, and on the surface of peripheral blood T cells derived from patients with early RA(239). In the CIA model, administration of an IL-15 mutant/Fcγ2a fusion protein, which binds with the IL-15 receptor with high affinity but does not signal through it, had both protective and therapeutic actions(240). In humans, neutralizing studies are focused on a monoclonal IgG1 antibody, AMG714, which inhibits the activity of both bound and soluble IL-15 in vitro and has promising results in clinical studies(241).

The IL-12/23 and IL-17 family of cytokines: The IL-12 superfamily includes a number of structurally related cytokines such as IL-12, IL-23 and IL-27, which are mainly produced by dendritic cells and macrophages.

IL-12 is a heterodimeric cytokine consisting of the p40 and p35(242) subunits that signals though a receptor complex composed of IL-12Rβ1 and IL-12Rβ2(243). It activates STAT4 and it is crucial for the development of a Th1 response(244-246). Its role in arthritis is not very clear. IL-12 has been detected in synovial membrane, produced mainly by macrophages, where it was suggested to induce IFNγ production by CD4 cells(247). Whereas blocking IL-12 during CIA induction reduces the severity of the pathology and low doses of the cytokine during the induction phase increase the CIA severity, high doses of IL-12 are protective(248). In addition, anti-sense therapy against STAT4, the main transcription factor initiated by IL-12, proved to be protective for CIA(249). However, in this case the role of other cytokines such as IL-15 and IL-23 that also activate STAT4 should be considered(249). On the other hand, IL-12 seems to be dispensable for the development of arthritis in the KxB/N model, whereas IL-12p35 deficient mice that specifically do not produce the full IL-12 molecule, but produce IL-23, show increased susceptibility to CIA, suggesting a regulatory role for IL-12(9)'(250).

IL-23 is composed by the p40 subunit of IL-12 and the unique p19 subunit(251). It is mainly produced by macrophages and dendritic cells in response to various

inflammatory stimuli(251-253). It signals through a heterodimeric receptor, which compromises of the common with IL-12, IL-12Rβ1 subunit and the unique IL-23R(254). Contrary to IL-12 that induces the development of Th1 cells, IL-23 binds to memory T cells that produce mainly IL-17, IL-17F, TNF, IL-6 and IL-22 and are termed Th17(8;255). IL-23 and IL-23R has been linked to many autoimmune diseases, such as psoriasis(256) and Chron's disease(257) . A role in RA for this cytokine was proposed mainly based on the fact that IL-23 deficient mice were protected from CIA development due to their inability to develop IL-17-producing CD4 cells(9). In RA, the p19 subunit of IL-23 has been detected in RA synovial fluid in abundant levels, however only low levels of bioactive IL-23 were measured in these patients(258). It has been reported that PGE2 induces IL-17 production in an IL-23 dependent manner, mediating neutrophilia and tissue pathology in two models of RA, CIA and antigen-induced arthritis(259;260). While for diseases such as inflammatory bowel disease and psoriaris there is a strong association with IL-23R polymorphisms, this is not the case for RA(57;257;261). There are however some IL-23R variants that have been suggested to confer increased risk of RA, even though the association is not as strong as the aforementioned diseases(262;263). These data suggest that even though in animal models there seems to be a clear pathogenic role for this cytokine, its role in human disease is not very clear.

The IL-17 cytokine family was recently identified and it includes IL-17A, IL-17B, IL-17C, IL-17D, IL-17E (also known as IL-25) and IL-17F(264). IL-17A (from now termed IL-17) is the prototypic member of the family exerting its actions as a homodimer with a molecular weight around 35 kDa(265). IL-17 and IL-17F induce the production of antimicrobial peptides (defensins and S100 proteins) (255;266) , cytokines (IL-6, GM-CSF and G-CSF)(267-270), chemokines (IL-8, CXCL5, CCL20)(270-273) and matrix metaloproteinases(265). The proinflammatory functions of IL-17 expand to the induction of adhesion molecules, such as intracellular adhesion molecule-1 (ICAM-1) by keratinocytes (274) and IL-1 and TNFα by macrophages(275).

From the above it is suggested that IL-17 could be a potent inflammatory mediator in rheumatoid arthritis. This role is supported by the presence IL-17 and IL-23p19 in the sera, synovial fluid and synovial biopsies of RA patients but not osteoarthritis (OA) patients(276). Their production is increased via a phosphoinositide-3 kinase (PI3K) pathway and f NFκB dependent pathway(277;278). In addition, IL-17 activates the production of IL-6, IL-8 and VEGF by fibroblasts and thus promoting the recruitment and activation of inflammatory cells (189;279).There is an established role for IL-17 in cartilage and bone destruction in RA. Specifically, it enhances the production of IL-6, cartilage destruction and cartilage generation by RA synovial explants and causes bone erosion in RA bone explants(280). Furthermore, it induces metalloproteinases in synoviocytes and chondorocytes(281) and thus it plays a role in cartilage degradation(282). In addition it has been shown to have a direct catabolic effect on cartilage(283). The destructive effect of IL-17 is independent of IL-1 production as IL-1Ra cannot inhibit matrix degradation initiated by IL-17(284). The downstream signalling of IL-17 and IL-1 are distinct and deferential pathways of AP-1 initiated by IL-17 and IL-1 have been described(12). Furthermore, IL-17 induces the expression of RANKL in cultures of osteoblasts(12). As mentioned previously RANKL binds to its receptor RANK and the RANK/RANKL pathway is crucial in osteoclastogenesis and bone erosion process(225). On the other hand the decoy receptor OPG acts as a negative regulator of osteoclastogenesis(225). It has been reported that in the CIA model, IL-17 overexpression enhanced RANKL expression and also strongly up-regulated the RANKL/OPG ratio in the synovium(11). These could justify a role for Th17 in RA pathology as these cells are a major source of IL-17(285). However, it should be stressed that Th17 cells are not the only source of IL-17. Other cell types such as $CD8^+$ T cells, NK cells, $\gamma\delta$ T cells, mast cells and neutrophils have also been reported to produce IL-17 (286-290). It is thus important not to denote all IL-17 related inflammatory events to Th17 cells.

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1.6 T helper subsets

The induction of an adaptive immune response begins when an antigen is ingested by immature DCs in the presence of PAMPs or DAMPs(291-293). The activated DC are carried away from the involved tissue in lymph, along with their antigen cargo to enter peripheral lymphoid tissue, in which they can interact with naïve T cells and initiate the adaptive immune response(293;294). Activated DCs will interact with antigen specific T cells, which in response will proliferate and differentiate in to effector Th cells(138;293;295;296) (Fig 1.3). Many different Th subsets with different functions have been proposed, with Th1, Th2, Th17 and T follicular helper (TFH) cells being involved in inflammatory responses whereas regulatory T cells are engaged in maintaining peripheral tolerance and immune suppression.
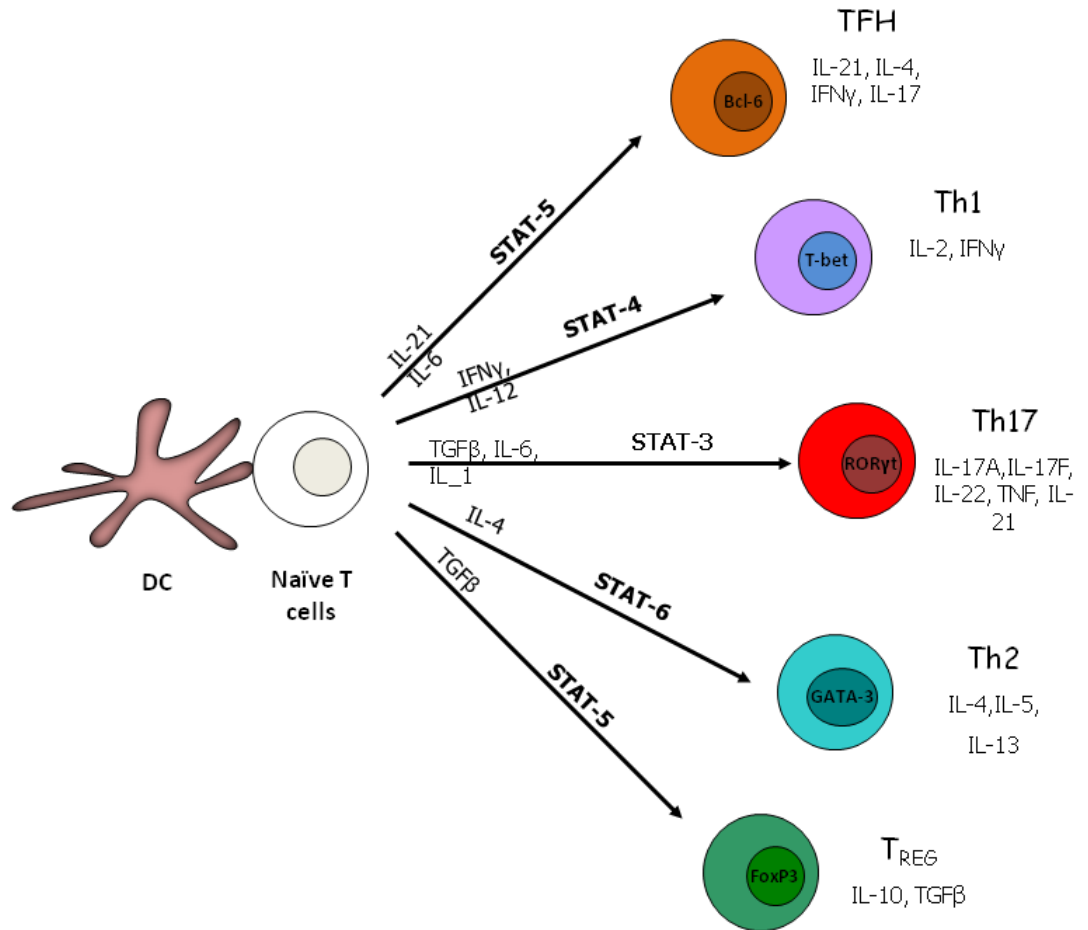
Fig 1.3: Th cell subsets

When a naïve T cells encounters a DCs bearing its cognate antigen it is activated and in the presence of the right environmental cues it can polarise to a Th subtype. The different Th cell lineages are characterised by the production of a selective set of cytokines that affect the immunological outcome. In addition, these lineages express characteristic transcription factors that regulate this selective cytokine production.

1.6.1 Th1 cell subset

More than twenty years ago it was proposed that Th cells could be subdivided into two populations, Th1 and Th2(297). Th1 cells are characterized by the production of IL-2 and IFNγ and induce responses that tend to be dominated by cell-mediated forms of immunity (298;299). A prime example for their role in conferring immunity against intracellular pathogens is the parasite *Leishmania major*. An infection with this pathogen is lethal to genetically susceptible mouse strains, such as BALB/c, which correlates with the development of an inappropriate IL-4/Th2 response(300-302). On the other hand mouse strains resistant to the infection, such as C57/BL6, mount an IFNγ/Th1 response that is able to clear the pathogen(300). Furthermore, adoptive transfer of pathogen-specific T cell lines that produced IFNγ could confer protection to susceptible BaLB/c mice, whereas IL-4 producing lines could not(302). Similar results have been reported for other pathogens, such as *Mycobacterium avium*, *Salmonella typhimurium, Listeria monocytogenes* and *herpes simplex viru*s(303-306). In addition to their role in clearing intracellular pathogens Th1 cells have been proposed to be important in tumour rejection(307;308). The cytokine milieu is probably the most dominant determinant for Th differentiation, and for Th1 commitment the role of IL-12 is pivotal(309;310). TcR stimulation induces upregulation of IL-12R, whose expression is sustained only under Th1, but not under Th2 conditions(311;312). Mice deficient in IL-12p40 are impaired in IFNγ production and mounting Th1 responses, whereas patients with defective IL-12R signalling are susceptible to mycobacterial infections(313;314). However, the fact that IL-12 deficient mice are still capable of generating Th1 cells, albeit in a reduced capacity(315;316), suggests that other factor are able to promote Th1 generation. Indeed, cytokines such as IFNγ and IL-27 have been suggested to promote *de novo* Th1 generation, whereas IL-18 and IL-27 has been reported to synergizes with IL-12 to enhance Th1 differentiation(298;317-324). Commitment to the Th1 lineage is linked with the expression of specific transcription factors, the most important of which is T-bet (T-box expressed in T cells)(325). T-bet is specifically expressed by Th1 cells and it is induced by TcR and IFNγR/STAT-1 signalling(325-327). Retroviral induction

of T-bet into primary T cells induced IFNγ expression, whereas its induction in Th2 polarised T cells resulted in IFNγ production and repression of IL-4 expression(325). Furthermore, CD4 cells from T-bet deficient mice fail to differentiate towards a Th1 phenotype *in vitro* and *in vivo*, whereas their phenotype skews towards a Th2 profile(328). Its importance was also demonstrated by the failure of T-bet deficient mice to control a Th1 protozoan infection and the spontaneous development of a Th2-mediated airway hypersensitivity that resembles human asthma(328;329). Apart from T-bet other, transcription factors, such as Hlx and STAT-4 have also been linked to Th1 cell polarisation. Hlx-1 seems to be downstream of T-bet, and in synergy with it, promotes IFNγ production(330). On the other hand STAT-4 is critical for IL-12 signalling, which has a pivotal role in Th1 generation(315). Due to their role in cell mediated immunity and tumour rejection, Th1 cells have been linked to the development of chronic inflammatory conditions and autoimmunity(331-334). In animal models of inflammatory bowel disease and autoimmune diabetes it has been reported that the pathology is mediated by IFNγ-producing CD4 cells(331-335). In the CIA model of rheumatoid arthritis, administration of factors that promote Th1 immunity, such as IL-12 and IL-18, exacerbated the pathology, increased the production of CII-specific antibodies and lead to enhanced production of pro-inflammatory cytokines, such as IFNγ, TNF and IL-6(333). In rheumatoid arthritis patients, the presence of IFNγ-producing CD4^{+} clones from synovial fuid and membranes that would suggest an involvement of Th1 cells in disease pathogenesis(336-339). The discovery, however, of the Th17 subset forced a reassessment of the established views relating to the role of Th1 cells in the development of some autoimmune syndromes as it will be discussed later.

1.6.2 Th2 cell subset

Th2 cells are characterized by the production of IL-4, IL-5, IL-5, IL-10 and IL-13(297). Th2 cells have been linked to humoral immune responses and IgG1 and IgE class switching(340). They are considered the cells responsible for immunity

against large extracellular pathogens such as helminths(341-344). On the other hand an over-exuberant Th2 responses leads to pathogenic conditions, such as atopic airway hypersensitivity and asthma(345;346). The most dominant cytokine responsible for the induction of the Th2 phenotype is IL-4(347-349). As with Th1 cells, specific transcription factors are linked to Th2 lineage commitment, mainly GATA-3, STAT-6 and c-Maf(341;350-352). Indeed, forced expression of GATA-3 into Th1 cells induces IL-4 production, whereas CD4 cells from GATA-3 deficient mice are unable to fully differentiate towards a Th2 phenotype(350). *In vitro*, STAT-6 has been shown to be activated by IL-4-signaling and subsequently to activate GATA-3(352). C-Maf on the other hand has been reported to control IL-4 production in Th2 cells(353).

Recently a new subtype of Th cells was proposed, termed Th9, which produce high levels of IL-9 and IL-10, but not IL-4(354;355). Functionally they appear to be related to the Th2 subtype as there are also involved in expulsion of intestinal helminths(356). Their differentiation seems to be driven by TGFβ and IL-4(354;355), however much more studies are needed to definitely distinguish whether this is a distinct subset.

1.6.3 TFH cell subset

The role of T cells in supporting B cell responses, in the form of antibody class switching, affinity maturation and generation of B cell memory is a well established phenomenon(138;357-360). Even though Th1 and Th2 cells have been shown to support B cell responses(361;362), it is now widely accepted that CD4 T cells that migrate to the follicles to provide help to B cells are a unique subset of Th cells termed TFH cells. These cells sustainably express the chemokine receptor CXCR5 and various co-stimulatory molecules, such as ICOS, CD40L, OX-40 and PD-1 that allow follicular localisation and B cell help(363-365). TFH cells have been reported to produce various cytokines, amongst them IL-21, IL-4, IFNγ and IL-17(366-368). Cytokines, such as IL-21 and IL-6 has been suggested to be important in TFH cell

generation(369;370). Type I IFN signaling in DCs and non-hematopoietic cells has also been shown to drive TFH cell generation(371). In addition, their localisation and the follicular area provides to these T cells unique environmental cues deriving either from cognate B cells or stromal cells that could promote their differentiation(372;373) (Fig 1.4). As with other Th subtypes, commitment to the TFH cell lineage is regulated at the transcriptional level and the transcription factor B cell lymphoma (Bcl)-6 appears to act as the master switch determining TFH cell development. Bcl-6 is a transcriptional repressor, binds to the promoter region of T-bet and RORγt, and suppresses Th1 and Th17 differentiation(374-376). In addition, it regulates GATA-3 protein levels, repressing Th2 cell differentiation(377). As many autoimmune diseases are characterised by the presence of class switched autoantibodies a role for TFH cells in these conditions has been suggested. Indeed, cells displaying TFH cell phenotypes are evident in human autoimmune diseases and in numerous animal models. Their presence and activity is of particular note in systemic lupus erythematosus (SLE), where patients demonstrate higher levels of $ICOS^+CD4^+$ T cells in peripheral blood and spleen(378;379). A similar phenomenon is also observed in the *Roquin^{san/san}* mice, which exhibit a lupus-like syndrome due to an inability to post-translationaly repress ICOS expression resulting in an excessive TFH activity(380;381). Interestingly, expression of TFH cell phenotypes by circulating peripheral blood cells in autoimmune patients correlates with disease severity(379). One possible effect of these cells might be the development of ectopic GC, which is not uncommon in autoimmunity, and have been reported in rheumatoid synovium, diabetogenic islets and inflamed meninges(382-384). However, the role of these structures in disease severity and chronicity is still debatable.
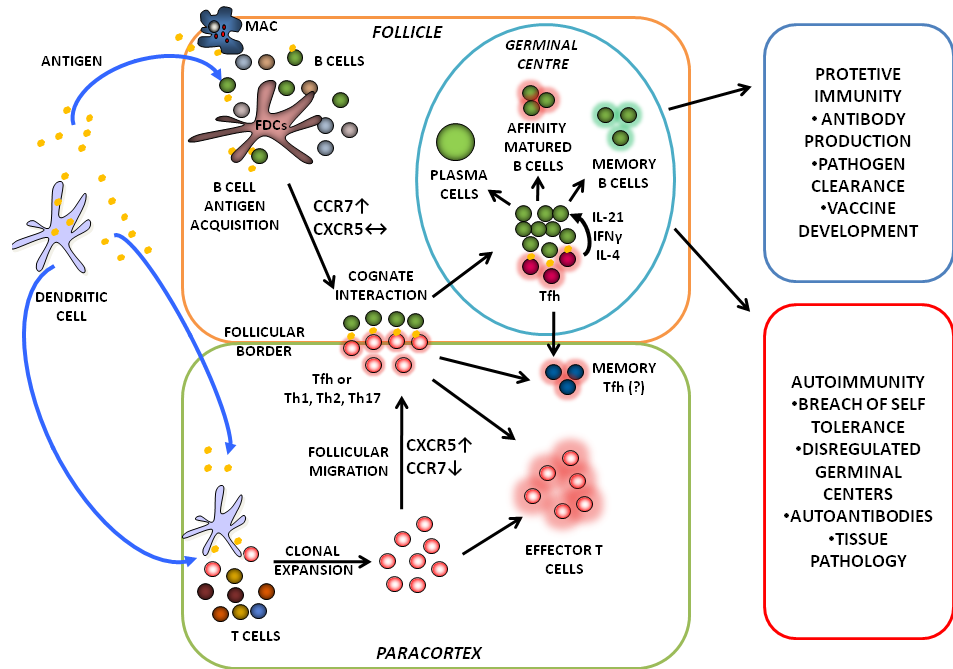
Figure 1.4: B-T cell interactions and their role in the generation of protective or pathogenic humoral responses

Dendritic cells present antigen to T cells in the paracortex leading to a cognate interaction that results in the priming and clonal expansion of antigen-specific T cells. Activated T cells downregulate CCR7 and upregulate CXCR5, which allows them to migrate to the follicular border. At the same time, B cells encounter antigen in the follicle and are activated through their BCR. This leads to the upregulation of CCR7 and migration to the follicular border. At the follicular border the meeting of an antigen-specific B cell with its cognate T cell has bilateral effects on both cell types. B cells receive co-stimulatory and cytokine signals that lead to the formation of the germinal centre that regulates the humoral immune response. At the same time, T cells receive signals from B cells that potentially drive them to a specific phenotype (Tfh, Th1, Th2, Th17). Understanding this bilateral relationship could reveal targets for improved vaccine development and for developing treatment for autoimmune and chronic inflammatory conditions.

1.6.4 Regulatory T cells

In contrast to effector Th cells that promote inflammation, T_{REG} cells are crucial in maintaining peripheral tolerance and immune homeostasis(385). Naturally occurring T_{REG} (nT_{REG}) cells constitute 5-10% of peripheral $CD4^+$ T cells and are characterized by high expression of the IL-2R (CD25), CTLA-4 and glucocorticoid-induced tumor necrosis factor receptor family−related gene (GITR)(386-388). As with the effector Th cells the lineage commitment of T_{REG} cells is controlled at the transcriptional level by specific transcription factors, most notably by FoxP3(389-391). These cells produce high levels of IL-10 and membrane bound of TGFβ and seem to exert their suppressive effect by cell-cell contact(392). Apart from the nT_{REG} cells that are generated in the thymus, $CD4^+CD25^-$ cells after TCR stimulation in the presence of TGFβ and IL-2 can give rise to $CD4^+CD25^+FoxP3^+$ inducible (i)T_{REG} cells with similar functions as the $nT_{REG(393;394)}$. It has been suggested that nT_{REG} are primarily develop in respond to self antigens expressed in the thymus whereas iT_{REG} are induced in response to environmental antigens presented to them by DCs in secondary lymphoid organs(395;396). FoxP3 expressing cells are very important in maintaining peripheral tolerance as mice deficient in this transcription factor develop a fatal lympho-proliferative autoimmune syndrome that affects multiple organs(397). Similarly, in humans mutations in the FoxP3 gene results in the immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX), a inflammatory conditions that can manifest as diabetes-mellitus and psoriasis-like dermatitis(398). In addition, reduction in number or reduced activity of T_{REG} cells has been reported in various autoimmune diseases, such as diabetes, SLE and multiple sclerosis (MS)(399-401), and in animal models, such as the EAE model of MS, which is characterise by defective myelin specific T_{REG}s. Other subsets of regulatory T cells have been described, that also express IL-10 and TGFβ, but do not express FoxP3 and are termed Tr1 cells(402). The generation of these cells *in vitro* requires IL-10 and they have similar suppressive functions as the other T_{REG} cells(403).

1.6.5 Th17 cell subset

Recent studies have defined a previously unknown Th subset, the Th17 lineage. As stated previously, the prototypic cytokine expressed by this lineage is IL-17. The breakthrough for the discovery of the Th17 subset was given from studies in animal models of autoimmunity, mainly EAE and CIA that were traditionally considered as Th1 mediated(9;333;404-406). Paradoxically, IFNγ receptor deficient mice were more susceptible to CIA(407;408) , IFNγ deficient mice were susceptible to EAE development(409) and IFNγ itself played a negative regulatory role in the initiatory and effector phase of myelin oligodendrocyte glycoprotein-induced EAE(410). In addition, IL-12 specific depletion did not protect mice from CIA or EAE development(8;9). On the other hand mice deficient in IL-23 production were resistant to both CIA and EAE development, a phenomenon that was linked to the absence of IL-17–producing $CD4^+$ T cells despite normal induction of autoreactive-interferon-γ–producing Th1 cells(8;9). At the same time data were presented that favoured the presence of a distinct effector lineage that was termed Th17. It was reported that IL-23 induced naïve precursor cells to differentiate to the Th17 lineage, whereas their development was potently inhibited by IFNγ and IL-4(411). Most importantly, fully differentiated Th17 were resistant to suppression by Th1 or Th2 cytokines (IFNγ, IL-4)(411). Even though initially IL-23 was considered as a critical cytokine for polarisation towards the Th17 lineage, subsequent studies reported that IL-23 is not the crucial differentiation factor for the generation of Th17 cells and instead IL-6 and TGFβ can induce the differentiation of naïve T cells to Th17 effector cells(412-414). On the other hand, even though IL-23 was dispensable in Th17 differentiation it was important for their expansion and survival(413). Recent studies suggest that the source of TGFβ is T cells and more specifically, Th17 cells in an autocrine manner, but not T_{REG} cells(415;416). T_{REG} cells seem to promote Th17 differentiation not by providing TGFβ, but limiting the availability of IL-2, which has an inhibitory role in Th17 lineage commitment(417-419). Studies, however have challenged the role of TGFβ

in driving Th17 lineage polarisation(420). It was suggested that TGFβ is not directly promoting Th17 polarisation, but instead inhibits the generation of Th1 and Th2 cells by blocking the expression of the transcription factors STAT4 and GATA3(420) respectively. Apart from TGFβ and IL-6, IL-21 was found to be produced by Th17 and act in an autocrine manner amplifying the Th17 axis(421;422). Human Th17 were identified later than their mouse counterpart. Two studies have demonstrated the existence of memory CD4$^+$ T cells that produced IL-17 after polyclonal stimulation in human peripheral blood and in gut from healthy individuals or patients with Crohn's disease(423-425).Both studies reported that the human Th17 expressed IL-23R and the chemokine receptor CCR6. Further studies revealed that presence of Th17 clones specific for Candida Albicans hyphae, which exhibited poor proliferative and cytotoxic capacity and could induce production of IgG, IgM and IgA, but not IgE(423). Initially the development of human Th17 was considered completely different from mouse Th17. It was reported that TGFβ was not essential for human Th17 differentiation and IL-1, IL-23 and IL-6 were the critical cytokines inducing IL—17 production by human CD4$^+$ T cells(426-428). However more recent studies reported that TGFβ is required for differentiation of human Th17 cells alongside with either IL-1, IL-23 or IL-21 (429;430).

Apart from IL-17, Th17 express IL-17F, IL-6, TNF, GM-CSF, IL-21, IL-22 and in humans also IL-26(8;255;422;427). The major function of the cytokines produced by these cells is to chemo-attract other cells through the induction of other cytokines and chemokines(425). IL-17A and IL-17F act on different cell types and induce the production of IL-6, IL-8, GM-CSF, G-CSF, CXCL1, and CCL20 and in this way attract neutrophils(431). Th17 produce IL-21 that has B cell differentiating role(432). These suggest an important role for these cells in mediating host immunity against various pathogens, which will be discussed in more details later in this chapter.

1.6.6 Transcriptional regulation of Th17 cells

As for Th1 and Th2 cells, Th17 development and function is governed by specific transcription factors, which are either lineage specific such as retinoic acid-related orphan receptor (ROR)γt or non specific such as nuclear factor of activated T cells (NFAT)(433). Similarly to the other Th cell subtypes, the first step of Th17 differentiation and activation is TcR engagement. One of the first events after TcR engagement is the production of intracellular calcium and the activation of NFAT(434). It is not a surprise thus that the human IL-17 promoter has two putative NFAT binding sites, which bind to NFATc1 and NFATc2(433). In addition, signalling through the TcR differentially regulates the expression of Th17 cytokines(435). More specifically, the inducible T cell kinase (Itk) deficient mice exhibit decreased expression *in vitro* and *in vivo* of IL-17A, despite normal expression of RORγt and IL-17F(435). Itk is required for sufficient TCR-induced activation of the phospolipase C-γ (PLC-γ1) pathway, which leads to NFAT activation(434). The absence of a NFAT binding site from the IL-17F promoter, that the IL-17A promoter possesses, explains the differential regulation of these cytokines by the TcR (435).

Similarly to other Th cells, Th17 cells selectively express a lineage specific transcription factor, Retinoic Orphan Receptor (ROR)-γt. RORγt is a member of the nuclear hormone receptors superfamily and its mouse form is encoded by the *RORc* gene, which in mice is located on chromosome 3 and in humans at the chromosome region 1q21(436;437). *RORc* encodes two isoform, RORγ and RORγt(438). Whereas RORγ was found to be expressed in a variety of tissues such as thymus, muscle, brain, heart, kidney, lung and liver, RORγt initially, was found to be expressed only in the thymus (and thus the name RORγt)(438). RORγt was predominantly considered to be important in the early development of the adaptive immune system. It is essential for survival of CD4$^+$CD8$^+$ double positive thymocytes and the formation of lymph nodes and Peyer's patches(439). Interestingly, RORγt is highly expressed in a subpopulation of CD3$^-$CD4$^+$ CD45$^+$IL-7Ra$^+$ cells(439). These cells, termed lymphoid inducer cells (LTi), have

been shown to be associated with lymphoid organogenesis(440;441) and require RORγt for their generation(442). It was only when IL-17A was discovered to be a lineage specific cytokine, that this transcription factor was associated with Th17 development. By using a RORγt-EGFP-reporter mouse it was demonstrated that in the lamina propria apart from LTi cells, there is a subpopulation of TcRαβ$^+$ and TcRγδ$^+$ cells that express RORγt(443). More, importantly when the RORγt-expressing TcRαβ$^+$ cells were isolated and stimulated they produced IL-17, in contrast to their non-RORγt expressing counterparts(443). In addition, in vitro Th17 polarisation was greatly inhibited in RORγt deficient mice, whereas forced expression of RORγt in highly purified naïve T cells resulted in IL-17, IL-17F and IL-22 production(443). However, of considerable importance is the fact that even when RORγt was absent there was residual IL-17A and F production(437;443). This suggests that RORγt is sufficient to induce some parts of the Th17 programming however there are co-factors that may compensate its absence. Supporting, this hypothesis, another member of the ROR-family, RORα acts synergistically with RORγt to promote Th17 differentiation(444). Other factors that control Th17 development include interferon regulatory factor-4 (IRF-4)(445). IRF-4 deficient T cells fail to differentiate to a Th17 phenotype, have less expression of RORγt and increased expression of FoxP3, whereas IRF-4 deficient mice are resistant to EAE(445). On the other hand, transcription factors such as FoxP3 inhibit Th17 development(446;447). TGFβ induces both RORγt and FoxP3, however it is unable to induce IL-17 without the presence of pro-inflammatory cytokines, such as IL-6 or IL-21, which suggests that the cytokine-regulated balance between FoxP3 and RORγt controls Th17 or iT$_{REG}$ generation(446). The interaction of FoxP3 with Runx1 is essential for the negative effect of FoxP3 on Th17 differentiation(447).

As mentioned previously cytokines such as IL-6, IL-21 and IL-23 are critical for the development and expansion of Th17 cells(411;421;448). All of these cytokines have in common that they preferentially activate STAT3(449-451). There is much evidence that support a central role of STAT-3 in the Th17 biology. Firstly, in vitro Th17 differentiation is greatly impaired in STAT-3 deficient T cells(419). In

addition, the expression of the signature transcription factor for Th17, RORγt, is also dramatically reduced in STAT3 deficient T cells(452). Furthermore the IL-17a/f locus has putative STAT binding sites and STAT-3 has been shown to directly bind to the IL-17a/f and IL-21 promoter(453)ˈ(454). Apart from the direct effect that on IL-17 production, STAT-3 activation has other indirect effects that promote Th17 development and survival. For example, both IL-6 induced production of IL-21 expression and IL-21 induced IL-17 production is STAT-3 dependent(455). In addition, all IL-6, IL-21 and IL-23 up-regulate the IL-23R in a STAT-3 dependent manner(422;428;455). Furthermore, deletion of suppressor of cytokine signalling 3 (SOCS3), which is a negative regulator of STAT-3 signalling, leads to increased STAT-3 phosphorylation, IL-17 production and Th17 generation, further supporting a role for STAT-3 in Th17 development(453;456).

1.6.7 The role of Th17 cells in immunity

Even though the initial description of the Th17 lineage was made using mouse models of autoimmunity and their function has been linked to various autoimmune diseases, there is accumulative evidence for their role in host defence against extracellular bacteria, fungi and even viruses, especially in mucosal surfaces such as the gut and the lung(457). Various bacteria, fungi, fungal products and viruses, such as *Klebsiella pneumoniae*(458), *Mycobacteria tuberculosis*(459), *Helicobacter pylori*(460), *Fransicella tularensis*(461), *Citrobacter rodentium* and *Escherichia coli* (462), *Candida albican*, β-glucans(463), and herpes simplex virus(464) can condition DCs to produce Th17 polarising cytokines, TGFβ, IL-6, IL-1β and IL-23. The various cytokines produced by the Th17 cells has been reported to have specific roles in host defence. One of the first documented effects of IL-17A is the promotion of neutrophil differentiation by CD34^{+} progenitor cells and the induction of cytokines such as IL-6, IL-8, G-CSF and PGE2(270). IL-17RA, which binds IL-17A and IL-17F, and IL-17A deficient mice are susceptible to *Klebsiella pneumoniae* pulmonary infection, a phenomenon linked to reduced chemokine

production (CXCL2) and neutrophil recruitment(465). IL-17A was also found to induce the production of β-defensin-2, S-100 proteins and various chemokines, such as CXCL1 and CXCL5, in lung epithelial cells(466;467). Furthermore, CD4-derived IL-17 was demonstrated to be important for intra-abdominal abscess formation in response to bacteria such as *Staphylococcus aureus* and *Bacteroides fragilis*(468). Mechanistically, it was also reported that bacteria alone and not viruses, conditioned DCs through the nucleotide oligomerization domain-2 (NOD2) pathway to promote IL-17 production from memory T cells(469). Apart from IL-17A and IL-17F another Th17 cytokine, IL-22, has been reported to play important role in mucosal immunity. IL-22 induces production of antimicrobial peptides, such as RegIIIβ and RegIIIγ, defensins and chemokines by host epithelial cells and it increase their proliferation and resistance to injury(470)'(471). The above data signify the importance of Th17 derived cytokines in mucosal defence against mucosal pathogens. These cytokines do not seem to be so critical for immunity against intracellular pathogens such as *Mycobacterium tuberculosis* and *Listeria monocytogenes*(472). These cytokines and Th17 cells are also important for anti-fungi immunity. When the phenotype of human Th17 cells was initially described it was reported that memory T cells specific for Candida albicans were mainly present at the $CCR6^+CCR4^+$ Th17 subset, whereas memory T cells specific for *Mycobacterium tuberculosis* were found mainly at the $CCR6^+$ $CXCR3^+$ Th1 subset(473). In addition, patients with autosomal-dominant hyper-IgE syndrome (Job's syndrome), which is caused by a mutation in the STAT-3 gene, have been show to have impaired Th17 responses. Interestingly these patients are particularly susceptible to mucocutaneous infections caused by *Candida albicans*(474). To further support these data it was reported that a C-type lectin, Dectin-2 acts as a pattern recognition receptor (PRR), through which DCs are conditioned by fungi derived molecules to promote Th17 responses(475). All of these facts demonstrate a clear role for Th17 immunity against extracellular bacteria and fungi especial in mucosal surfaces.

1.6.8 The role of Th17 cells in RA

It is still not clear wheather RA is a Th1 or Th17 mediated disease. Studies that specifically inhibited either the IL-12/Th1 axis or the IL-23/Th17 axis demonstrated that at least in the CIA model, animal deficient in generating Th1 responses were more susceptible to disease development, and this was associated with the presence of Th17 cells, whereas IL-23 or IL-17 deficient mice were protected from the disease development(9;476) . In addition, in other RA models, such as the SKG and IL-1Ra KO animals, the active phase of the disease seems to be mediated by Th17 cells, and in addition the synovial membrane expresses chemokines (CCL20) that can attract these cells to the pathological site(477-479). In patients with RA and ankylosing spondylitis, IL-17$^+$ and IL-22$^+$ CD4$^+$ T cells could be detected in the circulation and were increased compared to healthy controls(480).   Furthermore, as mentioned previously, there is a well established role for IL-17 and Th17 in joint destruction and remodeling. In the CIA model it has been demonstrated that Th17 cells promote osteoclastogenesis an ability absent from Th1 and Th2 cells(176). IL-17, which is highly expressed in RA synovial membrane and fluid, acts on osteoblasts, stimulating the production of pro-osteoclastogenic factors, such as osteclastogenesis differentiation factor (ODF), promoting this way differentiation of osteoclast maturation(481;482). Other Th17-related cytokines have been reported to be involved in RA pathology. IL-21-blockade ameliorates CIA in mice and rats, IL-21 receptor (IL-21R) deficient mice are protected from the development of arthritis in the autoimmune prone K/BxN model and in humans the IL-21R is expressed by RA synovial macrophages and fibroblasts(483-485). It should be noted however, that many of the Th17-related cytokines, such as IL-21, IL-22 and even IL-17 it-self, are not exclusively produced by Th17 cells, but from other cell types and effector T cells(286;486;487). Even though the above studies suggest a role for Th17 in RA, other studies in human and animals, report a less significant role for these cells. The proteoglycan-induced model of arthritis, for example, is mediated by IFNγ-producing cells, and mice

deficient for this cytokine develop significantly less severe pathology(488). In humans, a study in Japan revealed that the frequency of Th17 cells was neither increased in RA patients nor correlated with disease severity, and was significantly decreased in joints compared to peripheral blood, unlike Th1 cells that were more abundant in the joint(489). Furthermore, Th17 frequency was reported to be increased only in seronegative arthropathies, such as psoriatic arthritis, but not in RA(490). It remains thus possible that the role of Th1/Th17 can differ according to disease subtype. All the aforementioned studies, in animal model and humans, are mainly focused in the articular phase of the disease. On the other hand, the role of Th17 cells in the events that lead to the breach of self tolerance, however, are ill defined and under-studied.

1.6.9 T helper cell plasticity

The reductionist approach, that two Th subsets, Th1 and Th2, regulate host immunity against pathogens, collapsed in a certain degree with the discovery of regulatory T cells, Th17 and TFH cells (Fig 1.3). These subsets fit the lineage paradigm and, as mentioned previously, produce and express specific sets of cytokines and transcription factors. However recent studies suggest that cytokine expression is not as stable as it was initially thought (Fig 1.5). For instance, Th17 cells have been shown to produce the Th1 signature cytokine IFNγ, especially *in vivo*(427;491). In some cases also, Th17 cells seem to totally cancel the production of their signature cytokine, becoming selective IFNγ producers(492;493). In addition both Th1 and Th17 cells have been reported to produce IL-10, which has been suggested to regulate the inflammatory responses initiated by IFNγ and IL-17 respectively(494;495). The phenomenon of cytokine plasticity can also be expanded to Th2 cells. Indeed, Th2 cells can produce IL-9 under the influence of TGFβ(355). More importantly, *in vitro* polarised Th2 cells specific for lymphocytic choriomeningitis virus (LCMV) are protective *in vivo* through the production of IFNγ(496). This plasticity can be also observed in the transcription factor level.

FoxP3 expression can be turned off and former regulatory cells can acquire pro-inflammatory phenotype, producing either IL-17 or IFNγ, depending on the site investigated(497;498). Alternatively, FoxP3-expressing T cells can acquire a TFH phenotype and support B cell responses and IgA antibody production in Peyer's patches in the gut(499). All these suggest that the phenotype of regulatory and effector T cells is not as stable as originally believed, and this might serve the functions of the immune system, either by allowing regulation of ongoing immune responses or by using the memory repertoire in the most appropriate way, especially as becomes more limited with age(500). This also can open opportunities for intervention, as altering the phenotype of the immune response could be an effective therapy for conditions such as asthma and various autoimmune syndromes.
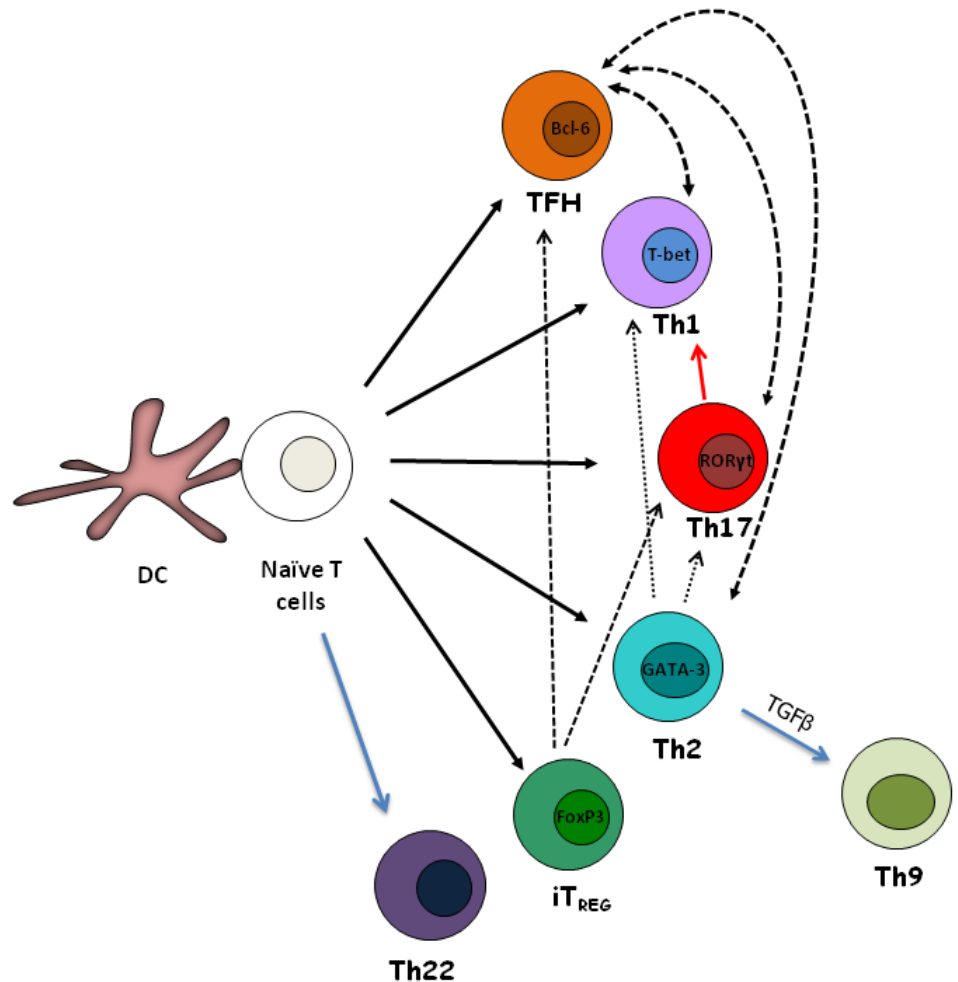
Fig 1.5: Flexibility and plasticity of Th subsets

Recent studies have revealed that Th subset are more flexible both in cytokine production and transcription factor expression, than originally considered. CD4^{+} cells can change their cytokine profile, regulatory cells can become inflammatory, whereas effector cells can acquire regulatory functions. In addition there are circumstances where expression of transcriptional master regulators is transient or cells express more that one at the same time (e.g T-bet/RORγt or FoxP3/RORγt).

1.7 Animal models of RA

RA is a very complicated multifactorial inflammatory disease. In this concept animal models are instrumental for understanding the pathology and aetiology of RA. The advantages of using animal models are mainly:

- Animals can be genetically controlled. Laboratory mice and rats have been inbred which dramatically reduces variations that are very common in human studies.
- Their environment can be easily controlled
- The genetic background of the animals can be manipulated

Animal models of inflammatory arthritis can be subdivided to induced models, whose development is based on immunising animals with an autoantigen or protein in the presence of an adjuvant or spontaneous models whose development is based on genetic manipulations (Table 3). Probably the most widely used model of arthritis is CIA. It was first described in rats, but was subsequently found to develop in genetically susceptible DBA/1 mice that carry the MHC Class II I-A^q haplotype(123;501). It is based on the immunisation of heterologous CII in CFA and its development is characterised by anti-CII B and T cell responses(502).

Table 3: Animal models of inflammatory arthritis[ii]

Model	Species	Disease characteristics
Arthritis caused by infection		
Mycoplasma induced arthritis(503)	Rat and mice	Mild chronic arthritis
Borrelia induced arthritis(504)	Rat and mice	Severe erosive arthritis
Stapylococus induced arthritis(505)	Rat and mice	Severe arthritis
Arthritis caused by fragments of bacteria persisting in the joints		
Adjuvant (mycobacterium cell wall) induced arthritis(505)	Rats	Acute general inflammatory disease with erosive arthritis
Streptococcal cell wall induced arthritis(506)	Rats and mice	Severe and erosive arthritis
Adjuvant induced arthritis		
Mineral oil induced arthritis(507)	Rats	Acute, self limited inflammation in the peripheral joints
Pristane induced arthritis(508)	Rats and mice	Chronic and generalized inflammatory disease mainly affecting the joints
Arthritis induced by cartiladge protein immunisation		
Collagen induced arthritis(123;501)	Rats and mice	Chronic and erosive arthritis in peripheral joints
Human proteglycan (in CFA)-induced arthritis(509)	Mice	Chronic arthritis
CXI (in CFA)-induced arthritis(510)	Rats	Mild, acute arthritis
'Spontaneous' arthritis models		
TNFa transgenic mice (overproducing TNFa)(219)	Mice	Erosive arthritis as well as generalized tissue inflammation
Mice with ZAP-70 mutation (SKG mice)(66)	Mice	Chronic erosive arthritis
TcR transgenic mouse (T cell autoreactivity)(27)	Mice	Severe arthritis

Antibodies are active drivers of the pathology in this model, as immunoglobulin concentrates of sera from CIA mice can transfer disease even to resistant mouse strains(512). Similar protocols are now used to induce arthritis and are collectively

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[ii] This table was adapted from ref: (511)

termed collagen antibody-induced arthritis (CAIA). Apart from CII, immunisation with other joint derived antigens, such as cartilage-proteoglycan, induces arthritis in mice, which is accompanied by B and T cell responses against these antigens(509). A limitation of these models is that they rely on breaching existing tolerance to a single self-antigen based on aggressive immunisation protocols utilizing this same antigen. Spontaneously arising autoimmune models could be more beneficial in understanding how breach of tolerance is likely to occur in human RA. Genetic manipulation has lead to the development of various spontaneous developing arthritis models (Table 3). In the K/BxN model, disease occurs in the F1 progeny of NOD mice crossed with the KRN TcR transgenic mouse(27). In this system the transgenic TCR shows reactivity with GPI in the context of I-Ag7 [27]. Pathogenesis relies on T cell activation of B cells and their production of complement fixing GPI specific antibody(27). The fact however that this model utilises a single specificity TCR transgenic to initiate/maintain disease against an antigen limits its physiological relevance as probably multiple autoimmune clones are involved in RA development. Multiple autoreactive T cell clones are involved in the development of the SKG model of arthritis(66). The development of this model is based on a point mutation in the gene encoding the TcR signalling molecule ZAP-70 resulting in altered thymic selection(66). These mice have high titres of rheumatoid factor, anti-type II collagen, ACPA and heat shock protein reactive antibodies, demonstrating multiple antigen specific response(66).

The importance of cytokines, such as IL-1 and TNF in development of arthritis has been demonstrated in animal models. Mice that overexpress TNF(66;219) or deficient in the IL-1 receptor antagonist(66;479) develop spontaneous arthritis and have been instrumental in investigating the role of these cytokines in RA development.

Most of these models resemble the active, destructive phase of the disease, and thus do not allow the delineation of the immunological mechanisms that lead to the underlying autoimmunity that characterises RA patients even years before disease development or the mechanisms that initiate the articular phase of the disease. To

understand this phase of the disease an animal model that resembles it needs to be utilised.

1.8 OVA-TcR-induced model of early arthritis

In our lab, a novel model of experimental arthritis has been developed that highly resembles the underlying autoimmunity that characterizes the preclinical stage of RA(5). Transfer of Th1-polarised OVA-specific-TcR-transgenic CD4$^+$ T cells, induces transient arthritis in mice challenged in the footpad with heat-aggregated OVA (HAO). This is characterized by a transient paw swelling, which lasts around 7-9 days, synovial hyperplasia and cartilage erosion proximal to the HAO challenged paw(5). These clinical and histopathological signs of disease are mild compared to the aggressive polyarthritis of other models, such as CIA and SKG, which resemble more the advanced human disease(5;66;123). However, the most important feature of this model is the unbiased breach of self-tolerance. Indeed, even though these animals never encountered autoantigen in an immunogenic way, as for example in the CIA model, they develop self reactive T and B cell responses. More specifically animals in this model develop a number of class-switched (IgG) autoantibodies, namely anti-CII antibodies, ACPA, RF and anti-DNA antibodies, and T cell responses against CII(5;6;168). Interestingly, the anti-CII antibodies in this model recognise the U1 peptide, which is one of the epitopes recognised by antibodies in the CIA model(513) (Conigliaro P. *et al* manuscript submitted). Importantly, our group has demonstrated that even-though non-specific inflammation could recapitulate the clinical and histopathological signs of the disease, it was not able to lead to the development of autoreactivety, suggesting that this is dependent on eliciting an antigen specific T cell response of irrelevant-specificity proximal to the joint(6). Furthermore, using this model we have demonstrated that pDCs have a regulatory role, limiting self reactivity and the developing pathology(168). Crucially, we have identified that the CD11c$^+$ APCs mediate the breach of self tolerance, as these cells can substitute HAO challenge, and their absence inhibits the development of autoreactivity(7). More recently, we have demonstrated the importance of co-stimulation on the development of auto-

reactivity, as CTLA-4-Ig (Abatacept) was able to inhibit the development of autoantibodies, through an effect on TFH differentiation(514). All of these data demonstrate the usefulness of this model in delineating the early immunological events that lead to the breach of self tolerance.

This model will be employed to answers question relating to the role of Th17 effector cells in the events that lead to the breach of self tolerance as it will be described in the result chapters of this thesis. This thesis will start by describing the establishment of a reproducible and efficient Th17 protocol. It will continue by investigating the involvement of Th17 in the OVA-TcR arthritis model and their ability to induce breach of self tolerance. In the next chapter, the relative ability of Th17 compared to Th1 population in supporting B cell responses, as this might be in the development of autoantibodies. Finally, in the last chapter the role of Siglec-G in the development of autoreactivity will be investigated. As these molecules are involved in the negative-regulation of sterile inflammation, it would be interesting to investigating their involvement in autoimmunity development.

Chapter 2: Material and methods

2.1 Mice

BALB/c (H-$2^{d/d}$) mice, between 6-12 weeks old, were either bred by the University of Strathclyde Biological Procedures Unit or purchased from Harlan, UK. C57BL/6 (H-$2^{b/b}$) mice were purchased from Harlan, UK. Homozygous DO11.10 BALB/c (H-$2^{d/d}$) mice, expressing the DO11.10 TcR specific for chicken OVA peptide 323-339/I-A^d, were used as CD4^+ cell donors(515). In other experiments homozygous C57BL/6 OT-II mice that express a TcR that recognises the OVA peptide 323-339 in the context of I-A^b were used as transgenic T cell donors(516). DO11.10 BALB/c (H-$2^{d/d}$) SCID mice were bred by the University of Strathclyde Biological Procedures Unit or by the Glasgow University Central Research Facility. In some experiments Sigleg G deficient mice (517) on the BALB/c (H-$2^{d/d}$) background were used as recipient of transgenic DO11.10 cells. These mice were kindly donated by Prof. Paul Crocker from Dundee University.

Mice heterozygous for the anti-hen egg lysozyme (HEL) IgM^a and IgD^a transgenes on the BALB/c background (MD4) were screened by flow cytomentry for their ability to bind HEL and positive animals were used as donors of transgenic B cells(518). IgH^b BALB/c (H-$2^{d/d}$, IgM^b) mice(519) were used as recipients of the transgenic B cells. All animals were maintained at either the University of Strathclyde Biological Procedure Unit or the University of Glasgow Central Research Facility in accordance with Home Office regulations, in SPF cages, or filter-top cages, as appropriate.

2.2 Preparation of single cell suspensions from LNs and Spleens

Mice were euthanized by cervical dislocation and various lymph nodes (LNs) (cervical, inguinal, popliteal, auxiliary, brachial, cervical, mesenteric and para-aortic LNs) and/or spleen were extracted in RPMI complete media (for composition, refer to the appendix). Single cell suspensions were prepared by passing them through a 40μm sieve (BD Biosciences) into RPMI complete media using the plunger of a sterile 5ml syringe (BD Biosciences). Cell suspensions were washed with complete RPMI media and were centrifuged at 400xg for 5min at 4°C. In the case of the spleen cell suspension the pellet was resuspended into 2-5ml of red blood cell (RBC) lysis buffer (ebioscience) and cells were incubated for 5min on ice. 20-40ml of complete RPMI media was added to stop the reaction and the cells were centrifuged (400xg, 5mins, 4°C) and resuspended in complete RPMI media. Cells were counted using a haemocytometer with non-viable cells excluded on the basis of trypan blue staining.

2.3 Flow cytometric analysis

LNs and/or spleens were made into a single cell suspension as described in 2.1.2. For cell surface staining, $2x10^5$ to $1x10^6$ cells per well were transferred to a 96-well round bottom microtitre plate (Costar), washed with 250μl of FACS buffer (for composition refer to appendix in the end of the chapter) and centrifuged (400xg, 5mins, 4°C). Cells were resuspended in 50μl of FcR blocking buffer (for composition refer to the appendix) were incubated for 15min at 4-8°C. Antibodies for extracellular staining were diluted in FcR blocking buffer in concentration from 1-5μg/ml, 50μl were added to each well and incubated for 30min at 4-8°C in the dark. Cells were washed twice with 250μl of FACS buffer and centrifuged (400xg, 5mins, 4°C). In cases where biotin-conjugated antibodies were used a fluorochrome-labelled streptavidin secondary reagent was necessary. The labelled

streptavidin was diluted in FACS buffer and used at a concentration of 1μg/ml for 15min at 4-8°C. Cells were washed twice and were either resuspended in FACS buffer or were fixed with 4% paraformaldehyde (PFA) (100μl per tube, 20min, at room temperature in the dark).

For intracellular cytokine staining, 2×10^5 cells per well were added in a 96-well round bottom microtitre plate and incubated with 50ng/ml of Phorbol-12-Myristat-13-Acetate (PMA)(Sigma) , 500ng/ml Ionomycin (Sigma) for 5 hours at 37°C, 5% CO_2. Golgi-Plug (BD Biosciences) (diluted 1/1000) was added for the last 4 hours of the stimulation. After the incubation the cells were centrifuged (400xg, 5min, 4°C) and then stained for extracellular markers as described before. Cells were then fixed with 100μl of 4% PFA for 20min at room temperature in the dark, washed with 250μl of permeabilisation buffer (for composition refer to the appendix), centrifuged (400xg, 5min, 4°C) and resuspended in the same buffer. Cells were permeabilised for 20min at 4°C in the dark, centrifuged (400xg, 5min, 4°C) and incubated with the antibody against the cytokine of interest. The antibodies were diluted in permeabilisation buffer at a concentration of 5μg/ml and cells were incubated with them for 30min in room temperature in the dark. The cells were then washed with permeabilisation buffer, centrifuged (400xg, 5min, 4°C) and resuspended in FACS flow (BD biosciences). Antibodies for extracellular and intracellular staining and the composition of the buffers used are listed in the appendix of this chapter. Data were acquired on a FACS Canto (BD), using the Diva software, or FACSCalibur (BD) using Cell Quest Pro software and analyzed with FlowJo software (Treestar).

2. 4 Magnetic-activated cell sorting (MACS)

Mice were euthanized by cervical dislocation and peripheral LNs (cervical, inguinal, popliteal, axillary, brachial, cervical, mesenteric and para-aortic LNs) and spleen were extracted in RPMI complete media. For CD4$^+$ isolation, the CD4$^+$ T cell isolation kit from Miltenyi Biotec (#130-095-248) was used and the manufacturer's

instructions were followed. In detail, spleen and LNs were made to single cell suspension as described in 2.1.2. The cells were then centrifuged (300xg, 10min, 4°C), resuspended in 40μl of MACS buffer (for composition refer to the appendix) per 10^7 cells and 10μl of antibody cocktail per 10^7 cells and incubated for 10min at 4-8°C. According to the manufacturer, the antibodies of the cocktail were directed against CD8a, CD11b, CD11c, CD19, CD45R (B220), CD49b (DX5), CD105, MHC-class II and Ter-119 (an erythroid cell marker). This incubation was followed by the addition of 30μl of MACS buffer per 10^7 cells and 20μl of anti-biotin labelled magnetic beads per 10^7 cells to the cell suspension and incubation for 15min at 4-8°C. Cells were then washed with MACS buffer (30-40ml), centrifuged (300xg, 10min, 4°C) and resuspended for cell sorting in the appropriate volume of MACS buffer (500μl per 10^8 cells). LS columns (Myltenyi Biotec) were fitted to a magnet (Miltenyi Biotec), primed with 3ml of MACS buffer and the cells were applied onto them (up to $2x10^9$ per column). Columns were washed 4 times with 3ml of MACS buffer, and the negative fraction ($CD4^+$ enriched fraction) was used for Th1 and Th17 polarisation. The positive fraction was flushed out with 5ml of MACS buffer and used as a source of antigen presenting cells for the Th1 and Th17 polarisations. The cell in the positive and negative fraction were counted using a haemocytometer (Hawksley) and trypan blue (Sigma) for non-viable cell exclusion. Cells were washed and resuspended in complete RPMI or complete IMDM medium (for compositions refer to the appendix) depending on future use. In the case of the positive fraction and spleen cells used as a source of APCs, cells were treated with mitomycin C (50μg/ml, Sigma) for 60min at 37°C, 5% CO_2 and were then washed twice with complete media.

2.5 In vitro Th1 and Th17 polarisation

Th1 polarisation were based on the protocol used by Maffia *et al*(5). In detail, MACS sorted $CD4^+$ T cells from DO11.10 or OT-II mice at a concentration of $5x10^5$ cells/ml were co-cultured with mitomycin C treated splenocytes at a concentration of $5x10^6$ cells/ml in complete RPMI media in the presence of

0.5μg/ml OVA$_{323-339}$ (Cambridge Biosciences) and the following cytokines and neutralizing antibodies: IL-12 (10ng/ml, RnD Systems), anti-IL-4 (clone 30340, 2μg/ml, RnD Systems)(246;520). Cells were culture in 75T tissue culture flasks (Nunc) for 3 days at 37°C, 5% CO_2. For Th17 polarisation, MACS sorted CD4$^+$ T cells from DO11.10 or OT-II mice at a concentration of 3×10^5cells/ml were co-cultured with mitomycin C treated splenocytes at a concentration of 3×10^5 cells/ml in complete IMDM media (for composition refer to appendix) in the presence of 1μg/ml OVA$_{323-339}$ and the following cytokines and neutralising antibodies: IL-6 (20ng/ml, RnD Systems), TGFβ (1ng/ml, RnD Systems), IL-23 (10ng/ml, RnD Systems), IL-1β (10ng/ml, RnD Systems), anti-IL-4 (clone 30340, 10μg/ml, RnD Systems), anti-IFNγ (clone XMG1.2, 10μg/ml, BD Biosciences)(8;412;414;455;521-523). Cells were cultured for 4 days at 37°C, 5% CO_2. The phenotype of the polarised population was assessed by intracellular cytokine flow cytometric staining or enzyme-linked immunosorbent assay (ELISA) of the culture supernatans.

2.6 Proliferation assay

To measure the relative ability of CD4$^+$ T cells to proliferate in response to various antigens we directly measured the incorporation of the nucleoside analogue 5-ethynyl-2´-deoxyuridine (EDU) during active DNA synthesis using the Click-iT®EdU Alexa Fluor® 488 Cytometry assay kit (Invitrogen). Detection is based on a click reaction, a copper catalyzed reaction between an azide and an alkyne(524;525). In this case the EDU contains the alkyne and the Alexa Fluor® 488 dye contains the azide. Mice were euthanized by cervical dislocation and popliteal lymph nodes were extracted into complete RPMI media. Single cell suspensions were prepared from the popliteal LNs as described in section 2.1.2. 2.5×10^5 cells were added in each well of a 96-well microtitre plate that contained either complete RPMI media, or complete RPMI media with 1mg/ml of chicken OVA or 50μg/ml of CII (Sigma) and were incubated for 72hrs at 37°C, 5% CO_2. After 48hrs, EDU (Invitrogen) was added to each well at a concentration of 5μg/ml.

After 72hrs the cells were centrifuged (400xg, 5min, 4°C), washed twice with FACS buffer, and stained for surface markers as described previously (2.1.3). They were then washed twice with 1% bovine serum albumin (BSA) /PBS, fixed with 4% PFA (20min at room temperature in the dark), washed with 1% BSA/PBS and centrifuged (400xg, 5min, 4°C). Following this, they were resuspended in the Click-iT™ reaction cocktail prepared according to manufacturer's instructions and incubated for 30min at room temperature in the dark. Cells were then washed with 1% BSA/PBS, centrifuged (400xg, 5min, 4°C) and resuspended in FACS flow (BD). Data were acquired using a FACS Canto (BD), using the Diva software, or FACSCalibur (BD) using Cell Quest Pro software, and analyzed with FlowJo software (Treestar).

2.7 Enzyme-linked immunosorbent assay (ELISA)

96-well microtitre plates (Costar) were coated with antigen or capture antibody in carbonate buffer pH 9.6(for composition refer to the appendix) (50μl per well), overnight at 4°C. Plates were washed with ELISA wash buffer (3x), and non-specific protein binding was blocked by incubation with blocking buffer (200μl per well, 10% FCS in PBS, 37°C, 1hr). Following this, the plates were washed with wash buffer (0.05% Tween-20, PBS, 3x), and the serum samples were added (50μl/well) and incubated for 2hrs at 37°C. In the case of anti-OVA, anti-CII, or anti-CCP antibody detection, serial dilutions of the mouse sera were employed. After incubation, plates were washed with wash buffer (4x), and were incubated with the detection antibody (50μl per well, diluted in dilution buffer:0.2% FCS, 0.05% Tween-20, PBS) for 1hr at 37°C. Plates were washed (4x) with wash buffer, and in the case were the detection antibody was biotinylated, were incubated with horse-radish-peroxidase (HRP)-conjugated streptavidin (50μl per well, diluted in dilution buffer) for 30min at room temperature. Plates were then washed (4x) with wash buffer, and incubated with SureBlue TMB Microwell Substrate (KPL) for the appropriate time at room temperature. The reaction was terminated by the addition of 10% H_2SO_4 and the absorption was determined at OD_{450} using an ELISA plate

reader (Molecular Devices). The antigen-antibody and antibody pairs used, their concentrations and the composition of the buffers used are listed in the appendix of this chapter.

2. 8 Immunohistochemistry

The protocols used in this thesis were adopted and/or modified from protocols developed by Grierson *et al(526)*. The composition of buffers and antibodies used are listed in the appendix in the end of this chapter. In detail, mice were euthanised by cervical dislocation and draining lymph nodes were removed and snap frozen in OCT embedding medium (VWR). 6-8μm thick sections were cut using a cryotome (Thermo Scientific) and mounted on superfrost plus microscopy slides (VWR). Slides were then stored at -20°C. Prior to staining sections were brought to room temperature and were fixed in acetone for 10min. Slides were allowed to dry and sections were marked using an ImmEdge hydrophobic barrier pen (Vector). From this point on all samples were kept in a darkened, humidified box at room temperature, unless differently specified. The sections were rehydrated with PBS for 5min and endogenous peroxidase activity was inhibited by incubation with the endogenous peroxidase blocking buffer (for composition refer to the appendix) for 15min. This step was repeated three times. Sections were washed with PBS (3x, 3min). Non-specific FcR binding was blocked with incubation with FcR blocking buffer (for composition refer to the appendix). All endogenous biotin, biotin receptors or avidin binding sites present in the tissue sections were blocked using an avidin/biotin blocking kit (Vector) according to manufacturer's instructions. In detail, sections were incubated with diluted avidin (100μl per section, 4 drops in 1 ml of PBS) for 15min, washed with PBS (1x, 5min), incubated with diluted biotin (100μl per section, 4 drops in 1 ml of PBS) and washed again with PBS (1x, 5min).

When samples were stained only for extracellular markers, sections were initially incubated with biotinylated antibodies diluted in 1% blocking buffer for 30min

(100μl per sample). Samples were washed in TNT wash buffer (3x, 5min) and were incubated with HRP-conjugated streptavidin (Perkin Elmer) diluted 1:100 in TNB blocking buffer (for compositions refer to the appendix). They were then washed with TNT buffer (3x, 5min) and incubated with biotinylated tyramide (Perkin Elmer) diluted in 0.015% H_2O_2 /amplification buffer (Perkin Elmer) for 10min. Tissues were washed with TNT (3x, 5min) and AlexaFluor®647-labelled streptavidin (2μg/ml, Invitrogen) diluted in TNB was added for 30min. Following this, sections were washed with TNT buffer (3x, 5min) and incubated with directly fluorochrome-labeled antibodies diluted in 1% blocking buffer overnight at 4°C. The samples were then washed with PBS (3x, 5min) and in some cases incubated with AlexaFluor®488-labelled anti-fluorescein antibody (Invirtogen) diluted in 1% blocking buffer (2μg/ml) for 30min. They were then washed with PBS (3x, 5min), allowed to air dry (5-10min, in the dark), mounted in vectashield (Vector) and sealed with a coverglass (VWR) and clear nail varnish.

For detection of the intracellular transcription factor RORγt or for IL-17, samples were incubated for 30min with permeabilisation buffer A (50μl per sample, for composition refer to the appendix), washed with PBS (3x,10sec) and were then incubated with 50μl of 1% blocking buffer/0.1% saponin for 30min. The sections were washed with PBS (3x, 10sec) and incubated with the antibody against the intracellular marker diluted in permeabilisation buffer B (for composition, refer to appendix) overnight at 4°C. Samples were then washed with TNT buffer (3x, 5min) and were incubated with biotynilated secondary antibodies diluted in 1% blocking buffer/0.1% saponin for 30min. They were then washed with TNT buffer (3x, 5min) and incubated with biotynilated tyramide (Perkin Elmer) diluted in 0.015% H_2O_2 /amplification buffer (Perkin Elmer) for 10min. Tissues were washed with TNT (3x, 5min) and AlexaFluor®647-labelled streptavidin (2μg/ml, Invitrogen) diluted in TNB was added for 30min. Samples were then stained for cell surface markers as described previously.

Images were acquired using a Carl Zeiss LSM510 META Confocal Imaging System and analyzed using Volocity™ software (Impovision, Perkin Elmer). Using the tile scan function of the LSM510 confocal microscope, the full area of the section was imaged. Usually 3 random sections per sample were imaged. Colour levels of the acquired images were optimized and noise was reduced using Volocity™ software employing the "contrast enhancement" and "Remove Noise" tools. For quantification of transferred T and B cells, cells were tracked using the "Find Objects Using SD Intensity" tool. This tracks cells based on the mean of the intensities in each pixel and selecting standard deviations about that mean. Objects smaller than $30\mu m^2$ and larger than $350 \ \mu m^2$ were excluded using the "exclude objects by size". Touching objects were separated using $100\mu m^2$ as a guide size.

2.9 Chicken Ovalbumin (OVA)-Hen Egg Lysozyme (HEL) chemical conjugation

The OVA-HEL conjugate antigen was prepared by using glutaraldehyde to couple HEL (Bioenzyme laboratories) to OVA (Sigma) as described before(138;361;362). One hundred and thirty micrograms of OVA-HEL was estimated to contain the equivalent of 100μg of OVA and 30μg of HEL. In details 450mg of OVA and 126mg of HEL were separately diluted in 18ml of phosphate buffer each. The two solutions were combined in a 50ml tube, centrifuged at 450xg for 5min and the supernatant was transferred to a bottle wrapped in tinfoil. 78.6μl of glutaraldehyde were added to 24ml of phosphate buffer and 14.4ml of this solution were added to the OVA/HEL solution (glutaraldhyde concentration 1mM). The HEL/OVA/glutaraldehyde solution was stirred for 1hr at room temperature after which it was centrifuged at 450xg for 5min. The supernatant was transferred to dialysis cassettes (Thermo Scientific) and dialysed overnight in PBS at 4°C. The dialysed product was transferred to concentrators (Amicon) and was centrifuged at 3600xg for 30min at 4°C. The concentration of the HEL-OVA conjugate was determined using a NanoDrop 1000 spectrophotometer (Nanodrop) at 280nm.

2.10 Preparation of Heat aggregated ovalbumin (HAO)

Chicken ovalbumin (Sigma) was diluted in PBS at a concentration of 20mg/ml and was incubated at 100°C for 2hrs. The denatured solidified ovalbumin was washed with PBS, centrifuged (450xg, 5min, 4°C) and resuspended in PBS. HAO was stored at -20°C. Before use, HAO was homogenized in a gentle-MACS Dissociator (formely Dispomax, Miltenyi) in order to be injected.

2. 11 OVA-TcR induced animal model of arthritis

The OVA-TcR induced animal model of arthritis was initially developed by Maffia *et al(5)* (Fig 2.1). Peripheral lymph nodes (LNs) (axillary, cervical, inguinal, popliteal, para-aortic), mesenteric LNs and spleen from DO11.10 mice were made to a single cell suspension and $CD4^+$ cells were MACS sorted from them as described in section 2.1.2. $CD4^+$ from DO11.10 mice were polarised to a Th1 or Th17 phenotype as described in section 2.1.5. $2x10^6$ transgenic T polarised under Th1 or Th17 condition were transferred intra-venously (i.v.) into BALB/c recipients. One day following adoptive transfer, recipients were immunised subcutaneously (s.c.) on the back with 100μg of chicken OVA (Sigma) in complete Freund's adjuvant (CFA, Sigma). Ten days after immunisation all recipient animals were injected subcutaneously proximal to their ankle joints with 100μg of HAO. Control mice received PBS instead of HAO. The mice were monitored daily for signs of arthritis and were scored according to table (2.1). Paw thickness was measured using a dial calliper (Kroeplin). Seven days post footpad challenge recipient mice were euthanized by cervical dislocation and popliteal LN draining the challenged paw, blood, and the challenged paw were extracted. Cells from the popliteal LN were made into a single cell suspension, counted using a

haemocytometer in the presence of trypan blue to exclude non-viable cells, cultured for 72hrs with OVA (1mg/ml), CII (50μg/ml) or complete RPMI and their ability to proliferate and produce cytokines was assessed by flow cytometry employing the Click-iT EDU proliferation assay and intracellular cytokine staining respectively as described in sections 2.1.3 and 2.1.6. In addition, cells from popliteal LNs were analyzed phenotypically by flow cytometry as described in section 2.1.3. Serum samples were extracted by centrifuging the blood at 13200rpm (15575xg) for 5min. These were analyzed for anti-CII and anti-OVA antibodies (IgG, IgG1, IgG2a) by ELISA as described in section 2.1.7. For histological analysis hind limbs were fixed in 10% neutral-buffered formalin (Sigma) for 14 days and sent to the Histopathological Department of the Veterinary School of Glasgow University to be stained with Heamatoxylin and Eosin (H&E) or toluidine blue.

Table (2.1): Clinical scoring system of arthritis

Each limb could receive a score of ≤4 points were:	
Score 0:	No reaction, normal
Score 1:	Mild, but definite redness and swelling of the ankle
Score 2:	Moderate redness and swelling of the ankle
Score 3:	Severe redness and swelling of the entire paw including digits
Score 4:	Maximally inflamed limb with involvement of multiple joints

2.12 B-T cell co-transfer model

This model was initially developed by Garside *et al* to visualize the development of antigen specific T cell-dependent B cell responses(138) (Fig 2.3). MACS sorted CD4$^+$ T cells were polarised towards a Th1 or Th17 phenotype and spleens from MD4 BALB/c mice were made into a single cell suspension as described in section 2.1.2. The percentage of HEL-specific B220$^+$ MD4 cells or KJ1.26$^+$CD4$^+$ DO11.10 T cells in these preparations was determined by flow cytometric analysis. Cell suspensions containing $2x10^6$ transgenic T cells polarised under Th1 or Th17 conditions and $2x10^6$ of transgenic B cells were co-transferred by i.v. injection into congenic age-matched IgH^b BALB/c recipients. One day after adoptive transfer the mice were immunised s.c. in the back of the neck with 130μg of OVA-HEL conjugate antigen in CFA. OVA-HEL was prepared using glutaraldehyde to couple OVA with HEL as described in section 2.1.9. Mice were euthanised at days 3, 7 and 10 after immunisation and draining LNs (axillary and bronchial) and blood were extracted. LNs were either snap frozen in OCT embedding medium for immunohistochemical analysis or used to analyze the phenotype of the cells by flow cytometry. From the blood sera was extracted by centrifugation and analyzed by ELISA for the presence of anti-OVA and anti-HEL antibodies.

2.13 Preparation of bone marrow derived dendritic cells (DCs)

DCs were prepared from bone marrow (of BALB/c mice) as previously described[527]. Bone marrow was flashed out from the femur and tibia of BALB/c mice using a syringe filled with complete RPMI media. Cells were passed through nitex mesh (Cadisch & Sons Ltd. London, UK) to filter any bone particles and were washed in complete RPMI, centrifuged and counted. Bone marrow cells were plated at a concentration of 0.5x106 cells/well in 6 well plates (Costar) in complete RPMI media supplemented with the supernatant of X63 myeloma cells transfected with mouse GM-CSF cDNA (10%v/v).

2.14 Assessment of viability of Th1 and Th17 polarised populations

MACS sorted CD4$^+$ T cells from DO11.10 mice were polarised towards a Th1 or Th17 phenotype as described in 2.1.5. Cells were rested for 24hrs in the absence of polarising cytokines and antigenic stimulus and 10^5 of them were co-cultured with $3x10^4$ bone marrow DCs in the presence or absence of OVA$_{323-339}$ for two time points, 24hr and 48hrs. DCs were either in resting condition or activated with LPS (Sigma, 1µg/ml) for 24hrs. The viability of the CD4 cells was assessed using the Annexin-V FITC kit (Miltenyi) according to manufacturer instructions. The kit includes Annexin-V FITC that binds phospatidylserine (PS) and propidium iodine (PI) that binds DNA and thus dead cells. In normal cells PS is located in the cytosolic leaflet of the plasma membrane, however during apoptosis and necrosis it redistributes and becomes available for binding with Annexin-V(528;529). Live cells are negative both for PI and Annexin-V staining. In detail, cells were harvested and their number was determined using a haemocytometer employing toluidine blue to exclude dead cells. They were then stained for cell surface markers, namely CD4 and the DO11.10 TcR as described in 2.1.3. Cells were washed in binding buffer (provided by the kit) and centrifuged at 300xg for 10min. They were then resuspended in 100µl of binding buffer per 10^6 cells, 10µl of annexin V were added to them and incubated in the dark at room temperature for 15min. Cells were washed with 1ml annexin-V and centrifuged at 300xg for 10min. The cell pellet was resuspended in 250µl of FACS flow (BD) and 1µl of PI was added prior to analysis with flow cytometry.

2.15 Statistics

Data were analysed using the GraphPad Prism$^®$ software. To test normality of the data sets the D' Agostino and Pearson omnibus test was used. To test if the means of two samples are different the Student's t-test or Mann Whitney test was used, for

normally and non-normally distributed data sets respectively. To compare the means of two or more samples one-way analysis of variance (ANOVA) was used. When the interaction of two independent variables was tested two-way ANOVA was employed. A value of $P<0.05$ was considered as significant.
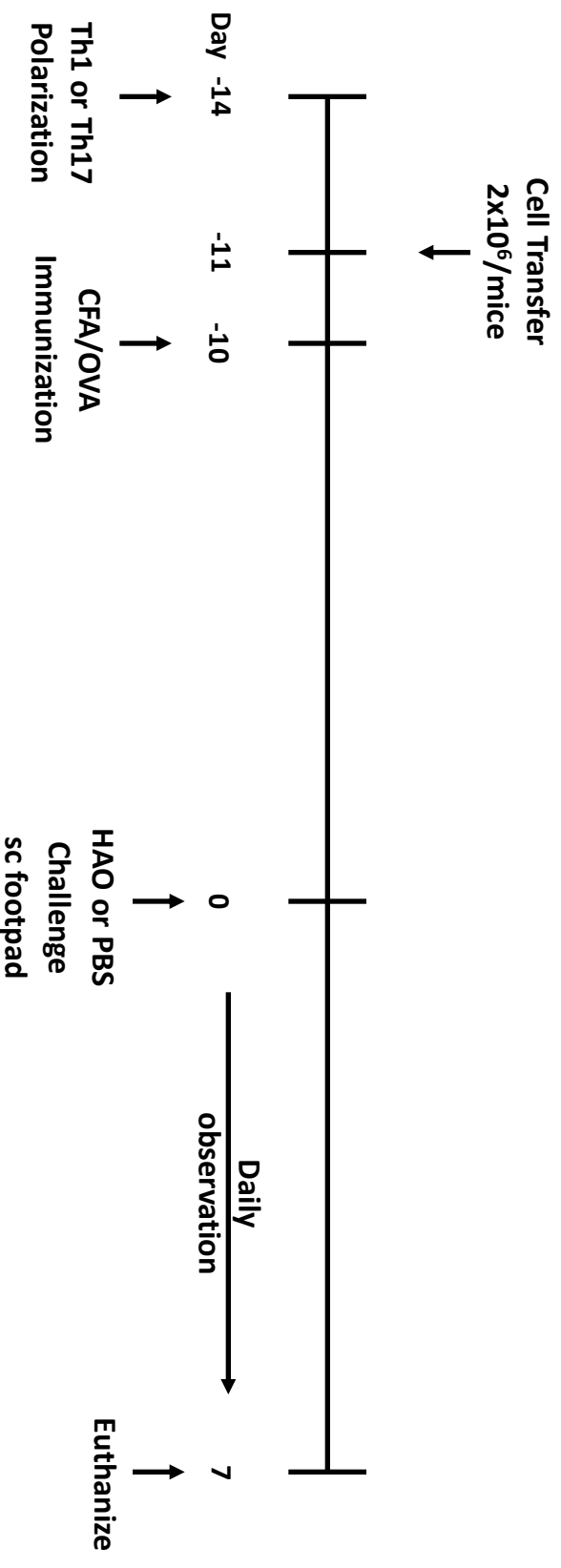
Fig 2.1: OVA TcR induced animal model of arthritis.

MACS sorted CD4+ T cells from DO11.10 or OT-2 mice were polarized under Th1 or Th17 conditions and were adoptively transferred into congenic BALB/c or C57/BL6 mice respectively. Recipient mice were immunized 24hrs post- transfer with CFA/OVA and 10 days after were rechallenged in the footpad with HAO. Control mice received PBS. Mice were observed daily for the development of arthritis and their paw thickness was measured with a calliper. Seven days after challenged mice were euthanized by cervical dislocation. On the cull day draining LNs were removed and cells were used to assess proliferation against OVA and CII and production of cytokines by fluorescence cytometry. The hind limbs were used for histological analysis and blood serum was analyzed for the presence of antibodies against OVA, CII and ACPA.

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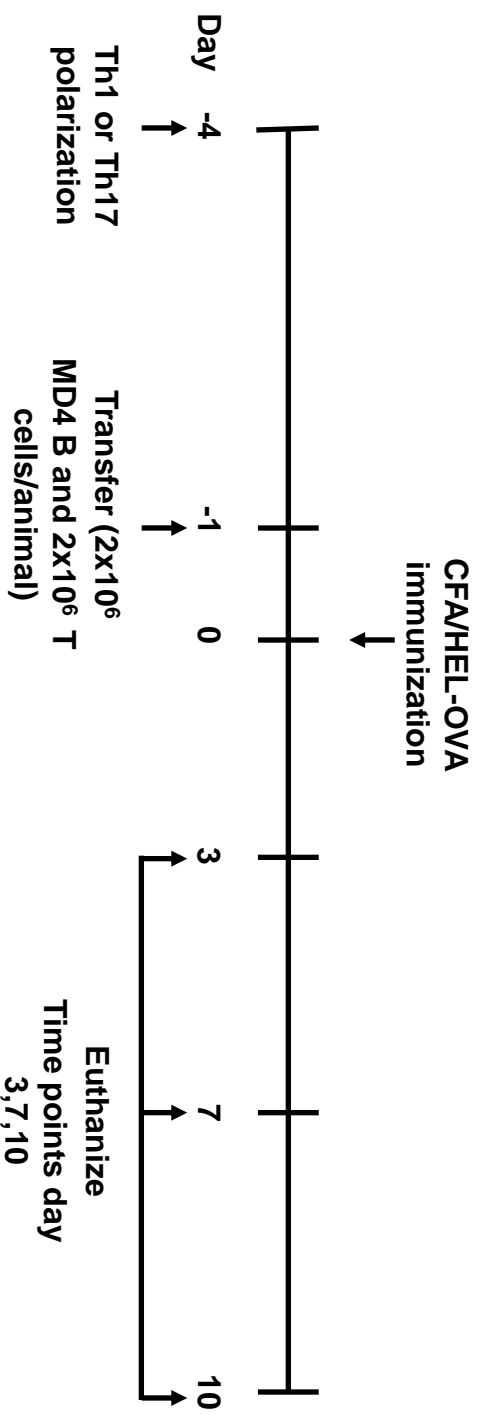
Fig 2.2: B-T cell co-transfer model.

MACS sorted CD4+ T cells from DO11.10 mice polarized under Th1 or Th17 conditions were adoptively transferred alongside with splenocytes from MD4 mice. The percentage of TCR and BCR transgenic cells was determined before transfer by FACS so that 2×10^6 T and B cells respectively would be transferred per animal. One day post transfer mice were immunized with HEL-OVA conjugate in CFA in the back of the neck. Control mice received PBS. Mice were euthanized at days 3, 7 and 10 post immunization. On cull days draining LNs were either snap frozen in OCT cryo-media for immunohistological analysis or were used to assess the number and the phenotype of transferred and host B and T cells by fluorescence cytometry. Serum from blood was used for the detection of anti-HEL and anti-OVA antibody titers by ELISA.

Chapter 3: Development of Th17 Polarisation protocol

3.1 Aim and rationale

In this chapter the development of a robust and reproducible protocol for the generation of Th17 cells is presented. We have recently developed a model of breach of self tolerance in the context of RA that is based on the adoptive transfer of OVA-specific Th1 cells(5). Due to the mounting evidence relating Th17 cells to various autoimmunity animal model (8;9;477) we hypothesised that a Th17 induced RA model could lead to a more potent breach of self tolerance that could possibly be accompanied by more severe clinical image than the current Th1 induced model. In order to test this model, a reliable and consistent protocol for Th17 polarisation had to be developed. In addition, in this chapter the phenotype of the transferred Th1 and Th17 population was investigated, and specifically the presence of T_{Reg} and TFH cells, as the presence of these cell types could potentially give information relating to the pathology of the models.

3.2 Introduction

3.2.1 Cytokine regulation of Th17 polarisation

Th17 is a recently discovered effector CD4$^+$ subtype that is identified by its ability to secrete IL-17A and other cytokines, such as IL-17F, IL-21, IL-22 and TNF(255;422;454;530). Various cytokines have been involved in Th17 lineage commitment. When Th17 cells were initially characterized in models of autoimmunity, the dendritic cell derived cytokine IL-23 was considered to be critical in lineage commitment(8). However, the fact that IL-23 induced only small percentages of IL-17$^+$ CD4$^+$ cells and was not sufficient to generate Th17 from naïve cells *in vitro* (8;521) suggested that other factors must be more important for the *de novo* generation of naïve T cells to the Th17 phenotype. In is now accepted thal IL-6 and TGFβ, are responsible for the *de novo* differentiation of Th17

cells(412-414). IL-6 or TGFβ alone can only modestly, if at all, induce generation of IL-17 producing CD4$^+$ cells, however their combination is highly effective in generating Th17 cells from naïve precursors(413;414;521). TGFβ is a pleiotropic cytokine with functions important from T cell development and homeostasis to tolerance(531). Its importance in Th17 polarisation *in vivo* was revealed in mice that were either deficient for the TGFβ receptor or possessed T cells that over-expressed TGFβ. The first do not respond to TGFβ, do not generate Th17 cells and are protected from EAE, whereas the latter develop more severe EAE and have elevated Th17 responses(414;523). TGFβ has the potential to induce both Th17 and T$_{Reg}$ cells. In combination with pro-inflammatory cytokines such as IL-6 and IL-21, TGFβ induces upregulation of IL-23R and the production of IL-17 by TCR activated CD4$^+$ cells(446). In addition, TGFβ, regardless of the presence of IL-6, can rapidly induce the prototypical Th17 transcription factor RORγt(446;532). On the other hand high concentration of TGFβ favours the development of Foxp3$^+$ T cells and the repression of the IL-23R(446). The fact that CD4$^+$ cell-specific TGFβ ablation leads to inhibition of *in vivo* Th17 development suggests an autocrine or paracrine role for this cytokine(415).

The role of IL-6 in Th17 differentiation was initially discovered when an antibody against IL-6 could inhibit the production of IL-17 by anti-CD3/CD28 stimulated naïve CD4$^+$ T cells cultured in the presence of LPS conditioned DC media(413). In addition, recombinant IL-6 was able to inhibit TGFβ induced Foxp3 upregulation and induced IL-17 production by CD4$^+$ T cells(414). Lamina propria CD4$^+$ T cells from IL-6 deficient mice failed to express RORγt, IL-17F and the IL-23 specific chain of the IL-23R, which suggests that IL-6 is required for the *in vivo* generation of Th17 cells in the gut(443). Activation of the IL-6R leads to activation of STAT3(533). STAT-3 deficient mice have a greatly reduced capacity to produce IL-17 and have a decreased RORγt and RORα induction(444;451;534). Also, mice with a conditional CD4$^+$ deletion of STAT-3 are resistant in the induction of EAE(534).

Apart from IL-6 and TGFβ other cytokines were found to be important for Th17 differentiation. Three independent studies revealed an important autocrine role for IL-21 in Th17 differentiation(421;422;455). IL-21 is highly expressed by Th17 cells and its expression is induced by IL-6 and IL-21 but not IL-23 or TGFβ(422). In addition, IL-21 alone or in combination with TGFβ resulted in upregulation of RORγt, IL-23R and Th17 cytokines such as IL-17, IL-17F and IL-22 by anti-CD3 activated naïve CD4$^+$ T cells in STAT-3 dependent manner (422;455). Another cytokine with an important role in Th17 differentiation is IL-1. IL-1 receptor 1 (IL-1R1) is expressed in higher amounts in Th17 cells compared with Th1 cells a phenomenon that is mediated by the IL-6/STAT-3 axis (535). *In vivo* IL-1R1 deficiency protected mice from the development of EAE, which was correlated with a failure of development of autoantigen specific Th17 responses and their ability to migrate to the site of inflammation(522;535) . This was not due to a secondary effect of IL-1 on another cell type (e.g APCs) but a direct failure of IL-1 signalling on CD4$^+$ T cells as CD4$^+$ specific IL-1 signalling deficiency protects mice from EAE(535) and transfer of IL-1-competent autoantigen specific T cells could re-establish the disease(522). Furthermore, IL-1 was found to enhance IL-23 induced IL-17 production(522), retain the production of IL-17 by Th17 polarised cells even in the absence of TcR stimulus, promote the transformation of T$_{Reg}$ to Th17 cells(535), and abrogate the inhibitory effect of IL-2 in IL-17 production(536).

As well as cytokines that promote Th17 differentiation there are a number of cytokines that inhibit their differentiation. *In vitro*, IFNγ and IL-4 the prototypical cytokines of Th1 and Th2 cells have been shown to inhibit Th17 differentiation(412). However the presence of IFNγ and IL-17 double positive cells *in vivo* in models of autoimmunity potentially contradicts this fact(494). IL-2 which is a growth factor for activated T cells and T$_{Reg(537-539)}$, inhibits Th17 development via a STAT-5 dependent mechanism(419). IL-27, a member of the IL-12 family of cytokines has a regulatory role in Th17 development that is mediated indirectly via TGFβ and IL-6 *in vitro* and *in vivo* or through the generation of Tr1-like cells that produce IL-10(540-542).

3.2.2 AhR and Th17 polarisation

Another modifier of Th17 development is the aryl-hydrocarbon receptor (AhR). AhR is a highly conserved molecule that is expressed by various cell types and it is considered to have a dual role in the metabolism of small molecules and in the modulation of the immune system(543). Dioxin is the prototypical ligand for AhR, however there are a vast array of possible endogenous ligands, such as indoles (e.g. 6-formylindolo[3,2-b]carbazole (FICZ)), tetrapyroles and arachidonic acid metabolites(544). Th17 cells have been found to express high levels of AhR and administration of the AhR ligand FICZ leads to a significant worsening of EAE(545;546). AhR is not indispensable for Th17 development as AhR deficient CD4$^+$ can still be polarised to a Th17 phenotype, however they are impaired in their ability to produce IL-22(546).

3.2.3 Signal 1 and Signal 2 in Th17 polarisation

For any T cell, the first step of activation is initiated by the binding of the TcR to its cognate antigen in the context of an MHC molecule (signal 1), and the second by co-stimulatory molecules expressed on the activated DC (signal 2)(434;547-549). Previously, studies have suggested that TcR signal strength is an important factor for *in vivo* Th1/Th2 differentiation. It was reported that peptide/MHC complexes that bind strongly to the TcR, in the absence of polarising cytokines, drive T cells to a Th1 phenotype whereas peptide/MHC complexes that bind weakly induce Th2 cells(550;551). In addition, antigen dose has also been reported to play a significant role in the *in vitro* Th1/Th2 polarisation, with low doses favoring development of Th2-like cells and high doses Th1-like cells(552). It is not very clear how these factors affect Th17 polarisation. Signals through the TcR induce IL-17 production from memory and naïve CD4$^+$ cells(491). Qualitatively functional avidity of the stimulating peptide has been reported to affect significantly Th17 generation *in vivo*, with the high avidity peptides favoring higher Th17:Th1 ratio,

compared to lower avidity peptides(553). Furthermore, it has also been demonstrated that *in vitro* optimal production of IL-17, under Th17 polarising conditions, requires high doses of anti-CD3 antibody, an IL-17 specific phenomenon as IL-17F production was not affected(435). Contrary to these data, it has been reported that low concentration of anti-CD3, in the presence of anti-CD28 and polarising cytokines, results in higher proportion and number of IL-17$^+$CD4$^+$ compared with high concentration(554). In any case, these two studies only give information relating to the efficacy of Th17 polarisation under different conditions of TcR signaling as they are performed in the presence of polarising cytokines, unlike the initial studies investigating the role of TcR signal strength in Th1/Th2 polarisation where no polarising cytokines were used(550;552). The role that the various co-stimulatory molecules play in Th17 polarisation is equally understudied. When Th17 cells were initially discovered it was reported that their generation requires the co-stimulatory molecules ICOS and CD28(285). However, this study employed IL-23 expanded Th17 cells, which were probably memory cells and not *de novo* generated Th17 cells. More recently, it has been reported that CD28 co-stimulation reduced the frequency of *in vitro* generated IL-17 producing CD4$^+$cells[555]. They suggest that this is mediated by CD28-induced IL-2 and IFNγ production(555). However, there are no other studies that investigate other co-stimulatory molecules or the effect that the Th17-inducing cytokines have on APCs, as most studies so far utilize anti-CD3/anti-CD28 antibodies. In addition, it is quite questionable how physiologically relevant these issues are in an *in vivo* setting. TcR cross-reactivity or degeneracy, where a TcR binds and responds to multiple peptide-MHC ligands is a well accepted concept(556-558). Estimates of the peptide repertoire have shown that the number of potential immunogenic peptides in the environment far exceeds the total number of TCR specificities in an individual at any given point(557). In addition, in the thymus T cells must recognise, with low affinity, MHC molecules bearing self peptides(559). From this point of view, cytokine regulation of T cell polarisation would probably be hierarchically more significant, at least from TcR signal "strength" as "strong" signal for one T cell clone could be a weak or intermediate for another, thus leading to the development

of unwanted Th phenotypes. Based on the above data, the development of a protocol that would produce a highly polarised Th17 population, which could be employed for the development of a Th17-induced RA model and for the investigation of the role of these cells in B cell responses, was pursued.

3.3 Results

3.3.1 The APC:T cell ratio is crucial for the effectiveness of *in vitro* Th17 polarisation

The Th17 protocol initially employed was based on the Th1 protocol established in our group and used by Maffia *et al* to develop the OVA-TcR induced RA model(5). This involved the culture of MACS sorted $CD4^+$ cells from DO11.10 mice with mitomycin C treated splenocytes as APCs, in an APC:T ratio of 10:1 (for detailed description refer to Chapter 2: Materials and Methods, Section 2.1.5) . Based on the published data relating to the *in vitro* Th17 polarisation we used a cytokine and antibody cocktail consisting of anti-IFNγ, anti-IL-4, IL-6, TGFβ, IL-23 and IL-1β(413;521;535) (for concentrations refer to Chapter 2: Materials and Methods, Section 2.1.8). When $CD4^+$ cells from DO11.10 mice were polarised under these conditions the effectiveness of Th17 polarisation was very poor and was characterised by very low number of IL-17$^+$ $CD4^+$ cells (Fig 3.1, lower panel). This was specific for Th17 polarisation as Th1 polarisation with the same APC:T cell ratio resulted in a high number of IFNγ$^+$ $CD4^+$ cells (Fig 3.1, top panel). Following this, a small panel of cytokines that included IL-2, IFNγ and IL-17 (Fig 3.2a-c) was analysed in the supernatants of the Th1 and Th17 polarisation cultures by ELISA. As expected the Th1 cultures were characterized by high production of IFNγ and low production of IL-17, whereas Th17 cultures were characterized by high production of IL-17 and low production of IFNγ (Fig 3.2a-b). In both Th1 and Th17 polarising conditions the levels of IL-2 production were similar (Fig 3.2c). In addition there was no difference in the expansion of the two populations (Fig 3.2d).

IL-2 has been reported to have an inhibitory function in Th17 generation through the preferential promotion of TREG cells(419). The fact that the supernatants of the Th17 polarisations contained high amounts of IL-2 could suggest that this cytokine constrains the generation of IL-17+ CD4+cells. To definitively determine the role of IL-2 in this system CD4+ T cells were cultured under Th17 conditions as in Fig 3.1 in the presence or absence of an IL-2 blocking antibody. Unlike the published reports(419), in our system IL-2 blocking did not have any effect in the percentage of IL-17$^+$CD4$^+$ cells (Fig 3.3a and b), which remained low (<5%).

Studies in the past have shown that the availability of MHCII-peptide complexes or co-stimulatory molecules, controlled by the APC:T cell ratio, has a profound effect in T cell activation, proliferation and even functional differentiation(552;560;561). For example, it has been reported that T cell activation is decreased as APC:T ratio decreases(561). As mentioned above, the peptide availability could affect polarisation, with high doses of antigen favouring Th1 generation and low doses Th2(552). In order to investigate if the APC:T cell ratio has an effect in the *in vitro* Th17 polarisation, MACS sorted CD4$^+$ T cells from DO11.10 mice were cultured under Th17 conditions in an APC:T cell ratio of either 10:1 or 1:1 (Fig 3.4). Interestingly, when the APC:T cell ratio was 1:1 the cultured CD4$^+$ population was consistently characterized by a higher percentage of IL-17$^+$ CD4$^+$ T cells (Fig 3.4 a and b). This was not a generalized effect in cytokine production by the activated CD4$^+$ T cells as the percentage of IFNγ$^+$ CD4$^+$ cells had not increased significantly (Fig 3.4b). In addition, Th1 polarisation was not influenced by the APC:T cell ratio as shown by the percentage of IFNγ$^+$ CD4$^+$ cells (Fig 3.4c).

These data show that the efficiency of the *in-vitro* Th17 polarisation is crucially affected by the APC:T cell ratio. As *in vitro* Th17 polarisation has been reported to be modified by both the quality of co-stimulation and TcR signal(554;555), this effect could be either related to the availability of MHCII-peptide complexes or the amount of co-stimulation given to the proliferating T cells, however more experiments are required to validate this.

3.3.2 The effect of culture media on Th17 differentiation

Even though the modulation of the APC:T ratio from 10:1 to 1:1 resulted in a dramatic increase of the effectiveness of Th17 polarisation, the percentage of IL-17$^+$ CD4$^+$ varied significantly between experiments, ranging from ~10% to ~40%. As mentioned previously, Th17 differentiation is dependent on IL-6 and TGFβ and is modulated by the expression of the AhR receptor. AhR is highly expressed by Th17 cells and its activation by high affinity ligands during Th17 development markedly increases the proportion of IL-17$^+$ cells(545;546). In early 2009 it was reported that RPMI medium contains relatively low levels of AhR agonists, which results in poor Th17 polarisation when this cell culture medium is used. On the other hand the same group reported that IMDM, a medium richer in aromatic amino acids, which give rise to AhR agonists, when used in Th17 polarisation, results in higher Th17 polarisation efficiency(562). All Th17 polarisations so far were conducted in RPMI culture medium, which could suggest that, the high variability in Th17 efficiency was due to low concentration of natural AhR ligands in the culture media. In order to confirm that the variability of Th17 polarisation efficiency was due to the use of RPMI medium, MACS sorted CD4$^+$ T cell from DO11.10 were cultured under Th17 polarising conditions, at 1:1 APC/T cell ratio, either in RPMI or IMDM culture medium. Consistent with the published reports(562) CD4$^+$ cells cultured in IMDM medium under Th17 polarising conditions were characterized by significantly higher percentages of IL-17$^+$ compared to cells cultured in RPMI media. More importantly, Th17 polarisation conducted in IMDM medium was consistently characterised by percentages of IL-17$^+$CD4$^+$ cells higher than 35% (Fig 3.5). In addition, the increase in IL-17$^+$ CD4$^+$ cells was not associated with an increase in the percentage of IFNγ$^+$ CD4$^+$ cells (Fig3.5a). These data demonstrate that apart from the polarising cytokines and blocking antibodies, the culture media has a crucial effect in Th17 generation efficacy, with RPMI media being only able to support sub-optimal Th17 polarisation.

3.3.3 The effect of the mouse strain in Th17 polarisation

Different mouse genetic backgrounds are known to significantly alter the direction of Th subset development. BALB/c and C57BL/6 mice are known to express different type of immune responses, with the first to be considered Th2 prone and the latter Th1(300;563;564). In response to *Leishmania major* infection susceptible BALB/c mice develop a Th2 response that fails to clear the pathogen, whereas resistant C57BL/6 mice develop a protective Th1 type response(300). Similar reports exists for helminth parasites, such as *Trichuris muris*, were susceptible inbred mice cannot mount an effective Th2 response, unlike resistant strains(565). In addition, C57BL/6 mice are more susceptible than BALB/c to experimental autoimmune diseases such as experimental autoimmune myasthenia gravis and experimental autoimmune uveitis(563;564). Even though some of these differences could be attributed to factors apart from Th phenotype, such as the MHCII haplotype, these reports demonstrate the role of genetic background in the quality of the immunological response.

There is no evidence relating to how the genetic background modulates the *in vitro* or *in vivo* development of Th17 cells. In order to investigate the relative effect the genetic background has on *in vitro* Th17 polarisation, the relative ability of DO11.10 or OT-II CD4+ T cells was compared. It should be noted at this point that apart from differences relating to the genetic background these two transgenic mouse strains differ in the affinity with which their TcR receptor recognises the same peptide (OVA$_{323-339}$). The OT-II mouse strain has a low affinity TcR, and recognises the same antigen as the transgenic TCR carried by DO11.10 transgenic mice, albeit presented by a different MHC II molecule (I-A^b). The DO11.10 TCR carries the transgenic αβ TCR (Vα13/Vβ8) that also recognises OVA$_{323-339}$, however, in the context of MHC class II I-A^d and has approximately 50 fold higher affinity for peptide/MHCII than OT-II(566;567). MACS sorted CD4$^+$ cells from DO11.10 mice (BALB/c) or OT-II mice (C57BL/6) were cultured under Th17 polarising conditions (1:1 APC:T cell ratio, IMDM culture media) and their

phenotype was assessed by intracellular cytokine staining (Fig 3.6). There was no difference in the percentage of IL-17$^+$ CD4$^+$ T cells between DO11.10 and OT-II mice. In both cases the percentage of Th17 cells was consistently higher than 35% (Fig 3.6 b) suggesting that CD4$^+$ T cells from both mouse strains have the same potential to polarise *in vitro* to a Th17 phenotype.

3.3.4 Phenotypic characteristics of the in vitro Th1 and Th17 populations

Now that a reliable protocol for Th17 polarisation was established, the phenotypic characteristics of the polarised population were analysed in more detail. More specifically the presence of two other T helper subtypes, inducible T$_{Reg}$ and TFH cells, was investigated. This is important as the presence or absence of either phenotype could have important impact in the pathogenesis of experimental arthritis, with T$_{Reg}$ cells regulating the development of autoreactive responses and TFH supporting and exacerbating B cell responses(568-572).

Immunological self tolerance is maintained at least in part by regulatory T cells that actively control potentially autoreactive T cells(385). There are various subtypes of regulatory T cells, such as naturally CD4$^+$CD25$^+$Foxp3$^+$ T$_{Reg}$, IL-10 secreting Tr1 cells, inducible CD4$^+$CD25$^+$Foxp3$^+$ T$_{Reg}$ cells, and TFGβ producing Th3 cells(530). Th17 and T$_{Reg}$ developmental pathways share a reciprocal connection. Naïve T cells after TCR stimulation in the presence of TGFβ express Foxp3 and become T$_{Reg.}$ However, as mentioned above, in the presence of TGFβ and IL-6/IL-21 these cells polarise to a Th17 phenotype. In order to investigate the presence of T$_{Reg}$ in the Th1 or Th17 polarised population, polarised cells were analyzed for the expression of the transcription factor Foxp3 by intracellular flow cytometric analysis. Both Th1 and Th17 population included cells that expressed Foxp3 (Fig 3.7). In addition there was no difference in the percentage of Foxp3$^+$ cells between the two populations.

TFH cells are defined by their mobilization from the T cell zone to the B cell follicle following antigenic priming, their unique cytokine signature and provision of B cell help(573). TFH cells can be distinguished from other T helper subsets by their sustained expression of CXCR5. The co-expression of CXCR5 with ICOS and/or PD-1 has proven a useful phenotypic profile to distinguish this T helper cell subset(573). In order to investigate the presence of follicular homing markers in the Th1 and Th17 polarised population, polarised cells were analyzed for the co-expression of CXCR5 and ICOS (Fig 3.8). In both cases only a very small percentage (~2%) exhibited a TFH phenotype (Fig 3.8). These data demonstrate that both Th1 and Th17 polarised population are relatively free of other contaminating T helper subsets and only a very small percentage express markers specific for T_{Reg} and TFH cells. However, it should be noted that especially in the case of Th17 polarisation a significant proportion of the $CD4^+$ did not produce either IL-17 or IFNγ (30-55%).
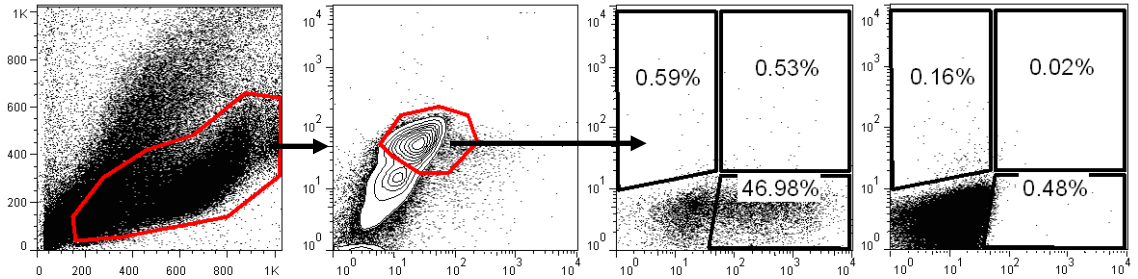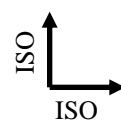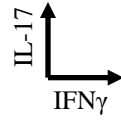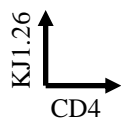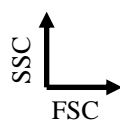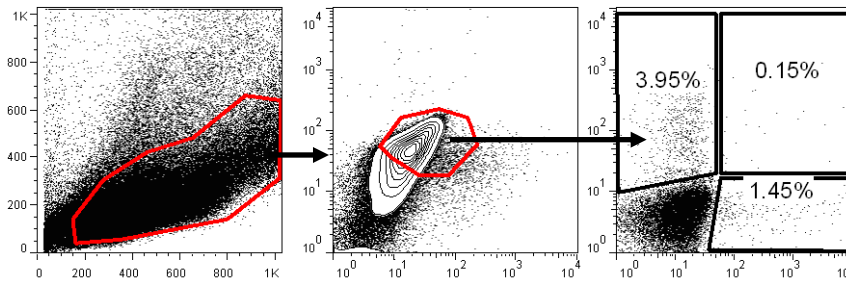
Fig 3.1: Th1 and Th17 polarisation using a 10:1 APC to T cell ratio.

CD4^{+} T cells from DO11.10 mice were cultured under or Th17 polarising conditions for 72hrs at a 10:1 APC:T cell ratio. The ability to produce IL-17 and/or IFNγ was assessed by intracellular flow cytometry staining. Lymphocytes were identified based on the FSC and SSC profile and transgenic T cells based on KJ1.26 staining and CD4 expression. Similar results were obtained in three independent experiments.
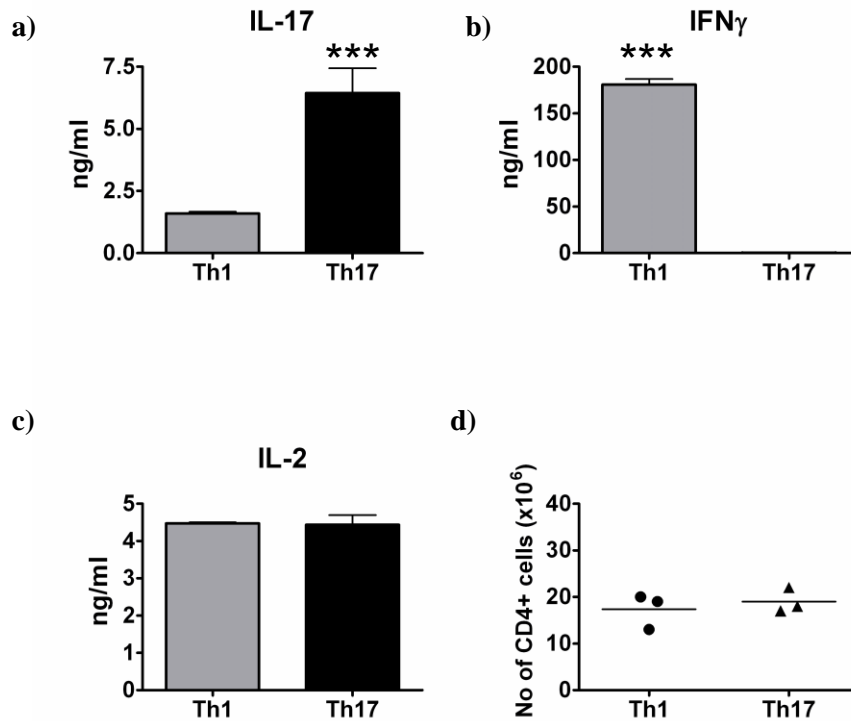
Fig 3.2: Th1 and Th17 polarisation using a 10:1 APC to T cell ratio.

CD4^{+} T cells from DO11.10 mice were cultured under or Th17 polarising conditions for 72hrs at a 10:1 APC:T cell ratio. Their ability to produce IL-17 (a), IFNγ (b) and IL-2 (c) was assessed by ELISA of the culture supernatants.   At the same time point the cells were harvested and their number was determined using a heamocytometer (d). Data are presented as mean±SE (a-c) or as mean (d). Similar results were acquired in two independent experiments,***: p<0.001.
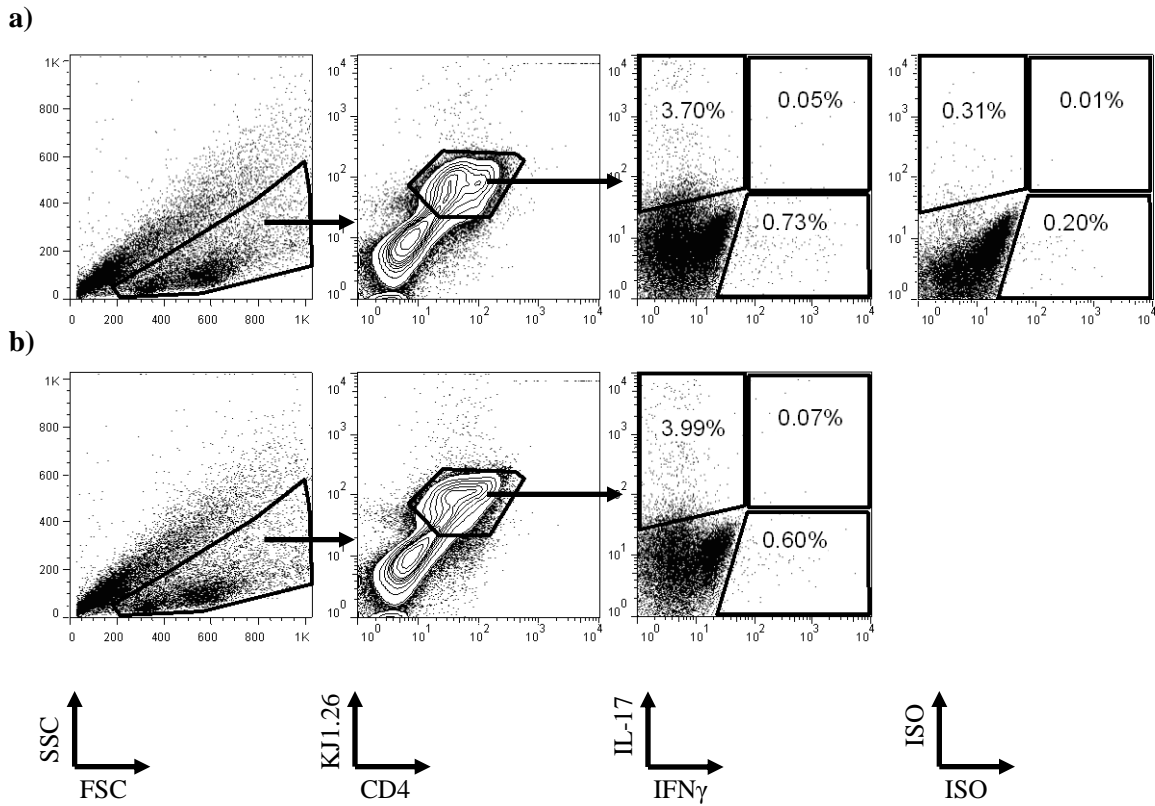
Fig 3.3: IL-2 blockade does not increase the percentage of CD4+IL-17+ cells.
CD4+ cells from DO11.10 mice were cultured under Th17 polarising conditions at a 10:1 APC:T cell ratio in the absence (a) or presence (b) of anti-IL-2 antibody. Their ability to produce IL-17 and/or IFNγ was assessed by intracellular flow cytometry. Lymphocytes were identified based on the FSC and SSC profile and transgenic T cells based on KJ1.26 staining and CD4 expression. Similar results were acquired in two independent experiments.
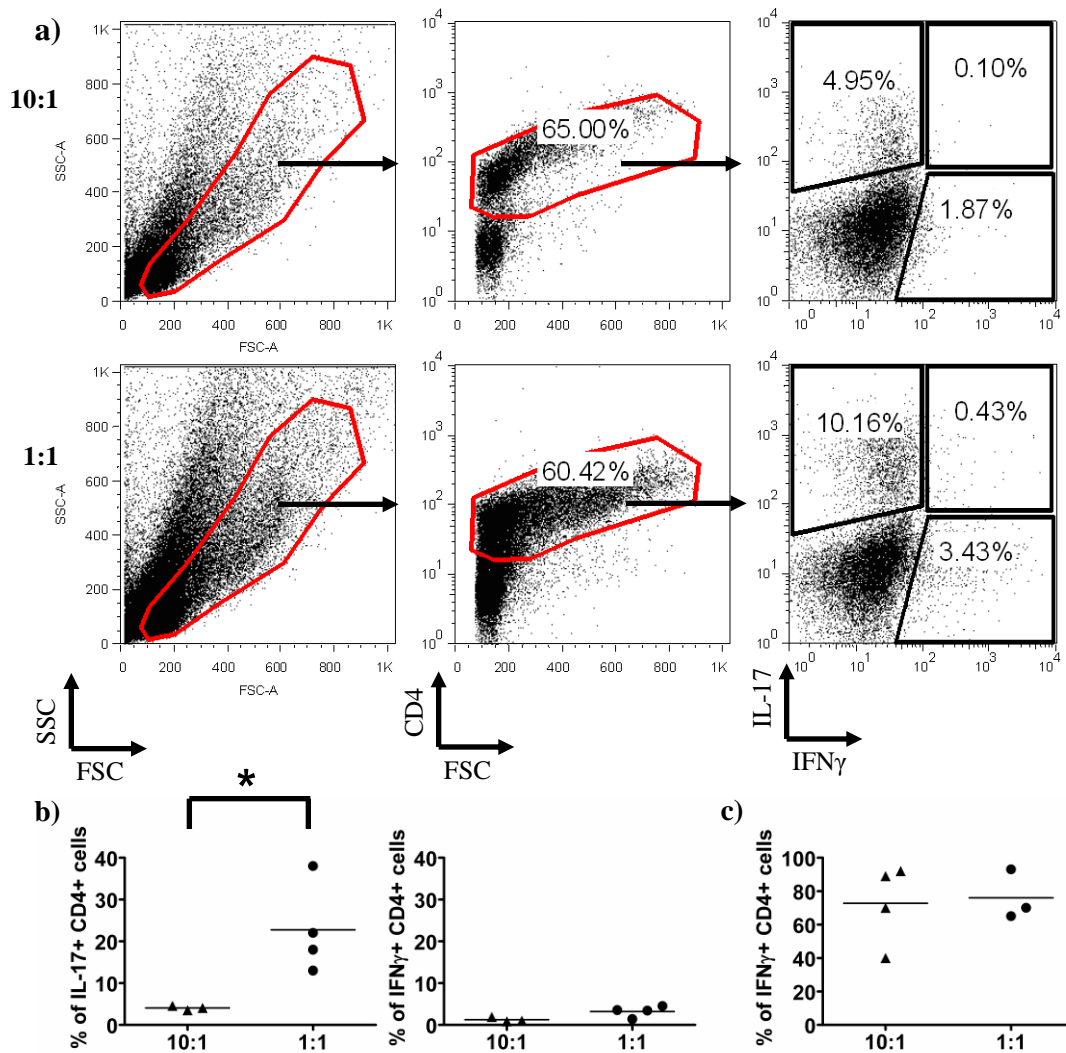
Fig 3.4: The APC:T cell ratio is critical for Th17 polarisation.

a) MACS sorted CD4+ cells from DO11.10 mice were cultured under Th17 polarising conditions at an APC:T cell ratio of either 10:1 (top panel) or 1:1 (lower panel). Their ability to produce IL-17 and/or IFNγ was assessed by intracellular flow cytometry. b) Scatter plot from individual experiments with CD4+ T cells from DO11.10 mice polarised under Th17 conditions showing the percentage of IL-17+ CD4+ cells when the APC:T cell ratio is 10:1 or 1:1 ; *:p<0.05.c) Scatter plot of from individual experiments with CD4+ cells from DO11.10 mice polarised under Th1 conditions showing the percentage of IFNγ+CD4+ cells when the APC:T cell ratio is 10:1(triangle) or 1:1(circle).
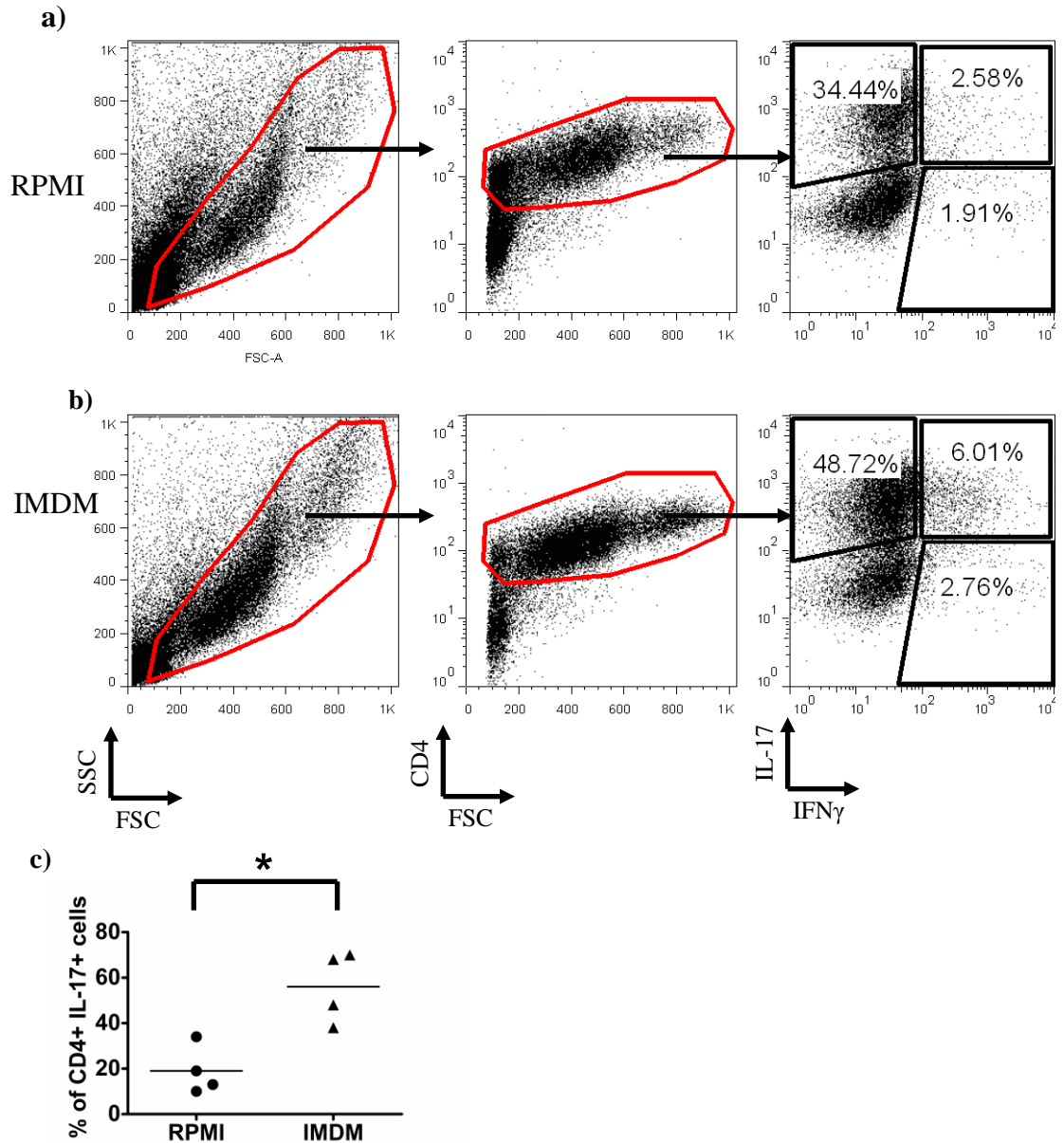
Fig 3.5: IMDM culture media induces higher percentage of CD4+IL-17+ cells under Th17 polarising conditions.

MACS sorted CD4+ cells from DO11.10 mice were cultured towards a Th17 phenotype either in RPMI (a) or IMDM (b) complete media. Cells were harvested and their ability to produce IL-17 and/or IFNγ was assessed by intracellular flow cytometry. c) Scatter plot from individual experiments with CD4 T cells from DO11.10 mice polarised under Th17 conditions showing the percentage of IL-17+ CD4+ cells in the presence of RPMI (circle) or IMDM (triangle), * p<0.05

a)

DO11.10

34.97%   3.76%

6.92%

OT-II

46.99%   3.74%

3.33%

SSC / FSC

CD4 / FSC

IL-17 / IFNγ

FSC-A

b)
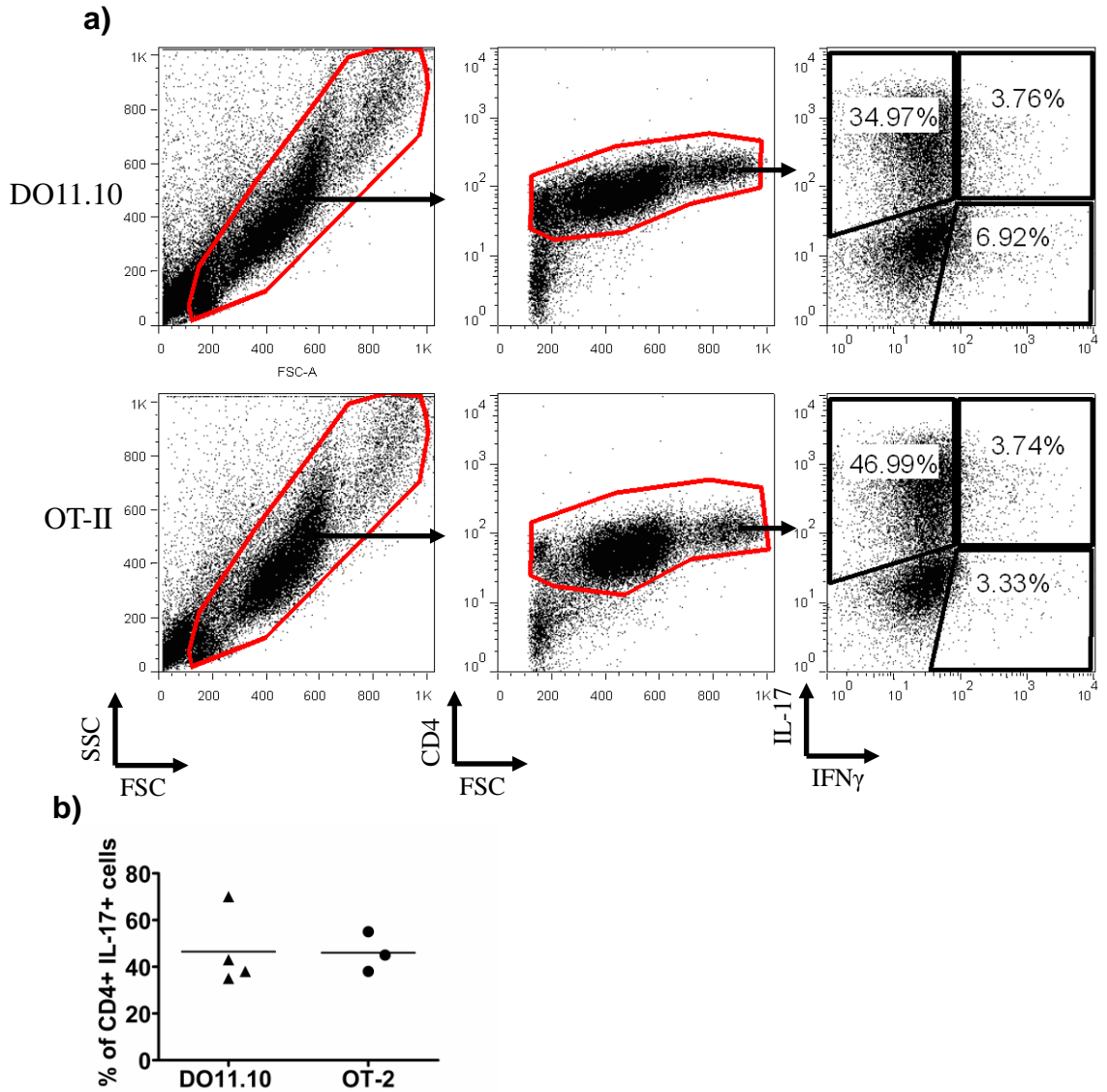
Fig 3.6: OT-2 and DO11.10 CD4$^+$ T cells have equal ability to polarise to a Th17 phenotype.

a) MACS sorted CD4$^+$ cells from DO11.10 (top panel) or OT-II (bottom panel) mice were cultured under Th17 conditions. Cells were harvested and their ability to produce IL-17 and/or IFNγ was assessed by intracellular flow cytometry. b) Scatter plot from individual experiments with CD4 T cells from DO11.10 (triangles) or OT-II (circles) mice polarised under Th17 conditions showing the percentage of IL-17$^+$ CD4$^+$ cells.
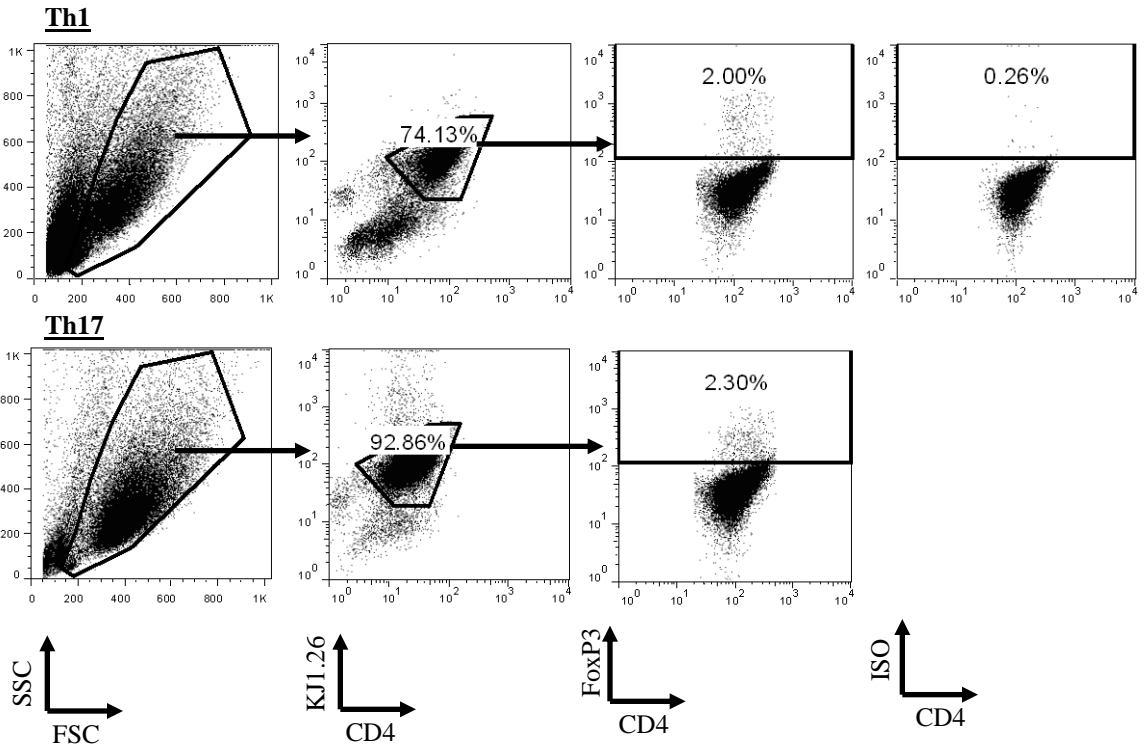
Fig 3.7: T_{REG} marker expression in the Th1 and Th17 polarised populations.
MACS sorted CD4+ cells from DO11.10 mice were polarised under Th1 (top panel) or Th17 (bottom panel) conditions. Cells were harvested and the expression of the T$_{Reg}$ specific transcription factor FoxP3 was analyzed by intracellular fluorescent cytometry. Similar results were acquired by two independent experiments.
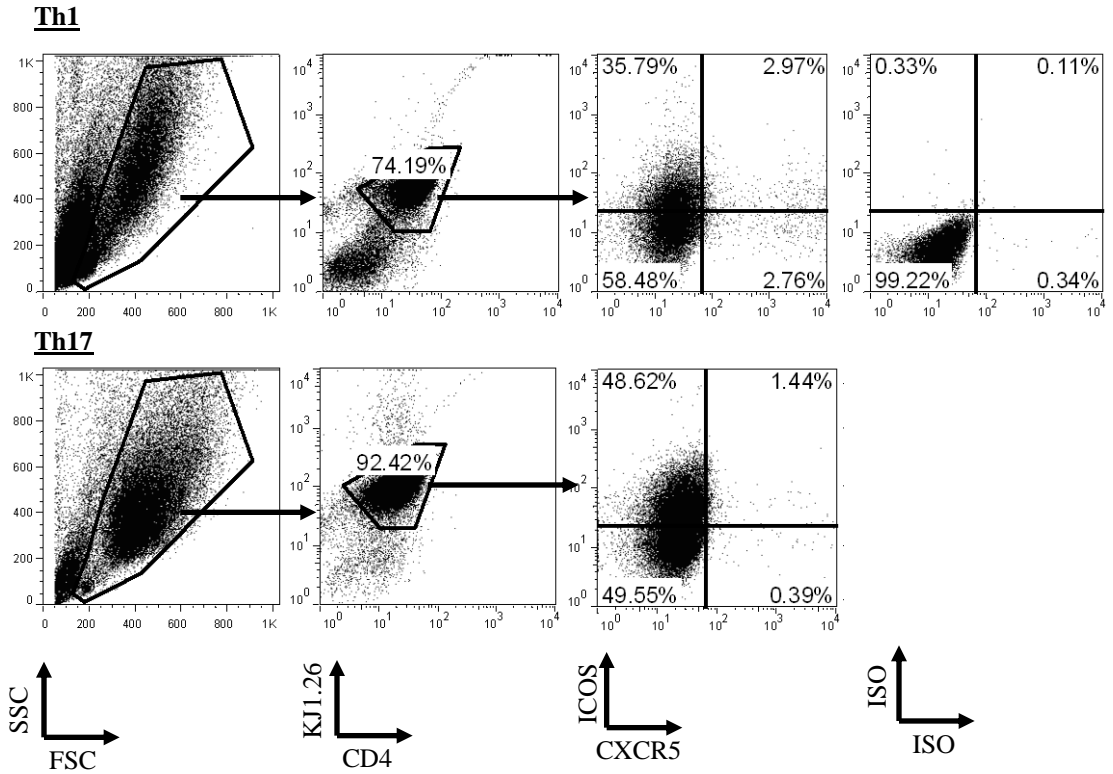
Fig 3.8: TFH marker expression in the Th1 and Th17 polarised populations.
MACS sorted CD4+ cells from DO11.10 mice were polarised under Th1 (top panel) or Th17 (bottom panel) conditions. Cells were harvested and were analysed for the expression of ICOS and CXCR5 by flow cytometry. Transgenic T cells that were double positive for ICOS and CXCR5 were considered as TFH cells. These are representative plots from two independent experiments.

3.4 Discussion

In this chapter, a reliable, consistent and robust Th17 polarisation protocol was developed. This process gave insights to the mechanistics of *in vitro* Th17 generation. Firstly, it was demonstrated that Th17 generation is crucially dependent on the APC:T cell ratio, as high APC:T cell ratio inhibited the generation of Th17 cells. This effect was not IL-2 dependent as inhibition of this cytokine did not increase the generation IL-17$^+$ CD4$^+$ cells. Only when the APC:T ratio was reduced to 1:1 there was a significant increase in Th17 cell generation. However, even in this case, the percentage of IL-17$^+$ cells was highly variable. This was probably due to the use of RPMI media as when this was replaced by IMDM this dramatically increased the efficacy of Th17 differentiation and reduced its variability. In addition, it was confirmed that, at least *in vitro*, differences in the genetic background between BALB/c and C57BL/6 mouse strains do not have a significant impact in Th17 polarisation. Finally, it was demonstrated that the polarised population are mostly free of other Th subtypes and specifically T$_{REG}$ and TFH cells.

The dramatic effect that the APC:T cell ratio had on Th17 polarisation could be attributed to the potency of TCR signaling, availability of MHCII-peptide complexes and/or co-stimulatory molecules. All of these factors have a crucial influence on the activation and proliferation of CD4+ T cells(560;561) and could potentially have an effect on Th cell differentiation. Relating to co-stimulatory molecule availability, it has been reported that CD28 co-stimulation exerts a negative regulation of Th17 differentiation and IL-17 production(555). This could potentially explain the effect the APC:T cell ratio has on Th17 polarisation, as in low APC:T cell ratio there will be greater competition for co-stimulatory molecules, and thus less CD28-ligation. In addition, when the same group used APC/T cell cultures, mature dendritic cells were less efficient than immature dendritic cells in their ability to support Th17 differentiation(555). Interestingly, they also showed that the inhibitory activity of CD28 was most potent when the TCR stimulus (anti-

CD3 antibody in this case) was at its highest concentration, conditions that resemble a high APC:T cell ratio. It should be noted that early studies on Th17 generation have suggested an essential role for CD28 and ICOS in this process(285). However these studies employed only IL-23 driven Th17 cells which probably constitute a memory population and not *de novo* polarised T cells. In a system using human cells it was demonstrated that low TCR stimulation favors greater *in vitro* Th17 stimulation compared with high, with more efficient Th17 polarisation at lower APC:T ratios(554). Contrary to this published report, there are other studies, which indicate that at least IL-17 production from Th17 cells requires strong TCR stimulation(435). CD4$^+$ deficient in Itk, a tyrosine kinase required for full TCR-induced phospholipase-Cγ activation, exhibit a reduced IL-17 production both *in vivo* and *in vitro*, event though IL-17F production was not affected(435). In addition they demonstrated that optimum IL-17 production required TCR stimulation with high concentration of anti-CD3 antibody(435). Notably, IL-2 blocking did not have any effect on the percentage of IL-17$^+$ CD4$^+$ cells when a 10:1 APC:T cell ratio was used. It has been reported that IL-2 inhibits the generation of Th17 cells and promotes generation of T$_{Reg(419)}$. In addition, CD28-mediated inhibition of Th17 generation is IL-2-dependent(555). Probably in our system other factors, mainly APC:T cell ratio and culture media, are more important, rendering IL-2 inhibition unconsequential.

Even though the modification of the APC:T cell ratio to 1:1 resulted in a significant increase of Th17 polarisation efficiency the percentage of IL-17$^+$CD4$^+$ cells was highly variable, ranging from ~10% to 40%. This could be overcome by the use of IMDM media, which resulted in significant increase of the efficacy of Th17 polarisation and reduced variability. These data are in agreement with recent a recent study which reports that RPMI media supports low levels of Th17 polarisation, whereas use of IMDM results in a higher Th17 expansion due to the higher concentration of aromatic amino acids that give rise to AhR agonists(562). The same group and others have reported that ligation of the AhR by agonists promotes Th17 differentiation(545;546). The link between the AhR and Th17

pathway could be of significant physiological importance. Autoimmune diseases are multifactorial conditions, where genetic background and environmental factors have central role. As AhR is a responsive to many environmental pollutants it is intriguing to hypothesize that these factors may be involved in the development of autoimmune diseases through the enhancement of Th17 responses.

After the establishment of a reliable Th17 protocol, the effect of the genetic differences between BALB/c and C57/BL6 mice was investigated. As the OVA TcR induced RA model can be employed in both strains it was very important to investigate how the genetic background of each mouse strain can influence *in vitro* Th17 polarisation. There was no difference in the percentage of IL-17$^+$ CD4$^+$ cells between the two mouse strains, suggesting that at least under strong polarising conditions the genetic characteristic of each mouse strain had little effect on Th17 generation efficiency. These data are in agreement with studies investigating the effect of the genetic background on Th1/Th2 polarisation. In these studies it was shown that *in vitro* under strong polarising conditions the effect of genetic background is negligible(574). Under neutral conditions however, a predisposition towards Th1 or Th2 phenotype could be revealed(574). Thus it remains possible that under neutral conditions differences between the two strains may be detected. Apart from the genetic background, DO11.10 and OT-II mice differ in the affinity in which they recognise the OVA$_{323-339}$ in the context of their respective MHCII molecules(566), with DO11.10 having higher affinity for the peptide compare with OT-II(566). This suggests that at least *in vitro* in highly polarising conditions the affinity of the TcR does not have any effect on Th17 polarisation. It should be noted, however, that *in vivo* it has been reported that as the functional avidity of the immunising peptide for the TCR/MHCII increases the Th17:Th1 ratio increases(553).

From the experiment presented in this chapter it is very difficult to conclude which is the most critical factor that affects Th17 polarisation efficacy. *In vitro*, it is probably a combination of factors relating to cytokine stimulation, TcR signaling

and quality of co-stimulation. It is most probable that a plethora of factors, ranging from location of APC activation, the type of pathogen or damage associated molecules present, availability and type of antigen, cytokine milieu, and quality and kinetics of co-stimulation and TcR stimulation, would have a collective role in Th17 generation.

Finally, in this chapter, the presence of TFH and T_{REG} cells, in the polarised Th1 and Th17 population were investigated. Especially in the case of Th17 polarisation, cytokines such as TGFβ and IL-6 have been reported to be important for the generation of inducible T_{Reg} and TFH cells respectively(385;575). IL-6 promotes the production of IL-21 from CD4$^+$ cells which has been reported to promote both Th17 and TFH generation(370;454;576). However, in both Th1 and Th17 polarised population, there was minimal contamination from $T_{REG}$and TFH cells. It would be interesting however to determine the cytokine, chemokine and chemokine receptor profile of the two population as this would give us clues for the functional and localisation potential after transfer.

The Th17 protocol developed in this chapter was used to generate the Th17 populations used for all the subsequent studies of this thesis.

Chapter 4: Potential role of Th17 effector cells in the initial events that lead to breach of self tolerance

4.1 Aim and rationale

In this chapter the potential role of the Th17 effector T cells in the initial events that lead to the breach of self tolerance in experimental arthritis was investigated. While many studies on rheumatoid arthritis have focused on the active phase of the disease(219;501;512;577) the initial immunological events that lead to the underlying autoimmunity that precedes joint patholog are relatively understudied. We previously developed a model of breach of self tolerance where a Th1 response to irrelevant antigen (OVA) results in arthropathy associated with spontaneous induction of autoreactive T and B cell responses(5). Due to the mounting evidence relating to Th17 cells in various autoimmunity models(8;9;285;477;578) we hypothesized that if Th17 played a significant role in the breach of self tolerance in experimental arthritis, the auto-antigen specific immune response in the Th1-induced RA model would be characterized by IL-17-producing CD4$^+$ cells. In addition, we hypothesized that a model induced by Th17 effector cells would be characterized by more potent autoimmune B and T cell responses that potentially could lead to a more severe clinical and histopathological image compared to the Th1 model. Furthermore, in this chapter, the phenotype, the kinetic characteristics, distribution and viability of the Th1 and Th17 transferred populations was analyzed. As such, in this chapter, the disease induced by Th1 cells was compared with that caused by Th17 cells and the immunological parameters associated with this were characterised.

4.2 Introduction

The pathogenesis of rheumatoid arthritis can be grossly subdivided into three phases(3). Genetically susceptible individuals, under the influence of various environmental factors develop an underlying autoimmunity which manifests with the production of various autoantibodies, such as rheumatoid factor and ACPA(3;130). This phase precedes any clinical manifestation in some cases even by 10 years(3;4). The mechanisms that mediate this are ill-defined, but the

association of molecules such as PTN22 and CTLA-4 with diseases pathogenesis(57;579), suggest a failure in aspects of both central and peripheral tolerance. This asymptomatic autoimmune phase is followed by a transitional stage, which leads to the development of the clinical symptoms of rheumatoid arthritis(3). The onset of the clinical disease leads the relative acellular synovial membrane to become hyperplastic and be infiltrated by a plethora of immune cells(1;130). Most studies of RA have focused their attention at the active, articular, phase of the disease. This is due to the availability of tissue from patients with active RA and from the development of a number of animal models, such as CIA, TNF transgenic mice, and AIA(219;501;577), that highly resemble this stage of the pathology. In addition, most treatments of RA are symptomatic and do not re-establish immunological tolerance. Thus, it would be more useful to understand the early events that lead to breach of self tolerance, with a target to re-educate the immune system and re-establish tolerance.

As mentioned previously, the ultimate goal in all autoimmune disorders is to re-establish immunological tolerance. This is very difficult, as patient studies are practically impossible to dissect the critical events mediating the initiation of self-reactivity because these take place some times many years prior of the clinical diagnosis. Furthermore, many of the animal models of the disease are based on the aggressive immunisation of putative self-antigens (e.g. CIA), which do not resemble the early pre-articular phase of the disease and do not permit its analysis(123;580).   As mentioned previously in this thesis, a novel model of experimental arthritis has been developed in our lab that highly resembles the preclinical stage of the disease(5). Transfer of Th1-polarised OVA-specific-TcR-transgenic CD4$^+$ T cells, induces transient arthritis in mice challenged in the footpad with HAO, thus avoiding immunisation with a self antigen. This is characterized by a transient paw swelling, which lasts around 7-9 days, synovial hyperplasia and cartilage erosion proximal to the HAO challenged paw(5). However, the most important characteristic of this model is the breach of self-

tolerance that is manifested by the generation of class-switched (IgG) autoantibodies, namely anti-CII antibodies, ACPA, RF and anti-DNA antibodies, and T cell responses against CII(5;6;168). It represents a model of preclinical or early arthritis, showing high similarities with the underlying autoimmunity that characterizes these stages of the disease. As such, it is a very useful tool in delineating the early immunological events that lead to the breach of self tolerance and was used through out this thesis to investigate the role of Th17 cells in these events.

It is still not clear whether RA is a Th1 or Th17 mediated disease. Studies in models such as the CIA, SKG and IL-1Ra KO revealed that the IL-23/Th17 axis is mediating pathogenesis though the production of cytokines such as IL-17 and IL-22 (9;476-479). Furthermore, there is a well established role for IL-17 and Th17 in joint destruction and remodelling through the promotion of osteoclastogenesis and production of tissue degrading enzymes, such as MMP-1(176;481;482). Other Th17-related cytokines have been reported to be involved in RA pathology. IL-21-blockade ameliorates CIA in mice and rats, IL-21 receptor (IL-21R) deficient mice are protected from the development of arthritis in the autoimmune prone K/BxN model and in humans the IL-21R is expressed by RA synovial macrophages and fibroblasts(483-485). It should be noted however, that many of the Th17-related cytokines, such as IL-21, IL-22 and even IL-17 it-self, are not exclusively produced by Th17 cells, but from other cell types and effector T cells(286;486;487). Even, though the above studies suggest a role for Th17 in RA, other reports in human and animals, indicate a less significant role for these cells. The proteoglycan-induced model of arthritis, for example, is mediated by IFNγ-producing cells, and mice deficient for this cytokine develop significantly less severe pathology(488). In humans, a study in Japan revealed that the frequency of Th17 cells was neither increased in RA patients nor correlated with arthritis severity, and was significantly decreased in joints compared to peripheral blood, unlike Th1 cells that were more abundant in the joint(489). It remains thus possible that the role of Th1/Th17 can differ according to disease subtype. All the aforementioned studies, in animal

model and humans, are mainly focused in the articular phase of the disease. The role of Th17 cells in the events that lead to the breach of self tolerance, however, are ill defined and under-studied, as this stage of the disease is very difficult to be investigated.

In this chapter, the model of breach of self tolerance was employed to investigate the role of Th17 in this phase of the disease. The phenotype of the autoimmune response was characterized and the relative ability of Th17 effector population compared to a Th1 to induce breach of self tolerance was investigated. Lastly, the phenotype, distribution and clonal expansion of the transferred Th1 and Th17 populations were analyzed.

4.3 Results

3.3.1 Phenotype of the Collagen-specific response in the Th1 OVA TcR-induced RA model

As mentioned above, there are a few studies that support a role for Th17 cells in the active phase of RA, and especially about the role of these cells and the cytokines they produce in joint damage and remodelling(9;12;478;581). On the other hand there is very little known about the role of these cells in the events that lead to the underlying autoimmunity that characterizes the early pathology of RA. Therefore, first of all, the involvement of Th17 responses was examined. It was hypothesised that if Th17 were involved in the events leading to the breach of self tolerance in this system, then it would be possible to detect self-antigen specific Th17 cells. In order to test this hypothesis, the phenotype of the anti-CII response developed in the Th1 OVA-TcR induced RA model was analyzed, in respect of IL-17 and/or IFNγ production. The model was employed as described in materials and methods and previously(5-7;514). In agreement with published studies(5-7), the mice developed a transient mono-arthritis, measured as paw swelling, which lasted approximately 7 days (Fig 4.1a). Seven days post footpad challenge mice were euthanized and B and T cell responses against OVA and CII were assessed (Fig 4.1b-d). Both PBS and HAO challenged mice developed robust B cell responses against OVA in the form of anti-OVA IgG antibodies (Fig 4.1c), as both groups were immunised with OVA/CFA. More importantly, HAO challenged mice developed auto-reactivity in the form of B and T cell responses against CII that did not develop in the PBS challenged group (Fig 4.1b, d and Fig 4.2). In detail, HAO challenged mice developed significantly higher anti-CII IgG antibodies compared to the PBS challenged mice (Fig 4.1c). Furthermore, in an *ex-vivo* recall assay only CD4$^+$ from draining LNs of HAO challenged mice proliferated when cultured in the presence of CII (Fig 4.1d and 4.2), whereas cells from PBS challenged mice did not. As expected, CD4$^+$ cells from HAO challenged mice had a robust recall

response against OVA (Fig 4.1d and4.2). These data, demonstrate that by day 7 post challenge the mice have already developed autoimmune B and T cell responses confirming previous studies(7). In order to characterize the phenotype of the CII specific T cells, cells from the draining LN were cultured in the presence of media, OVA or CII and their ability to produce IFNγ and/or IL-17 in response to these antigens was investigated by intracellular flow cytometry. CD4$^+$ cells from PBS challenged mice did not produce either IFNγ or IL-17 in response to OVA or CII (Fig 4.3 and 4.4). The CD4$^+$ response against OVA in the HAO challenged mice was characterized exclusively by IFNγ producing cells, whereas the percentage of IL-17$^+$ CD4$^+$ or IL-17$^+$IFNγ$^+$ CD4$^+$ cells was not significantly higher from either the media control or the PBS-challenged mice (Fig 4.4a-c). Similarly, the CII CD4$^+$was characterized only by IFNγ producing cells as the percentage of IL-17 or IL-17/IFNγ producers were not different from controls (Fig 4.4a-c). These results demonstrate that the phenotype of the self-specific response in the Th1 OVA TcR-induced RA model is of a Th1 type with no apparent involvement of Th17 cells.

4.3.2 Effect of adjuvant in the development the Th1 OVA-TCR-induced RA model

The fact that the OVA-TcR-induced arthritis model is mediated by a highly polarised Th1 population might bias the developing primary auto-immune response towards a Th1 phenotype, inhibiting any developing Th17 cells. Indeed, IFNγ has been reported to inhibit development of Th17 cells(412). APCs, such as DCs, have been proposed to be the source of Th17 polarising cytokines(413;582), however the nature of the stimuli that drives the production of this cytokines is not very clear. It has been reported that mouse DCs stimulated via TLR4 or dectin-1 induced Th17 polarisation(413;463). The latter especially is particularly interesting in the context of experimental arthritis. Dectin-1 is a C-type lectin, which when binds to yeast β-glucans, such as curdlan, induces DC maturation and the production of copious amounts of IL-6, TNF and IL-23, but little IL-12, promoting Th17 polarisation *in vitro*, and both Th1 and Th17 polarisation *in vivo*(463). More importantly, curdlan

induces robust arthritis in SKG mice kept in pathogen-free condition that are normally resistant to disease development(82). Interestingly, even BALB/c control mice developed arthritic symptoms after curdlan (CUR) administration, albeit in a mild form(82). Furthermore, the β-glucan mediated arthritis in the SKG model was accompanied by an increase in the percentage of IL-17$^+$CD4$^+$ cells(477). Based on this data it was hypothesized that substitution of CFA with CUR in the Th1 OVA-TcR-induced arthritis model could potentially skew the developing autoreactive response to a Th17 phenotype that would possibly be accompanied by more severe clinical and histological signs of disease. The Th1 OVA-TcR induced RA model was employed as before but in this case mice were immunised either with OVA/CFA or with OVA/CUR. Ten days after immunisation mice were challenged in the footpad with HAO and arthritis was assessed for 7 days. Both CFA and CUR immunised animals developed similar levels of arthritis that lasted approximately seven days (Fig 4.5a). In addition, histological analysis did not reveal any difference between the CFA and CUR/OVA immunised HAO challenged mice, with both developing only mild synovitis (Fig 4.5b and c). As expected, PBS challenged mice did not develop clinical or histological signs of arthritis (Fig 4.5). When sera from the blood was analyzed for anti-CII antibodies, both CUR and CFA/OVA immunised mice that were challenged with HAO developed anti-CII antibodies in titres significantly higher that the PBS challenged mice (Fig 4.6a). Furthermore, HAO challenged mice from both CFA and CUR/OVA immunised mice developed proliferative T cell responses against CII, significantly higher than the PBS challenged mice (Fig 4.6b). These data demonstrate that both models develop B and T cell autoreactivity. There was no difference between the two adjuvants in respect of the T cell response against OVA, suggesting an equal ability to prime an adaptive immune response for both CUR and CFA. Interestingly, when the supernatant from the proliferation assay was analyzed for the presence of IL-17, only very low levels of this cytokine could be detected in response to OVA or CII, in either CUR or CFA/OVA immunised mice. This suggests that CUR did not skew the autoreactive response to a Th17 phenotype (Fig 4.6c).

There are reports that suggest that CUR can induce both Th1 and Th17 cells *in vivo*(463). In order to investigate relative ability of CFA and CUR to induce *in vivo* Th17 generation, MACS sorted CD4$^+$ from DO11.10 mice were adoptively transferred to BALB/c mice and were then immunised with either CFA or CUR/OVA. Seven days after immunisation mice were euthanized and the ability of the transferred and host CD4$^+$ to produce IL-17 or/and IFNγ was investigated. Notably, only a very small percentage of the transferred transgenic T cells produced IL-17 or IFNγ in response to either CFA or CUR/OVA (Fig 4.7a-top panel, b and c). The two adjuvants had a similar effect in the host CD4$^+$population (i.e. CD4$^+$KJ1.26^{NEG}), inducing only a small percentage of IL- or IFNγ. These data demonstrate that CUR does not preferentially prime unpolarised T cells towards a Th17 phenotype *in vivo*. As the breach of tolerance in the arthritis model is mediated by polarised OVA-specific Th1 cells, the relative effect of CUR and CFA on the phenotype of the transferred transgenic T cells was investigated. Th1 polarised CD4$^+$ from DO11.10 mice were adoptively transferred to BALC/c mice, which were then immunised with OVA/CFA or OVA/CUR. Five days after immunisation mice were euthanized and cells from the draining LNs (axillary) were analyzed for the production of IFNγ and/or IL-17 by flow cytometry (Fig 4.8). The transferred Th1 population retained, albeit at lower percentage IFNγ production in response to both OVA/CFA and OVA/CUR (Fig 4.8b). More importantly, none of the adjuvants induced the production of IL-17 from the transferred T cells (Fig 4.8c).

These data demonstrate that CUR and CFA, when employed to the Th1 OVA-TcR induced RA model as adjuvants, result in the development of similar clinical and histological signs of arthritis. In both cases, HAO challenge results in the breach of self as demonstrated by CII B and T cell responses. In contrast to published reports(463), this data did not demonstrate any *in vivo* Th17 polarisation ability by CUR or CFA. In addition, CUR did not alter the phenotype of polarised Th1 cells, which retained IFNγ production and failed to produce any IL-17 after adoptive transfer.

4.3.3 Relative ability of Th1 and Th17 cell to induce breach of self tolerance in the OVA-TcR-induced arthritis model

So far in this chapter it has been confirmed that Th1 polarised CD4$^+$ of an irrelevant specificity can induce the development of transient arthritis, but most importantly autoreactivity, in the form of T and B cell responses against CII. The phenotype of the CII specific T cell response was characterized by the production of IFNγ, and by the absence of IL-17 producing CD4$^+$. This disproves part of our original hypothesis, which stated that if Th17 cells were playing a part in the breach of self tolerance that develops in the Th1 model, the autoreactive T cell response would be partly or fully of a Th17 type. It is however possible that the highly polarised Th1 cells that mediate the autoreactivity might skew the developing T cell self-response towards a Th1 phenotype. Using curdlan, a yeast β-glucan that has been reported to act as a Th17 adjuvant and to induce arthritis(82;463), did lead to breach of self tolerance, but did not alter the Th1 phenotype of the autoreactive response. As in some other models, such as the CIA, IFNγ responses are considered regulatory, it was hypothesized that if the model was induced by a Th17 polarised population, this would potentially mediate a more robust breach of self tolerance, e.g. higher anti-CII T-cell responses, and more severe and chronic clinical disease. In order to test this, the relative ability of Th1 and Th17 polarised populations in mediating the breach of self tolerance in the OVA-TcR-induced model was compared. Th1 or Th17 polarised CD4$^+$ population (Fig 4.9a) from DO11.10 mice were adoptively transferred into BALB/c mice, which were then immunised with OVA/CFA and challenged in the footpad with HAO. Control mice were challenged with PBS. Both Th1 and Th17 recipient HAO-challenged mice developed similar levels of transient mono-arthritis, as demonstrated by clinical score and paw swelling (Fig 4.9b-c). PBS challenged mice did not develop any clinical signs of arthritis (Fig 4.9b-c). Similarly, there were no histological differences between the two models, as only HAO challenged mice developed very mild synovitis, in both Th1 and Th17

recipient mice (Figd4.9d and 4.10). On the other hand, none of the PBS challenged mice developed histological signs of the disease (Fig 4.9d and 4.10).

The development of autoreactivity in the form of T cell responses against CII was then investigated (Fig 4.11). Cells from draining LNs from PBS or HAO challenged mice were cultured in the presence of OVA, CII or no antigen and their ability to proliferate was assessed using the Click-iT EDU proliferation assay. As expected cells from HAO challenged mice of Th1 or Th17 recipients proliferated robustly in response to OVA (Fig4.11a-b). Crucially, cells from both Th1 and Th17 recipients challenged with HAO proliferated to a similar degree in response to CII (Fig 4.11a-b). Cells from PBS challenged mice that either received Th1 or Th17 cells did not proliferate in response to either OVA or CII.

It has been previously demonstrated in this chapter that Th1 cells mediate breach of self tolerance in the form of IgG anti-CII antibodies, whereas our group has also reported the presence of ACPA in the Th1 OVA-TcR-induced arthritis model(6). Thus the presence of these antibodies was investigated in the Th17 OVA-TcR-induced model (Fig 4.12a and b). As in the Th1 model, Th17 recipients challenged with HAO developed both anti-CII and ACPA antibodies in titers significantly higher than PBS challenged mice (Fig 4.12). Unfortunately, due to inconsistency in the development of the autoreactive T and B cell responses in both Th1 and Th17 models, it was not possible to directly compare the titres of these antibodies between the two models.

These data clearly demonstrate that both Th1 and Th17 populations can mediate similar levels of pathology, but more importantly can both breach B and T cell self tolerance. However, our data do not demonstrate a relative advantage of Th17 in inducing more robust auto-reactive response or more severe pathology.

4.3.4 Phenotype of the Collagen-specific response in the Th17 OVA-TcR-induced RA model

Previously in this chapter, it was reported that the CII T cell response in the Th1 OVA-TcR-induced RA model is of a Th1 phenotype. Even though, these data so far demonstrate any differences between the two models, it was still possible that the phenotype of the auto-reactive T cell response might differ between them. Cells from draining LNs from HAO or PBS challenged Th17 recipient mice were cultured with OVA, CII or no antigen for 72 hours and the ability of the CD4$^+$ to produce either IL-17 and/or IFNγ was analyzed by flow cytometry (Fig 4.13). CD4$^+$ from PBS challenged mice failed to produce either cytokine in response to the stimulating antigens (Fig 4.13a-top panel, b-d). Surprisingly, CD4$^+$ cells from HAO challenged mice failed to produce IL-17 in response to OVA, however they produced high amounts of IFNγ (Fig 4.13a-bottom panel, b-d). More interestingly, in response to CII, CD4$^+$ produced only IFNγ, and no IL-17 or IL-17/IFNγ producers were detected. These data demonstrate that, as in the case of the Th1-induced model, the CII-specific CD4$^+$ response in the Th17-induced model is of a Th1 type. Moreover, unlike the Th1-model, the OVA-specific response, did not retain the phenotype of the Th17 transferred transgenic population, but acquired a Th1 phenotype.

4.3.5 Presence of FOXP3$^+$ cell in the Th1 and Th17 OVA-TcR-induced arthritis models

In both the Th1 and Th17 models, a B and T cell breach of self tolerance occurs, suggesting a failure in some aspect of peripheral tolerance. T_{REG} cells are crucial for preventing generalized autoimmunity and their importance have been demonstrated in various models of autoimmune disease such as multiple sclerosis and arthritis(570;583). Furthermore there is reciprocality in the development of T_{REG} or Th17 cells that depends on the cytokine milieu(413;414). In order to investigate if the breach of self tolerance in the Th1 and Th17 models is related to a failure of the development of T_{REG} cells the presence of these cells was assessed.

As mentioned in the previous chapter, the transcription factor FOXP3 is crucial for the commitment of cells to the T_{REG} lineage. Thus, this marker was used to identify these cells. Th1 or Th17 OVA-TcR-induced RA models were developed as previously described, and seven days post-footpad challenge cells from the dLNs were analyzed for the presence of FOXP3 expressing CD4$^+$ cells by flow cytometry (Fig 4.14). CD4$^+$ FOXP3$^+$ cell could be identified in both Th1 and Th17 models, irrespective if the mice were challenged with HAO or PBS. Even though the percentage of CD4$^+$FOXP3$^+$ cells did not differ between the groups, HAO challenged mice exhibited increased numbers of T_{REG} cells compared to PBS challenged mice (Fig 4.14b and c). More interestingly, Th17 HAO challenged recipients had significantly higher numbers of FOXP3$^+$ T cells than their Th1 counterparts (Fig 4.14c). These data demonstrate that in both Th1 and Th17 models the development of auto-reactivity cannot be attributed to a failure of T_{REG} cell generation. It should be noted however that the functionality of these cells was not formally tested, thus it remains possible that impaired regulatory activity of these cells might account for the development of auto-reactivity.

4.3.6 Phenotype, kinetics and distribution of the transferred Th1 and Th17 trangenic CD4$^+$ population

An observation of this chapter is the Th1 phenotype of both the OVA and CII CD4$^+$ response in the Th17-induced model. Especially the predominant IFNγ response against OVA is quite intriguing as these animals received trangenic Th17 cells specific for this antigen. As mentioned in the introduction of this thesis, a characteristic of *in vitro* polarised Th17 cells is their phenotypic plasticity, and their transformation to a Th1-like cell type(492;493). In addition, as the number of Th subtypes has increased the idea of CD4$^+$ plasticity is now considered an established concept for other subsets(584). It should be noted that for some subsets such as Treg reports have suggested that they have a stable phenotype *in vivo(585)*. It is thus important to investigate the phenotype of the Th1 and Th17 transferred

populations that mediate the breach of tolerance, as this may change after transfer. Furthermore, as the transgenic Th populations mediate the pathology and are necessary for the development of the breach of tolerance(5), it is important to investigate the kinetics and their distribution, before HAO challenge. This could give mechanistic information as to how these cells mediate breach of self tolerance.

 In order to investigate the phenotype of the transferred population, Th1 or Th17 polarised cells from DO11.10 mice were adoptively transferred in to BALB/c mice, which were then immunised with OVA/CFA or PBS. Mice were culled at days 3, 7 and 10 post-immunisation and draining LNs (axillary and brachial) were removed. Cells from the draining LNs were stimulated with PMA and ionomycin, to induce synchronous cytokine production and the ability of the transgenic T cells to produce IL-17 or/and IFNγ was assessed by flow cytometry (4.15). Transgenic T cells were identified based on the expression of CD4$^+$ and the DO11.10 TCR, which is identified by the KJ1.26 monoclonal antibody. In immunised animals, the transferred Th1 cells continued to produce IFNγ, in a reducing rate that was stabilized after day 7 (Fig 4.16). These cells produced minimal amounts of IL-17 the first two time points investigated, however at day 10 the percentage of IL-17$^+$ transgenic T cells was significantly higher than at day 3 and 7 (Day 3: 0.54± 0.25, Day 7: 1.01± 0.29, Day 10: 5.02± 0.63, Day 10 vs. Day 3: p<0.001, Day 10 vs. Day 7: p<0.001, n=3, data presented as mean±SD) (Fig 4.16b). In unimmunised mice, the kinetics of IFNγ production by the transferred population was similar to the immunised mice, contrary to IL-17 production which is minimal at all time points. On the other hand, the Th17 transferred population in OVA/CFA immunised mice experienced a sharp reduction in the percentage of IL-17$^+$ cells with an 83.01% reduction by day 3 (Day 0: 50.03± 17.17, Day 3: 8.4± 0.60) compared to the original percentage. After day 3 the percentage of IL-17$^+$ transgenic cells remained relatively stable at around 5% (Day 3: 8.4± 0.60, Day 7: 5.23± 1.77, Day 10: 5.77± 2.35, n=3 data presented as mean±SD) (Fig 4.12c). The proportion of IFNγ-producing transgenic T cells was not affected by the transfer and remained relatively low (Day 0: 2.88± 0.79, Day 3: 1.30± 0.21 Day 7: 2.41± 0.30 Day 10:

4.51± 1.98, n=3, data presented as mean±SD). Similarly, in unimmunised mice, the percentage of IL-17^{+} transgenic T cells was dramatically reduced (Day 0: 50.03± 17.17, Day 3: 4.67 ±1.12, Day 7: 1.12± 0.28, Day 10: 2.67± 2.09, n=3, data presented as mean±SD) after transfer (Fig 4.16c). Interestingly, however, the percentage of IFNγ^{+} cells in the transferred population gradually increased and by day 10 it was similar to the percentage of the Th1 transferred population (Day 0: 2.88± 0.79, Day 3: 1.10± 0.92, Day 7: 4.26± 0.59, Day 10: 24.98± 4.570, Day 0 vs. Day 10, p<0.001, Day 3 vs. Day 10, p<0.001, Day 7 vs. Day 10, p<0.001, n=3, data presented as mean±SD) (Fig 4.16b), suggesting a spontaneous IFNγ production by these cells at this time-point.

It was demonstrated previously in this study that CUR had similar effects to CFA in polarised Th1 cells (Fig 4.6). In addition, it was also that the adoptively transferred Th17 population rapidly loses its ability to produce IL-17. As CUR has been shown to drive Th17 responses *in vivo* and *in vitro* its effect on adoptively transferred *in vitro* polarised Th17 population was tested, hypothesising that it might stabilize their phenotype. As before, DO11.10 cells polarised under Th17 conditions were adoptively transferred to BALB/c mice that were then immunised with either OVA/CFA or OVA/CUR. The phenotype of the transferred population was assessed five days after immunisation by flow cytometry (Fig 4.17a and b). Similarly to CFA, CUR failed to maintain a high percentage of IL-17^{+} transgenic cells in the transferred population. As in the case of CFA, this was not followed by an increase on the percentage if IFNγ producing cells.

The relative expansion and distribution of the transferred Th1 and Th17 polarised population was then investigated. Three different secondary lymphoid organs were analyzed, the draining LNs (axillary and brachial), the spleen to investigate systemic responses and the mesenteric LNs (mLNs) as a more distal site. As before, Th1 or Th17 polarised CD4^{+} from DO11.10 mice were transferred to BALB/c mice, which were then immunised with OVA/CFA or PBS. At days 3, 7 and 10 post-immunisation cells from draining LNs, spleen and mLNs were analyzed for the

presence of the transferred transgenic T cells (Fig 4.18). Unfortunately, due to technical issues relating to the emulsion nature of the adjuvant (CFA) it was not possible to isolate any cells from the site of injection. As expected, in unimmunised mice that had received either Th1 or Th17 populations, in all sites investigated, there was no expansion of the transgenic T cells, which became almost undetectable by day 10 (Fig 4.18). In immunised, animals on the other hand, Th17 polarised cells expanded to a higher degree compared to the Th1 polarised population, as shown by both the number and percentage of transgenic T cells, in all organs examined. This was very prominent it the spleen, where cells polarised under Th17 conditions accumulated in high numbers and persisted at the site even at the latest time-point investigated (Fig 4.18b and e). Cells polarised under Th1 conditions accumulated mainly in the draining LNs where their numbers probably peacked between day 3 and day 7, and were reduced to levels of unimmunised mice by day 10 (Fig 4.18a and d). In contrast, cells polarised under Th17 conditions accumulated in the dLNs and persisted at the site in high number even at the last time-point investigated (Day 10) (Fig 4.18b and e). Interestingly in immunised mice, cells polarised under Th17 conditions could be detected in high numbers and constituted a significant percentage of CD4$^+$ cells in mLNs at days 3 and 7, something that was not observed with cells polarised under Th1 conditions. These data demonstrate that the two populations differ greatly in their distribution, expansion and kinetics, with cells polarised under Th17 conditions expanding in a greater degree than the Th1 population, distributing widely in all organs examined and persisting in the dLNs and spleen even ten days after immunisation.

4.3.7 Relative viability of cells polarised under Th1 and Th17 conditions

Differences in viability of the cells polarised under Th17 conditions relative to cells polarised under Th1 conditions, might be a possible explanation for the greater expansion and persistence of the former. In order to investigate this possibility, CD4$^+$ cells from DO11.10 mice were polarised under Th1 or Th17 conditions for

72hrs, rested in the absence of any TcR stimulus or polarising cytokine for 24hrs, and cultured in the absence or presence of bone marrow derived DCs. The bone marrow DCs were either in a resting state, with or with out antigen or LPS-activated in the presence of $OVA_{323-339}$ (Fig 4.19 and 4.20). The viability of the transgenic T cells was analyzed at two time-points, 24 and 48hrs, by annexin V and propidium iodide (PI) staining by flow cytometry. Cells negative for both annexin V and PI staining were considered viable. In the absence of antigen, independently of the presence of DCs, cells polarised under Th17 conditions were more viable than the Th1 counterparts, at both time-points investigated. Similarly, in the presence of antigen, cells polarised under Th17 conditions were more viable compared to the Th1 population, especially at 48hrs. In both Th1 and Th17 population the presence of antigen resulted in a significant reduction of the percentage of viable cells, compared to condition of antigen absence, suggesting activation-induced cell death (AICD)(Fig 4.14b and c). These data demonstrate, that cells polarised under Th17 conditions are more viable that Th1 polarised cells. This is possibly not only due to differences in AICD, as the Th17 population is more viable even in the absence of a TcR stimulus.
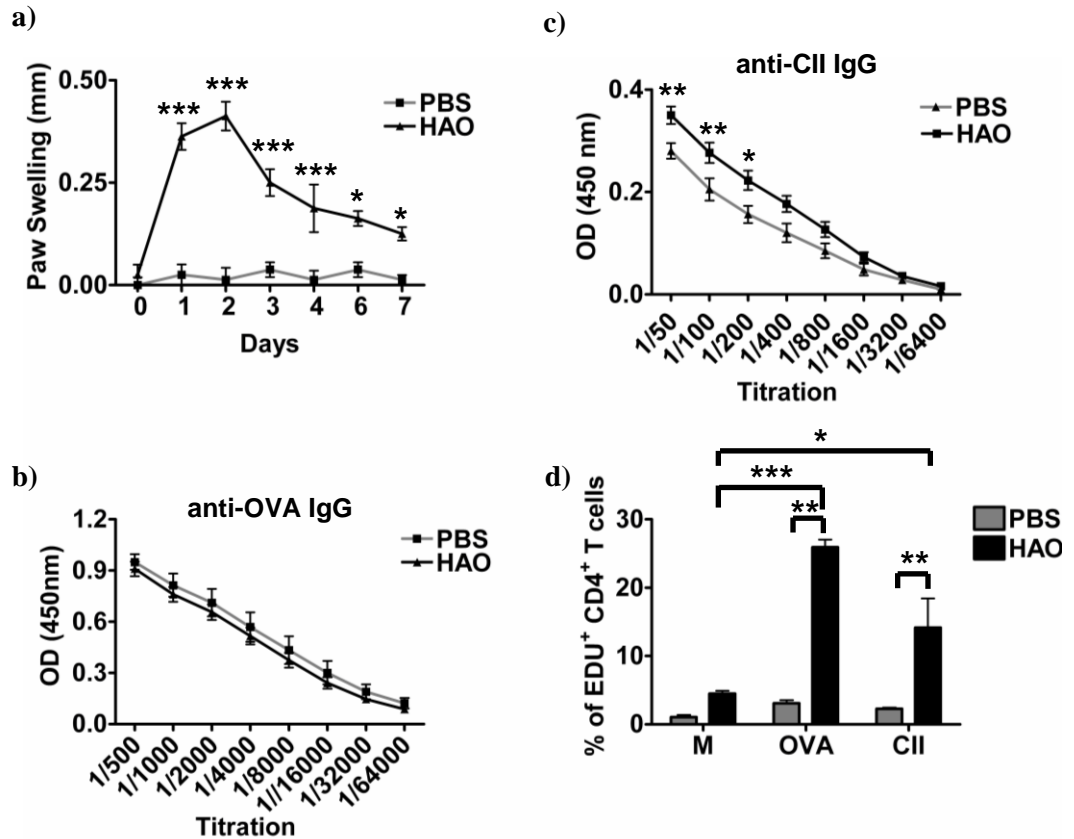
Fig 4.1: Breach of tolerance in the Th1 OVA-TcR induced RA model.

Th1 cells from DO11.10 mice were transferred to BALB/c recipients, which were then immunised with OVA/CFA. Ten days after immunisation recipient mice were challenged in the hind paw with HAO. Control mice received PBS. Arthritis was assessed for 7 days by measuring the difference in paw thickness between the challenged and unchallenged paw (a). Antibody responses against OVA (total IgG) and CII (total IgG) were analysed by ELISA (b and c). Cells from the draining LN were cultured for 72hrs in the presence of either media, OVA or CII and the ability of CD4$^+$ cells to proliferate in response to them was assessed using the Click-iT EDU proliferation assay by flow cytometry (d). Populations were gated on lymphocytes based on the FSC and SSC profile and then CD4$^+$ T cells based on CD4 expression. Data represent mean ±SEM.*$p<0.05$, **$p<0.01$, ***$p<0.001$ (n=5).
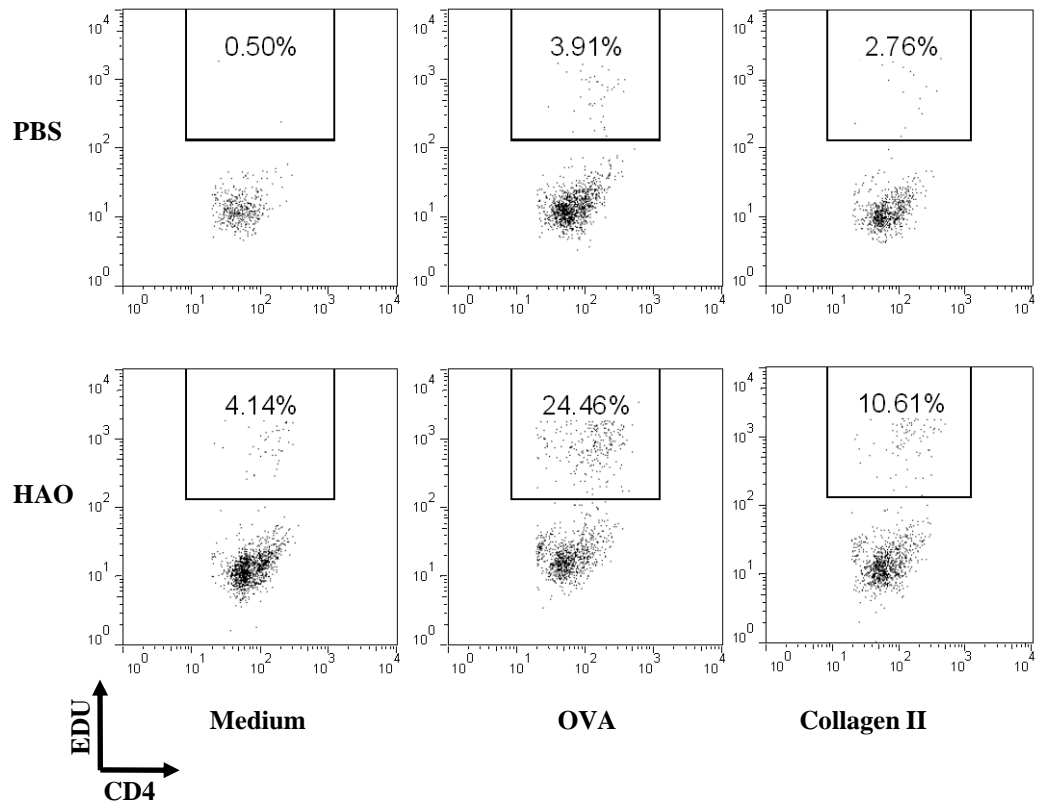
Fig 4.2: Breach of tolerance in the Th1 OVA-TcR induced RA model.

Th1 cells from DO11.10 mice were transferred to BALB/c recipients, which were then immunised with OVA/CFA. Ten days after immunisation recipient mice were challenged in the hind paw with HAO. Control mice received PBS. Cells from the draining LN were cultured for 72hrs in the presence of either media, OVA or CII and the ability of CD4$^+$ cells to proliferate in response to them was assessed using the Click-iT EDU proliferation assay by flow cytometry. Populations were gated on lymphocytes based on the FSC and SSC profile and then CD4$^+$ T cells based on CD4 expression. Similar results were obtained in 3independent experiments.

a)

Fig 4.3: Phenotype of Collagen II T cell response in the Th1 OVA-TCR induced RA model

Th1 cells from DO11.10 mice were transferred to BALB/c recipients, which were then immunised with OVA/CFA. Ten days after immunisation recipient mice were challenged in the hind paw with HAO. Control mice received PBS. Mice were culled 7 days after and cells from the draining LN were cultured for 72hrs in the presence of either media, OVA or CII and their ability to produce IL-17 and/or IFNγ was assessed by intracellular fluorescent cytometry staining. Representative fluorescent cytometry plots demonstrating the production of IL-17 and/or IFNγ by CD4+ cells from a draining LN of a PBS (top panel) or a HAO (bottom panel) challenged mouse. Populations were gated on lymphocytes based on the FSC and SSC profile and then CD4+ T cells based on CD4 expression. Similar results were obtained in one more experiment.
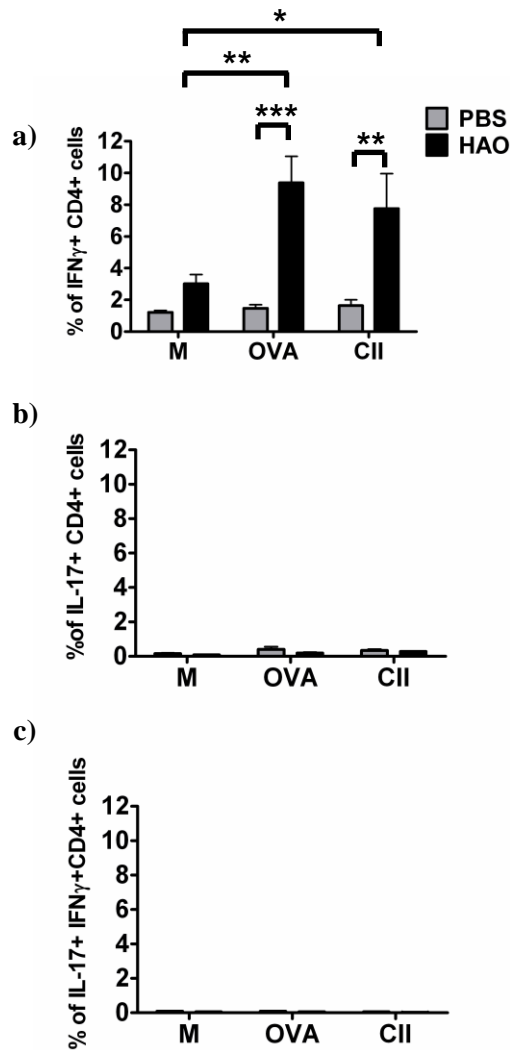
Fig 4.4: Phenotype of Collagen II T cell response in the Th1 OVA-TCR induced RA model

The Th1 OVA-TcR induced RA model was employed as described in materials and methods. Mice were culled 7 days after challenge and cells from the draining LN were cultured for 72hrs in the presence of either media, OVA or CII and their ability to produce IL-17 and/or IFNγ was assessed by intracellular fluorescent cytometry staining. Collective fluorescent cytometry data demonstrating the production of IFNγ (a), IL-17 (b), IFNγ and IL-17 (c) by CD4^{+} cells from draining LN of PBS (grey bars) or HAO (black bars) challenged mice. Data represent mean ±SEM.*p<0.05, **p<0.01, ***p<0.001 (n=5).
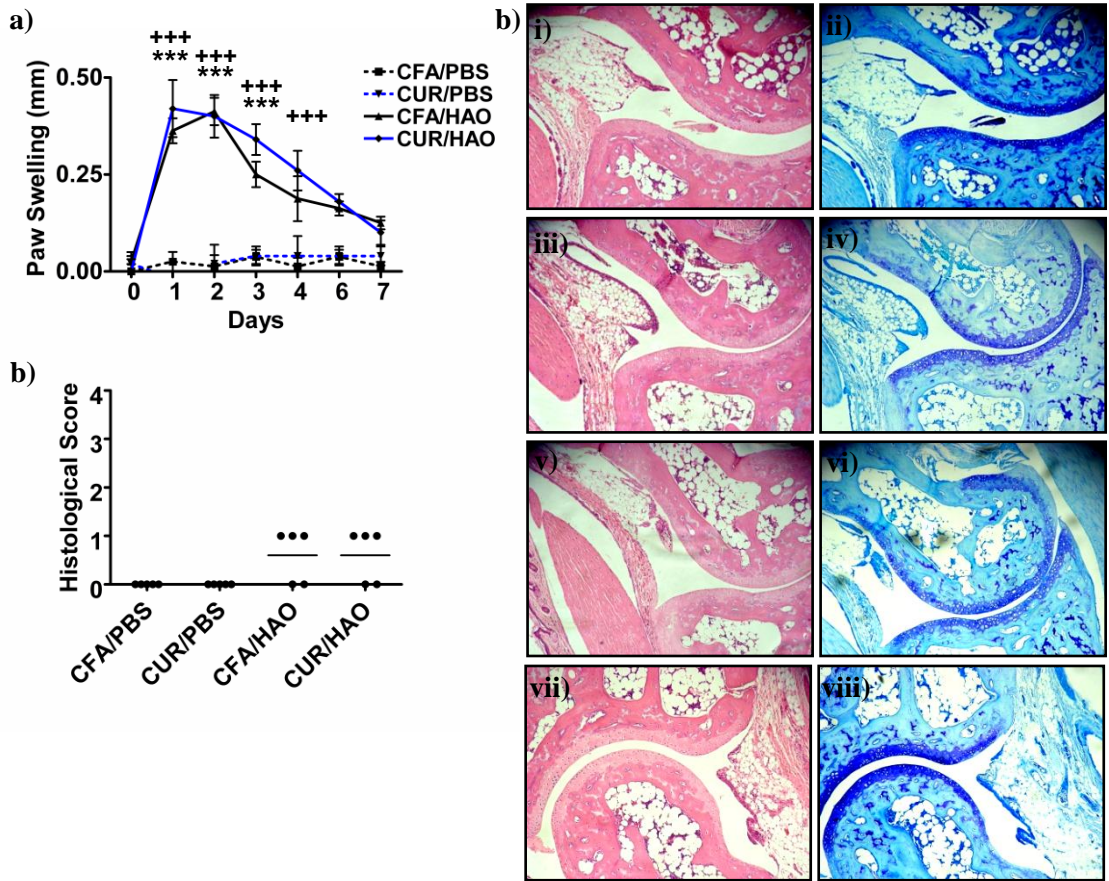
Fig 4.5: The relative effect of curdlan compared to CFA in the induction of breach of tolerance in experimental arthritis.

Th1 cells from DO11.10 mice (a) were transferred to BALB/c recipients, which were then immunised with OVA/CFA or OVA/CUR. Ten days after immunisation recipient mice were challenged in the hind paw with HAO. Control mice received PBS. Arthritis was assessed for 7 days by measuring the differencing in thickness between the challenged and unchallenged paw (a). Ankle joints from challenged hind paws were stained (H&E and toluidine blue) and section were assessed for histological signs of arthritis (b and c). Data represent mean, n=5. c) H&E (i,iii,v,vii) and toluidine blue (ii, iv, vi, viii) of ankle joints from CFA (i and ii) and CUR (iii and iv) immunised mice challenged with PBS or CFA (v and vi) and CUR (vii and viii) immunised mice challenged with HAO. Original magnification (x10). CFA/HAO vs.CFA/PBS:*, ***: p<0.001, CUR/HAO vs. CUR/PBS:+, +++ :p<0.001, n=5.
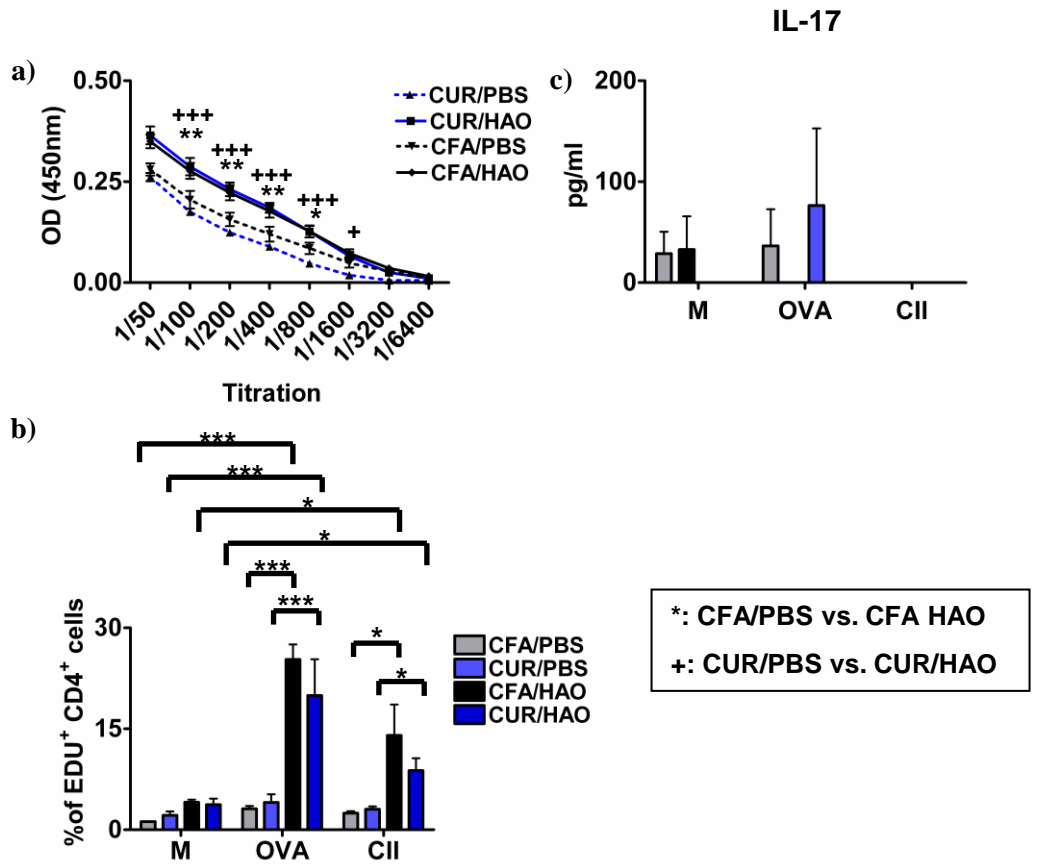
Fig 4.6: The relative effect of curdlan compared to CFA in the induction of breach of tolerance in experimental arthritis.

Th1 cells from DO11.10 mice were transferred to BALB/c recipients, which were then immunised with OVA/CFA or Cur/OVA. Ten days after immunisation recipient mice were challenged in the hind paw with HAO. Control mice received PBS. a) Antibody responses against CII (total IgG) were analysed by ELISA of the blood serum. b) Cells from the draining LN were cultured for 72hrs in the presence of either media, OVA or CII and the ability to proliferate was analyzed using the Click-iT EDU proliferation assay. Lymphocytes were identified based on the FSC and SSC profile and then CD4^{+} T cells based on CD4 expression. c) Cells from the draining LN were cultured for 72hrs in the presence of either media, OVA or CII and the ability to produce IL-17 was assessed by ELISA analysis of the culture supernatants Data represent mean ±SEM.*,+ $p<0.05$, **, ++$p<0.01$, ***, +++$p<0.001$ (n=5).

a)

b)

c)

d)

e)
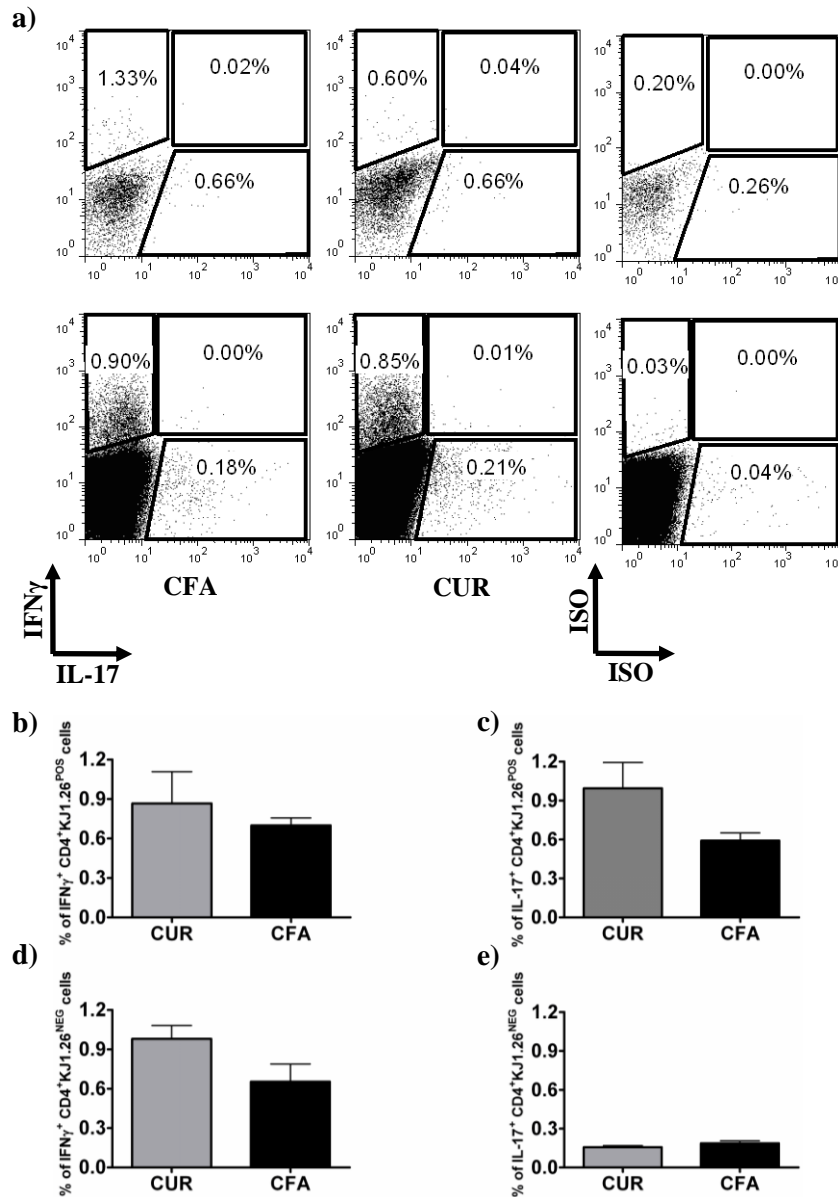
Fig 4.7: Relative ability of curdlan and CFA in promoting *in vivo* Th1/Th17 responses

$CD4^+$ cells from DO11.10 mice were transferred to BALB/c recipients, which were then immunised with OVA/CFA or Cur/OVA. Seven days after immunisation the mice were euthanised and cells from draining LNs were stimulated with PMA/ionomycin and their ability to produce IL-17 and IFNγ was assessed by flow cytometry (a-e). Lymphocytes were identified based on the FSC and SSC profile and then were gated according to the expression of CD4 and KJ1.26 into $CD4^+KJ^{NEG}$ (a-bottom panel, d and e) or $CD4^+KJ^+$ (a-top panel, b and c) populations. Data represent mean ±SEM (n=3).

a)

Th1 Transferred
Population

IFNγ

IL-17

IFNγ

IL-17

CFA/OVA
Day 5

CUR/OVA
Day 5

b)

% of KJ1.26+ CD4+ IFNγ+ cells

c)

% of KJ1.26+ CD4+ IL-17+ cells
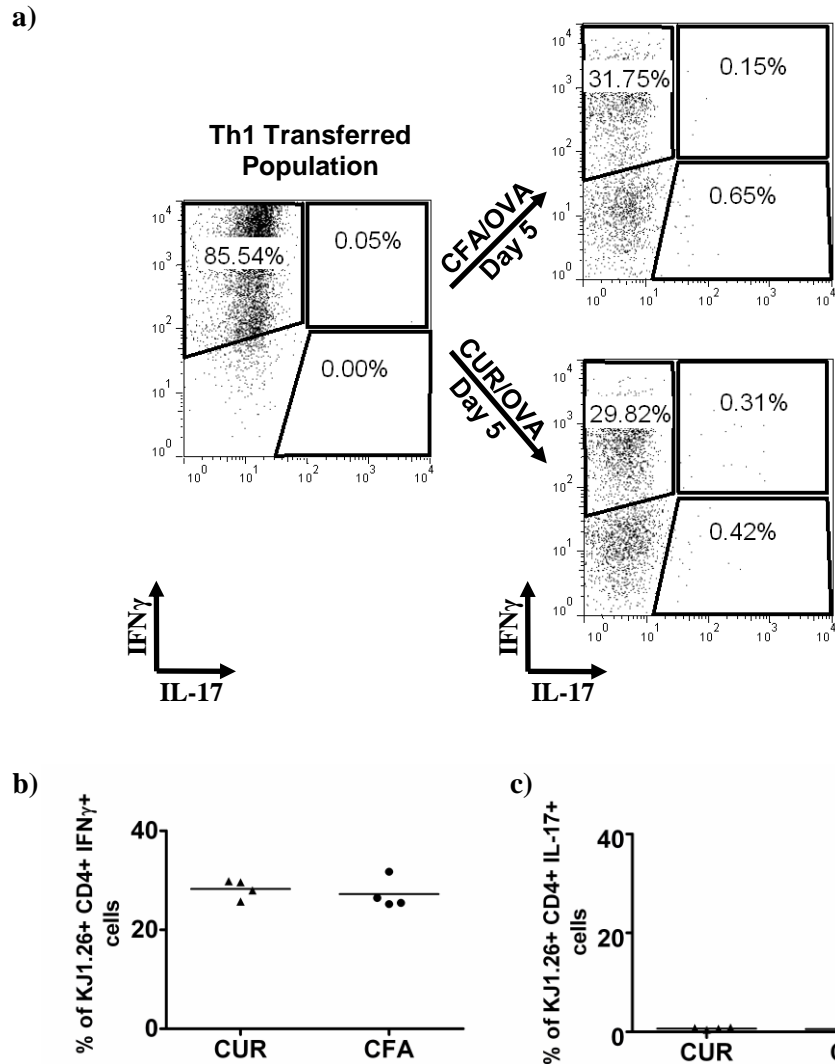
Fig 4.8: Effect on curdlan and CFA on adoptively transferred Th1 polarised cells

Th1 polarised CD4$^+$ cells ((a) left panel) from DO11.10 mice were transferred to BALB/c recipients, which were then immunised with OVA/CFA or Cur/OVA. Five days after immunisation the mice were euthanised and cells from draining LNs were stimulated with PMA/ionomycin and their ability to produce IL-17 and IFNγ was assessed by flow cytometry (a-c). Lymphocytes were identified based on the FSC and SSC profile and transgenic T cells based on KJ1.26 staining and CD4 expression. (n=4).
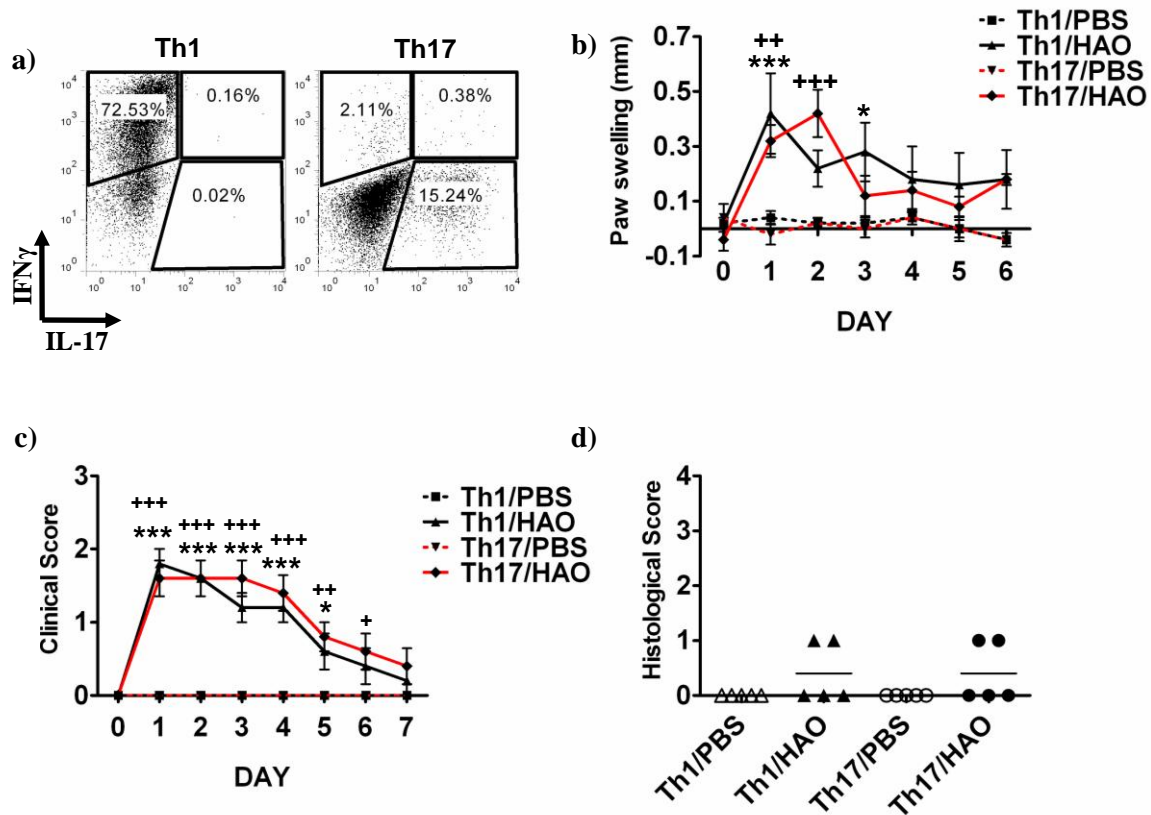
Fig 4.9: Relative ability of Th1 and Th17 cells to induce clinical and histological signs of experimental arthritis

Th1 or Th17 cells from DO11.10 mice (a) were transferred to BALB/c recipients, which were then immunised with OVA/CFA. Ten days after immunisation recipient mice were challenged in the hind paw with HAO. Control mice received PBS. b-c) Arthritis was assessed for 7 days by measuring the difference in paw thickness between the challenged and unchallenged paw (b) and clinical score (c). Data represent mean ±SEM, n=5. d)Ankle joints from challenged hind paws were stained (H&E and toluidine blue) and sections were assessed for histological signs of arthritis. Data represent mean, n=5. Th1/PBS vs. Th1/HAO:*,*<0.05, **<0.01, ***<0.001, Th17/PBS vs. Th17/HAO: +, +<0.05, ++<0.01, +++<0.001
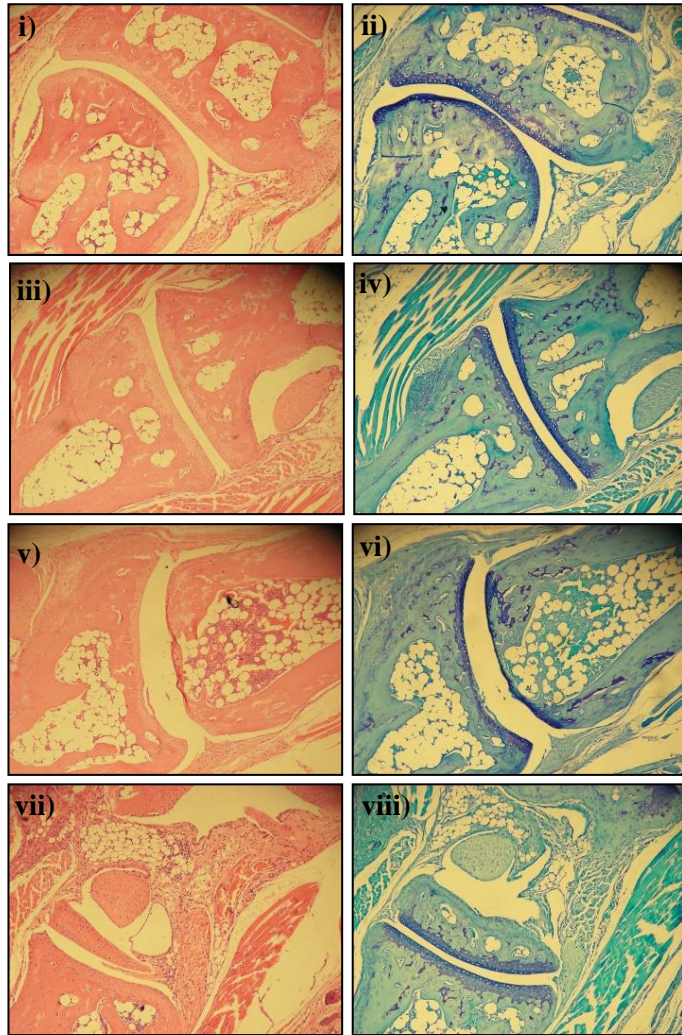
Fig 4.10: Histological signs of arthritis in the Th1 and Th17-induced models
H&E (i,iii,v,vii) and toluidine blue (ii, iv, vi, viii) of ankle joints from Th1 (i and ii) and
Th17 (iii and iv) recipient mice challenged with PBS or Th1 (v and vi) and Th17 (vii and
viii) recipient mice challenged with HAO. Original magnification (x10).
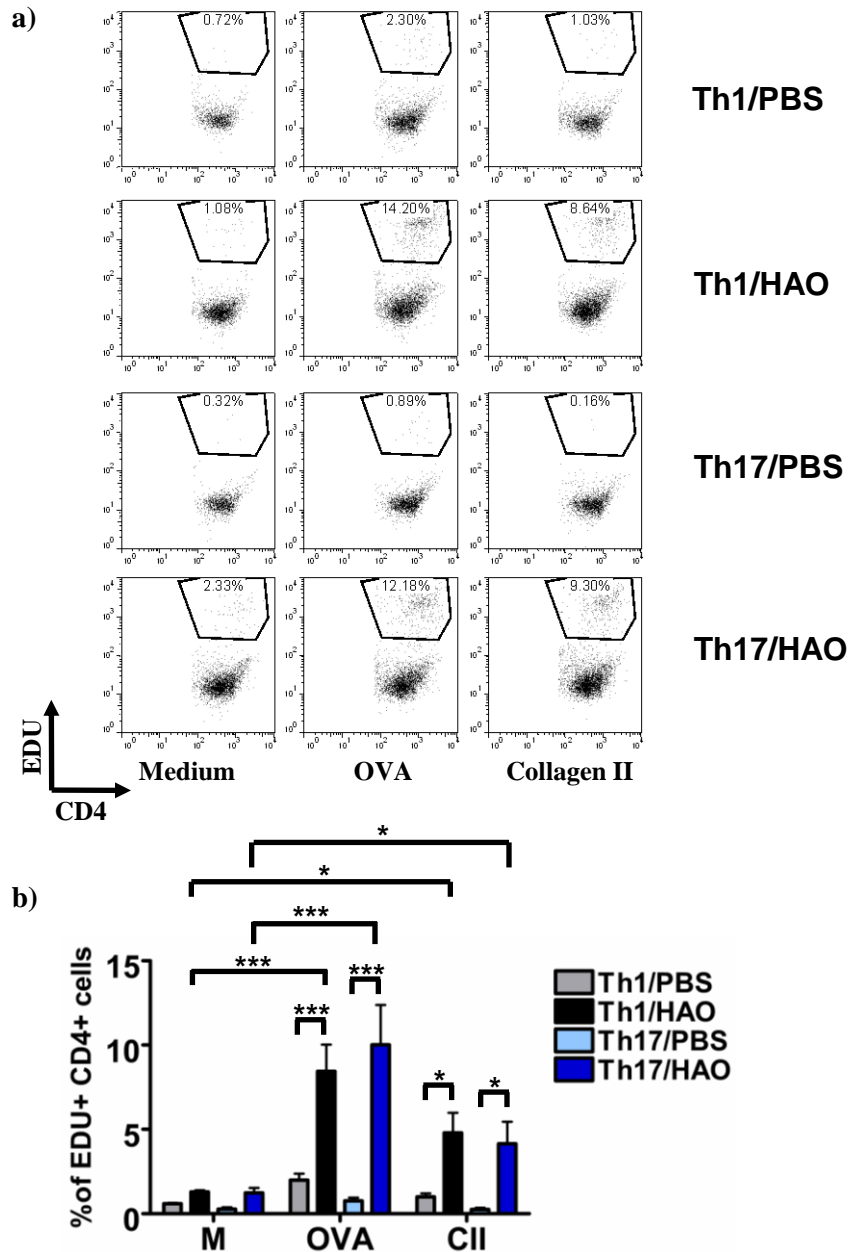
Fig 4.11: Relative ability of Th1 and Th17 cells to mediate breach of self tolerance in experimental arthritis

Th1 or Th17 cells from DO11.10 mice were transferred to BALB/c recipients, which were then immunised with OVA/CFA. Ten days after immunisation recipient mice were challenged in the hind paw with HAO. Control mice received PBS. Seven days after challenge cells from draining LNs were cultured for 72hrs in the presence of either media, OVA or CII and the ability of CD4$^+$ cells to proliferate in response to them was assessed using the Click-iT EDU proliferation assay by flow cytometry (a and b). Lymphocytes were identified based on the FSC and SSC profile and then CD4 T cells based on CD4 expression. Data represent mean ±SEM.*p<0.05, **p<0.01, ***p<0.001 (n=5).
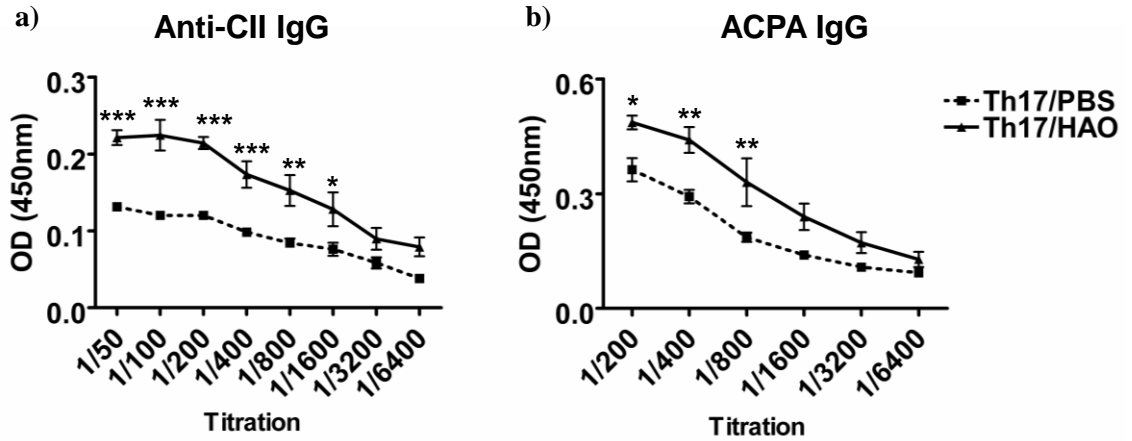
Fig 4.12: Breach of self tolerance in the Th17-induced RA model: anti-CII antibodies and ACPA

Th17 cells from DO11.10 mice were transferred to BALB/c recipients, which were then immunised with OVA/CFA. Ten days after immunisation recipient mice were challenged in the hind paw with HAO. Control mice received PBS. Seven days after challenge mice were euthanised and serum from blood was analysed for the presence of anti-CII (a) and ACPA (b) IgG antibodies by ELISA. Data represent mean ±SEM.*p<0.05, **p<0.01, ***p<0.001 (n=5).
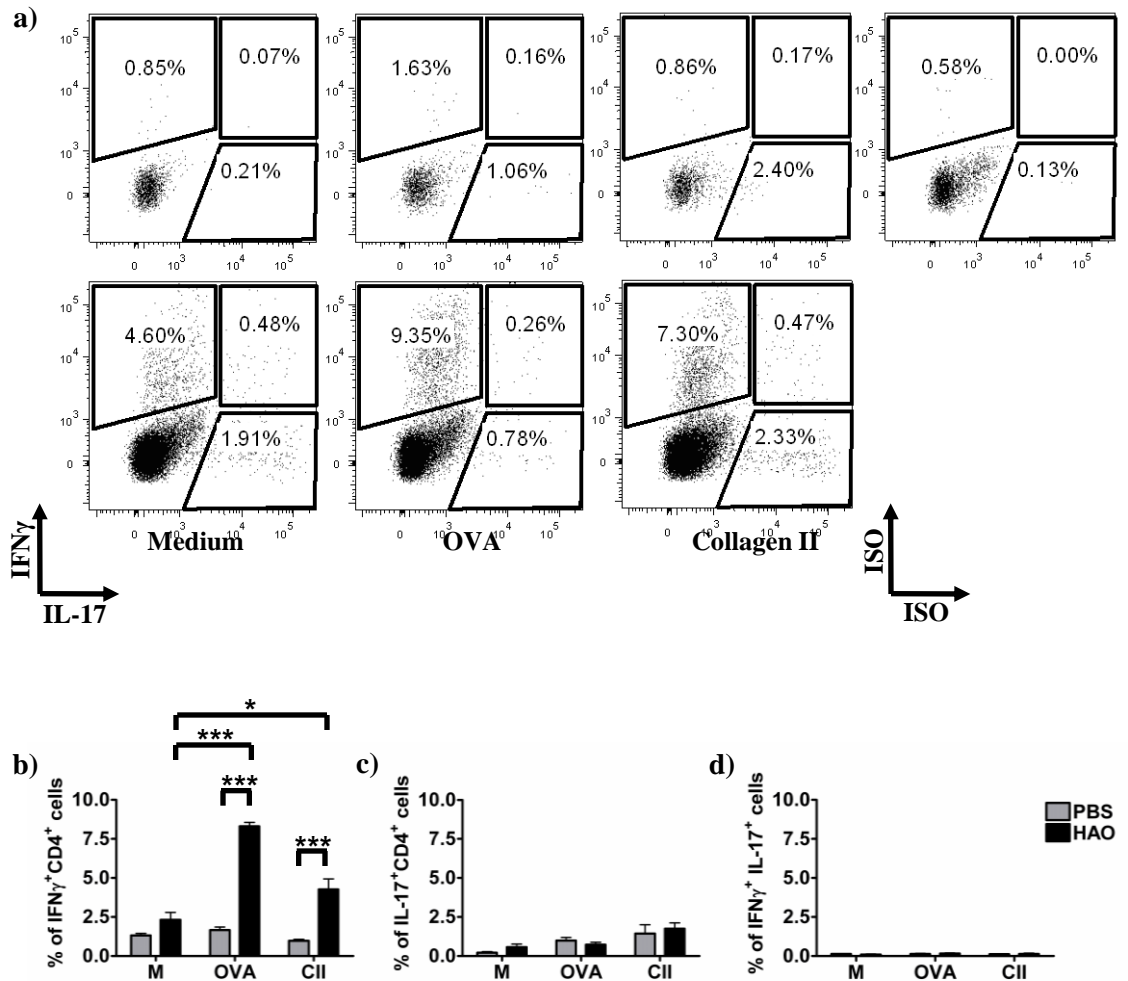
Fig 4.13: Phenotype of Collagen II T cell response in the Th17 OVA-TCR induced RA model

Th17 cells from DO11.10 mice were transferred to BALB/c recipients, which were then immunised with OVA/CFA. Ten days after immunisation recipient mice were challenged in the hind paw with HAO. Control mice received PBS. Mice were culled 7 days after and cells from the draining LN were cultured for 72hrs in the presence of either media, OVA or CII their ability to produce IL-17 and/or IFNγ was assessed by intracellular flow cytometry staining. a) Representative fluorescent cytometry plots demonstrating the production of IL-17 or/and IFNγ by CD4+ cells from a draining LN of a PBS (top panel) or a HAO (bottom panel) challenged mouse. Lymphocytes were identified based on the FSC and SSC profile and then CD4+ T cells based on CD4 expression.b-d) Collective fluorescent cytometry data demonstrating the production of IFNγ (b), IL-17 (c), IFNγ and IL-17 by CD4+ cells from draining LNs of PBS (grey bars) or HAO (black bars) challenged mice. Data represent mean ±SEM.*p<0.05, **p<0.01, ***p<0.001 (n=8).
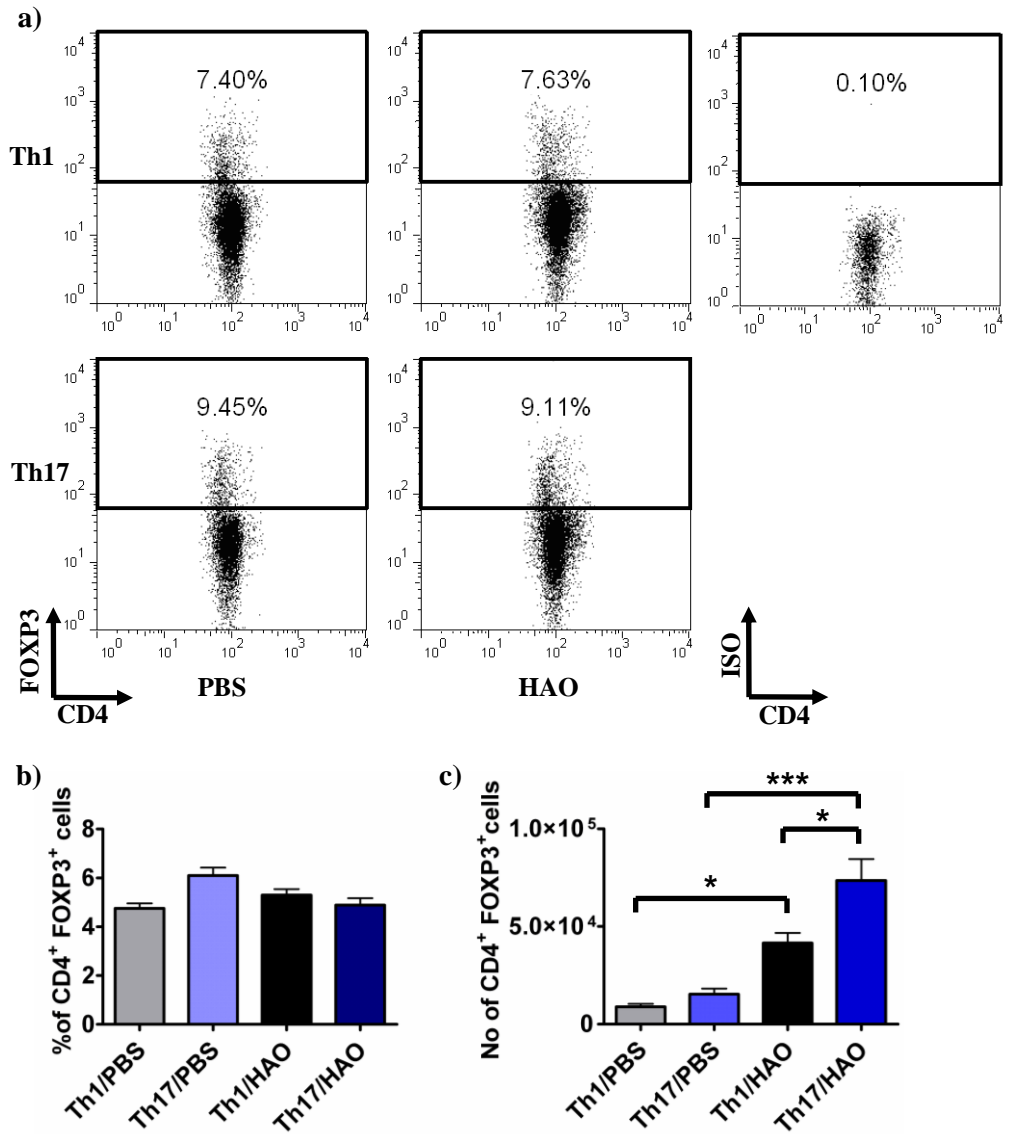
Fig 4.14:Presence of FOXP3$^+$ CD4$^+$ in the Th1 and Th17 OVA-TcR induced arthritis models

Th1 or Th17 cells from DO11.10 mice were transferred to BALB/c recipients, which were then immunised with OVA/CFA. Ten days after immunisation recipient mice were challenged in the hind paw with HAO. Control mice received PBS. At day 7 post challenge mice cells from draining LNs (popliteal) were analysed for the expression of the regulatory marker FOXP3 by flow cytometry. a) Representative flow cytometry plots demonstrating FOXP3 expression from CD4$^+$ cells from Th1 (top panel) or Th17 (bottom panel) recipient mice challenged with PBS (left panel) or HAO (right panel). Lymphocytes were identified based on the FSC and SSC profile and then CD4$^+$ T cells based on CD4 expression.b) Collective flow cytometry data demonstrating the % of CD4$^+$ FOXP3$^+$ cells. c) Number of CD4$^+$ FOXP3$^+$ cells in Th1 or Th17 recipients challenged with PBS or HAO. Data represent mean ±SEM.*p<0.05, **p<0.01, ***p<0.001 (n=5).
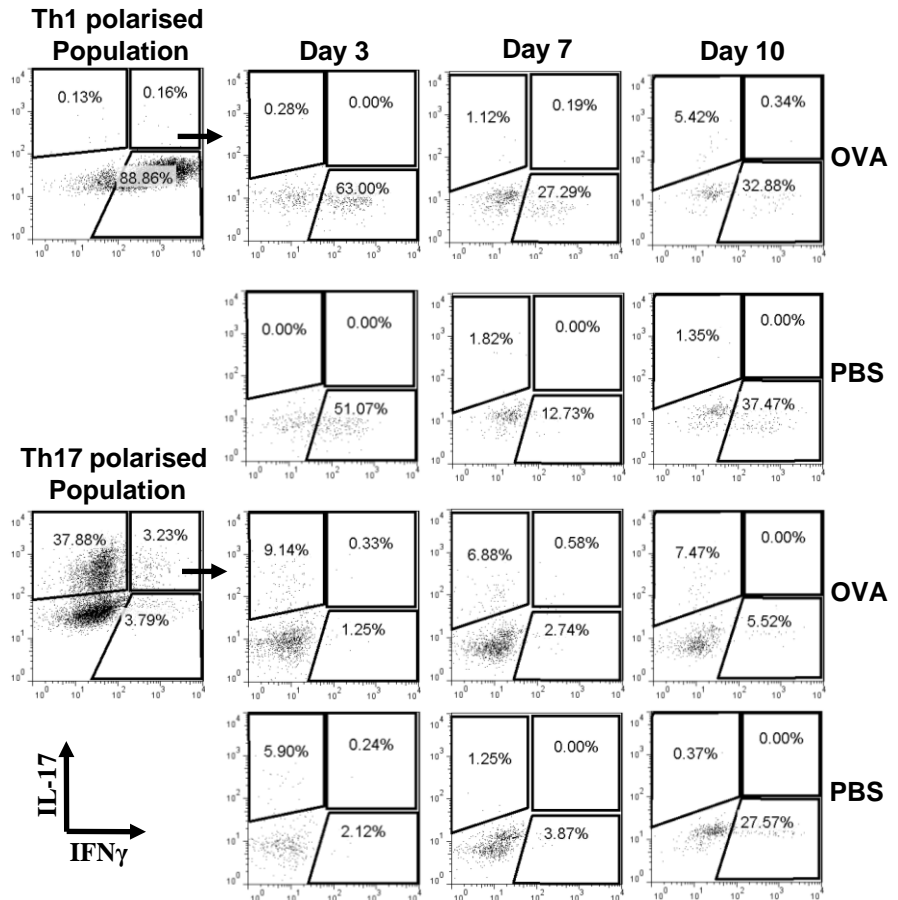
Fig 4.15: Phenotype of Th1 and Th17 polarised population after adoptive transfer.

Th1 or Th17 polarised CD4^{+} cells from DO11.10 mice were adoptively transferred to BALB/c mice, which where then immunised with OVA in CFA. Control mice received PBS. Mice were euthanised at days 3, 7 and 10 post immunisation and cells from draining LNs analysed for the expression of IL-17 and IFNγ by flow cytometry. Representative flow cytometry plots from Th1 recipient mice immunised with OVA/CFA or PBS (top two panels) or Th17 recipients immunised with OVA/CFA or PBS (bottom two panels). Lymphocytes were identified based on the FSC and SSC profile and transgenic T cells based on KJ1.26 staining and CD4 expression.
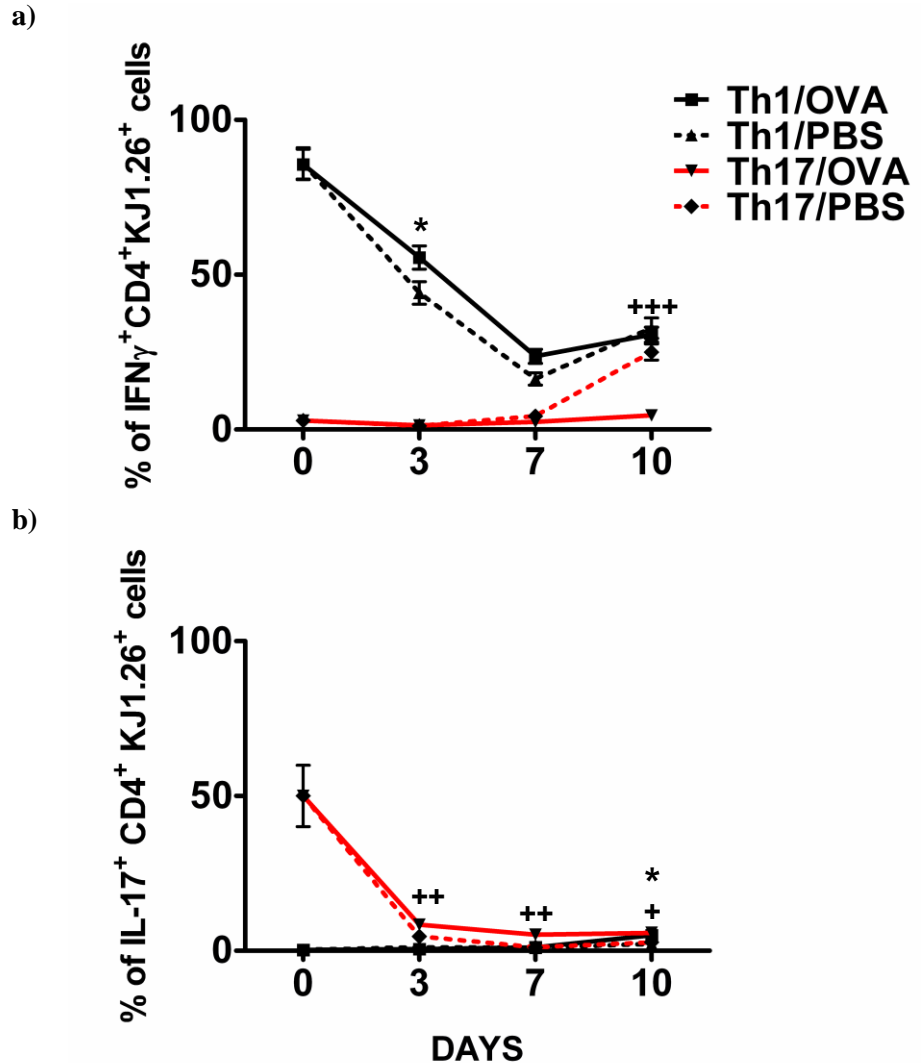
Fig 4.16: Phenotype of Th1 and Th17 polarised population after adoptive transfer.

Th1 or Th17 polarised CD4^{+} cells from DO11.10 mice were adoptively transferred to BALB/c mice, which where then immunised with OVA in CFA. Control mice received PBS. Mice were euthanised at days 3, 7 and 10 post immunisation and cells from draining LNs were stimulated with PMA/ionomycin and analysed for the expression of IL-17 and IFNγ by flow cytometry Time course of IFNγ (a) and IL-17 (b) expression from CD4^{+} KJ1.26^{+} cells from Th1 or Th17 recipient mice immunised with OVA/CFA or PBS. Day 0 represents the % of IFNγ and IL-17 expressing cells of the transferred Th1 and Th17 populations. Data represent mean ±SEM, *: Th1/OVA vs. Th1/PBS +: Th17/OVA vs. Th17/PBS.*,+$p<0.05$, **,++$p<0.01$, ***,+++$p<0.001$ (n=3).
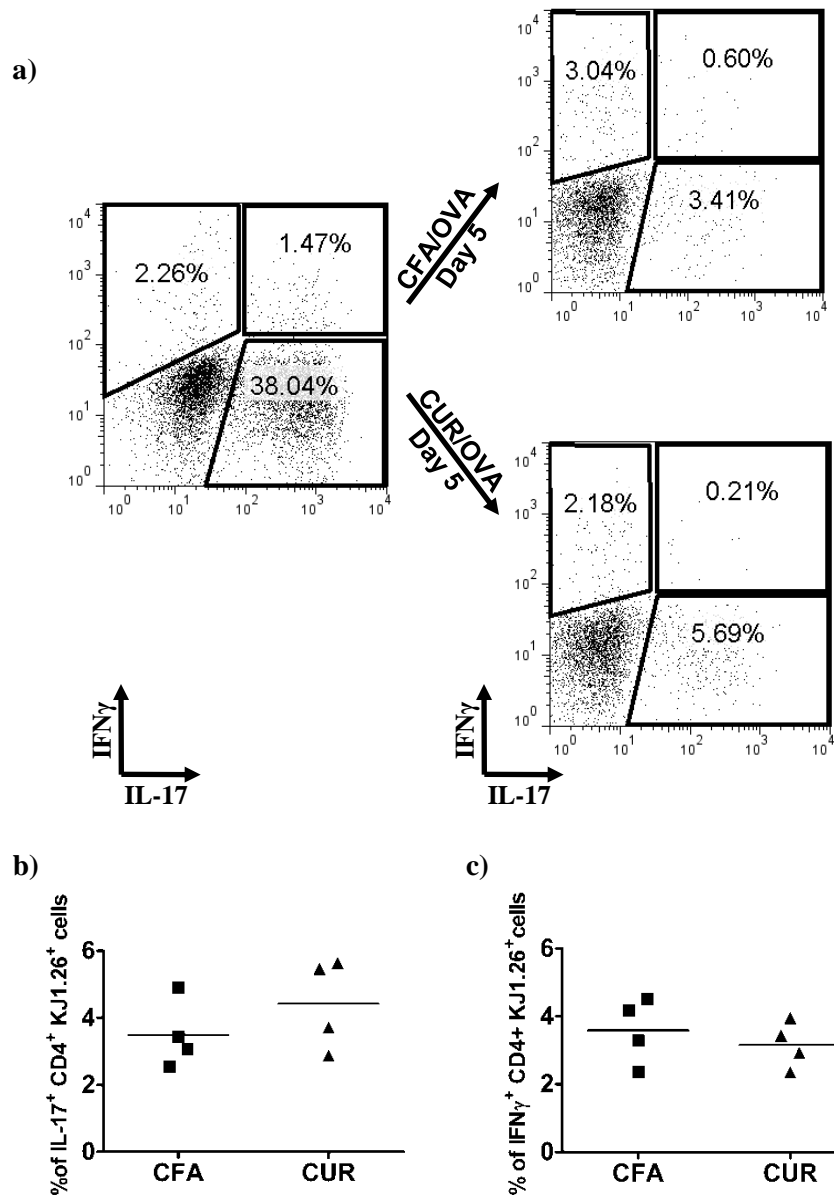
Fig 4.17: Effect on Curdlan and CFA on adoptively transferred Th1 polarised cells

Th17 polarised CD4^{+} cells ((a) left panel) from DO11.10 mice were transferred to BALB/c recipients, which were then immunised with OVA/CFA or Cur/OVA. Five days after immunisation the mice were euthanised and cells from draining LNs were stimulated with PMA/ionomycin and their ability to produce IL-17 or IFNγ was assessed by flow cytometry (a-c). Lymphocytes were identified based on the FSC and SSC profile and transgenic T cells based on KJ1.26 staining and CD4 expression. (n=4).
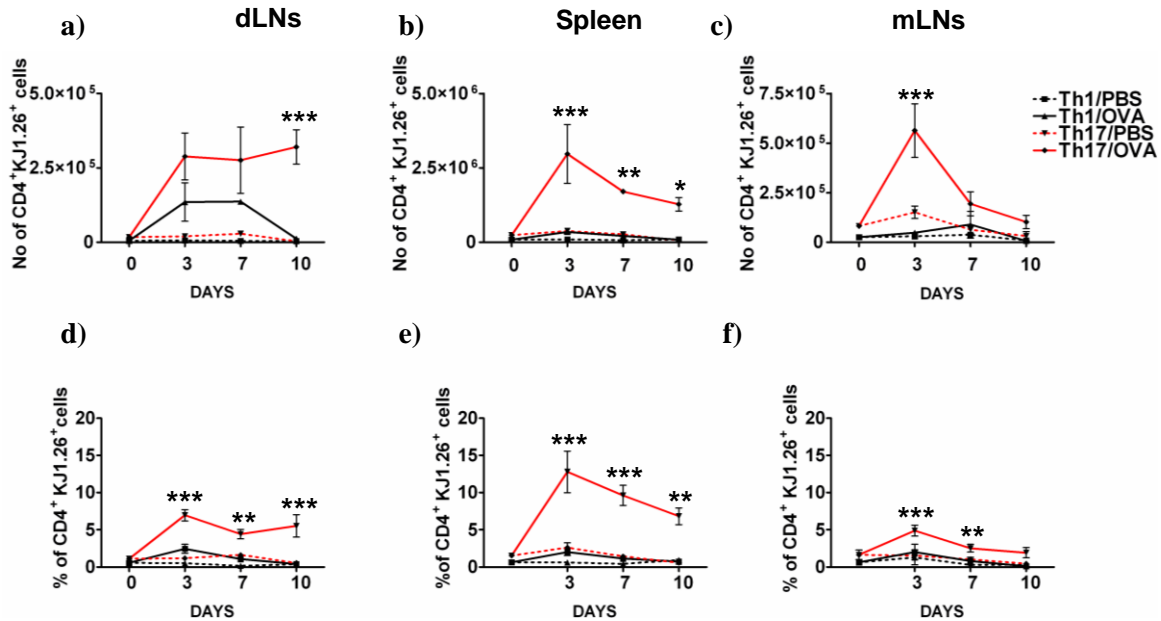
Fig 4.18: Distribution and expansion of Th1 and Th17 polarised population after adoptive transfer.

Th1 or Th17 polarised CD4+ cells from DO11.10 mice were adoptively transferred to BALB/c mice, which where then immunised with OVA in CFA. Control mice received PBS. Mice were euthanised at days 3, 7 and 10 post immunisation and cells from draining LNs (axillary and brachial, a and d), spleens (b and e), mesenteric LNs (c and f) were analyzed for the presence of transgenic T cells by flow cytometry. Lymphocytes were identified based on the FSC and SSC profile and transgenic T cells based on KJ1.26 staining and CD4 expression. a-c) Total number of transgenic T cells in the draining LN (a), spleen (b) and mesenteric LNs (c). d-f) Percentage of transgenic T cells in the draining LN (d), spleen (e) and mesenteric LNs (f). *: Th1/OVA vs.Th17/OVA. Data represent mean ±SEM.*p<0.05, **p<0.01, ***p<0.001 (n=3).
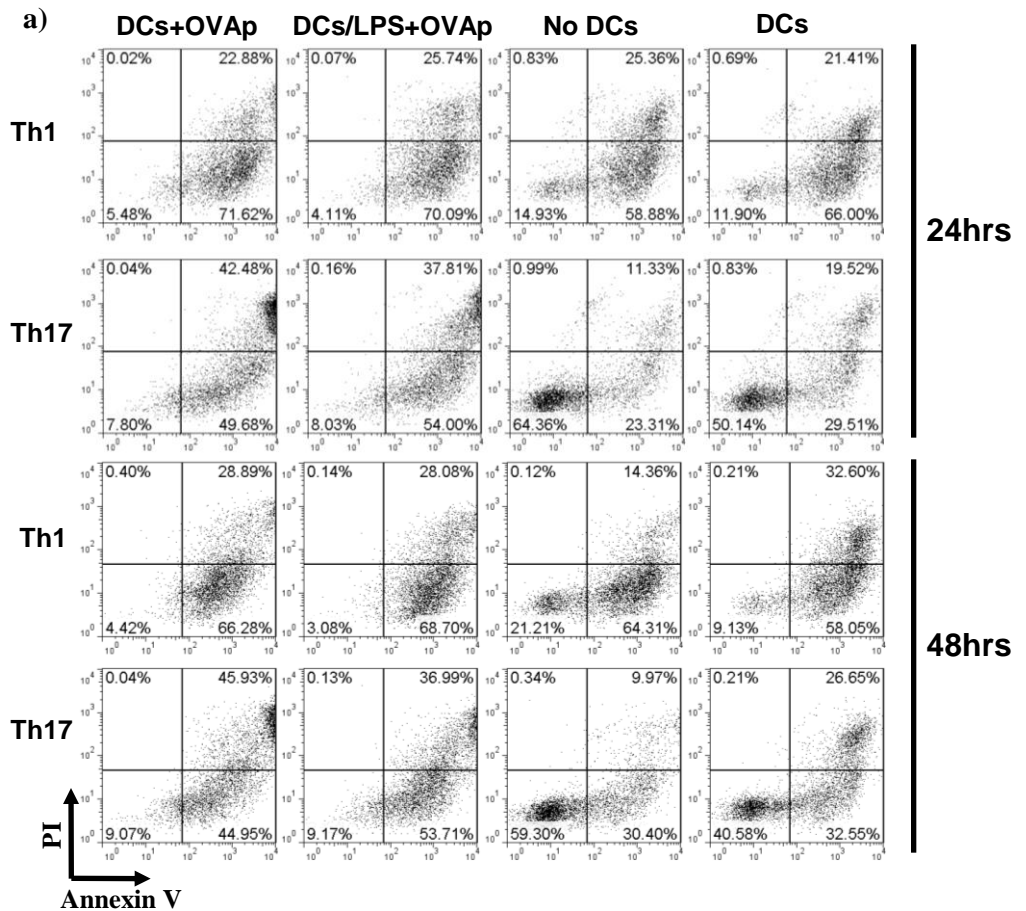
Fig 4.19: Viability of Th1 and Th17 polarised populations

MACS sorted CD4^{+} T cells from DO11.10 mice were polarised towards a Th1 or Th17 phenotype and were cultured either alone or with bone marrow-derived DCs, unpulsed or pulsed with $OVA_{323-339}$, or $OVA_{323-339}$ and LPS. Viability was assessed at two time-poinsts, 24hrs or 48hrs by PI and annexin V staining by flow cytometry Representative flow cytometry plots, gated on CD4^{+} KJ1.26^{+} cells, demonstrating viability staining of Th1 and Th17 polarised cells on 24hrs (top two panels) and 48hrs (bottom two panels).
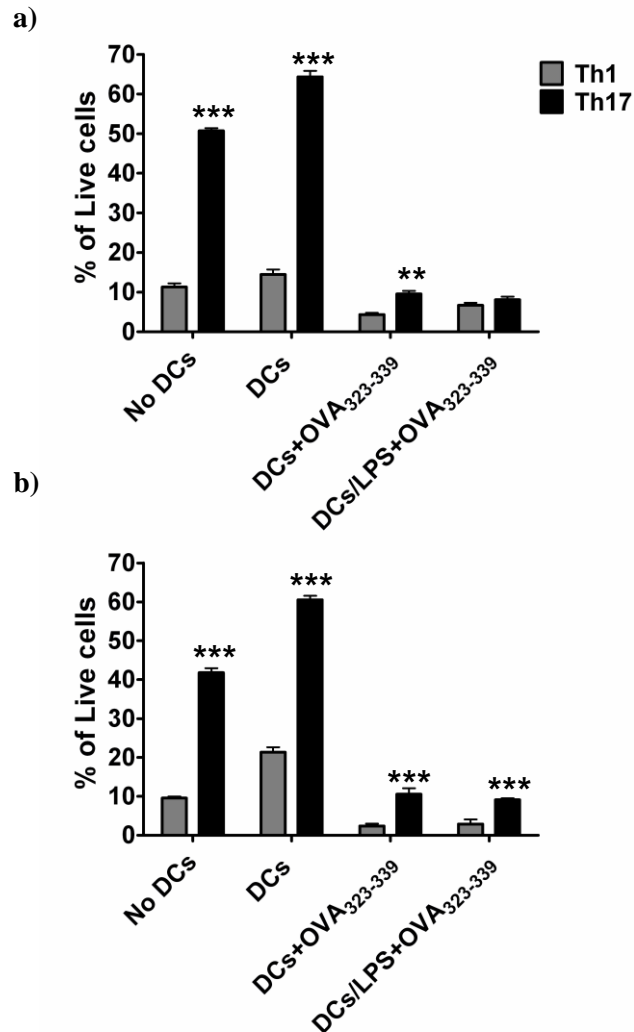
Fig 4.20: Viability of Th1 and Th17 polarised populations

MACS sorted CD4^{+} T cells from DO11.10 mice were polarised towards a Th1 or Th17 phenotype and cultured either alone or with bone marrow-derived DCs, unpulsed or pulsed with $OVA_{323-339}$, or $OVA_{323-339}$ and LPS. Viability was assessed at two time-poinsts, 24hrs (a) or 48hrs (b) by PI and annexin V staining by flow cytometry . Lymphocytes were identified based on the FSC and SSC profile and transgenic T cells based on KJ1.26 staining and CD4 expression. Collective fluorescent cytometry data demonstrating percentage of live cells (i.e. Annexin- V^{NEG}/PI^{NEG}). Data represent mean $\pm$SEM.*$p<0.05$, **$p<0.01$, ***$p<0.001$ (n=3).

4.4 Discussion

In this chapter the role of Th17 effector cells in the breach of self tolerance that characterize the early phase of RA pathogenesis was investigated. In order to do this a model of breach of self tolerance, in the context of arthritis, was employed, in which a Th1 response against an irrelevant antigen (OVA) results to arthropathy that is characterized by the development of auto-reactivity in the form of T and B cell responses against various auto-antigens, namely CII. As Th17 have been linked to various models of autoimmunity, we hypothesized that if these cells are involved in the breach of self tolerance observed in our model then self specific Th17 cells could be identified. When the CII specific response was characterized phenotypically, it was revealed that it was of a Th1 type. The phenotype of the CII specific response was not altered even when curdlan, a yeast-derived β-glucan adjuvant that has been reported to induce Th17 responses(463;478), was employed. As Th1-derived cytokines, such as IFNγ, have been shown to be regulatory in some autoimmunity models, it was postulated that a model induced by a Th17 population would induce more robust breach of self tolerance that would potentially result in more severe pathological signs. Th17 cells could induce breach of self tolerance in the form of B and T cell responses against CII. However, these responses were similar to the ones induced by the Th1 population. Morever, Th17 cells did not induce more severe or chronic clinical signs of the disease or enhanced histological damage. Interestingly, the phenotype of both the OVA and CII CD4$^+$ T cell responses in the Th17-induced model was characterized by IFNγ production. This could be due to the fact that the transferred Th1 population retained, even partly its IFNγ production, whereas the Th17 population was characterized by a sharp decline of its IL-17 production. Apart from these, it was demonstrated in this chapter that the two populations differ dramatically in their expansion, distribution and kinetics, with the Th17 population expanding in a greater degree and persisting for a longer time period in the secondary lymphoid tissues examined. This could partly be explained by the greater viability demonstrated by cells polarised under Th17 conditions compared to their Th1 counterparts.

Th17 cells have been linked to various autoimmunity models, such as the EAE model of multiple sclerosis and the CIA and SKG model of arthritis, to name a few(8;9;477;478). It was thus surprising to find that the auto-reactive response in both Th1 and Th17-induced models was characterized by the absence of IL-17$^+$CD4$^+$ cells. In the case of the Th1-induced arthritis model, it was postulated that the highly polarised initiating OVA-specific population might skew the emerging auto-reactive T cell responses towards a Th1 phenotype. Indeed, it has been reported that Th1 cytokines, namely IFNγ, inhibit the development of Th17 cells, whereas at the same time induce the generation of Th1 cells(285;586). In order to test this hypothesis curdlan was employed as a Th17-inducing adjuvant. As mentioned previously, curdlan is a yeast β-glucan that specifically acts through a C-type lectin, dectin-1, and conditions DCs to promote Th17 responses both *in vitro* and *in vivo*(463). More importantly, a single injection of curdlan can induce chronic arthritis in SKG mice, which are resistant to disease development when kept in a pathogen-free environment, and transient arthritis in normal BALB/c mice(82). Interestingly the clinical development of the disease is accompanied by the development of Th17 cells in the affected joints(477). It should be noted, however, that CFA also has been reported to induce IL-17-producing CD4$^+$ cells, through IL-6 production, and is widely used in models that have been linked with Th17 cells, such as EAE and CIA(448). In the Th1-induced model curdlan was equally effective as CFA in inducing breach of self tolerance, however its employment did not lead to increased pathology nor to an enhancement of the Th17 element of the auto-reactive response. It was speculated that this was due to failure of curdlan to induce *in vivo* Th17 polarisation of naïve CD4$^+$ T cells in our system and in addition it did not had any effect on the Th1 polarised population, which retained its IFNγ production and thus its potential to skew any emerging auto-reactive responses to a Th1 phenotype. This is no surprise as curdlan has been shown to promote both Th1 and Th17 responses *in vivo*(463). Interestingly, even in the Th17-induced model the phenotype of the auto-reactive CD4$^+$ response was characterized by IFNγ production. This could suggest that it is IFNγ producing

CD4$^+$ cells that promote autoimmunity in the OVA-TcR-induced arthritis model. There are various ways that IFNγ-producing CD4$^+$ T cells could promote breach of self tolerance. Our group has previously demonstrated that cDCs are the APCs that drive the arthritogenic autoimmunity in our model(7). We have proposed that the OVA-specific memory population creates an environment that alters the characteristic of the cDCs and allows reversal of their tolerogenic interaction with autoreactive T cells and priming of auto-reactivity(7). It is possible that this is an effect mediated by IFNγ produced by T cells. Indeed, IFNγ has been reported to up-regulate MHCII on DCs and alongside with co-stimulation signals such as CD40L might be required for optimal expression of IL-12 by these cells(587;588). On the other hand, other studies report that IFNγ treated DCs afford protection against the development of diabetes in the NOD mouse and reduce autoantibody production in a model of autoimmune myasthenia gravies(589;590). It will therefore be crucial to block IFNγ in both Th1 and Th17 OVA-TcR arthritis models to determine its role in the development of autoimmunity. An approach to do that would be to use an antibody against IFNγ, before the HAO challenge. This would neutralize any IFNγ produced by either transferred cells, emerging autoreactive CD4$^+$ cells, or any host cell that could produce this cytokine. Antibody blocking of IFNγ function was employed, however due to immunogenicity of the isotype control these results are not presented in this thesis. Even though this approach would reveal the significance of IFNγ in the model it would not reveal the cellular source of the cytokine. An approach using mice deficient in IFNγ production would be able to give an answer to this question. In detail, employment of IFNγ deficient DO11.10 would reveal if IFNγ derived from the transfer transgenic T cells is crucial for the breach of self-tolerance, whereas CD4-specific IFNγ deficient recipients would reveal if the IFNγ from the host CD4$^+$ cells mediates the breach of self tolerance. The latter could be achieved using a conditional knock-out system, where *loxP* sequences would flank the *IFNγ* gene and *Cre* recombinase would be promoted by a CD4$^+$ specific promoter. The absence of IL-17$^+$ CD4$^+$ T cells should not exclude the involvement of Th17 as this is not the only cytokine that these cells produce and in addition they may localise in a difference site than the Th1 cells. It has been

reported that apart from IL-17, Th17 cells produce IL-17F, TNF, IL-21 and IL-22(255;454;477;591). Similarly, Th1 cells also produce other cytokines apart from IFNγ, such as IL-2 and TNF(592;593). There are studies that involve this cytokines in the RA pathology(484-486;594), and specifically for TNF there is a very well established role in disease development, which is demonstrated by the efficacy of TNF blocking therapies(595). Our group has previously reported that the development of autoimmune B and T cell responses in the OVA-TcR-induced arthritis model are TNF-dependent(7). As both Th1 and Th17 induce breach of self tolerance, and have been reported to produce this cytokine, it is possible that both cell types mediate development of autoimmunity through TNF production. A more detailed analysis of the cytokine profile of the CII-specific response could give more evidence for the phenotype of the developing auto-reactivity. As Th17 cells have been reported to express CCR6 and selectively being recruited to the joint, via CCL20(478), it would be useful to investigate the phenotype of the CD4$^+$ T cell that localise in the joints in both Th1 and Th17 models, as this will give information for the tissue specific environment in which APCs are conditioned and acquire antigens and potentially auto-antigens. To definitely determine the role of Th1 and Th17 in the development of autoimmunity in our system approaches that will inhibit their generation must be employed. Development of CD4$^+$-specific conditional knock-out mice for key transcription factors such as RORγ for Th17 or T-bet for Th1, which would be used as recipients, could reveal if a host Th17 or Th1 response is required for the development of autoimmunity in our models. For Th17 cells a more approachable pharmacological method could be employed. The small molecule halofuginone, a derivative of the plant alkaloid febrifugine(596), has been reported to specifically inhibits mouse and human Th17 development by activating a cytoprotective response, the amino acid starvation response(597). This molecule could be employed in both Th1 and Th17 models, before HAO challenge, to selectively inhibit the development of any Th17 response.

Another important finding reported in this study is the failure of the cells polarised under Th17 conditions to retain their ability to produce IL-17 after adoptive transfer,

unlike cells polarised under Th1 conditions which retained at some degree their ability to produce IFNγ. The plasticity of the Th17 phenotype has been reported previously, where highly purified Th17 cells acquired a Th1-like phenotype after adoptive transfer in a model of diabetes(492). As in this study the populations used were not purified it is not possible to suggest plasticity as the only explanation for the reduced percentage of IL-17$^+$ cells. As it was not possible to investigate the phenotype of the cells in the OVA/CFA injection site, a preferential localisation of the IL-17$^+$ CD4$^+$ cells at this site cannot be excluded. Th1 and Th17 have a distinct chemokine receptor profile, with Th1 cells mainly expressing CXCR4, CXCR6 and CCR5 whereas Th17 have been reported to express CCR6, CCR4 and CCR2(478;598-601). Most of these chemokine receptors will drive cells to inflammatory sites, however a receptor profiling of the transferred population could reveal if there is a potential for preferential recruitment for the IL-17$^+$CD4$^+$ transgenic cells to injection site. Interestingly, in humans, CCR6 expression correlates highly with IL-17 expression, which would agree with a preferential localisation of IL-17-producing cells to sites of inflammation(601). This was not the only difference between the two populations as cells polarised under Th17 conditions expanded to a greater degree and persisted longer compared to cells polarised under Th1 conditions. One possible explanation for this difference is the greater viability of cells polarised under Th17 conditions demonstrated in this chapter. Published reports suggest that Th17 cells are more resistant to activation-induced cell death (AICD) compared to Th1 cells, a phenomenon possibly mediated by a reduced expression of FasL by cells polarised under Th17 conditions(602). In addition, there is a well documented role for IFNγ in AICD of effector T cells(603;604), which could suggest that this cytokine mediates increased cell death in the Th1 polarised cells. It would be useful to assess the proliferative capacity of the two populations after transfer as this could show if there is a relative advantage in cells polarised under Th17 conditions. A study that compared this used CFSE dilution and reported that cells polarised under Th1 conditions exhibit a faster pace of proliferation compared to Th17 cells(602), which seems to contradict to the significantly higher expansion of the cells polarised under Th17 reported in

this chapter. An alternative explanation would be a preferential recruitment of cells polarised under Th17 to secondary lymphoid tissues. This is supported by studies that report that *in vitro* polarised Th17 cells migrate poorly to inflammation and preferentially locate in the spleen(605) due to a lack of expression of CCR5 and CXCR3(605). Other studies suggest that the presence of TGFβ induces CCR7 expression which could lead to a preferential recruitment of the Th17 polarised population to the secondary lymphoid organs(599).   As previously mentioned, a profiling of the chemokine receptor expression of both transferred populations would give information that could explain the differences observed in this study.

In this chapter it has been demonstrated that both Th1 and Th17 induce similar levels of inflammation and breach of self tolerance, in the form of B and T cell responses against CII. As in both models the autoreactive T cell response was characterized by IFNγ production, it is possible that this cytokine mediates the early immunological events that lead to breach of self tolerance. It is of high priority however to employ methods that will block the Th1 and Th17 cells and the cytokines they produce to definitely define the role of these cells in the development of autoimmunity. As there is a well documented role for B cells in the development of autoimmunity, and both OVA-TcR-induced models are characterized by the presence of various autoantibodies the next question investigated in this thesis related to the role of Th17 cells in the T cell-dependent B cell responses.

Chapter 5: The role of Th17 cells in B cell responses

5.1 Aim and rationale

In this chapter the role of Th17 cells in T cell-dependent B cells responses was investigated. More specifically their relative ability, compared to a Th1 polarised population, to support antigen specific B cell responses was assessed. The role of $CD4^+$ T cells in antibody generation is well established and it is now widely accepted that a specific T helper subtype, named TFH, involved in regulating various aspects of antigen-specific B cell responses(606). Our group has previously reported that both *in vitro* and *in vivo* generated Th1 and Th2 cells have similar ability to support antibody responses *in vivo*(361;362). However the role of role of inflammatory Th17 cells in B cell responses is relatively understudied. While at first glance it might not be expected that such effector cells might provide B cell help it could be argued that they would be involved in driving B cell responses to deal with pathogens they target, such as fungi. The presence of class switched antibodies in RA patients suggests an active T-cell dependent B cell response. As Th17 cells have been suggested to be important in some aspects of RA pathology and in animal models of the disease(12;13) it is of considerable importance to investigate their ability to support antibody responses as this may allow to have a better understanding for their role in disease development. In order to do this an adoptive transfer approach was employed where *in vitro* Th1 or Th17 polarised antigen-specific TcR transgenic populations were transferred to congenic recipient mice along side antigen-specific B cell receptor (BcR) transgenic B cells(138;361;362). This approach provides the ability to examine the relative effect of Th1 and Th17 populations on antigen specific B cell expansion, antibody production and differentiation. In addition it allows the tracking and localisation of the antigen specific B and T *in situ*. This approach was also extended to investigate Th17 of TFH and germinal centre B cell responses in the pathogenesis of murine RA models.

5.2 Introduction

As mentioned previously RA is a disease that is characterised by the presence of a number of class switched auto-antibodies[1]. In addition the effectiveness of B cell depletion as a therapeutic intervention signifies even more the importance of B cell responses in the development of the disease[607]. Isotype switched, high affinity antibody responses to protein antigens require cognate interaction between the antigen-specific B cell and the activated antigen-specific Th cell within the microenviroment of the secondary lymphoid tissue[138;606]. Naïve T cell are activated in the paracortex by professional APCs, such as DCs, and move to the outer edge of the B cell follicle where they interact with an antigen specific B cell which has also previously encountered antigen and has moved to the same location[138;365;608]. When the Th1 and Th2 subset were originally discovered, a division in the quality of the immune response was proposed with Th2 cell mediating humoral and Th1 cells cell-mediated immunity[609]. This was suggested mainly because Th2 cells characteristically produce cytokines that have been implicated in various stages of B cell proliferation and differentiation(610-612). However, there are a number of studies that demonstrate that both Th1 and Th2 cells are able to support B cell responses both *in vitro* and *in vivo*(361;362;612). More specifically, our group has reported that both *in vitro* and *in vivo* Th1 and Th2 polarised cells are able to migrate to the follicle to support B cell clonal expansion, differentiation and antibody production to a similar degree. More importantly, it was demonstrated that IFNγ producing CD4 cells migrate into the B cell follicle to interact with antigen specific B cells[361;362]. With the expansion of the T helper subset beyond the Th1 and Th2 phenotypes it is now considered that the CD4^{+} T cells that migrate into follicles and support B cell responses constitute a distinct T helper subset termed TFH cells(613). These cells are characterized by the sustained expression of the chemokine receptor CXCR5, costimulatory molecules such as ICOS, PD-1, CD40L and OX40, and cytokines, most important amongst other IL-21[363;364;370;422;614]. How TFH cells relate to the other T helper subtype is not yet very well established. There is evidence that suggest that TFH cells constitute a

truly distinct T cell subset, which develops independently of Th1, Th2 and Th17 cells(369). This is supported by the distinct transcriptional regulation of TFH cells, with Bcl-6 acting as the master regulator of TFH development, while inhibiting the generation of other effector phenotypes[374;376]. However there is evidence that argue against a distinct TFH phenotype. Firstly, most activated CD4$^+$ cells up-regulate CXCR5 transiently. Furthermore, it was shown recently that after transfer into naïve mice and antigenic challenge, CXCR5$^-$ PD-1$^-$ IL-4/GFP$^+$ CD4$^+$ T cells could develop into TFH cells, whereas TFH cells have been reported to co-express the Th2 transcription factor GATA-3 and produce IL-4 and IFNγ[366;614]. This could suggest either that plasticity exists within Tfh cells, or that distinct subsets, like Th1/Th2/Th17 Tfh cells, exist within the Tfh cell compartment. Indeed, a study suggests that the human blood CXCR5$^+$CD4$^+$ cells constitute a memory TFH compartment that can be subdivided into Th1, Th2 and Th17-like cells[368].

The role of Th17 cells in supporting B cell responses is not extensively studied, however there are evidences that suggest a possible role of IL-17 producing CD4$^+$ in B cell responses. The autoimmune prone BXD2 mice, which express more IL-17 and have elevated Th17 cells compared to wild type, show spontaneous development of germinal centres, followed by the production of pathogenic autoantibodies[615]. Importantly, IL-17-producing cells, most of which were CD4$^+$, were localised near the germinal centre region, which was also characterized by the presence of IL-17R$^+$ germinal centre B cells(615). Interestingly, inhibition of IL-17 or IL-17R signalling resulted in reduced germinal centre formation(615). In addition, the existence of IL-17 producing TFH cells has been reported both in mice and humans[367;368]. Even though these studies implicate IL-17-producing CD4$^+$ cells in aspects of the B cell immunity none of them followed the development of an antigen specific Th17-dependent B cell response and only indirectly involves these cells in antibody production. This leaves many questions relating to the mechanistics of this function unanswered, especially whether antigen-specific IL-17-producing CD4 cells directly interact with cognate B cells, promote germinal centre formation and antibody production. In addition, the quality of a Th17-driven B cell response is understudied. It is very well established

that in mice Th1 immunity is characterized mainly by the production of IgG2a antibodies whereas in Th2 immunity by IgG1 and IgE[361;362;609-612], however the type of antibody response that characterizes Th17-mediated immune responses is relatively unknown.

As Th17 are suggested to be important in some aspects of RA, a disease characterized by the presence of class switched autoantibodies, it would be useful to investigate the role of these cells in supporting B cell responses.

5.3 Results

In order to investigate the relative ability of Th1 and Th17 cells to support B cells an adoptive transfer approach was employed that allowed the tracking of antigen-specific B and T cells. This is an adaptation of a previously described model in which the response of BcR transgenic cells depends on cognate help provided by antigen specific transgenic T cells[138;361;362]. In detail, OVA-specific T cells from DO11.10 mice were polarised under Th1 or Th17 polarising conditions and adoptively transferred with HEL-specific B cells from MD4 mice to IgH^b congenic recipient mice. The mice were immunised with a HEL-OVA conjugate in CFA. This facilitates the cognate interaction between the transgenic B and T cells, as B cell acquire the HEL-OVA antigen through their BcR, process it and present OVA$_{323-339}$ peptide to the T cells. Transgenic T cells were detected using the KJ1.26 monoclonal antibody that recognises their TcR. Transgenic B cells were tracked using an anti-IgM^a monoclonal antibody as host B cells express the IgM^b haplotype.

5.3.1 Clonal expansion of antigen specific T cells

CD4$^+$ T cells from DO11.10 mice were polarised under Th1 or Th17 conditions and their phenotype in respect to the expression of the clonotypic DO11.10 TcR receptor and the production of IL-17 and/or IFNγ was assessed by flow cytometry (Fig 5.1a). As expected, in both Th1 and Th17 polarisations the vast majority of T

cells were expressing the DO11.10 TCR. In addition, the intracellular staining confirmed the phenotype of the two transferred population, with Th1 characterized mainly by IFNγ$^+$ cells and Th17 by IL-17$^+$ cells. At the same time splenocytes from MD4 mice were analyzed for the presence of transgenic HEL-specific B cells by assessing their ability to bind a biotinylated-form of HEL (Fig 5.1b). Flow cytometric analysis revealed that MD4 mice carry exclusively transgenic B cells. Polarised trangenic T cells and HEL-specific transgenic B cells were then transferred into congenic IgHb mice which were immunised with HEL-OVA/CFA. Control mice were injected with PBS. The draining LNs were removed from recipient mice and the presence of the transferred T cells was analyzed by flow cytometry (Fig 5.2 and Fig 5.3). In agreement with results presented in the previous chapter Th17 expanded in higher degree and persisted longer in the draining LN compared to the cells polarised under Th1 conditions. As expected all immunised groups displayed expansion above unimmunised.

5.3.2 Ability of Th1 and Th17 polarised populations to support transgenic B cells

B cell clonal expansion: The relative ability of Th1 and Th17 polarised cells to provide B cell help would be reflected in the clonal expansion of the cognate transgenic B cells. In response to HEL-OVA/CFA, mice that received T cells polarised under Th17 conditions exhibited significantly higher antigen specific B cell clonal expansion compared to Th1 recipient mice (Fig 5.4 and 5.5). In both Th1 and Th17 recipient mice, clonal expansion peaked between day 3 and 7. However in the case of Th17 recipients B cell numbers did not decline to unimmunised levels even at the last time point investigated as they did in Th1 recipients (Fig 5.5a and b). In the absence of immunisation, as anticipated, transgenic B cells did not expand in either Th1 or Th17 recipient mice.

HEL-specific antibody production: In order to have a measure of the functional status of the transgenic B cells, their ability to produce antibodies against HEL was assessed. As transgenic B cells do not class switch(518) the presence of anti-HEL

anti-IgM[a] antibodies was investigated. Serum was sampled on days 3, 7 and 10 post immunisation and anti-HEL antibody titres assessed by ELISA. T cells polarised under either Th1 or Th17 conditions could support antibody production, however the Th17 population induced higher levels of HEL-specific antibodies, at all time-points investigated (Fig 5.6). In the absence of immunological stimulus there was no antibody production in either Th1 or Th17 recipient mice. These results suggest that cells polarised under Th17 conditions have a relative advantage in supporting antigen specific antibody production.

OVA-Specific antibody production: In addition to the anti-HEL antibody production by the transferred transgenic cells, the production of antibodies against OVA by the host B cells also allowed the evaluation of the relative ability of Th1 and Th17 population to provide B cell help. As transgenic B cells from MD4 mice do not class switch, investigation of the anti-OVA antibody response, gave more qualitative information relating to the isotype class the two populations preferentially promote. Serum from days 3, 7 and 10 post immunisation was analyzed for the presence of anti-OVA IgM[b], IgG1 and IgG2a. In both Th1 and Th17 recipient mice immunised with OVA-HEL/CFA there were very low levels of anti-OVA IgM[b] antibodies that did not differ from control levels at all time points investigated (Fig 5.7). More prominent differences were noted in the anti-OVA IgG response. As expected at day 3 there were undetectable levels of either IgG1 or IgG2a anti-OVA antibodies. In the case of the IgG1 response, mice that have received cells polarised under Th17 conditions exhibited higher antibody titres from as early as day 7 compared to animals received cells polarised under Th1 conditions. This difference was still evident at day 10 (Fig 5.8a-c). On the other hand, recipients of Th1 polarised population demonstrated significantly higher titres of anti-OVA IgG2a antibodies in response to immunisation compared to recipients of cells polarised under Th17 conditions. This was observed from as early as day 7 and was evident even at the last time point investigated (Fig 5.8d-f). Only at day 10 anti-OVA IgG2a antibodies could be detected in Th17 recipients, albeit at levels much lower than Th1 recipients. In the absence of immunological stimulus there was no IgG response. These results suggest that T cells polarised

under Th17 conditions induce mainly an IgG1 response, whereas T cells polarised under Th1 conditions induce high IgG2a antibody levels.

Germinal Centre B cells: The germinal centre is known to be associated with T cell dependent antibody responses and is considered to be the site where phenomena such as clonal selection and expansion, class switching and affinity maturation occur and where high-affinity antibody-secreting plasma cells and memory B cells are generated(616). Based on that, the ability of cells polarised under Th1 or Th17 conditions to induce the generation of germinal centre B cells was used as a measure of B cell help. Germinal centre B cells were identified by the expression of GL-7 and FAS by flow cytometry as done previously by our group and others(514;617) (Fig 5.9). In both Th1 and Th17 recipient mice germinal centre B cells were first identified in higher proportion than unimmunised controls at day 7 (Fig 5.10b). Interestingly, the number and proportion of germinal centre B cells was significantly higher in mice that have received cells polarised under Th17 condition compared to the ones that had received Th1 polarised cells (Fig 5.10a and b). This was noticeable both at day 7 and 10. In unimmunised mice only a very small number of germinal centre B cells could be detected. These data further support a higher ability of the Th17 polarised population relative to the Th1 population in supporting B cell clonal expansion.

5.3.3 Evidence of follicular migration markers in the transferred Th1 and Th17 population

An important requirement of T cell-dependent B cell responses is the migration of activated antigen-specific T cell to the follicular region where they interact with their cognate B cell[138;613]. It has been reported that this is mediated by the downregulation of the chemokine receptor CCR7 and the upregulation of CXCR5 on T cells[363;618;619]. CXCR5 defines follicular localisation as in response to its ligand, CXCL13, it facilitates follicular migration[620]. As mentioned previously it is now accepted that TFH cells are the ones that regulate B cell responses, which are characterised by the expression of CXCR5 and costimulatory molecules, such as ICOS[364;368]. In order to measure the ability of follicular migration and provision of co-stimulatory signals by the transferred Th1 and Th17 polarised populations the expression of CXCR5 and ICOS was investigated (Fig 5.11). At days 3, 7 and 10 the transgenic T cells from the draining LNs were analyzed for the expression of CXCR5 and ICOS. Interestingly, the proportion of $ICOS^+CXCR5^+$ transgenic T cells was not different between the immunised and unimmunised groups of either Th1 or Th17 recipients (Fig 5.12b). This might suggest that cells activated under either Th1 or Th17 polarising environment are conditioned for follicular migration. On the other hand, the number of transgenic $ICOS^+$ $CXCR5^+$ T cells was significantly higher in the immunised mice. Notably, in the Th1 recipients the number of transgenic T cell with a TFH phenotype peaked approximately at day 3 and declined to unimmunised levels by day 10. On the other hand, in mice injected with cells polarised under Th17 conditions the number of transgenic cells did not peak at day 3 and was significantly higher than the Th1 counterparts both at days 7 and 10 (Fig 5.12a). In order to quantify differences in the levels of expression of CXCR5 and ICOS by the transgenic T cells the MFI for these markers was calculated. Transferred T cells in unimmunised mice expressed significantly lower levels of ICOS compared their counterparts in immunised mice. Remarkably cells polarised under Th17 conditions expressed significantly higher levels of ICOS compared to Th1 polarised cells at days 7 and 10, suggesting a higher ability to

provide costimulatory help (Fig 5.13a). In addition, there were no differences between the groups relating to levels of CXCR5 expression, which suggest that both populations have equal potential to migrate to the follicle, a phenomenon independent on the presence of antigenic stimulus (5.13b).

5.3.4 Localisation of antigen specific T cells within the draining LN

The differences in expression of markers associated with a TFH phenotype between the two populations prompted the investigation of possible differences in localisation of the transgenic T cells in the LN in situ. More specifically the relative ability of T cells polarised under Th1 or Th17 conditions to localise to the follicular region was investigated. The same experimental setting as before was employed and the localisation of the transgenic T cells was analyzed at days 3, 7 and 10 post-immunisation by fluorescent-based immunohistochemistry (Fig 5.14). The tile scan function of the Carl Zeiss LSM510 META Confocal Imaging System allowed imaging of the full surface of the LN section. Transgenic T cells were detected using the KJ1.26 monoclonal antibody against their TcR, whereas staining for B220 revealed the B cell follicles. The quantification of the localisation of the transgenic T cells was achieved using the Volocity© software (Fig 5.15). Areas of interest were drawn around the borders of the sections or around the follicular regions. This allowed the calculation of the surface of LN section and follicular area respectively. In the same time the number of transgenic T cells in the section and in the follicular areas could be calculated. The localisation of T cells in the follicular area was calculated as a fraction of the proportion of $KJ1.26^{+}$ cells in the follicular area ($KJ_{follicle}/KJ_{total}$) to the proportion of the follicular surface ($area_{follicle}/area_{total}$) (Fig 5.15b). This gave a number that was normalised for both T cell expansion (KJ_{total}) and follicular area ($area_{follicle}$), and thus differences observed would be due to follicular localisation and not due to higher clonal expansion or larger follicular area in a specific section. Interestingly, there were no differences between the Th1 and Th17 population in respect to follicular localisation (Fig 5.16). In addition, only

on day 3 there was increased localisation in the follicular area in immunised mice compared to unimmunised, suggesting that recruitment in the follicular area takes places in early time points after antigen encounter and the differences observed from that point after are due to greater clonal expansion in the immunised mice (Fig 5.16a-c). Indeed, when the proportion of transgenic T cells that reside in the follicle was calculated in all time points it was higher in the immunised mice compared to the unimmunised (Fig 5.17a-c). Furthermore, the number of transgenic cells per unit of follicular area was significantly increased in immunised groups (Fig 5.18a-c). Interestingly, in Th17 recipients this was observed even at day 10, unlike Th1 recipients which were at unimmunised levels at this point (Fig 5.18c). This suggests that cells polarised under Th17 conditions, due to higher clonal expansion, persist in the follicular area for longer time period compared to their Th1 counterparts.

5.3.5 Development of TFH cells and germinal centre B cells in the Th1 and Th17 OVA TcR-induced models of arthritis

Both the Th1 and Th17 OVA TcR-induced models of arthritis are characterized by breach of self tolerance, which is manifested with the presence of various autoantibodies. Evidence support the hypothesis that dysregulated germinal centre responses give rise to autoantibodies, a phenomenon supported by numerous autoimmune prone mouse strains that spontaneously develop germinal centre reactions[615;621;622]. In addition somatic hypermutation taking place in the germinal centre dark zones can lead to the development of autoantibodies(623). Given the importance of T cell help in supporting germinal centre generation it is not a surprise that the aberrant expression of TFH associated molecules such as ICOS, SAP, Bcl-6, c-maf and IL-21 impacts on autoantibody productions in murine models[367;621;624-626]. It is thus of considerable importance to investigate the generation of these cells in the OVA TcR arthritis models. Furthermore, as results so far in this chapter demonstrate a relative advantage for cells polarised under Th17 conditions to support B cell response it would be interesting to compare the Th1 and Th17 models both for the generation of TFH cells and germinal centre B cells. CD4$^+$ cells from DO11.10 mice were polarised under Th1 or Th17 condition and were adoptively transferred to BALB/c congenic mice, which were then immunised with OVA/CFA. Ten days after immunisation recipient mice were challenged proximal to the ankle joint with HAO. Control mice were injected with PBS. Seven post-challenge cells from the draining LNs were analyzed for the presence of TFH cells by flow cytometry. TFH cells were identified as CD4$^+$ cells co-expressing the chemokine receptor CXCR5 and the costimulatory molecule ICOS (Fig 5.19). In both Th1 and Th17 models cells with TFH phenotype were identified. Approximately 5-10% of the CD4 population in both Th1 and Th17 recipients challenged with HAO had a TFH phenotype. (Fig 5.20a) Interestingly, the percentage and number of TFH cells was similar between Th1 and Th17 models (Fig 5.20a-b). Mice injected with PBS had a significantly lower proportion and number of TFH cells compared to the HAO challenged mice. As the result of T cell

help is a germinal centre reaction the generation of germinal centre B cells was investigated. This could give another functional readout for the developing humoral response. Cells from draining LNs were analyzed for the presence of germinal centre B cells by flow cytometry. As previously in this chapter, germinal center B cells were identified as B220$^+$ cells co-expressing GL-7 and FAS (Fig 5.21). Around 20% of the B cells in the draining LNs of challenged mice had a germinal B cell phenotype; however as in the case of TFH cells, there was no difference in the number and proportion of germinal centre B cells between Th1 and Th17 recipients (Fig 5.22). HAO challenged mice had significantly higher proportion and number of germinal centre B cells compared to PBS control mice, demonstrating an active B cell response (Fig 5.22a-b). These data suggest that in challenged mice an active T cell-dependent B cell response is taking place, which might be responsible for the generation of the autoantibodies that characterize these models.
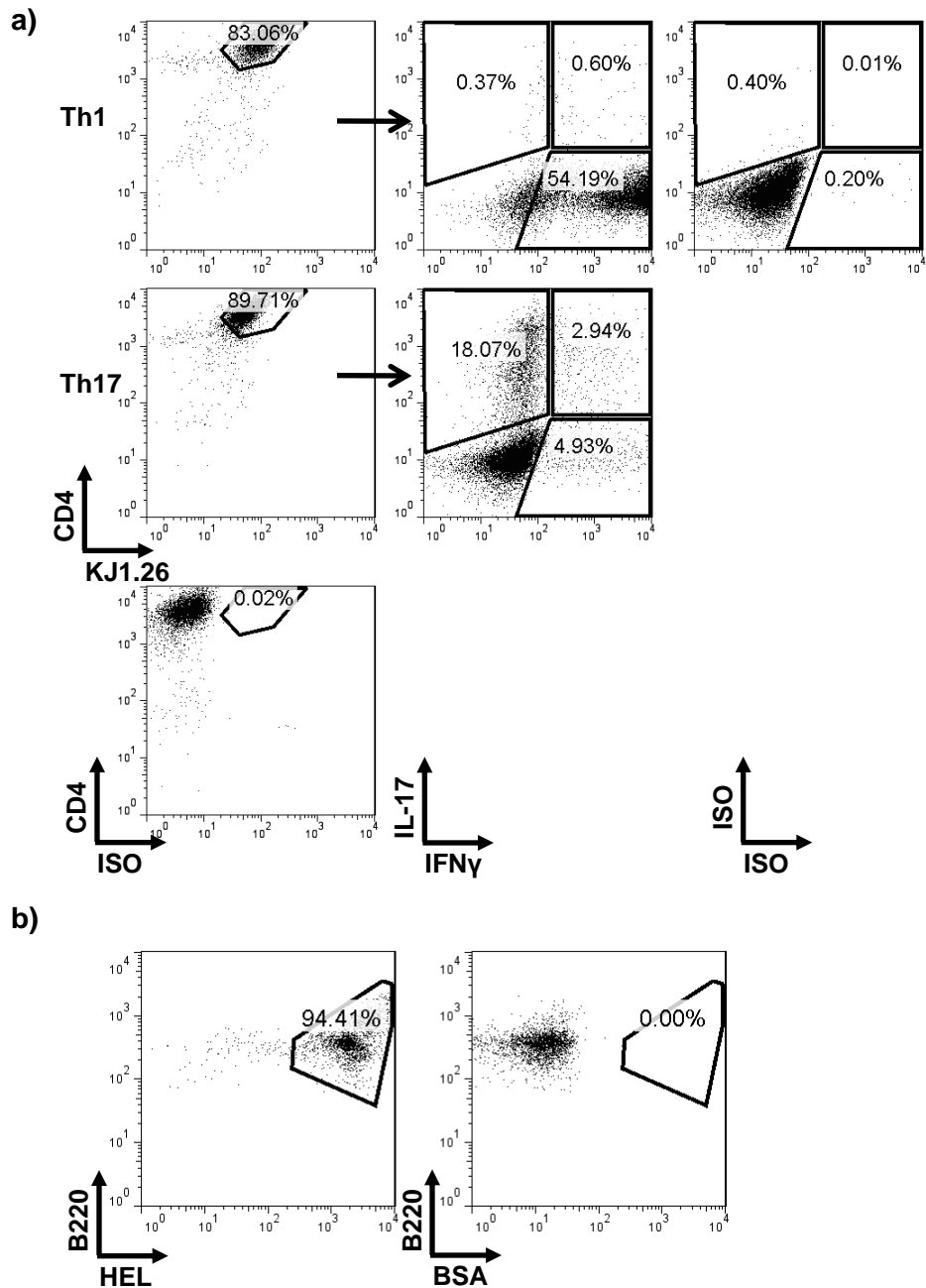
Fig 5.1: Phenotype of transferred transgenic B and T cells

Cells from DO11.10 mice were polarised under Th1 or Th17 conditions and transferred to congenic IgH^b recipient mice together with $2x10^6$ HEL-specific B cells from MD4 mice. The phenotype of the Th1 and Th17 population was assessed by intracellular flow cytometry (a). Lymphocytes were identified based on the FSC and SSC profile and transgenic T cells based on KJ1.26 staining and CD4 expression. The proportion of HEL- specific MD4 B cells was assessed by flow cytometry(b). Lymphocytes were identified based on the FSC and SSC profile and transgenic B cells based on B220 expression and the ability to bind biotinylated HEL.
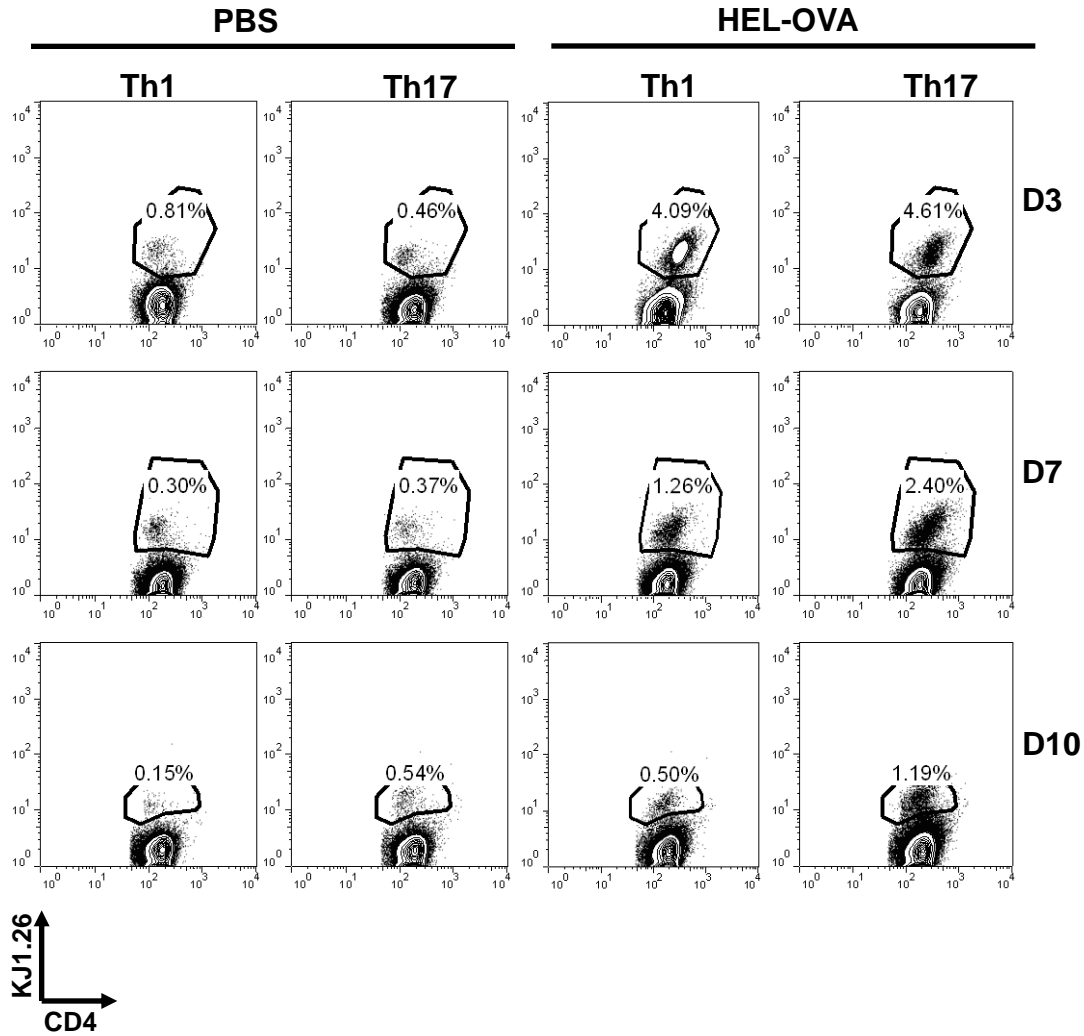
Fig 5.2: Kinetics of the KJ1.26+ CD4+ population after immunisation

$2x10^6$ Th1 or Th17 polarised CD4+ T cells from DO11.10 mice were adoptively transferred to IgH^b congenic recipient mice along side with $2x10^6$ HEL-specific B cells. On day 0 recipients were immunised s.c. with 130μg/ml of OVA-HEL. The presence of CD4+KJ1.26+ T cells in the draining lymph nodes (axillary and brachial) of recipient mice was assessed by flow cytometry on days 3, 5, and 10 after immunisation. Lymphocytes were identified based on the FSC and SSC profile and transgenic T cells based on KJ1.26 staining and CD4 expression. Figure demonstrate representative FACS plots of days 3 (top panel), day 7 (middle panel) and day 10 (low panel).
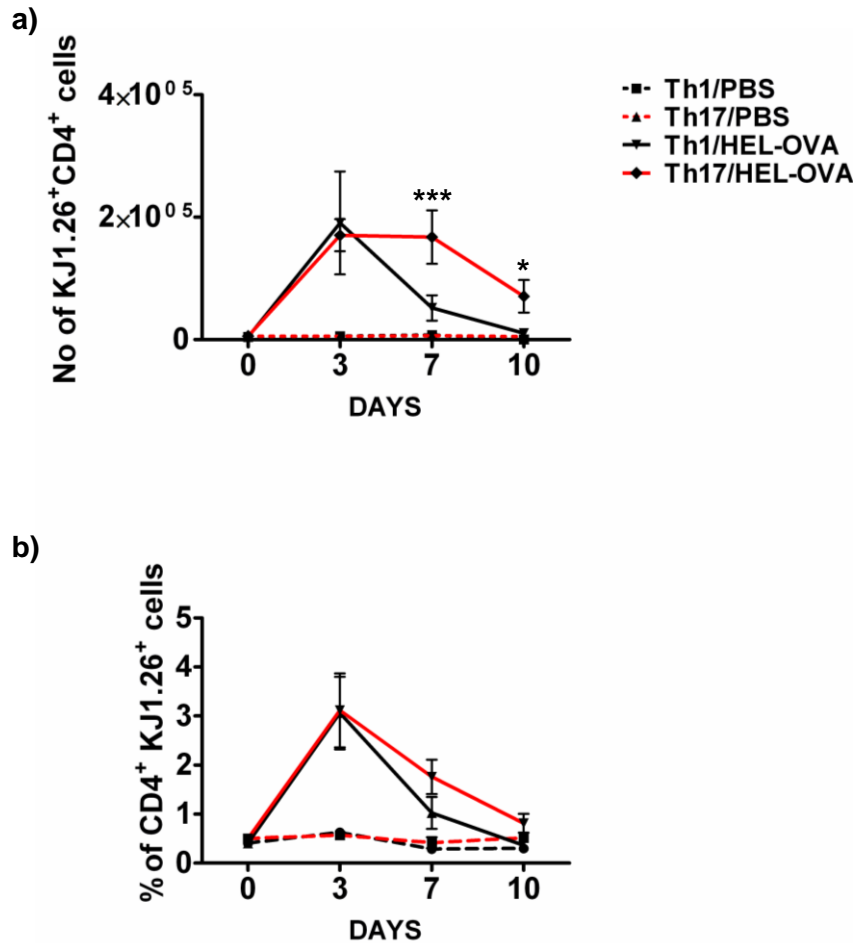
Fig 5.3: Kinetics of the KJ1.26$^+$ CD4$^+$ population after immunisation

2×10^6 Th1 or Th17 polarised CD4+ T cells from DO11.10 mice were adoptively transferred to IgH^b congenic recipient mice together with 2×10^6 HEL-specific B cells. On day 0 recipients were immunised s.c. with 130μg/ml of OVA-HEL. The number (a) and percentage (b) of CD4$^+$KJ1.26$^+$ T cells in the draining lymph nodes (axillary and brachial) of recipient mice was assessed by flow cytometry on days 3, 5, and 10 after immunisation. Lymphocytes were identified based on the FSC and SSC profile and transgenic T cells based on KJ1.26 staining and CD4 expression. Unimmunised controls from each time point were averaged and represented as day 0. Data represent mean ±SEM. *: Th1/HEL-OVA vs. Th17/HEL-OVA, *p<0.05, **p<0.01, ***p<0.001 (n=3). Similar results were obtained in one additional experiment.

a)

Fig 5.4: Relative ability of in vitro polarised Th1 and Th17 populations to support antigen specific B cell expansion

$2x10^6$ Th1 or Th17 polarised CD4^{+} T cells from DO11.10 mice were adoptively transferred to IgH[b] congenic recipient mice along side with $2x10^6$ HEL-specific B cells. One day post-transfer recipients were immunised s.c. with 130μg/ml of OVA-HEL. The presence of trangenic B cells in the draining lymph nodes (axillary and brachial) of recipient mice was assessed by flow cytometry on days 3, 5, and 10 after immunisation. Lymphocytes were identified based on the FSC and SSC profile and transgenic B cells based on IgM[a] and B220 expression. Figure demonstrates representative FACS plots of days 3 (top panel), day 7 (middle panel) and day 10 (low panel). Similar results were acquired in one additional experiment.

Fig 5.5: Relative ability of in vitro polarised Th1 and Th17 populations to support antigen specific B cell expansion

$2x10^6$ CD4^{+} T cells from DO11.10 mice were polarised under Th1 or Th17 conditions and were adoptively transferred to congenic IgH[b] recipient mice along side with $2x10^6$ HEL-specific B cells. One day post-transfer recipient mice were immunised s.c. with 130μg/ml of OVA-HEL/CFA. The presence of trangenic B cells in the draining lymph nodes (axillary and brachial) of recipient mice was assessed by flow cytometry on days 3, 5, and 10 after immunisation. Lymphocytes were identified based on the FSC and SSC profile and transgenic B cells based on the IgM[a] and B220 expression. Unimmunised controls from each time point were averaged and represented as day 0. Data represent mean ±SEM. *: Th1/HEL-OVA vs. Th17/HEL-OVA, *p<0.05, **p<0.01, ***p<0.001 (n=3). Similar results were obtained in one additional experiment.
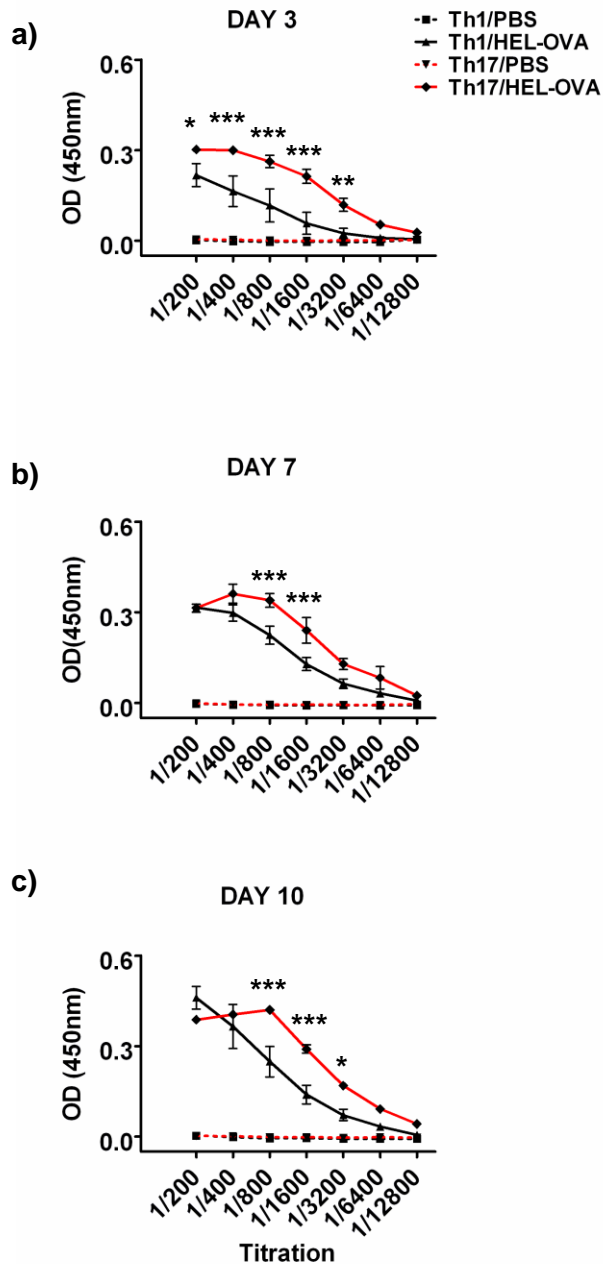
Fig 5.6:Relative ability of Th1 and Th17 populations to support the production of HEL-specific antibodies

Cells from DO11.10 mice were polarised under Th1 or Th17 conditions and transferred to congenic IgH[b] recipient mice along side with $2x10^6$ HEL-specific B cells from MD4 mice. One day post transfer recipient mice were immunised with HEL-OVA. Control mice were injected with PBS. Mice were euthanized at days 3, 7 and 10 after immunisation. Serum was taken from the animals and was assessed for the presence of HEL-specific IgM[a] antibodies. Data represent mean ±SEM. *: Th1/HEL-OVA vs. Th17/HEL-OVA, *p<0.05, **p<0.01, ***p<0.001 (n=3). Similar results were obtained in one additional experiment.
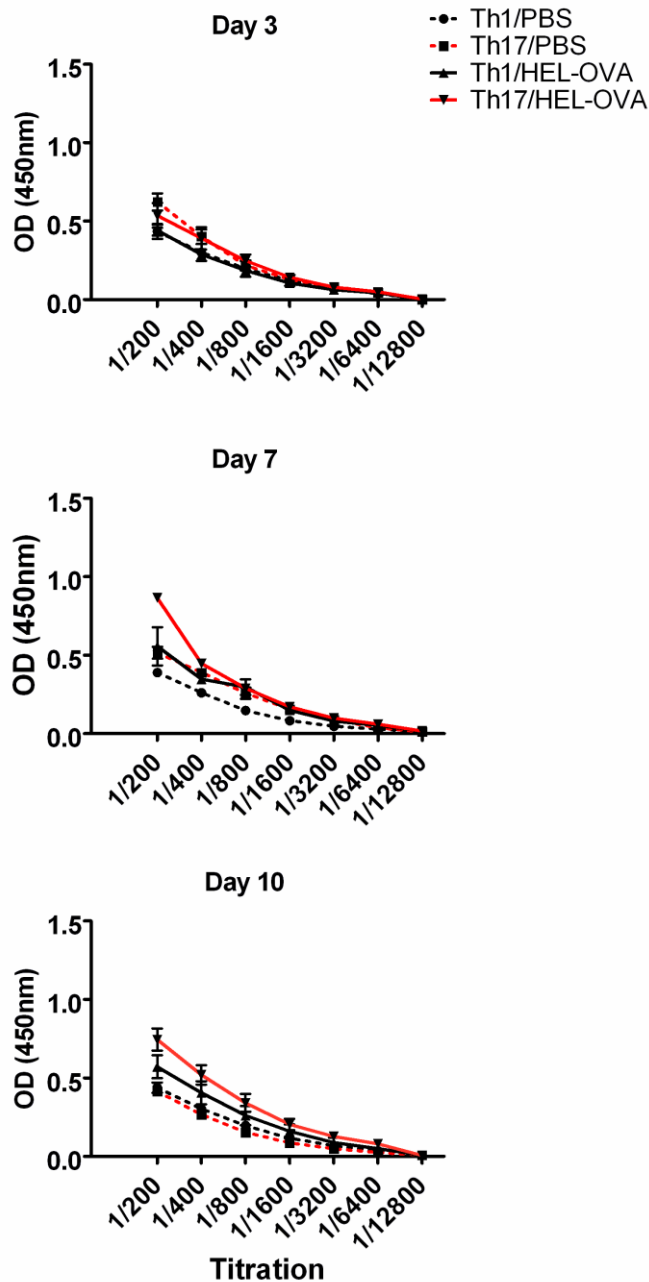
Fig 5.7:Relative ability of Th1 and Th17 populations to support the production of OVA-specific IgM^b antibodies

Cells from DO11.10 mice were polarised under Th1 or Th17 conditions and transferred to congenic IgH^b recipient mice along side with $2x10^6$ HEL-specific B cells from MD4 mice. One day post transfer recipient mice were immunised with HEL-OVA. Control mice were injected with PBS. Mice were euthanized at days 3, 7 and 10 after immunisation. Serum was taken from the animals and was assessed for the presence of OVA-specific IgM^{b}antibodies. Data represent mean ±SEM.(n=3). Similar results were obtained in one additional experiment.
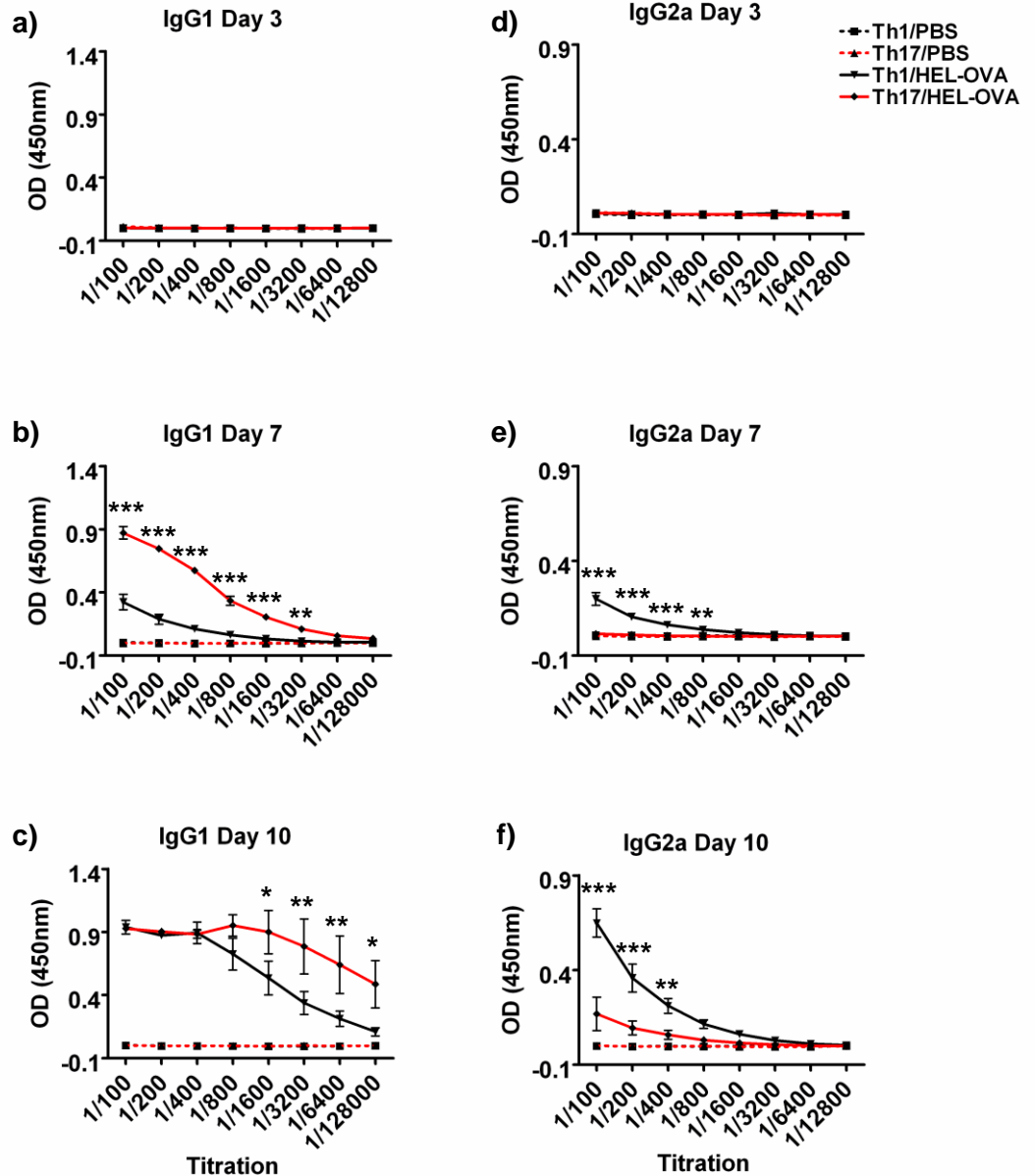
Fig 5.8:Relative ability of Th1 and Th17 populations to support the production of anti-OVA IgG1 and IgG2a antibodies

Cells from DO11.10 mice were polarised under Th1 or Th17 conditions and transferred to congenic IgH[b] recipient mice along side with $2x10^6$ HEL-specific B cells from MD4 mice. One day post transfer recipient mice were immunised with HEL-OVA/CFA. Control mice were injected with PBS. Mice were euthanized at days 3, 7 and 10 after immunisation. Serum was taken from the animals and was assessed for the presence of anti-OVA IgG1 (a-c) and IgG2a (d-f) antibodies. Data represent mean ±SEM. *: Th1/HEL-OVA vs. Th17/HEL-OVA, *p<0.05, **p<0.01, ***p<0.001 (n=3). Similar results were obtained in one additional experiment.

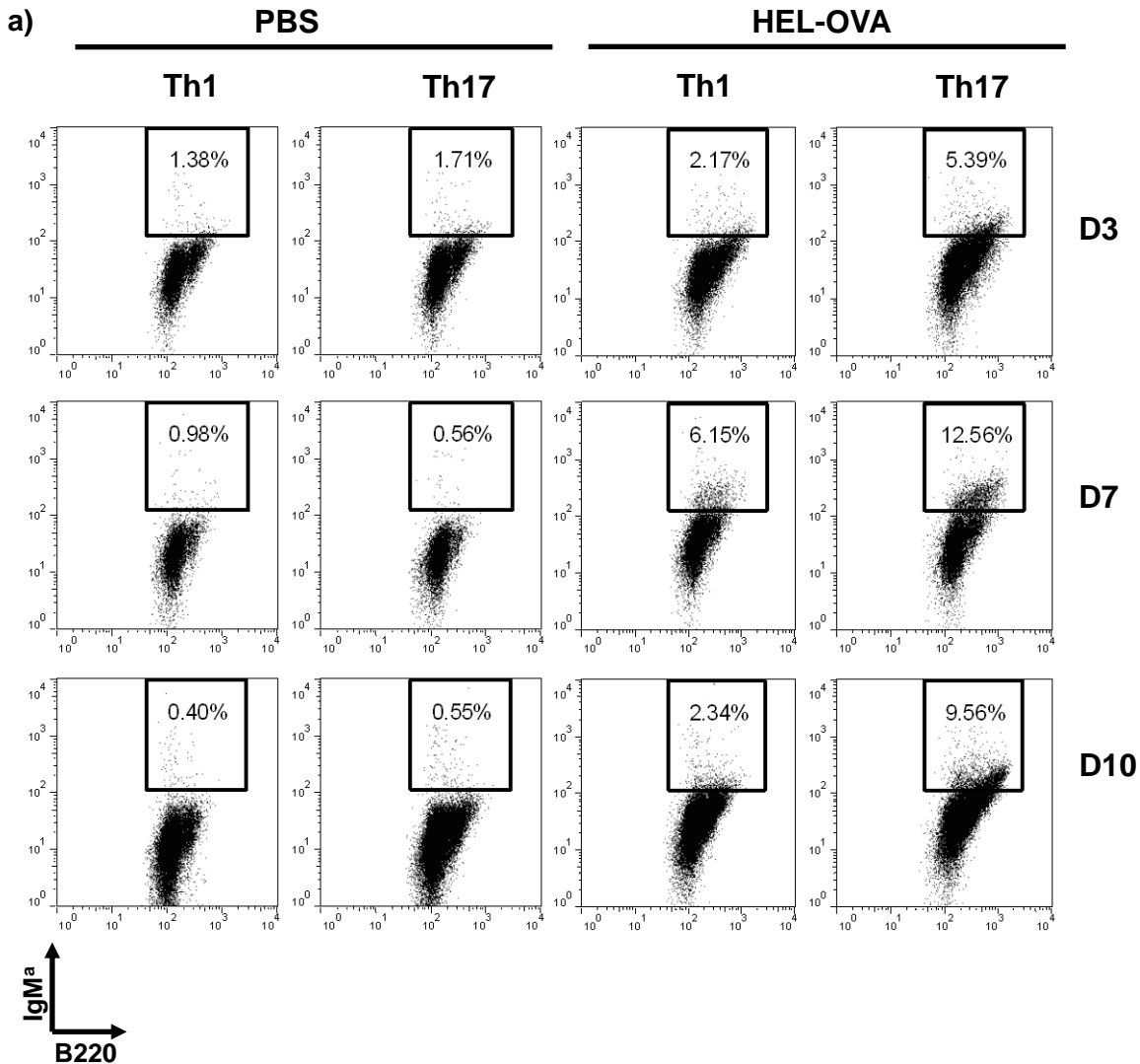**Fig 5.9: Relative ability of in vitro polarised Th1 and Th17 populations to support generation of germinal centre B cells.**

$2x10^6$ CD4^{+} T cells from DO11.10 mice were polarised under Th1 or Th17 conditions and were adoptively transferred to congenic IgH[b] recipient mice along side with $2x10^6$ HEL-specific B cells. One day post-transfer recipient mice were immunised s.c. with 130μg/ml of OVA-HEL/CFA. On days 3, 7 and 10 mice were euthanised and the presence of germinal centre B cells in the draining LNs were assessed by flow cytometry. Lymphocytes were identified based on the FSC and SSC profile and B cells based on B220 expression. Germinal centre B cells were identified by expression of GL-7 and FAS. Figure demonstrates representative FACS plots gated on B220^{+} cells of days 3 (top panel), day 7 (middle panel) and day 10 (low panel). Similar results were acquired in one additional experiment.

a)

b)
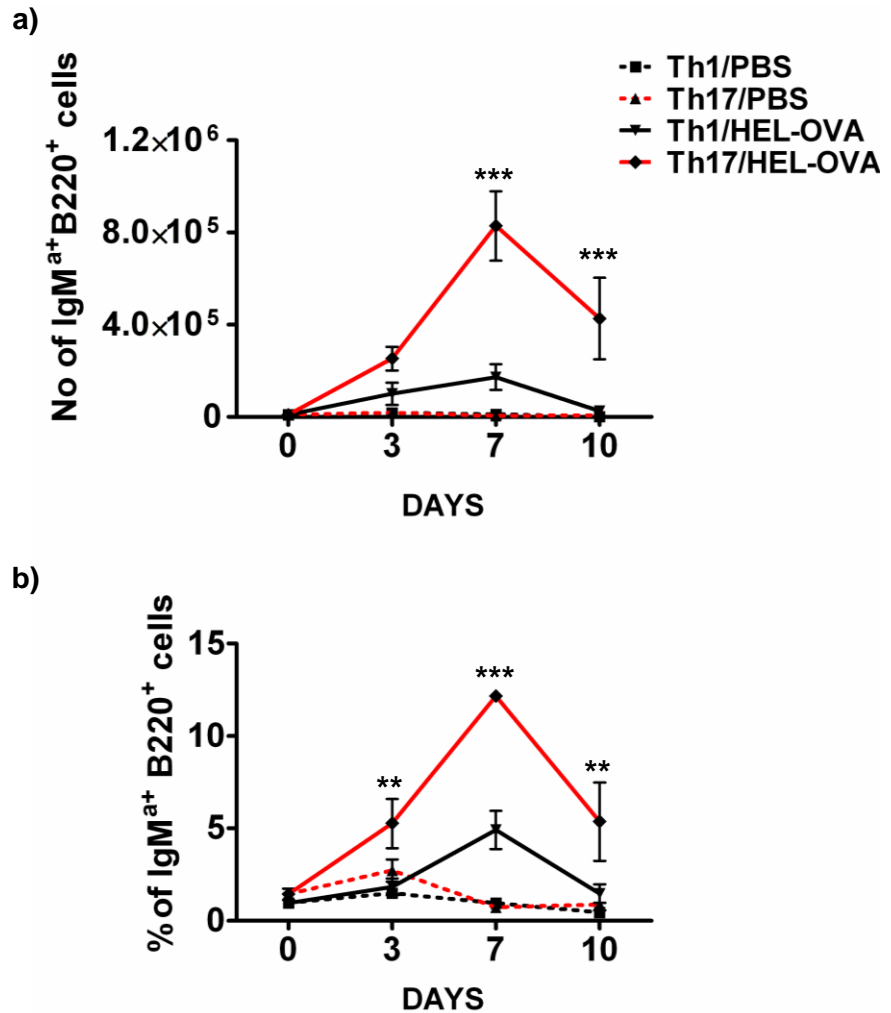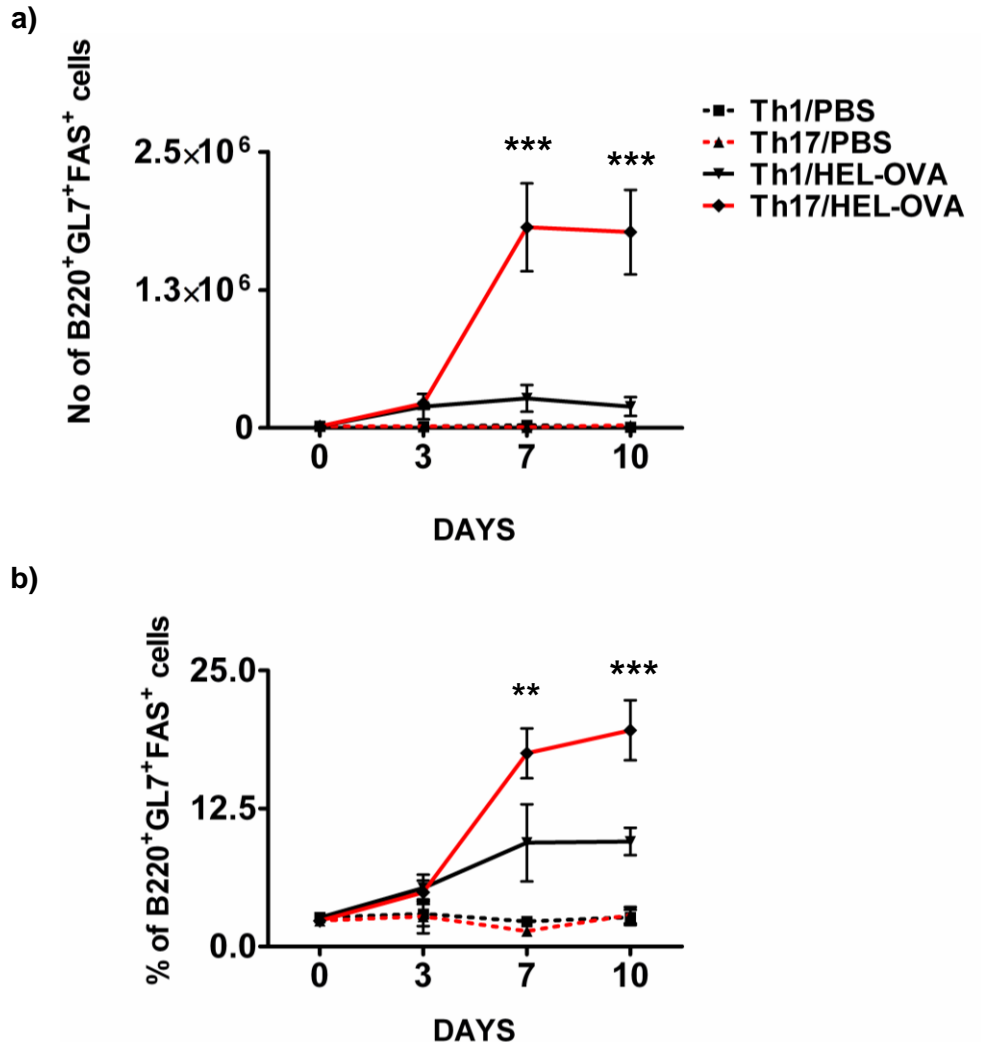

Fig 5.10: Relative ability of in vitro polarised Th1 and Th17 populations to support generation of germinal centre B cells.

2×10^6 CD4^{+} T cells from DO11.10 mice were polarised under Th1 or Th17 conditions and were adoptively transferred to congenic IgH[b] recipient mice along side with 2×10^6 HEL-specific B cells. One day post-transfer recipient mice were immunised s.c. with 130μg/ml of OVA-HEL/CFA. On days 3, 7 and 10 mice were euthanised and the presence of germinal centre B cells in the draining LNs were assessed by flow cytometry. Lymphocytes were identified based on the FSC and SSC profile and B cells based on B220 expression. Germinal centre B cells were identified by expression of GL-7 and FAS. Figure demonstrates the number (a) and percentage (b) of germinal centre B cells on days 3, 7 and 10 post immunisation. Unimmunised controls from each time point were averaged and represented as day 0. Data represent mean ±SEM. *: Th1/HEL-OVA vs. Th17/HEL-OVA, *p<0.05, **p<0.01, ***p<0.001 (n=3). Similar results were obtained in one additional experiment.
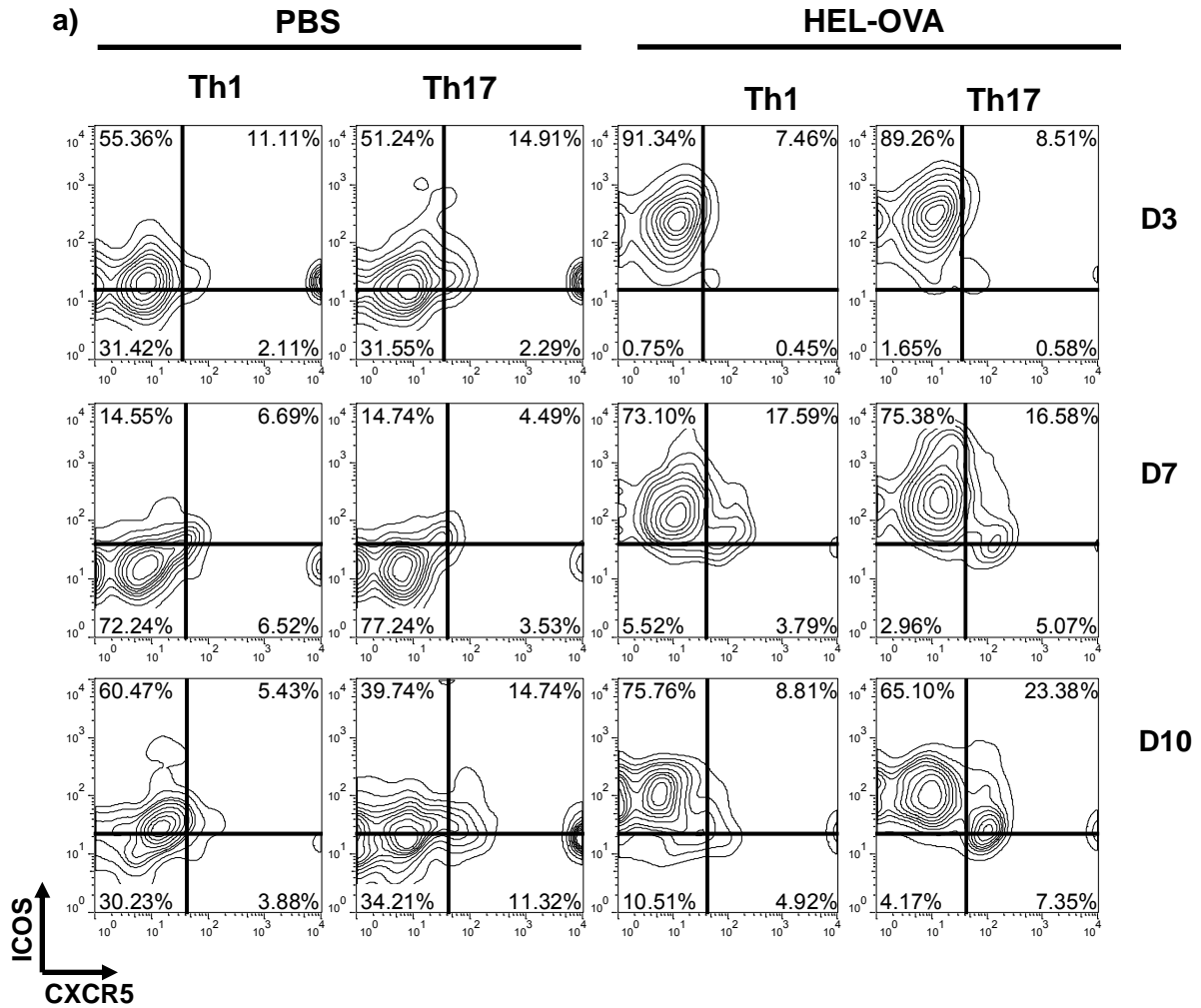
Fig 5.11: TFH phenotype acquisition by the adoptively transferred Th1 and Th17 populations

CD4^{+} cells from DO11.10 mice were polarised under Th1 or Th17 conditions and were adoptively transferred to IgH[b] congenic mice along side with HEL-specific B cells. The ability of the transferred T cell to acquire a TFH phenotype in the draining LNs was assessed by flow cytometry on 3 time-points (day 3,7 and 10) based on the expression of ICOS and CXCR5. Lymphocytes were identified based on the FSC and SSC profile and trangenic T cells based on the expression of CD4 and KJ1.26 staining. Figure demonstrates representative FACS plots gated on transgenic T cells. TFH were identified by expression of ICOS and CXCR5. Similar results were obtained in one additional experiment.
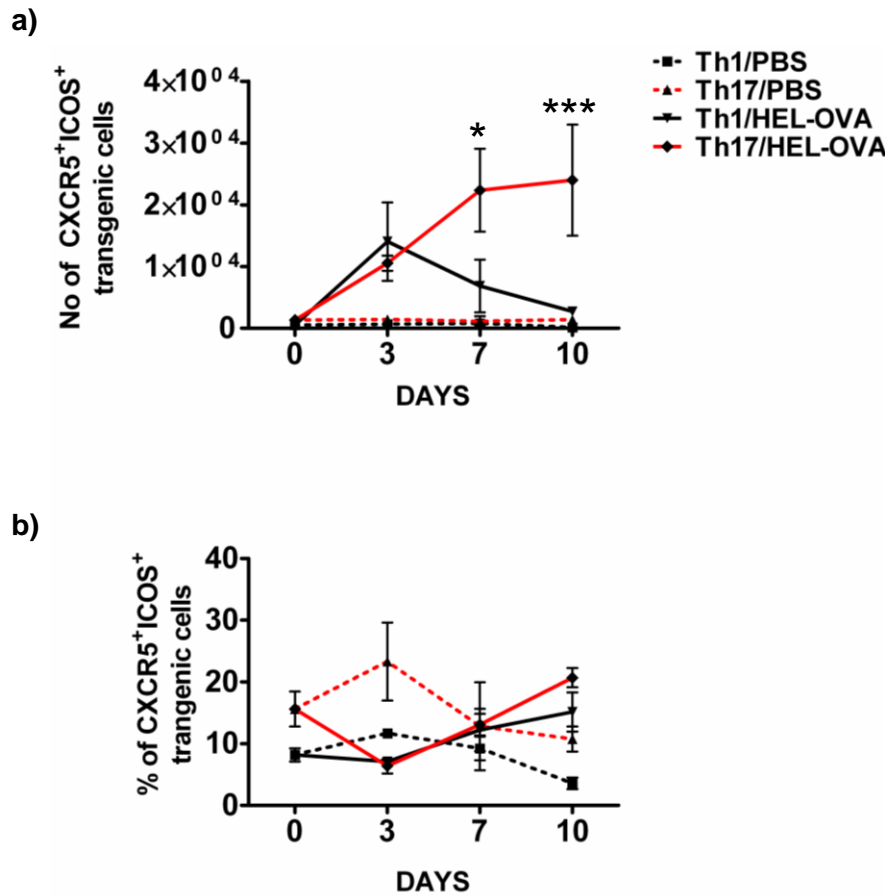
Fig 5.12: TFH phenotype acquisition by the adoptively transferred Th1 and Th17 populations

CD4^{+} cells from DO11.10 mice were polarised under Th1 or Th17 conditions and were adoptively transferred to IgH[b] congenic mice along side with HEL-specific B cells. The ability of the transferred T cell to acquire a TFH phenotype in the draining LNs was assessed by flow cytometry on 3 time-points (day 3,7 and 10) based on the expression of ICOS and CXCR5. Lymphocytes were identified based on the FSC and SSC profile and trangenic T cells based on the expression of CD4 and KJ1.26 staining. TFH were identified by expression of ICOS and CXCR5. Figure demonstrates the number (a) and percentage (b) of TFH cells on days 3, 7 and 10 post immunisation. Unimmunised controls from each time point were averaged and represented as day 0. Data represent mean $\pm$SEM. *: Th1/HEL-OVA vs. Th17/HEL-OVA, *$p<0.05$, **$p<0.01$, ***$p<0.001$ (n=3). Similar results were obtained in one additional experiment.
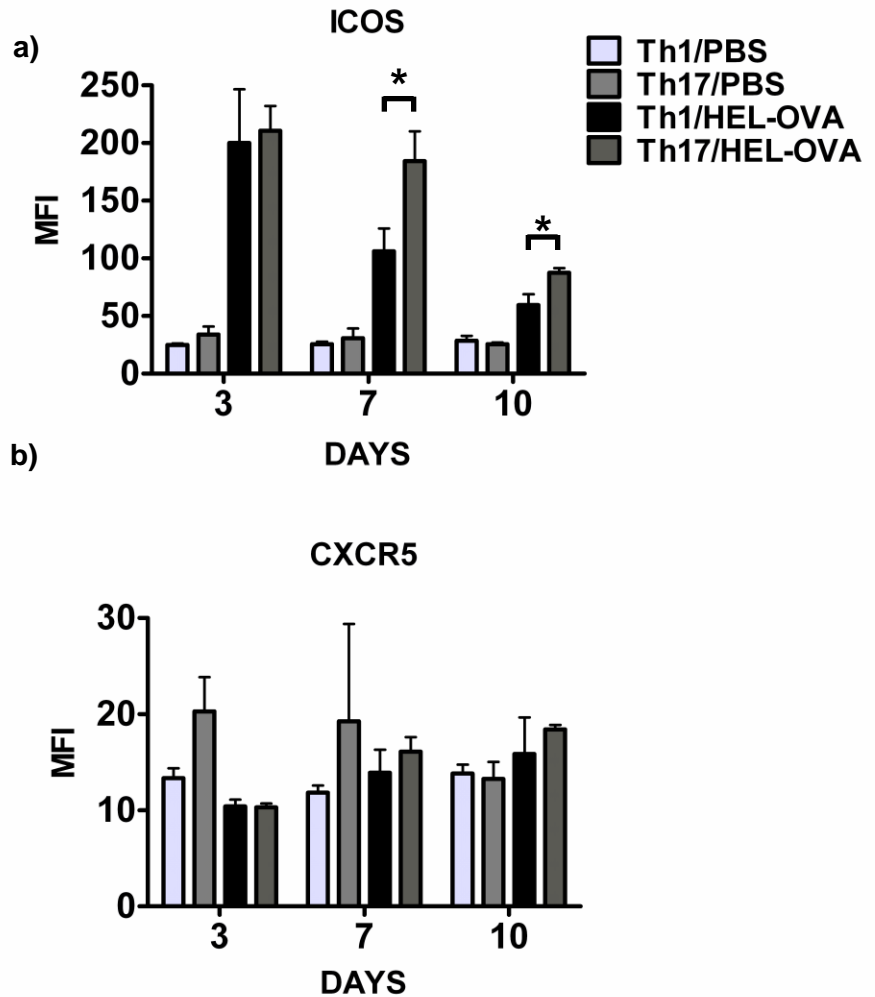
**Fig 5.13: Relative levels of ICOS and CXCR5 expression by the transferred Th1 and Th17 populations**

CD4^{+} cells from DO11.10 mice were polarised under Th1 or Th17 conditions and were adoptively transferred to IgH[b] congenic mice along side with HEL-specific B cells. Recipient mice were immunised with HEL-OVA/CFA. At days 3, 7 and 10 post challenge the mean fluorescence intensity of ICOS (a) and CXCR5 (b) on the CD4^{+} KJ1.26^{+} adoptively transferred T cell populations was calculated. Data represent mean ±SEM. *: Th1/HEL-OVA vs. Th17/HEL-OVA, *p<0.05, **p<0.01, ***p<0.001 (n=3).
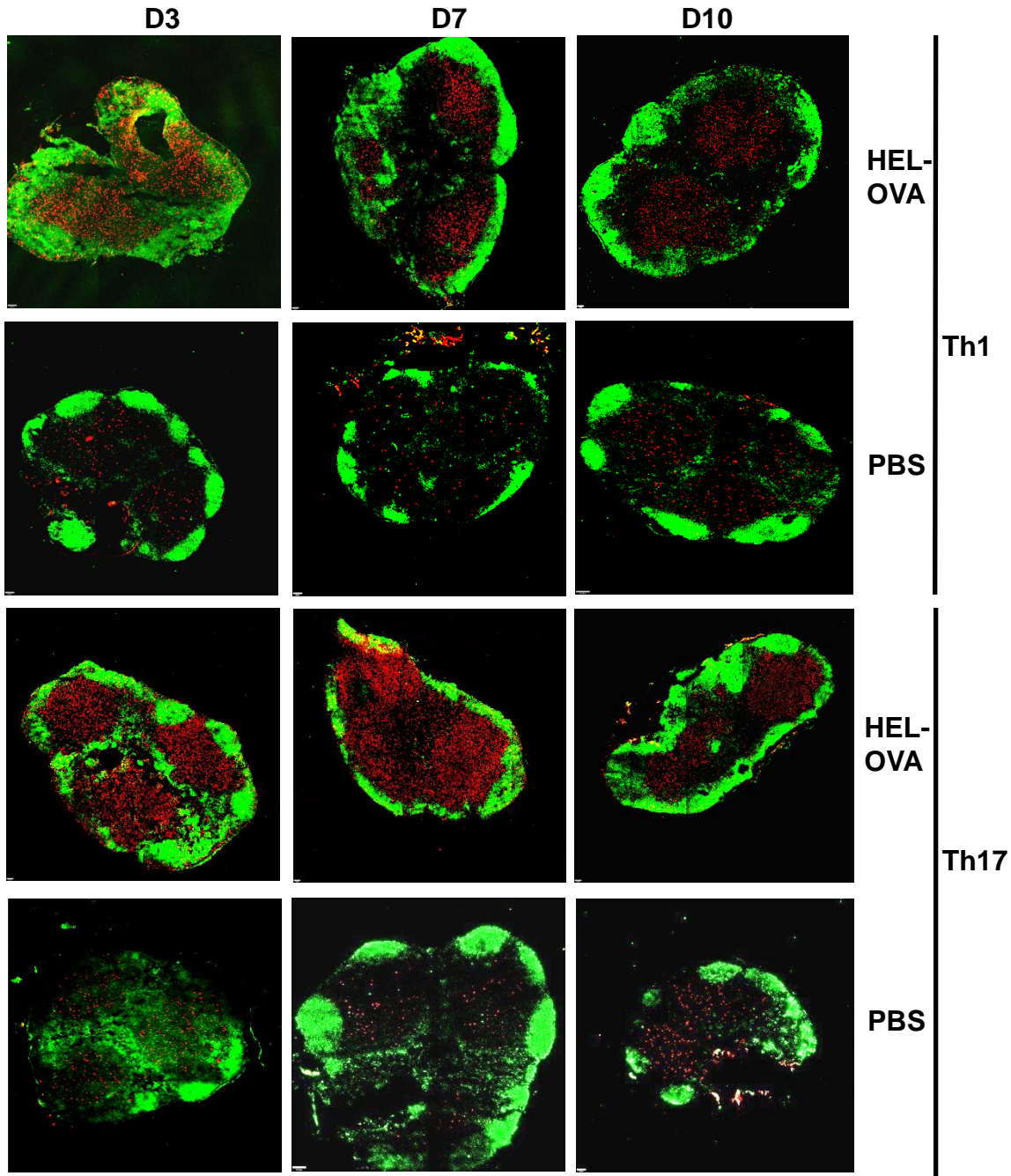
Fig 5.14: Localization of trangenic T cells in the draining LNs

CD4^{+} cells from DO11.10 mice were polarised under Th1 or Th17 conditions and were adoptively transferred to IgH[b] congenic mice along side with HEL-specific B cells. Recipient mice were inhected with HEL-OVA/CFA or PBS. The localization of transferred cells was determined by immunohistochemistry in draining LNs of Th1 (top two panels) or Th17 recipients (bottom two panels) on days 3,7 and 10. Trangenic T cells were detected using the KJ1.26 antibody against their TcR (RED) and B cell follicles using an antibody against B220 (GREEN). Pictures were taken using a confocal microscope with a 10x objective. The tile scan function was used to acquire the full surface of the LNs.

a)

B220
KJ1.26
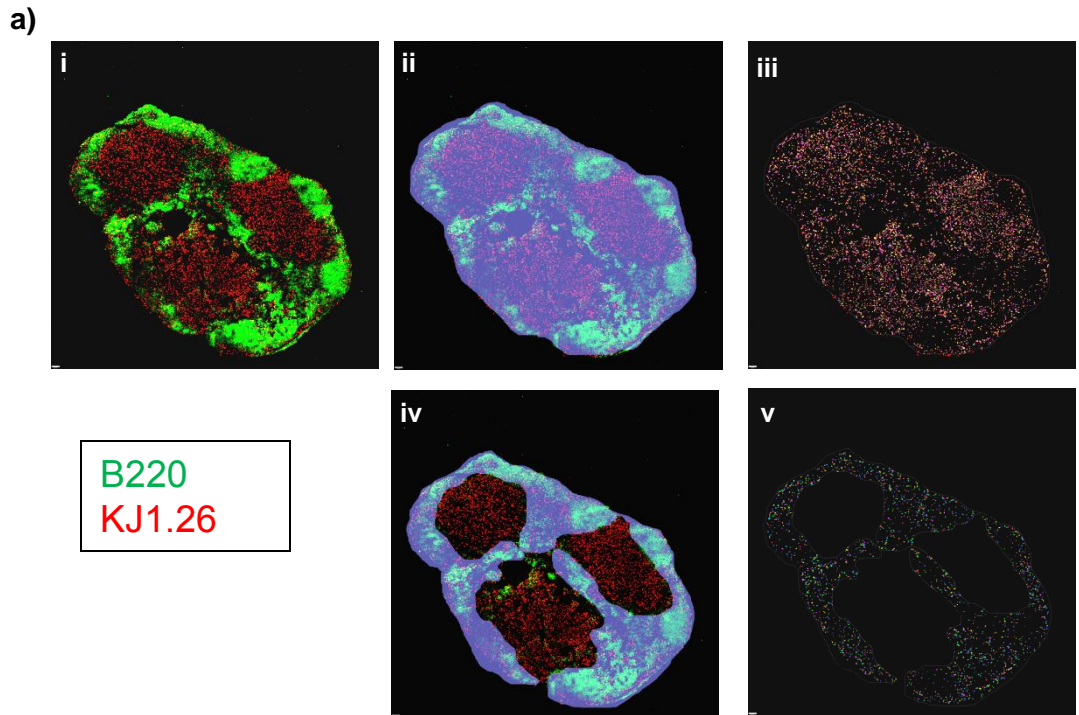
b)

$$\text{Transgenic cells per unit area} = \frac{(KJ_{follicle}/KJ_{total})}{area_{follicle}/area_{total})}$$

Fig 5.15: Analysis of localization of transgenic T cells in the draining LNs

Tile scan images of draining LN sections acquired by confocal microscopy were analyzed using Volocity® software. Areas of interest were drawn around the borders of the section (a-ii) or the B cell follicle based on B220 expression (GREEN) (a-iv), which allowed the calculation of the respective surfaces. The number of transgenic T cells was calculated based on the intensity of the KJ1.26 staining (RED). (a-iii and a-v) Objects smaller than 30μm and larger than 300μm were excluded. The proportion of transgenic T cells that reside in the follicle was normalized to the number of KJ1.26^{+} cells in the section and the surface of the section and follicle (b).
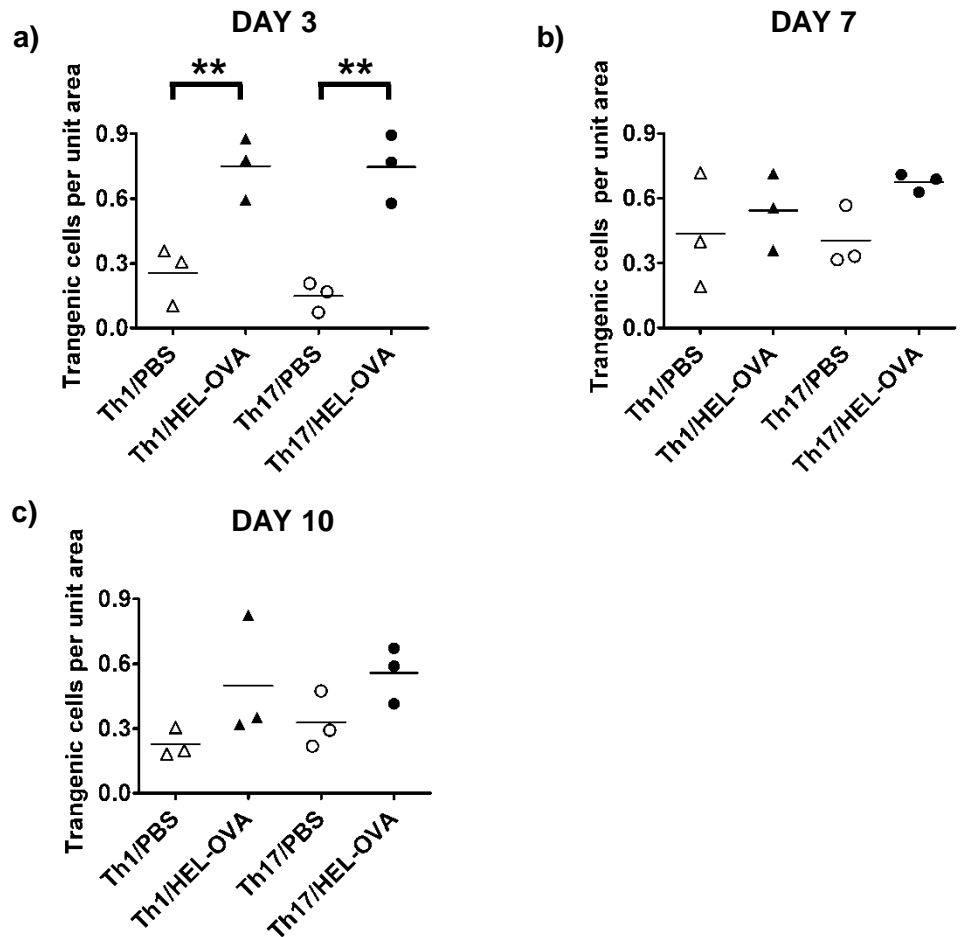
Fig 5.16: Follicular localization of transgenic T cells in the draining LNs

CD4^{+} cells from DO11.10 mice were polarised under Th1 or Th17 conditions and were adoptively transferred to IgH[b] congenic mice along side with HEL-specific B cells. Recipient mice were injected with HEL-OVA/CFA or PBS. On days 3 (a), 7(b) and 10 (c) section the relative ability of the transgenic T cells to localise in the follicular area was assessed by immunofluorescence using the volocity® software as described in fig 5.15. Up to three section were analysed from each animal and each point represents the mean of that.. *p<0.05, **p<0.01, ***p<0.001 (n=3).
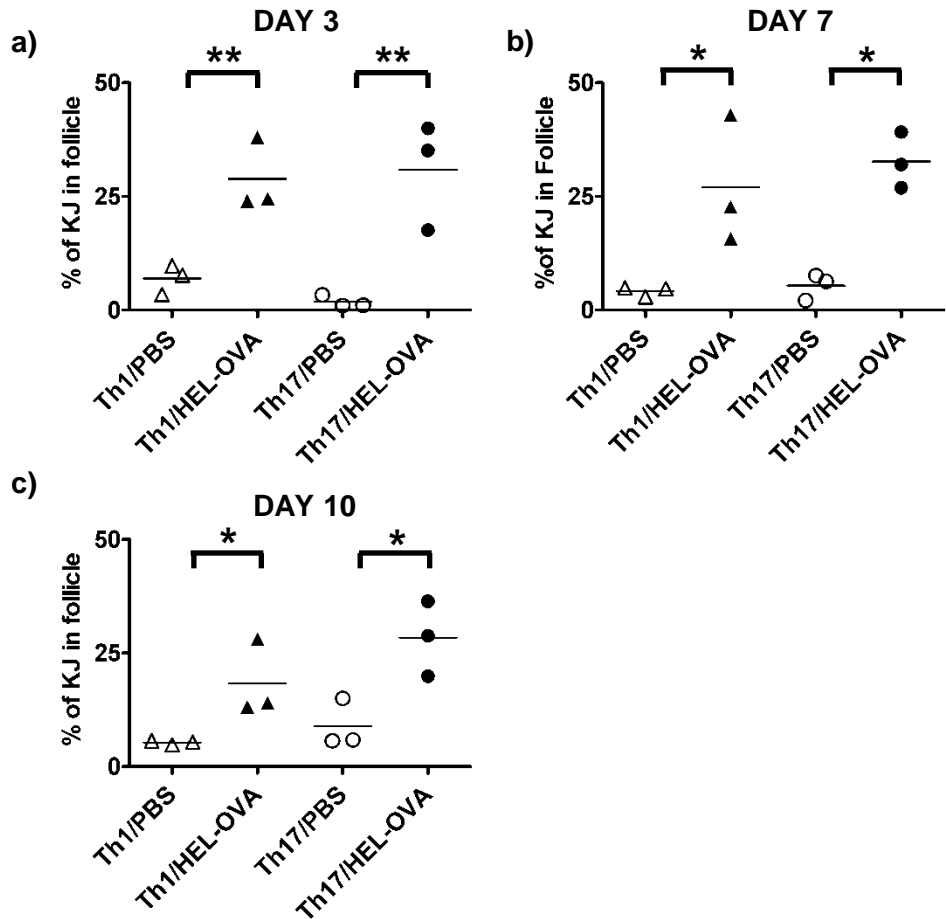
Fig 5.17: Follicular localization of transgenic T cells in the draining LNs (proportion of transferred T cells localizing in the follicle)

CD4^{+} cells from DO11.10 mice were polarised under Th1 or Th17 conditions and were adoptively transferred to IgH[b] congenic mice along side with HEL-specific B cells. Recipient mice were injected with HEL-OVA/CFA or PBS. On days 3 (a), 7(b) and 10 (c) the proportion of transgenic T cells that localize in the follicular area was assessed by immunofluorescence using the Volocity® software as described in fig 5.15. Up to three section were analyzed from each animal and each point represents the mean of that. *p<0.05, **p<0.01, ***p<0.001 (n=3).
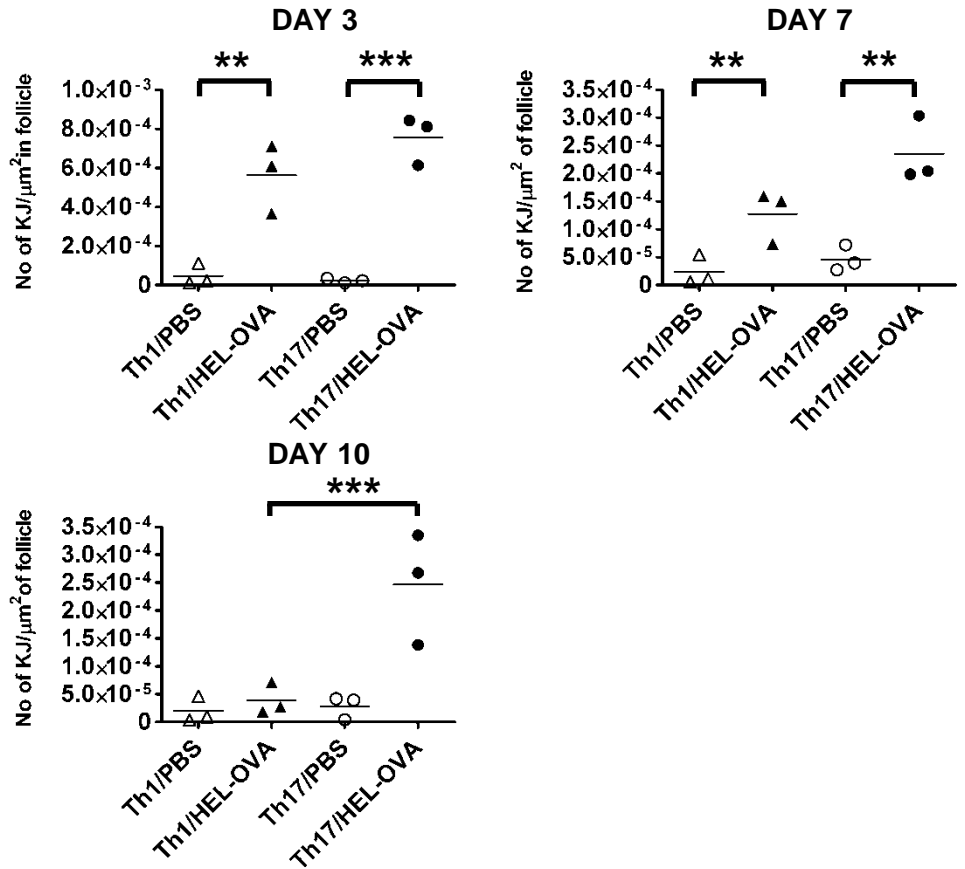
Fig 5.18: Follicular localization of trangenic T cells in the draining LNs (number of transferred T cells per unit of follicular area)

CD4^{+} cells from DO11.10 mice were polarised under Th1 or Th17 conditions and were adoptively transferred to IgH[b] congenic mice along side with HEL-specific B cells. Recipient mice were injected with HEL-OVA/CFA or PBS. On days 3 (a), 7(b) and 10 (c) the number of transgenic T cells per unit of follicular area was assessed by immunofluorescence using the Volocity® software. Up to three section were analyzed from each animal and each point represents the mean. *p<0.05, **p<0.01, ***p<0.001 (n=3).

a)
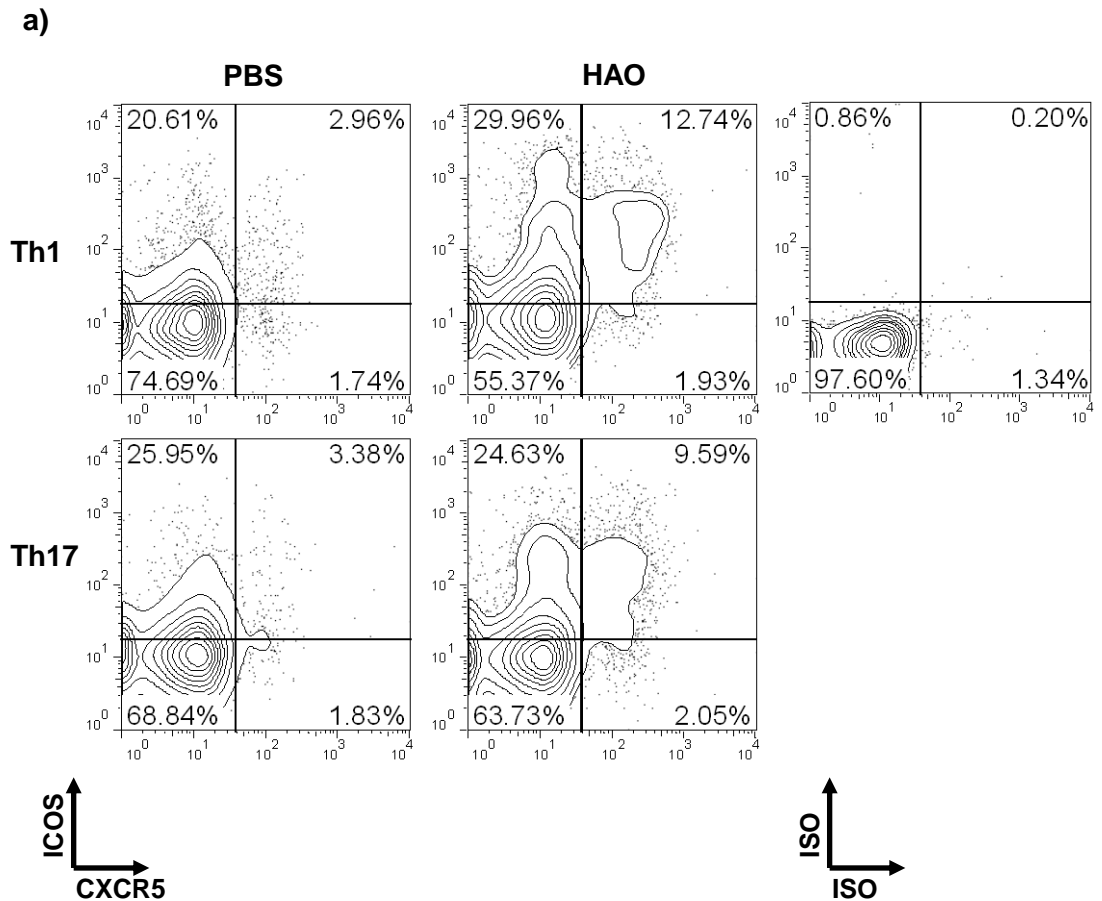
Fig 5.19: Presence of TFH cells in the Th1 and Th17 OVA-TcR-induced RA models.

Th1 or Th17 polarised CD4+ cells from DO11.10 mice were adoptively transferred to BALB/c mice, which where then immunised with OVA in CFA. Ten days after immunisation recipient mice were challenged in the hind paw with HAO. Control mice received PBS. Seven days post challenge cells from the draining LNs were analyzed for the presence of TFH cells. TFH cells were identified based on the expression of CD4, ICOS and CXCR5. The figure demonstrates representative FACS plots gated on CD4+ cells. Similar results were acquired in two additional experiments.

a)

b)
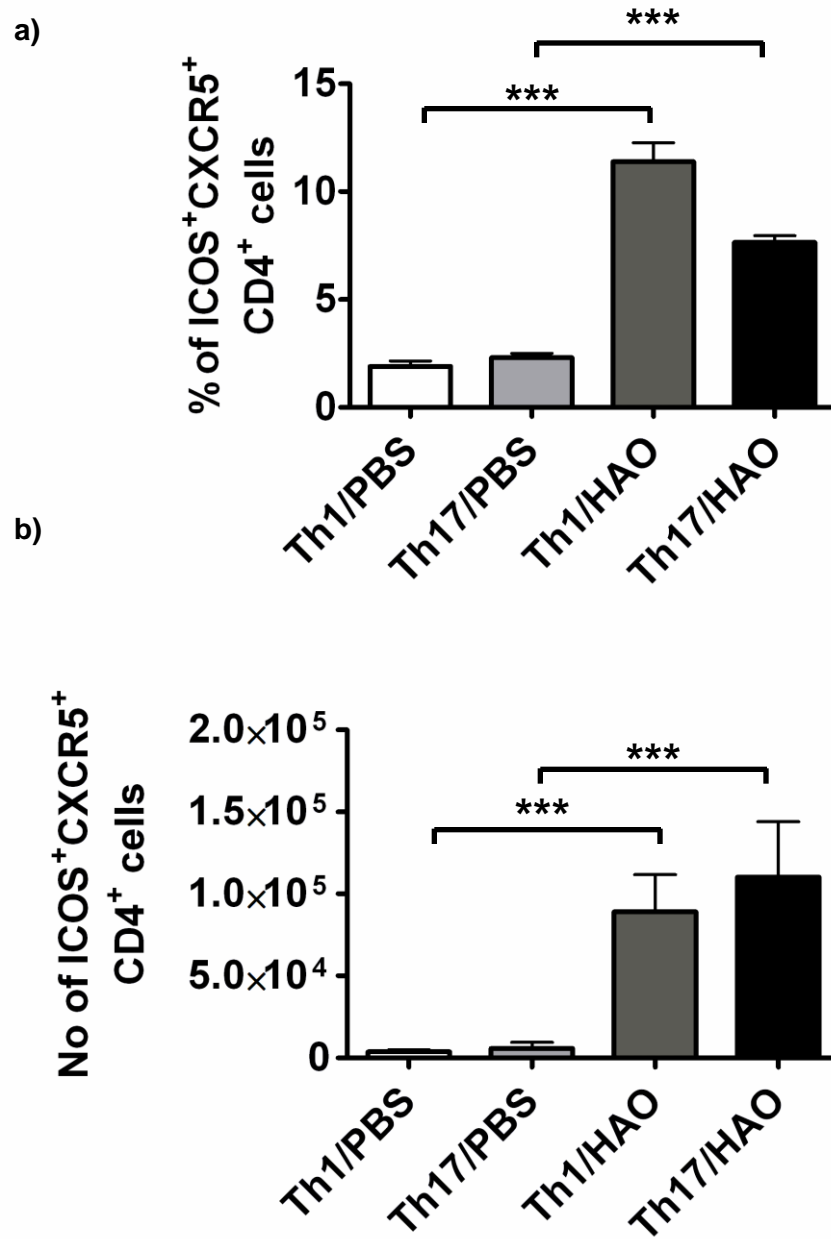
Fig 5.20: Quantification of TFH cells in the Th1 and Th17 OVA-TcR induced arthritis models

Th1 or Th17 polarised CD4^{+} cells from DO11.10 mice were adoptively transferred to BALB/c mice, which where then immunised with OVA in CFA. Ten days after immunisation recipient mice were challenged in the hind paw with HAO. Control mice received PBS. Seven days post challenge cells from the draining LNs were analyzed for the presence of TFH cells. The number (a) and percentage (b) of TFH cells in the draining lymph nodes (popliteal) was assessed by flow cytometry based on the co-expression of CD4, ICOS and CXCR5. Data represent mean ±SEM., *p<0.05, **p<0.01, ***p<0.001 (n=6).

Fig 5.21: Generation of germinal centre B cells in the Th1 and Th17 OVA-TcR arthritis models.

Th1 (top panel) or Th17 (bottom panel) polarised CD4^{+} cells from DO11.10 mice were adoptively transferred to BALB/c mice, which where then immunised with OVA in CFA. Ten days after immunisation recipient mice were challenged in the hind paw with HAO. Control mice received PBS. Seven days post challenge cells from the draining LNs were analyzed for the presence of germinal centre B cells. Germinal centre B cells were identified based on the expression of B220, GL-7 and FAS. Figure demonstrates representative FACS plots gated on B220^{+} cells. Similar results were acquired in two additional experiments.

a)

b)
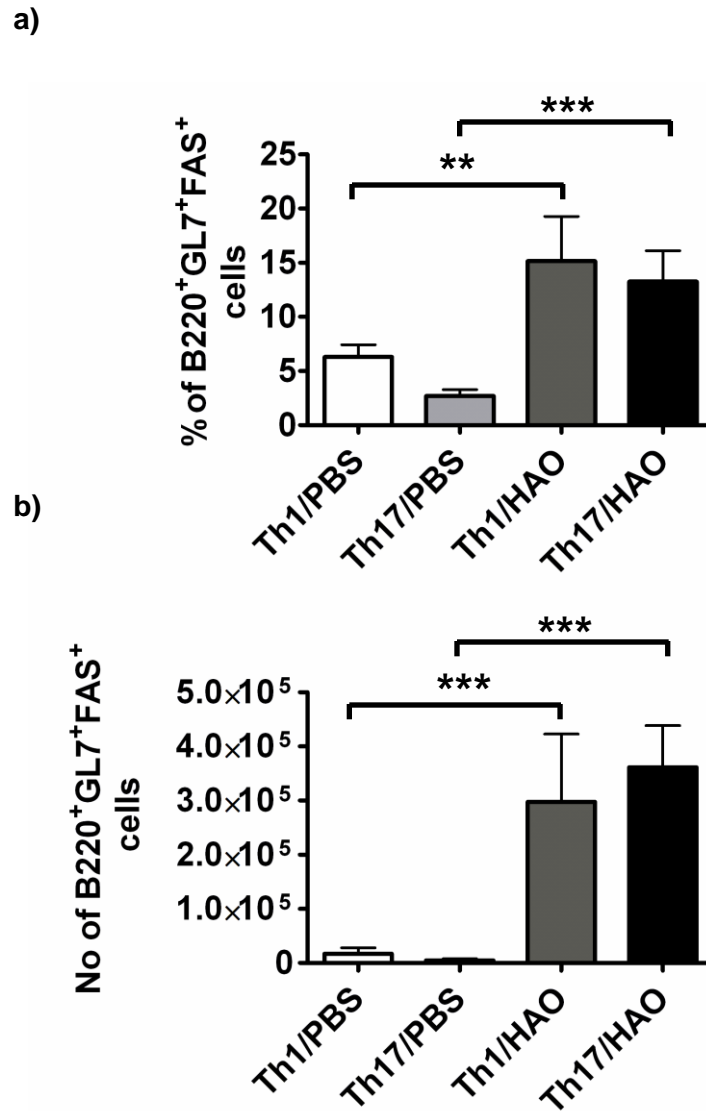
Fig 5.22: Quantification of germinal centre B cells in the Th1 and Th17 OVA-TcR arthritis models.

Th1 or Th17 polarised CD4+ cells from DO11.10 mice were adoptively transferred to BALB/c mice, which where then immunised with OVA in CFA. Ten days after immunisation recipient mice were challenged in the hind paw with HAO. Control mice received PBS. Seven days post challenge cells from the draining LNs were analyzed for the presence of germinal centre B cells. The number (a) and percentage (b) of germinal centre B cells in the draining lymph nodes (popliteal) was assessed by flow cytometry based on the co-expression of B220, FAS and GL-7. Data are presented as mean $\pm$SEM., *p<0.05, **p<0.01, ***p<0.001 (n=6). .

5.4 Discussion

In this chapter the relative ability of Th1 and Th17 polarised populations to support B cells responses was investigated. An adoptive transfer approach was employed that allowed the analysis of the cognate T cell help to antigen specific B cells and the subsequent developing humoral response. In this study transgenic CD4 cells were polarised *in vitro* towards a Th1 or Th17 phenotype and adoptively transferred to recipient mice where they clonally expanded. Similarly to results from the previous chapter, Th17 cells expanded in a greater degree and persisted for a greater time length in the draining LN than their Th1 counterparts. More importantly, it was demonstrated that cells polarised under Th17 conditions have a relative advantage in supporting antibody responses compared to Th1 polarised populations. The Th17 population supported a greater B cell clonal expansion and higher HEL-specific antibody production compared to cells polarised under Th1 conditions. Furthermore, the Th17 population supported higher titres of anti-OVA IgG1 antibodies. On the other hand, only Th1 polarised cells support the production of high titres of IgG2a anti-OVA antibodies. Interestingly, cells from both Th1 and Th17 population acquired a phenotype that supported follicular migration and B cell help, however due to a higher *in vivo* expansion of the Th17 population there was a greater number of cells with a TFH phenotype in Th17 recipients. Nonetheless, both populations had similar ability to migrate and localise into the B cell follicle. Even though, these results demonstrate a higher ability of cells polarised under Th17 conditions to support B cells responses, this was not evident in the Th17 OVA-TcR model of arthritis as both number and proportion of TFH cells and germinal centre B cells were similar.

The data from this chapter suggest a potential advantage of cells polarised under Th17 conditions in supporting B cell responses compared to Th1 polarised cells. Th17 cells have been so far considered pro-inflammatory mediators that cause tissue inflammation through the production of cytokines, such as IL-17 and IL-22(266;273;465;470). However their role in B cell responses has not been thoroughly investigated. An elegant study in the BXD2 autoimmune prone mouse

strain that have elevated levels of IL-17 production has demonstrated the presence of IL-17 producing CD4$^+$ cells in germinal centers, suggesting a potential role for Th17 in promoting autoreactive antibody production. However, the role of Th17 cells in an immunological setting that is not affected by the genetic abnormalities present in the inbred BXD2 mice has never been performed. This was attempted in this chapter using the B-T co-transferred adoptive transfer model. This did not only allow the tracking of both the B and T cell transgenic cells but gave the ability to modulate the phenotype of the transferred T cells and thus to directly compared cells polarised under Th1 and Th17 conditions. Using this model it was demonstrated that cells under Th17 conditions expanded and persisted longer in the draining LN. These results agree with previous data from this thesis and could potentially be the reason for the higher ability of the Th17 polarised population in supporting greater germinal centre formation and antibody production. Indeed dynamic imaging studies using multiphoton microscopy have revealed that most B and T cell interaction in the light zone of germinal centers are of short duration (<5min) and only around 4% of the them are long lasting suggesting that availability of T cell help is the limiting factor for B cell selection(627). This was further confirmed by studies targeting antigen to B cells using DEC205 antibody-antigen conjugates, where it was demonstrated that T cell help is the limiting factor for germinal centre intrazonal micration and B cell clonal expansion(628). It is thus possible that the higher clonal expansion of cells polarised under Th17 conditions increases the availability of T cell help to B cells both before and after the formation of the germinal centre resulting in more robust antibody responses. The microarchitecture of secondary lymphoid organs is critical for optimal cognate T-B cell interactions, which takes place in defined anatomical areas mainly the follicular border and the light zone of the germinal centres(138;616;627;628). As mentioned previously cells that migrate to the follicular region to provide B cell help constitute a distinct T helper phenotype termed TFH cells. Based on that, the relative ability of the Th1 and Th17 population to acquire a TFH phenotype was investigated. This would also be a measure for their potential to move to the follicle. Interestingly both populations had a similar ability to acquire a TFH phenotype, as demonstrated

by the percentage of ICOS and CXCR5 co-expressing cells. However due to a greater expansion of the Th17 population the number of transgenic cells with a TFH phenotype was significantly higher in the Th17 recipients. These data were in accordance with immunocytochemistry data presented in this chapter assessing the localisation of the transgenic T cells in the follicle. Indeed, there was no increased localisation of cells polarised under Th17 conditions in the follicle compared to their Th1 counterparts, when this was normalized to the clonal expansion of transgenic cells and surface of follicle and LN section. However, due to higher clonal expansion of cells polarised under Th17 conditions the number of transgenic cells per unit area of follicle was significantly higher in these mice compare to their Th1 counterparts. These data collectively suggest that the relative advantage of the Th17 population relies to one extent on sheer numbers.

Another possible contributory factor in the greater ability of Th17 population relative to their Th1 counterparts in supporting antibody production is the higher levels of ICOS expression. ICOS is a CD28-like molecule which is crucial for T cell dependent antibody responses and is highly expressed by TFH cells(363;364). In humans absence of ICOS leads to an immunodeficiency that is characterized by failure in memory B cell generation and immunoglobulin class switching, whereas in mice this is accompanied by failure in germinal centers generation (629;630). On the other hand overexpression of this molecule, as in SLE patients and in the *Roquin^{san/san}* mice (sanroque mice) leads to autoantibody related pathology and spontaneous development of germinal centers(378;381;621). It is thus possible that a combination of high number and a higher ability to provide costimulatory helps leads to a more robust B cells response in mice that received cells polarised under Th17 conditions.

Apart from differences in the magnitude of antibody response between the two groups a difference in the quality of the antibody response was observed. In mice that received cells polarised under Th17 conditions the IgG response was characterized by the IgG1 isotype and low levels of IgG2a, whereas Th1 recipients by IgG2a. The IgG2a profile of the Th1 response is not a surprise as the role of IFNγ in IgG2a class switching is well established and our group using the same co-

transfer model has demonstrated that *in vitro* and *in vivo* polarised Th1 cells promote IgG2a class switching(361;362;631). The data relating to the Th17 population agree with a recent report which demonstrated that the antibody class profile induced by cells polarised under Th17 conditions is characterised mainly by IgG1, secondly by IgG2b and low levels of IgG2a antibodies(632). Interestingly, a recent study in humans demonstrated that IL-17 producing TFH cells can induce *in vitro* naïve B cells to produce IgG, IgM and IgA(368). It would be interesting thus to investigate in the co-transfer system the presence of other isotypes such as IgA and IgE. As Th17 cells have been linked to immunity in mucosal surfaces, it would be intriguing to speculate that a Th17-B cell response would be characterised by the appropriate antibody profile, such as IgA.

Even though, it has been demonstrated in this chapter that cells polarised under Th17 conditions are able to support B cells responses, no direct evidence was presented that the cells that provide B cell help are actually Th17. It was demonstrated in the previous chapter that after adoptive transfer, the proportion of IL-17-producing cells in the Th17 population is reduced dramatically. This leaves open the possibility that cells other than Th17 are the ones that provide B cell help. So far most studies circumvent this issue by inhibiting IL-17 or IL-17R signalling which has as a consequence reduction in germinal centre formation. Even though this signifies the importance of IL-17 in germinal centre formation, it is not a direct proof of a Th17 cell providing help to its cognate B cell. In order to achieve this *in situ* staining for transgenic cells expressing Th17 markers (e.g. IL-17, RORγt) needs to be performed in conjunction with staining for the transgenic B cells. This will allow assessing whether Th17 cells are directly providing help to their cognate B cells. I am currently in the process of developing a protocol for *in situ* IL-17 and RORγt staining in order to perform this analysis.

As both Th1 and Th17 OVA-TcR models of arthritis are characterised by the generation of autoantibodies the presence of TFH cells and germinal centre B cells was investigated. In both models HAO challenged mice were characterized by the presence of a defined population of TFH and germinal centre B cells. This is expected as the antibody titres from these animals suggest an active germinal centre

reaction. Interestingly, unlike the co-transfer model were cells polarised under Th17 conditions induce a more robust antibody response, this is not the case in the OVA TcR model. This could be due to the fact that, as shown in the previous chapter, in both models the OVA and autoreactive T cell responses are characterised only by IFNγ producing cells. Unfortunately, it is difficult to analyse the specificity of the TFH cells. It is true that a proportion of TFH and the germinal centre B cells will be OVA-specific, however a proportion of them will be autoantigen specific. Using tools such as MHCII-tetramers it would be possible to isolate at least cells that are specific for CII peptides. Indeed, our group has reported that the CII-specific response in the Th1-OVA TcR model is predominately against the U1 CII peptide (Cogniliaro P, manuscript under review). This peptide has been reported to be one of the dominant epitopes recognised by anti-CII antibodies in the CIA model(513). Development of a MHCII-U1 peptide tetramer complex would allow the isolation of autoreactive T cells and the assessment of their functionally relative to the OVA-specific T cells.

In the next chapter, possible mechanisms that could lead to breach of self tolerance in autoimmune arthritis will be investigated. This will focus on the role of siglec G, a sialic acid receptor that has been involved in regulating danger associated signals, in this process.

Chapter 6: The Role of Siglec-G in the development of autoimmunity in experimental arthritis

6.1 Aim and rationale

The presence of class switch autoantibodies and autoreactive T cells in the OVA-TcR induced arthritis model suggests a failure in peripheral tolerance. Our group has suggested that local damage in the joint driven by polarised T cells of an irrelevant specificity condition DCs to promote autoreactive responses(7). It is thus possible that failure in regulation of signals induced by sterile damage might be important in developing autoimmunity. Siglecs are members of the immunoglobulin superfamily, which specifically recognise sialic acid on cell surface glycol-conjugates(633). Siglec-G has been reported to be part of a regulatory mechanism that discriminates damage (or danger)- and pathogen-associated molecular signals, thus preventing development of an excessive response against the former(634). Based on this, the role of this molecule was investigated in the OVA-TcR-induced model of arthritis, hypothesizing that its deficiency would lead to a more robust breach of self tolerance and potentially more severe pathology.

6.2 Introduction

It is now well accepted that the innate immune system recognises both DAMPS and PAMPS through the same set of receptors, such as TLRs and Nod-like receptors(635). This raises an important question as to how it differentially regulates damage and pathogen associated signals. Recent evidence suggests that the CD24/Siglec-G complex constitutes a regulatory mechanism that discriminates signals derived from sterile damage from those originating from pathogens. Most of siglecs are negative immunoregulators carrying ITIMs(636). These motifs, when phosphorylated in tyrosines, create binding sites for protein tyrosine phosphatases, such as SHP-1 and-2, which dephosphorylate various intracellular proteins leading to inhibition of many signalling pathways(637). Siglec-G is a member of the CD33-related siglec family in the mouse, has a clear orthologue in humans, Siglec-10(107) and carries an ITIM(638). It is highly expressed by all types of B cells, but also by

other immune cells such as CD11c^{+}, CD11b^{+} cells and T cells(639;640). Its role in immunological responses is not very clear. It has been reported that Siglec-G is a B1 cell regulatory receptor, which inhibits BcR-mediated calcium signalling(517). Its deficiency has as a result a cell intrinsic expansion of the B1a population, which results in higher titres of natural IgM antibodies, without any effect on the B2 cell population or IgG production(517). However, apart from its role in B1 cells, siglec-G has been reported to have a regulatory role in DAMP-mediated inflammatory responses(634). It has been reported that CD24 complexes with siglec-G to create a inhibitory signalling mechanism that specifically recognises DAMPs, such as HMGB1, heat-shock protein-70 and -90 (Hsp-70 and Hsp-90) and negatively regulates their stimulating activity(634). CD24 has a wide distribution on different cell types and was initially attributed with co-stimulatory activity for antigen specific T cells(641-643). Its costimulatory function seems to be redundant in cases where CD28 costimulation is abundant, however it seems to be important at sites as the central neural system where CD28 expression is poor(642;644). Consistent with this, CD24 deficient mice are protected from EAE development(645). This suggests an immune enhancing effect for this molecule, however in an acetaminophen-induced liver injury model CD24-deficiency resulted in an increased susceptibility to necrosis of liver cells(634). CD24 mediated this function through association with various DAMPs, namely HMGB1, Hsp-70 and Hsp-90, which were critical for liver necrosis. Interestingly, CD24 mediated its inhibitory function through Siglec-G, with which it physically associates, in an NFκB dependent manner(634). Indeed, Siglec-G deficient mice phenocopy CD24-deficient mice in the acetaminophen-induced liver injury model. This is a DAMP-specific effect as the proinflammatory signals initiated by LPS or poly-I:C are not regulated by this complex, making this pathway a possible discriminatory mechanism between DAMP and PAMP initiated signals. As DAMPs have been reported to possess adjuvant properties and activate DCs(646), which are the initiators of the adoptive immune response, it would be intriguing to hypothesise that absence of this regulatory mechanism might lead to aberrant immune responses against auto-antigens released during sterile damage.

Based on this, the effect of Siglec-G deficiency was investigated on the development of autoreactive responses in the Th1 induced model.

6.3 Results

6.3.1 Effect of Siglec-G deficiency on breach of self tolerance in the OVA-TcR-induced arthritis model

In order to investigate the effect of Siglec-G in breach of self tolerance the development of autoreactive responses in Siglec-G deficient mice was investigated, employing the Th1 OVA-TcR-induced model of arthritis. In both knock-out (KO) and wild type (WT) mice a transient arthritis developed as demonstrated by paw swelling and clinical score. Siglec-G KO mice exhibited more severe clinical signs of arthritis only at day 6, however by day 7 both paw swelling and clinical score were not different than control mice (Fig 6.1a-b). The development of autoreactive B cell responses was investigated by analyzing the presence of anti-CII IgG antibodies. Interestingly, HAO-challenged KO mice developed significantly lower anti-CII IgG antibody titres, compared to WT HAO-challenged mice (Fig 6.2b).Even though WT mice injected with PBS had a high background of anti-CII antibodies this was still lower than HAO challenged mice. This effect is CII-specific as both WT and KO mice develop equal levels of anti-OVA antibodies (Fig 6.2a). These suggest that Siglec-G might be involved in the development of autoreactive B cell responses. The development of autoreactive T cell responses was then investigated. Seven days post-challenge cells from draining LNs were cultured in the presence of media, OVA or CII and their ability to proliferate was assessed employing the Click-iT EDU proliferation assay. In both KO and WT animals challenged with HAO the T cell responses against OVA was significantly higher to PBS injected mice (Fig 6.3). Surprisingly there were no differences in CII-specific T cell responses between the KO and WT animals. In both cases, in HAO challenged mice the proportion of CD4$^+$ cells that proliferated in response to CII was much higher than the PBS challenged mice. It should be noted however that in both WT and KO HAO challenged mice there was a very high background,

as CD4 cells cultured in the absence of antigen exhibited a significantly higher proliferative response compared to CD4 cells from PBS challenged mice (Fig 6.3b). The phenotype of the OVA and CII specific T cell responses relating to production of IL-17 and IFNγ was then investigated. The phenotype of both OVA and CII responses did not differ between KO and WT mice, and as demonstrated in previous chapters they were characterized almost exclusively by IFNγ producing cells (Fig 6.4 and 6.5). There was minimal production of both IL-17 and IFNγ in PBS challenged mice. As in the proliferation assay, in HAO challenged mice there was a high background of IFNγ producing cells, such as that both OVA and CII responses are not significantly higher than media controls. It is thus of high importance to re-investigate the T cell responses in a separate experiment.

6.3.2 Effect of Siglec-G deficiency in the generation of TFH cells and germinal centre B cells

As there was a difference between KO and WT mice in the production of anti-CII antibodies their ability to develop effective T-cell dependent B cell responses mice was investigated. In order to achieve this, the relative ability of siglec-G KO compared to WT littermate mice to generate TFH cells and germinal centre B cells was investigated. At day 7 post challenge cells from draining LNs (popliteal) were analyzed for the presence of TFH cells by flow cytometry. As in previous chapters, TFH cells were identified as CD4 cells co-expressing CXCR5 and ICOS (Fig 6.6). In PBS challenged KO or WT mice there was a very small number of TFH cell in the draining LNs. On the other hand, in HAO challenged mice there was clear population of TFH cells. Interestingly, there was no difference between WT and KO mice in TFH cell development, as both the proportion and number of TFH cells were similar between the two groups (Fig 6.7). In order to have another measure of the ability of TFH cell to promote B cell responses the generation of germinal centre B cells was investigated. Germinal centre B cells were identified as B220$^+$ cells that co-expressed GL-7 and ICOS (Fig 6.8). Consistent with previous results in this thesis, in PBS challenged mice there was very small number of germinal centre B cells in the draining LN (Fig 6.9a-b). In HAO challenged mice there was a

clear population of germinal centre B cells. As in the case of TFH cells, there was no difference in the number and proportion of germinal centre B cells between KO and WT mice (Fig 6.9a-b). These data suggest that both KO and WT mice have a similar ability in generating T cell dependent B cell responses and thus the lower ability of KO mice to develop anti-CII antibodies is due to different reasons.
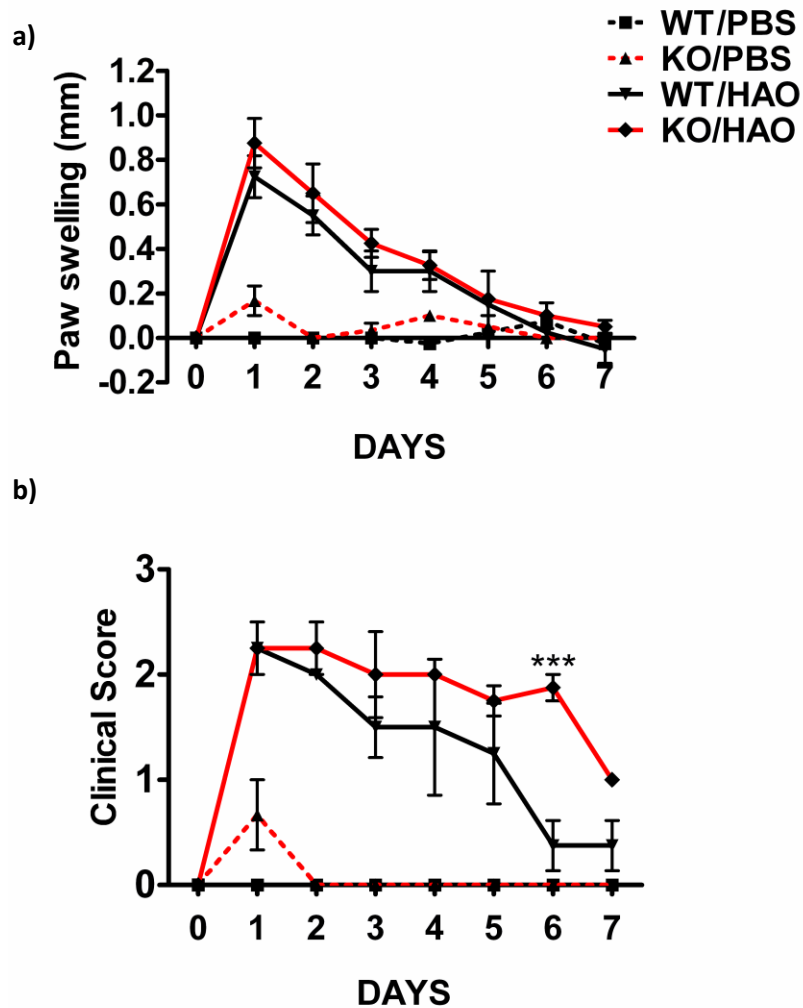
Fig 6.1: Effect of Siglec-G deficiency in the development of clinical signs of arthritis

The Th1 OVA-TcR-induced model of arthritis was employed using as recipient mice Siglec G KO or WT littermate mice. Arthritis was assessed for 7 days by measuring the difference in paw thickness between the challenged and unchallenged paw (a) or clinical score (b). Data represent mean ±SEM.*: WT/HAO vs. KO/HAO,*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ (n=4).
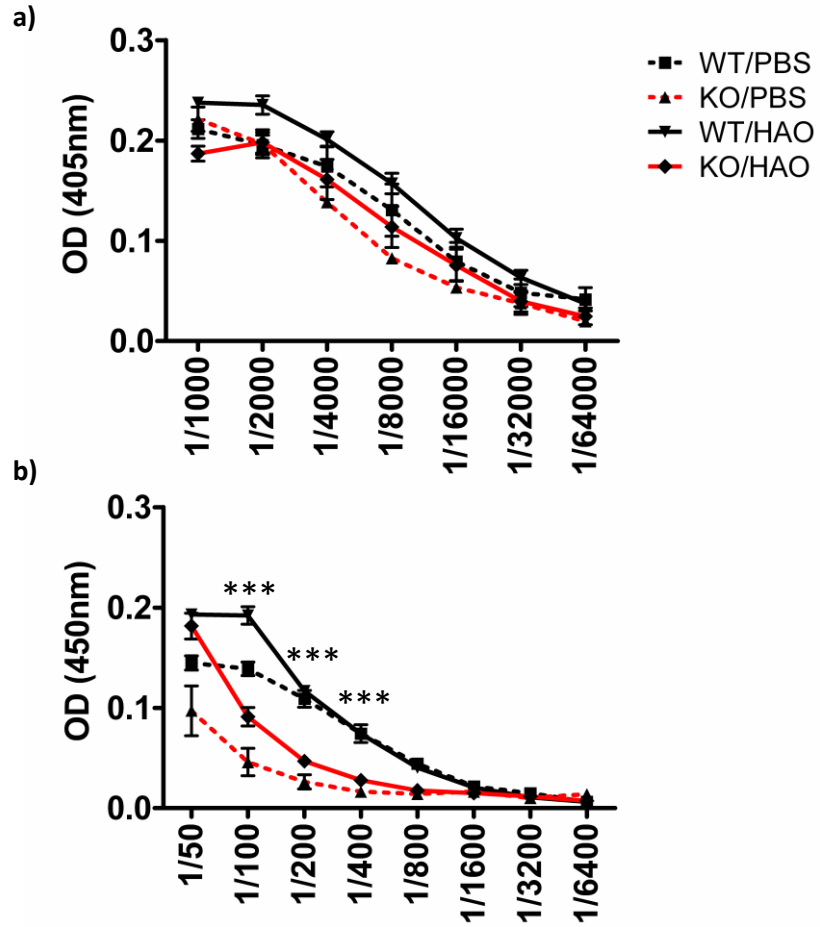
Fig 6.2: Effect of Siglec-G deletion in the development of autoantibodies
The Th1 OVA-TcR-induced model of arthritis was employed using as recipient mice Siglec G KO or WT littermate mice. At day 7 sera was sampled and the presence of anti-OVA (a) and anti-CII (b) IgG antibodies was analysed by ELISA. Data represent mean ±SEM.*: WT/HAO vs. KO/HAO,*p<0.05, **p<0.01, ***p<0.001 (n=4).
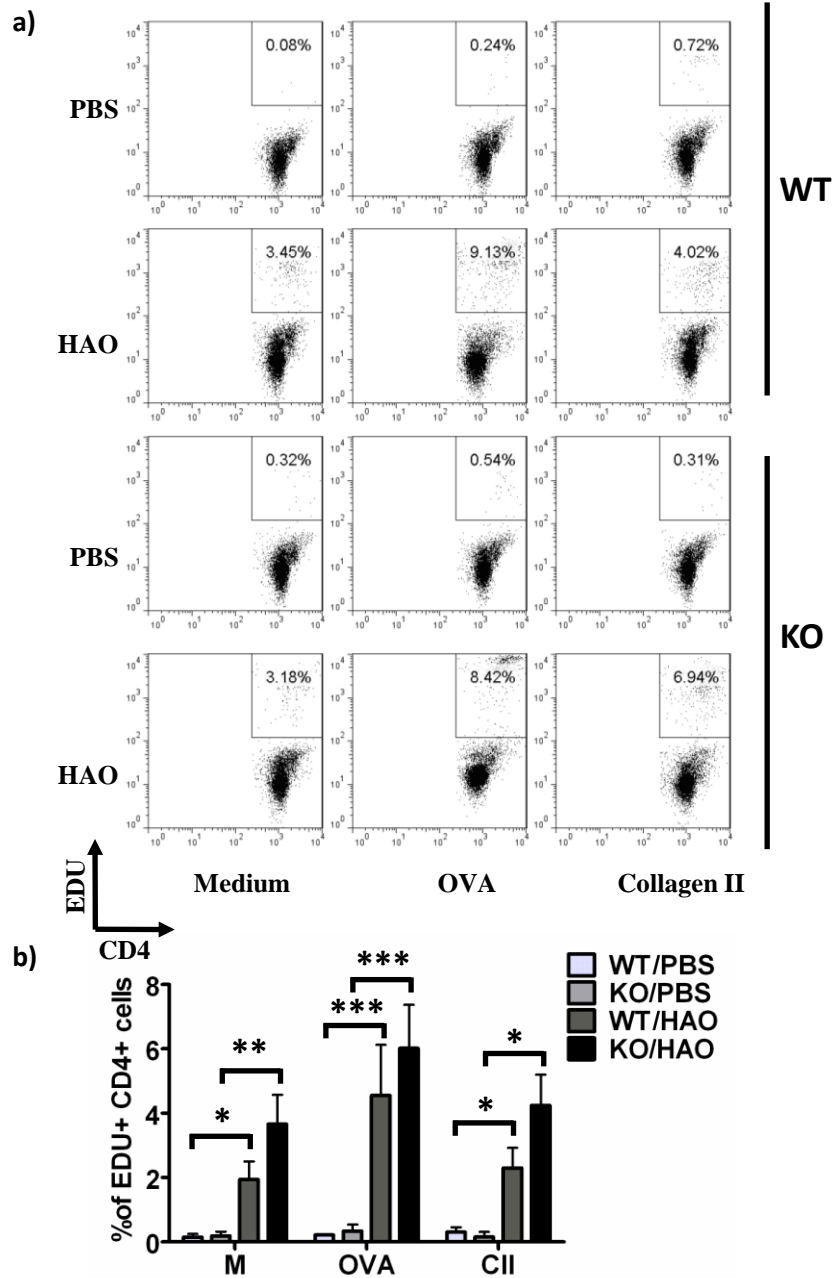
Fig 6.3: The effect of Siglec-G deletion on the development of autoimmunity

The Th1 OVA-TcR-induced model of arthritis was employed using as recipient mice Siglec G KO or WT littermate mice. At day 7 cells from the draining LN were harvested and cultured for 72hrs in the presence of either media, OVA or CII and the ability of CD4$^+$ cells to proliferate in response to them was assessed using the Click-iT EDU proliferation assay by flow cytometry. Populations were gated on lymphocytes based on the FSC and SSC profile and then CD4$^+$ T cells based on CD4 expression. a) Representative FACS plots of WT (top panel) or KO (bottom panel). b) Collective flow cytometry data of proliferation assay. Data represent mean ±SEM, ,*p<0.05, **p<0.01, ***p<0.001 (n=4).
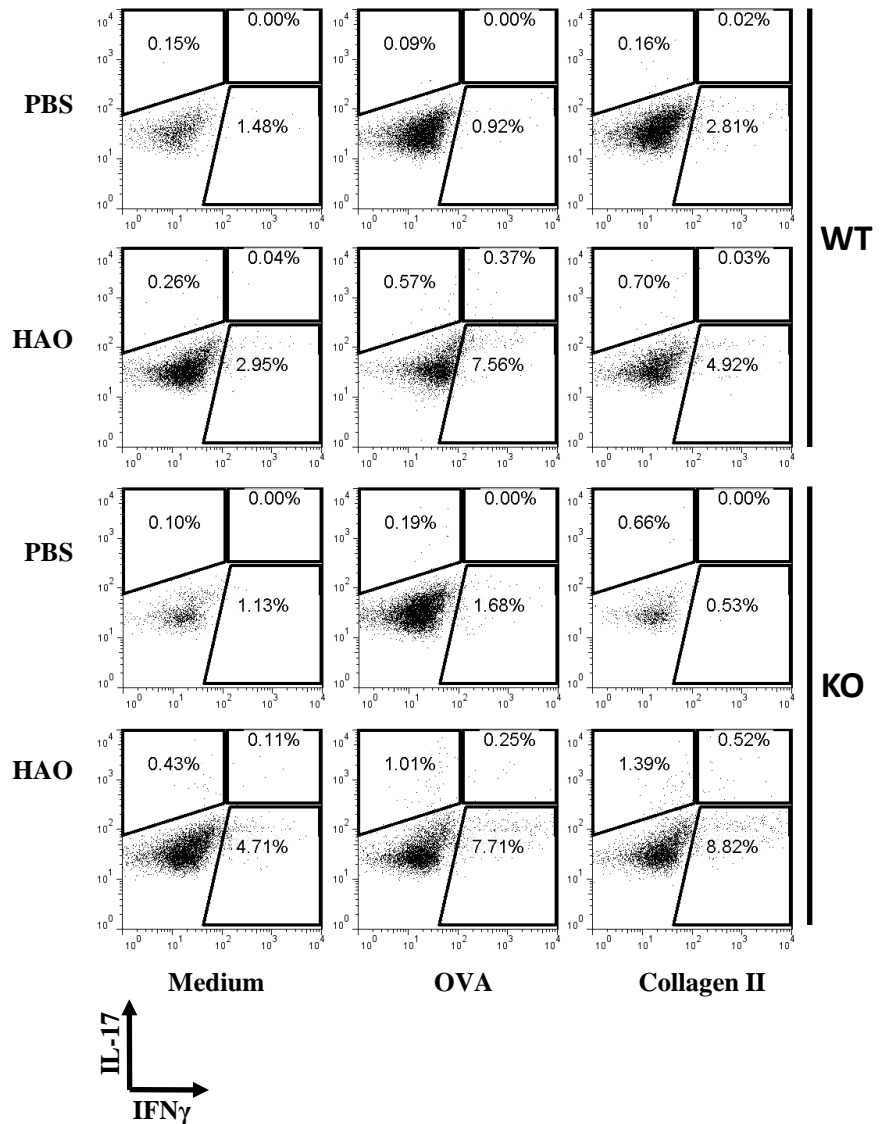
Fig 6.4: Effect of Siglec-G in the phenotype of the OVA and Collagen II T cell response

The Th1 OVA-TcR-induced model of arthritis was employed using as recipient mice Siglec G KO or WT littermate mice. At day 7 cells from the draining LN were harvested and cultured for 72hrs in the presence of either media, OVA or CII and the ability of CD4^{+} cells to cytometry staining. Representative fluorescent cytometry plots demonstrating the production of IL-17 or/and IFNγ by CD4^{+} cells of PBS or HAO challenged WT (top two panels) or KO (bottom two panels) mouse.
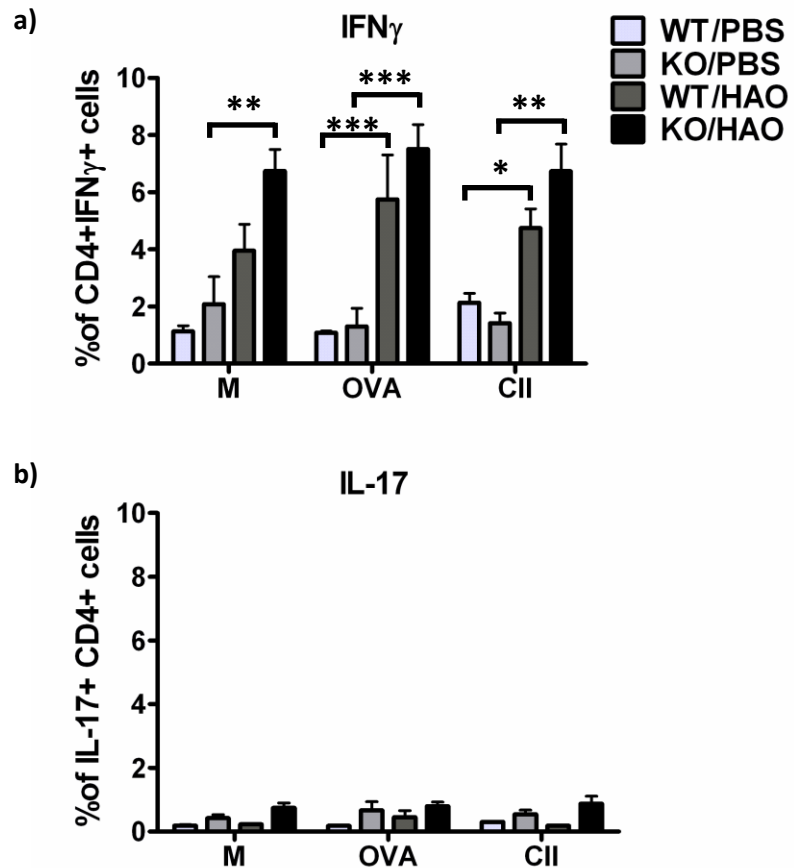
Fig 6.5: Effect of Siglec-G in the phenotype of the OVA and Collagen II T cell response

The Th1 OVA-TcR-induced model of arthritis was employed using as recipient mice Siglec G KO or WT littermate mice. At day 7 cells from the draining LN were harvested and cultured for 72hrs in the presence of either media, OVA or CII and the ability of CD4^{+} cells to cytometry staining. Fluorescent cytometry data demonstrating the production of IFNγ (a) or IL-17(b) by CD4^{+} cells of PBS or HAO challenged WT or KO mice. Data represent mean ±SEM, ,*p<0.05, **p<0.01, ***p<0.001 (n=4).
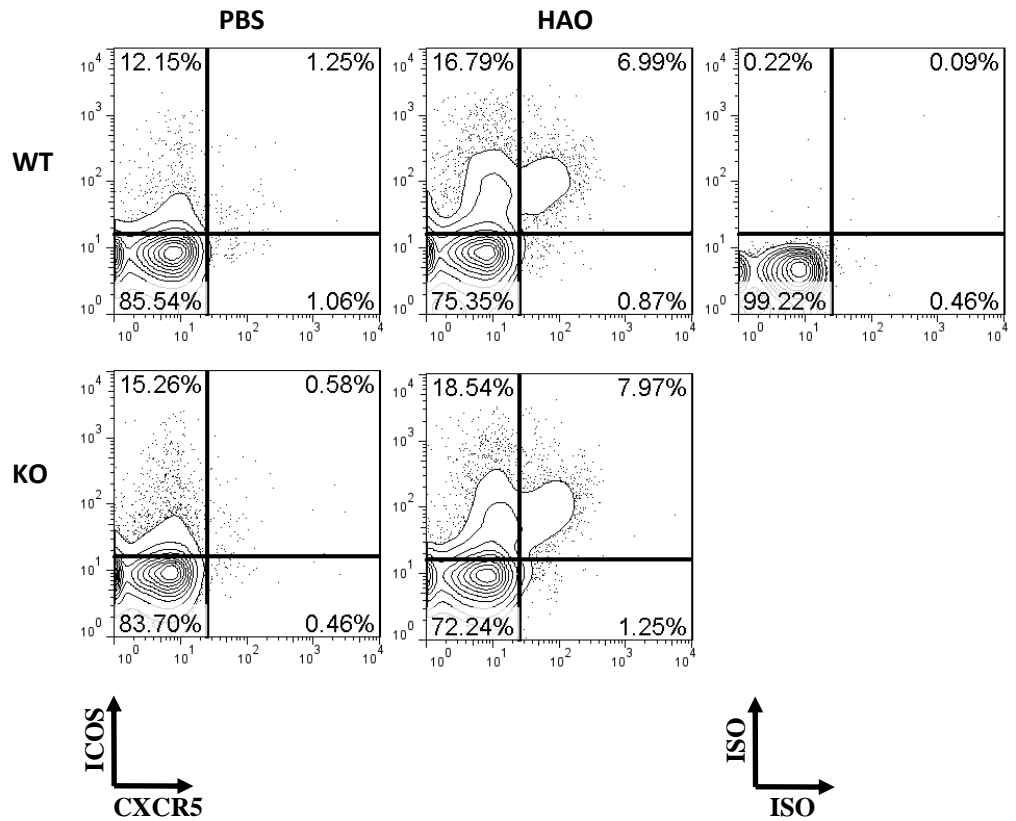
Fig 6.6 Effect of Siglec-G deficiency on TFH cell development

The Th1 OVA-TcR-induced model of arthritis was employed using as recipient mice Siglec G KO or WT littermate mice. Seven days post challenge cells from the draining LNs were analyzed for the presence of TFH cells. TFH cells were identified based on the expression of CD4, ICOS and CXCR5. The figure demonstrates representative FACS plots gated on CD4+ cells. Similar results were acquired in one additional experiments.
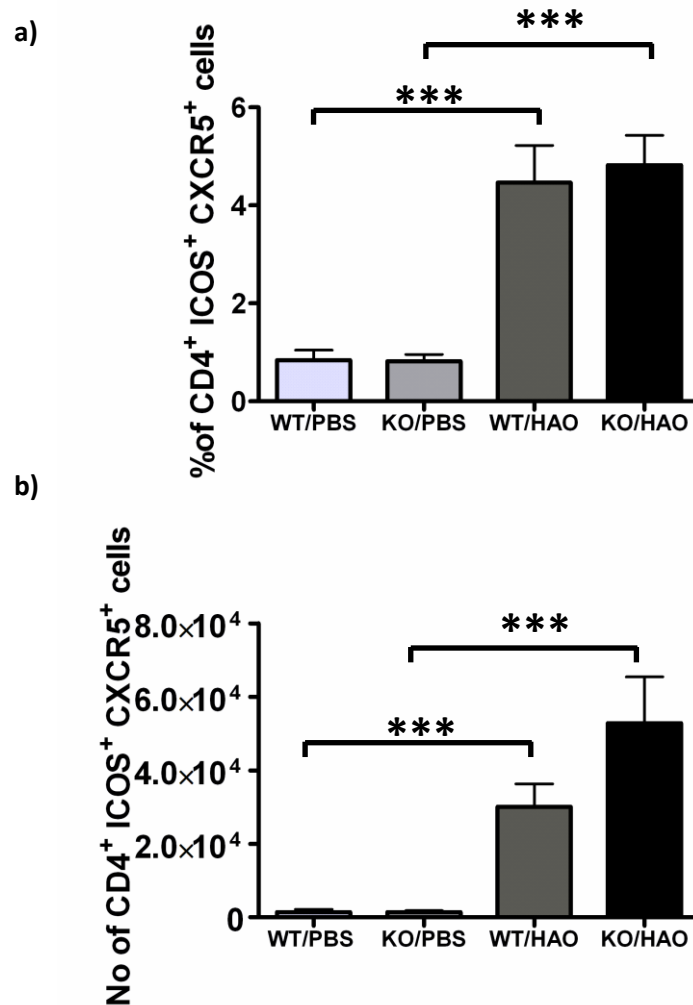
Fig 6.7 Effect of Siglec-G deficiency on TFH cell development

The Th1 OVA-TcR-induced model of arthritis was employed using as recipient mice Siglec G KO or WT littermate mice. Seven days post challenge cells from the draining LNs were analyzed for the presence of TFH cells. The number (a) and percentage (b) of TFH cells in the draining lymph nodes (popliteal) was assessed by flow cytometry based on the co-expression of CD4, ICOS and CXCR5. Data represent mean ±SEM., *p<0.05, **p<0.01, ***p<0.001 (n=6)
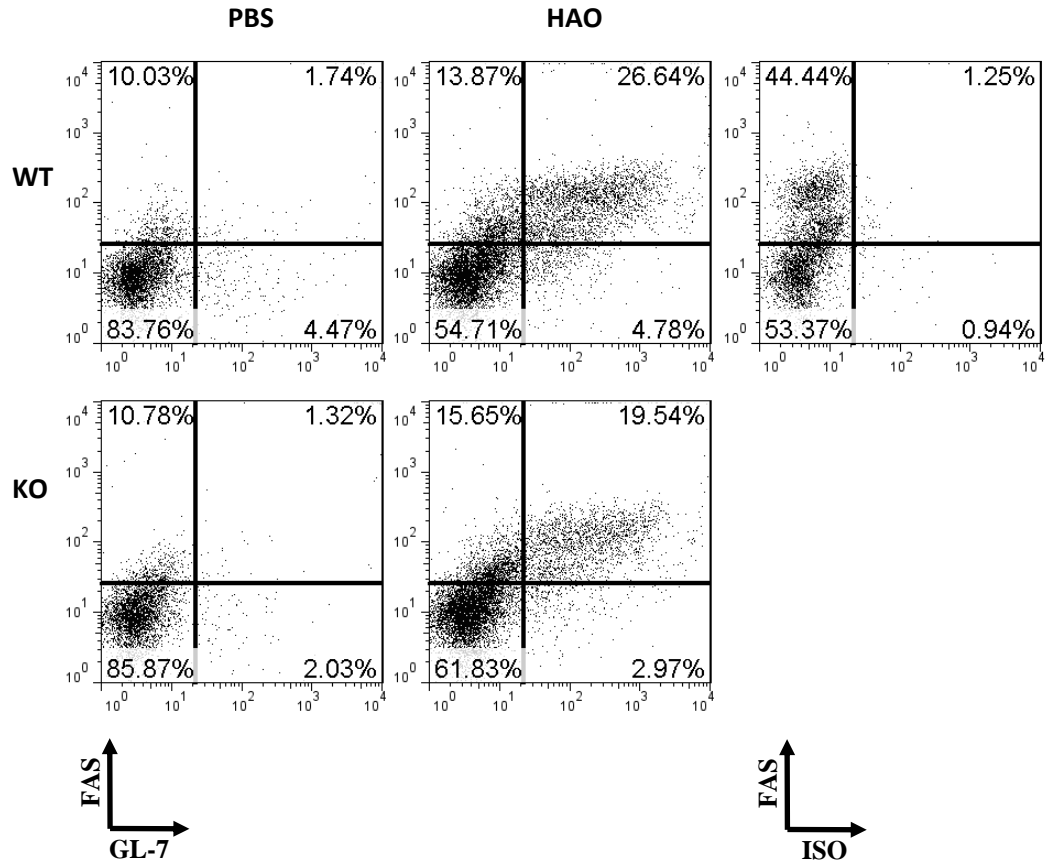
Fig 6.8 Effect of Siglec-G deficiency on germinal centre B cell development
The Th1 OVA-TcR-induced model of arthritis was employed using as recipient mice
Siglec G KO or WT littermate mice. Seven days post challenge cells from the draining
LNs were analyzed for the presence of germinal centre B cells. The figure
demonstrates representative FACS plots gated on B220+ cells. Germinal centre
B cells were identified as B220+ cells co-expressing GL-7 and FAS. Similar
results were acquired in one additional experiments.
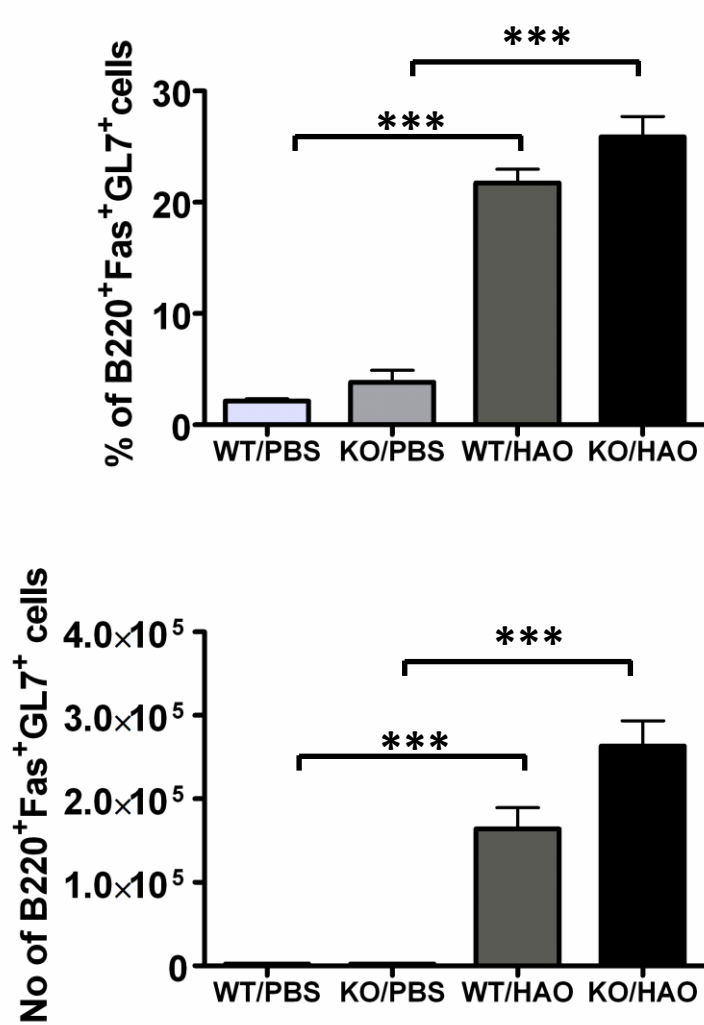
Fig 6.9 Effect of Siglec-G deficiency on germinal centre B cell development
The Th1 OVA-TcR-induced model of arthritis was employed using as recipient mice Siglec G KO or WT littermate mice. Seven days post challenge cells from the draining LNs were analyzed for the presence of TFH cells. The number (a) and percentage (b) of germinal centre B cells in the draining lymph nodes (popliteal) was assessed by flow cytometry based on the co-expression of B220, FAS and GL-7. Data represent mean ±SEM., *p<0.05, **p<0.01, ***p<0.001 (n=6)

6.4 Discussion

In this chapter the effect of Siglec-G deficiency on the breach of self tolerance was investigated. It was hypothesised that the absence of Siglec-G would result in a reduced ability to regulate damage related signals that would have as an effect more robust autoreactive responses. Surprisingly, however, Siglec-G deficiency protected animals from developing autoreactive B cell responses in the form of anti-CII IgG antibodies. This was an autoantibody specific phenomenon as the development of anti-OVA antibodies was not affected by Siglec-G deficiency. It was also demonstrated in this chapter that the absence of Siglec-G does not affect the phenotype of the OVA and CII T cell response, which predominantly is characterized by IFNγ-producing CD4 T cells. Lastly, it was demonstrated in this chapter that the inability of the Siglec-G KO mice to develop autoreactive B cell responses is not due to failure in generating TFH cell or effective germinal centre reactions.

The lower level of anti-CII antibodies in the Siglec-G KO mice is an unexpected result. As mentioned previously Siglec-G functions as a negative regulator of BCR signalling and it deficiency results in the expansion of the B1a subset of B cells(517).Indeed, Siglec-G KO mice have been reported to have 5 to 7 times higher titres of IgM antibodies compared to wild type littermates as B1 cells secrete natural antibodies of the IgM isotype mainly(517;647). Interestingly, 50- to 70-weeks old Siglec-G KO mice had higher titres of IgM RF and anti-erythrocyte IgM antibodies than wild types controls(517). In addition, mice deficient in both Siglec-G and CD22, another member of the Siglec family that also inhibits B cell signalling, spontaneously developed anti-DNA and anti-nuclear antibodies which resulted in moderate glomerulonephritis(648). Based on these it would be interesting to investigate the levels of anti-CII IgM antibodies as there is a possibility that these might predominate in the Siglec-G KO mice. If, however, the reason for lower anti-CII IgG antibodies in the KO mice is the predominance of the IgM isotype a similar effect would be observed in the anti-OVA response. On the contrary, in both WT and KO mice there are similar levels of anti-OVA IgG

antibodies. The ability of both Siglec-G KO mice to mount effective T-cell dependent B cell responses was also reconfirmed by the presence of TFH cells and germinal centre B cells. These data agree with reports that assessed the ability of Siglec-G KO mice to mount B cell responses against trinitrophenol (TNP)-ovalbumin, which demonstrated no defect in production of anti-TNP IgG1, IgG2b and IgM antibodies(649).

A possible explanation for the protection of Siglec-G KO mice from the production of anti-CII antibodies is the reduced acquisition or presentation of CII by antigen presenting cells. It is traditionally considered that pathogens have evolved the capacity to acquire sialic acid from the host in order to mimic the local microenvironment and to evade the immune response(107). However there are studies that suggest that sialic acids in pathogens are important for their recognition and the activation of the innate immune response. Indeed the recognition of various pathogens such as *Tryponosoma cruzi, Campylobacter jejuni*, *Neisseria meningitides* and *Porcine reproductive and respiratory syndrome virus (PRRSV)(107;650-653)*is to one extend mediated by sialic acid recognition through various siglecs (e.g. sialoadhesin). If a similar process can take place in humans and if this is mediated by Siglec-G is unknown. Sialylation has been reported to take place in the synovial membrane rheumatoid arthritis patients(654) so the presence of Siglec-G ligands in the joint is possible. A possible way to test the ability of Siglec-G KO DCs to acquire and present antigen is the employment of the Eα-GFP system. Previous studies have demonstrated that the Eα peptide derived from a self protein (I-E), can be detected in the context of MHC Class II by the antibody Y-Ae(655), thus allowing the quantification of antigen presentation. In addition GFP-acquisition allows the assessment of antigen uptake. Differentially sialylate Ea-GFP proteins could allow the comparison of the relative ability of Siglec-G KO compared to WT mice in acquiring and presenting antigen.

In this chapter preliminary data for a potential role of Siglec-G in the breach of self tolerance were demonstrated. However, further studies are required especially related to the effect of Siglec-G deficiency on T cell responses and antigen

presentation, as well as for potential ligands for this receptor in both our model and RA patients employing glycan microarrays(656).

Chapter 7: Conclusions-Future perspectives

As highlighted in chapter 1, RA is characterised by breach of self tolerance that is evident years before the onset of the disease. This is the least studied or understood stage of the disease due to limitation of existing animal models and inability to access tissue samples from patients at this phase. Understanding the timing, location and mechanisms that lead to autoimmunity might hold the key in preventing disease development and promoting re-establishment of immunological tolerance. This thesis aimed to investigate the role of the Th17 effector cells in the induction of autoimmunity by taking advantage an animal model that is characterised by the spontaneous breach of B and T cell tolerance(5). As mentioned previously these cells have been linked to animal models of autoimmunity(8;9) but their role in the breach of tolerance in the context of RA is ill defined.

The first question that was posed in this thesis related to the phenotype of the auto-reactive T cell responses that develop in the Th1 OVA TcR-induced arthritis model. This revealed that the CII-specific T cell response was characterised by the presence IFNγ and the absence of IL-17 producing CD4$^+$ cells. This did not change even in the presence of Th17 inducing adjuvants, such as curdlan or even when Th17 polarised transgenic T cells were used to induce the model. This could suggest that the early breach of self tolerance is characterised by Th1 type responses. On the other hand, cells polarised under Th17 conditions could induce similar breach of self tolerance as Th1 polarised populations. However, whereas the Th1 population retained its phenotype the Th17 population experienced a sharp decline in its ability to produce IL-17. It is thus possible that even in this case the IFNγ-producing cells mediate the immunological events that lead to breach of self tolerance. As already mentioned blocking IFNγ and IL-17 or Th1 and Th17 cells will give more clear answers related to their role in breach of self tolerance.

The absence of IL-17-producing auto-antigen specific cells in a model that is characterised by a variety of auto-antibodies(6) prompted the investigation of the ability of Th17 in supporting B cell responses. Interestingly, not only cells polarised under Th17 conditions were able to support B cell responses, but they have a relative advantage in this function compared to Th1 polarised populations. This is probably due to the significantly higher expansion of these cells *in vivo* and

higher expression of co-stimulatory molecules, such as ICOS. This, in first instance, seems to contradict the similar ability of Th1 and Th17 polarised populations in inducing breach of B cell tolerance. However, the fact that in both cases the CII-specific responses are mediated by Th1-like cells could explain this phenomenon. Even though cells polarised under Th17 conditions can support antibody production this does not necessarily mean that Th17 cells are the ones interacting with the cognate B cells, especially as this population rapidly loses its ability to produce IL-17. As mentioned in chapter 5, *in situ* staining for identification of Th17 cells interacting with cognate B cells will give more information about the role of these cells in antibody responses. Undoubtedly, however, the two transferred populations are distinct after transfer, as shown by differences in kinetics, expansion and functionality. Recent studies, employing fate mapping approaches using IL-17 reporter mice, suggest that Th17 cells can extinguish their ability to produce IL-17 and produce IFNγ(657). It is still unknown, whether these ex-Th17 cells have different functionality than the traditional Th1 cells, however it would be useful to investigate this possibility in the B-T cell co-transfer system and in the RA model. Understanding the fate of these cells through time is a very important question as it can offer opportunities for suitable temporal intervention. In fact, the possibility of a flexible program for effector T cells might have implications relating to both disease pathogenesis and therapeutic intervention. Indeed, based on data from this thesis, it could be possible that the initial breach of self tolerance, probably taking place in the secondary lymphoid organs, is mediated by Th1-like cells producing IFNγ. These cells could potentially acquire, under the influence of environmental factors, trauma or infection, a Th17 phenotype that could mediate enhanced antibody production, expansion of autoreactive B cells clones and direct tissue destruction, leading to the articular phase of the disease. On the other hand if Th cells are plastic, a resetting of their effector phenotype to a non-damaging one could be possible. So in the case of RA and diseases such as multiple sclerosis and type I diabetes, the destructive Th1/Th17 response could be altered to a more benign Th2 phenotype. There are also downsides in this approach, as in the case of

therapeutic use of T_{REG} cells, where their potential transformation to inflammatory cells could be detrimental.

One of the major limitations of the OVA-TcR arthritis model is that, even though it is characterised by the development of autoreactivity, it does not progress to the active phase of the disease. Indeed, in both Th1 and Th17 models the inflammation is self-resolving over a period of 14 days. In the Th1 model, despite minimal evidence for footpad inflammation after this point, autoantibody titres continue to rise, however mice do not spontaneously develop arthritis at a later timepoint (Conigliaro P. *et al*, submitted manuscript). This is very important for the human disease, as how and why the systemic autoimmunity, developed and expanded in the secondary lymphoid organs, is subsequently focused in the joint is a crucial question. We hypothesized that the answers may lay in the biomechanics of the joint itself and its ability to manage sterile damage. This is the reason that the role of Siglec-G was investigated in the breach of self tolerance, as it has been linked in the regulation of signals derived from sterile inflammation(658). Interestingly, Siglec-G$^{-/-}$mice are relatively protected from the development of anti-CII antibodies. It was speculated that this might be due to a possible defect in the ability of Siglec-G$^{-/-}$ DCs in acquiring autoantigen. Further studies are required to prove this; however the role of molecules such as Siglecs in RA might prove to be important. Siglecs, as mentioned previously, are a family of molecules that bind sialic acid and are thought to promote cell-cell interaction and regulate the function of innate and adaptive immune cells through recognition of glycans(107). They are categorized into two subsets, based on their sequence homology and evolutionary conservation, the CD-22 and CD33-related Siglecs(107). With the exception of resting T cells, most cell in the human and mouse immune system express one Siglec and some express several(107). All CD22- and most CD33-related Siglecs carry one or more ITIM, suggesting an immunoregulatory role for these molecules, whereas Siglec-H in mice and Siglec-14 and -15 in humans lack this motif(107). In RA, changes have been reported in glycosylation of synoviocytes, chondrocytes, in synovial fluid glycoproteins, and in IgG(659-662). It is thus possible that molecules, such as Siglec, which recognise glycan-moieties, to be involved in the pathogenesis of the

disease. Indeed, sialoadhesin, a CD22-related Siglec expressed by macrophages, has been found to be present in high levels in synovial membranes of RA patients(663). It would be thus useful to investigate the role of Siglecs in the development of autoimmunity and in conditions such as RA.

The events that lead to breach of self tolerance, progression to active disease and tissues destruction are highly dynamic in a temporal and spatial manner. Imaging techniques (e.g. PET, SPECT, MRI) have impacted on arthritis from a diagnostic and assessment of pathology point of view(664;665), whereas imaging at cellular resolution has a significant impact in the understanding of immunological processes(666;667). The most effective approach to acquire this type of data is through *in vivo* optical imaging(668). Optical imaging, employing techniques such as multi-photon laser scanning microscopy (MPLSM) will allow the undertaking of important, detailed, kinetic studies of cellular behaviour required in both lymphoid and disease relevant tissues during initiation, maintenance and resolution/regulation of autoimmunity and pathology. This could potentialy allow to identify where, when, and which cells are interacting, how they are interacting, thus facilitating the identification of new cellular and molecular targets. As these studies allow the temporal mapping of the development of an autoimmune response, they will potentially identify windows of opportunity for the most appropriate intervention. In the case of RA and experimental arthritis imaging modalities that maximize spatial resolution within the joint are required. Employing multiphoton endoscopy, using gradient reflective index lenses (GRIN) could allow unparalleled imaging within the joint. This approach has recently been applied to study morphological and structural alterations occurring in the joint during the onset of arthritis in the SKG murine model(669). As this imaging modality has already been employed in humans, it has a great potential as a diagnostic tool in assessing joint damage in a minimally invasive manner(670). Understanding the mechanisms that lead to autoimmunity in a spatio-temporal manner in vivo can provide data that will rationalize the use of existent therapeutics so that the right person can receive the right therapy at the right time and place.

In conclusion, this thesis, provided data relating to the ability of Th17 and Th1 cells in inducing breach of self tolerance. It was demonstrated that both Th1 and Th17 effector cells of an irrelevant specificity can induce breach of self tolerance, however in both cases these responses were mediated by Th1-like cells. In addition, in this thesis evidence is provided that demonstrate the relative advantage of cells polarised under Th17 condition in supporting B cell responses compared to Th1 cells, through their ability to expand and persist longer in the secondary lymphoid organs, even though they don't retain their ability to produce IL-17. Finally, some preliminary data were produced involving Siglec-G in the development of autoreactive B cell responses.

Appendix

I. Buffers

a) <u>Flow cytometry buffers</u>

<u>Phosphate Buffered Saline (PBS) 1x (1000ml)</u>

NaCl	8g	Sigma
KCl	0.2g	Sigma
Na_2HPO_4	1.44g	Sigma
KH_2PO_4	0.24g	Sigma

<u>Fixing Buffer</u>

PFA	4%	Sigma
PBS	1x	
NaOH	5N, 1-2 drops	Sigma

<u>Permeabilisation Buffer</u>

PBS	1x	
Saponin	0.5%	Sigma
FCS	1%	GIBCO, Invitrogen
NaN_3	0.05%	Sigma
EDTA	pH8, 2mM	Sigma

<u>Fc Receptor Blocking Buffer</u>
Supernatant from 2.4G2 hybridoma cultures

Mouse Serum	10%	Biosera
NaN_3	0.01%	Sigma

<u>Wash Buffer</u>

PBS	1x	
FCS	2%	Sigma
NaN3	0.1%	Sigma

b) <u>Magnetic-activated cell sorting (MACS) Buffer</u>

<u>MACS Buffer</u>

PBS	1x, pH 7.2	GIBCO, Invitrogen
FCS	2%	GIBCO, Invitrogen
EDTA	2mM	Sigma

c) <u>Tissue culture media</u>

<u>Roswell Park Memorial Institute (RPMI) complete media</u>

RPMI-1640	1x	GIBCO, Invitrogen
FCS	10%	GIBCO, Invitrogen
Penicillin	10,000 U/ml	GIBCO, Invitrogen
Streptomycin	10,000 µg/ml	GIBCO, Invitrogen
L-Glutamine	200mM	GIBCO, Invitrogen

<u>Iscove's complete media</u>

IMDM	1x	GIBCO, Invitrogen
FCS	10%	GIBCO, Invitrogen
Penicillin	10,000 U/ml	GIBCO, Invitrogen
Streptomycin	10,000 µg/ml	GIBCO, Invitrogen
L-Glutamine	200mM	GIBCO, Invitrogen

<u>Dendritc cell inducing culture media</u>

RPMI	1x	GIBCO, Invitrogen
Supernatant from the X63 GM-CSF producing cell line	10%	
FCS	10%	GIBCO, Invitrogen
Penicillin	10,000 U/ml	GIBCO, Invitrogen
Streptomycin	10,000 µg/ml	GIBCO, Invitrogen
L-Glutamine	200mM	GIBCO, Invitrogen

d) **Immunohistochemistry and immunocytochemistry Buffers**

TNT buffer
Distilled H_2O

Tris-HCl	0.1M, pH 7.5	Sigma
NaCl	0.15M	Sigma
Tween 20	0.05%	Sigma

TNB Blocking Buffer
TNT

Blocking reagent	1%	TSA, amplification kit, Molecular probes

Blocking Buffer
PBS

Blocking reagent	1%	TSA, amplification kit, Molecular probes

Fc Receptor (FcR)
Blocking Buffer
Supernatant from 2.4G2
hybridoma cultures

Mouse Serum	10%	Biosera
NaN_3	0.01%	Sigma

Permeabilisation Buffer A
(Cytospins and Tissue
sections)

PBS	1x	
Saponin	0.5%	Sigma
FCS	2%	GIBCO, Invitrogen
NaN_3	0.05%	Sigma
EDTA	pH8, 2mM	Sigma

Permeabilisation Buffer B
(Tissue sections)

PBS	1x	
Triton X-100	0.1%	Sigma
BSA	3%	Sigma

Endogenous peroxidase
blocking buffer

PBS	1x	
NaN_3	0.1%	Sigma
H_2O_2	3%	Sigma

e) Enzyme-linked immunosorbent assay (ELISA) buffers

Wash Buffer
PBS	1x	
Tween	0.05%	Sigma

Blocking Buffer
PBS	1x	
FCS	10%	GIBCO, Invitrogen

Dilution buffer
PBS	1x	
FCS	0.2%	GIBCO, Invitrogen
Tween	0.05%	Sigma

f) Buffer for OVA-HEL conjugation

Phosphate Buffer
Na_2HPO_4 (Dibasic)	2.77g in 300ml ddH2O	Sigma
NaH_2PO_4 (Monobasic)	0.78g in 100ml ddH2O	Sigma

Added 60ml of monobasic in 300ml of dibasic to have 65mM PO_4^{2-}, pH 7.5

II. Antibodies

a) <u>Flow cytometry antibodies</u>

<u>Target</u>	<u>Antibody</u>	<u>Isotype</u>	<u>Provider</u>
CD4	anti-CD4 PerCP (Clone RM4-5, 5μg/ml) anti-CD4 FITC (Clone RM4-5, 5μg/ml) anti-CD4 APC (Clone RM4-5, 5μg/ml) anti-CD4 PE (Clone RM4-5, 5μg/ml)	Rat-IgG2a,κ	BD Biosciences
CD45R/B220	Anti-CD45R/B220 APC (Clone RA3-6B2, 5μg/ml)	Rat-IgG2a,κ	ebioscience
ICOS	Anti-ICOS PE (Clone 7E.17G9, 2μg/ml)	Rat-IgG2b, κ	BD Biosciences
CXCR5	Biotynilated-anti-CXCR5 (Clone 2G8, 5μg/ml)	Rat-IgG2a,κ	BD Biosciences
IL-17	Anti-IL-17 PE (Clone TC11-18H10, 2μg/ml)	Rat-IgG1, κ	BD Biosciences
IFNγ	Anti-IFNγ APC (Clone XMG1.2, 5μg/ml)	Rat-IgG1, κ	BD Biosciences
FoxP3	Anti-FoxP3 APC (Clone FJK-16s, 2μg/ml)	Rat-IgG2a,κ	ebioscience
CD95	Anti-CD95 PE (Clone Jo2, 2μg/ml)	Hamster-IgG2, λ2	BD Biosciences

GL-7	Anti-GL7 FITC (Clone GL7, 5μg/ml)	Rat-IgM, κ	BD biosciences
DO11.10 TCR	Anti-DO11.10 TCR FITC (Clone KJ1.26, 2μg/ml)	Mouse-IgG2a	eBioscience
Vα2	Anti-Vα2 APC (Clone B20.1, 2μg/ml)	Rat-IgG2a,κ	eBioscience
Vβ5	Anti-Vβ5 FITC (Clone MR9-4, 2μg/ml)	Rat-IgG1, κ	BD Biosciences
IgMa	Anti-IgMa (Clone 5μg/ml)	Rat-IgG2a,κ	BD Biosciences
	Biotinylated-HEL	N/A	

b) <u>Immunohistochemistry antibodies</u>

<u>Target</u>	<u>Primary antibody</u>	<u>Secondary reagent</u>
CD4	Anti-CD4 eFluor®450 (Clone RM4-5, ebioscience, 5μg/ml)	N/A
B220	Anti-B220 FITC (Clone RA3-6B2, ebioscience, 5μg/ml)	Rabbit anti-FITC Alexa Fluor®488
B220	Anti-B220 eFluor®450 (Clone RA3-6B2, ebioscience, 5μg/ml)	N/A
DO11.10 TCR	Biotin-anti-DO11.10 TCR (Clone KJ1.26, 6μg/ml)	Tyramide signal amplification (TSA kit, Perkin Elmer) and streptavidin Alexa-Fluoe®647(Invitrogen, 2μg/ml)

c) **ELISA antibodies**

Factor to be determined	Coating antigen or detection antibody	Detection antibody
Anti-CII IgG	Collagen type II (4µg/ml, Sigma)	Goat anti-mouse IgG-HRP (1/5000, , Cell Signaling Technologies)
Anti-CII IgG2a	Collagen type II (4µg/ml, Sigma)	Goat anti-mouse IgG2a-HRP (1/5000, , Cell Signaling Technologies)
Anti-OVA IgG1	Chicken Ovalbumin (20µg/ml, Sigma)	Goat anti-mouse IgG1-HRP (1/5000, Cell Signaling Technologies)
Anti-OVA IgG2a	Chicken Ovalbumin (20µg/ml, Sigma)	Goat anti-mouse IgG2a-HRP (1/5000, Cell Signaling Technologies)
Anti-OVA IgG	Chicken Ovalbumin (20µg/ml, Sigma)	Goat anti-mouse IgG-HRP (1/5000, Cell Signaling Technologies)
IFNγ	Purirified anti-IFNγ antibody (Mouse IFN gamma ELISA Ready-SET-Go, ebioscience	Biotinylated anti-IFNγ antibody (Mouse IFN gamma ELISA Ready-SET-Go
IL-17	Purified anti-IL-17 antibody (1.5µg/ml, clone TC11-18H10, BD Biosciences)	Biotinylated anti-IL-17 antibody (1µg/ml, clone TC11-8H4., BD Biosciences)

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