The role of the intra-hepatic immune response following liver transplantation in allograft rejection and acceptance

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DECLARATION

I hereby declare, that all of the planning, experimental design, analysis and interpretation of data and writing of this thesis has been carried out by myself. I performed the experiments included in this thesis while holding a MRC Clinical Fellowship under the supervision of Professor David J. Harrison, Professor of Pathology, University of Edinburgh. The experiments in chapter five were carried out with the assistance of two undergraduate students, Alexey L. Glazyrin and Ben Tura, the RT-PCR with the technical assistance of Francis Rae (Department of Pathology, University of Edinburgh). All the work in this thesis has been presented at scientific meetings and has been published or submitted for publication as listed in the Appendix. I have not presented the studies included in this thesis in candidature for any other degree, diploma or qualification.

Matthias M. Dollinger

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With all my love to Elisabeth, Niklas and my parents

ABSTRACT

Compared to other solid organs following transplantation, the liver is unique as an allograft, more resistant to rejection and accepted without immunosuppression in many animal models. The aim of this thesis was to analyse the allogeneic immune response within the graft following liver transplantation and to examine the role of hepatic cells in graft rejection or acceptance. This might allow the better understanding of the specific features of the immune response towards liver grafts and open new therapeutic approaches to organ transplantation.

Firstly, the composition of the inflammatory infiltrate was analysed by immunohistochemistry in biopsies of patients following liver transplantation undergoing acute or chronic rejection in comparison with patients without rejection. Significant changes in the proportion of infiltrating cells were found for CD4+ T lymphocytes and NK cells. In contrast, the percentage of macrophages and CD8+ T lymphocytes did not vary and B lymphocytes were rarely present. Double-staining proved the CD4+ T cells to be naïve (CD45RA+) and memory (CD45RO+) T lymphocytes. Both subtypes were shown to proliferate within the graft irrespective of the severity of rejection, indicating a primary immune response within the liver allograft.

T lymphocyte-mediated cytotoxicity via perforin/granzyme B and Fas-Ligand/Fas was also studied in these biopsies. Granzyme B expression by CD8+ T lymphocytes was a prominent feature of rejection, implicating this pathway in the immune-mediated graft destruction. In contrast, the receptor Fas (CD95), which has to be present on the target cell for induction of the apoptotic signal, was differentially expressed in the biopsies. In grafts of patients without rejection, Fas expression was up-regulated by the inflammatory infiltrate, indicating a possible mechanism of deleting donor-reactive immune cells. However, in rejecting grafts Fas expression was increased in hepatocytes, which undergo apoptosis only during severe rejection. This was also associated with increased hepatocellular expression of the pro-apoptotic proteins bax, bcl-x and p53, intracellular signals of the apoptotic cascade

acting via mitochondrial depolarisation. In contrast, expression of the anti-apoptotic protein bcl-2 was restricted to the inflammatory infiltrate.

In vitro studies have demonstrated, that engagement of the Fas receptor alone is insufficient to induce large scale apoptosis of hepatocytes due to the intracellular regulation of the signal. Using murine hepatocytes, Fas-mediated cell death was studied and found to be amplified by the cytokine IFN-γ, an important mediator of allograft rejection. This increased sensitivity of hepatocytes to Fas-signalling was dependent on p53 activation as demonstrated in p53-/- hepatocytes, and blocked by cyclosporin A, a common immunosuppressive agent which also inhibits mitochondrial depolarisation. IFN-γ appears therefore to augment Fas-mediated cell death of hepatocytes by an intracellular amplification loop.

Dendritic cells (DC) are important regulators of immune responses including allograft rejection and are in particular equipped in activating naïve T lymphocytes. Using a mouse model, hepatic DC were isolated by adapting a novel method via immuno-magnetic separation, and compared to isolated renal and splenic DC. Although hepatic DC were found to have a unique composition with a high percentage of lymphoid-derived DC, functional differences between DC subpopulations in stimulating allogeneic T lymphocytes were not evident. The DC function was rather dependent on the cytokine environment with induction of a Th1 response following activation with GM-CSF and abrogation of the Th1 response after pre-incubation with TGF-β or CTLA-4Ig, but not IL-10.

In conclusion, these studies provide evidence for a modification of the allogeneic immune response within the liver allograft affecting the cell fate of the infiltrating cells as well as of the target cells. Although hepatic donor cells might contribute to this modification, their interaction with the infiltrating immune cells appears to be strongly dependent on the cytokine environment. This however would implicate, that the resistance of liver allografts to rejection and ultimately graft acceptance is open to manipulation and could be achieved in other organs, too.

TABLE OF CONTENTS

ABBREVIATIONS

Abbreviation	Full Form
-/-	knock-out, e.g. p53-/-
AICD	activation-induced cell death
ANOVA	Analysis of variance
APC	antigen-presenting cell
APT	adenosine triphosphate
bax	bcl-2-associated X protein
bcl-2	B cell lymphoma proto-oncogene 2
bcl-x	B cell lymphoma proto-oncogene X (long ($_L$) and short ($_S$) protein)
bp	base pair
BSA	bovine serum albumin (fraction V)
CD	cluster of differentiation, CD nomenclature, e.g. CD4
cDNA	complementary DNA
CMV	cytomegalovirus
cpm	counts per minute
CsA	cyclosporin A
CTL	cytotoxic lymphocyte
CTLA-4	cytotoxic T lymphocyte-associated molecule 4
CTLA-4Ig	CTLA-4 fused to human IgG
DC	dendritic cell
Dex	dexamethasone
DNA	deoxyribonucleic acid
dNTP	deoxynucleoside triphosphate
dT	deoxythymidine
DTT	dithiothreitol
ED_{50}	effective dose in 50% of a specific test assay by manufacturer
EDTA	ethylene di-amino tetra-acetic acid
EGF	epidermal growth factor
ELISA	enzyme-linked immunosorbent assay
FACS	fluorescence activated cell sorter/sorting
FADD	Fas-associated death domain
Fc	crystallisable fragment
FCS	foetal calf serum
FITC	fluorescein isothiocyanate conjugate
g	acceleration due to gravity (~ 10 ms^{-2})
GM-CSF	granulocyte-macrophage colony stimulating factor
^{3}[H]thymidine	tritiated thymidine
HLA	human leukocyte antigen
ICAM	intercellular adhesion molecule
ID	inner diameter
IgG, IgM	immunoglobulin class G, M
IFN	interferon
IL	interleukin
i.v.	intravenous
K DC	kidney-derived DC

kg	kilogramme
l	litres
L	ligand, e.g. CD95L
L DC	liver-derived DC
LFA	lymphocyte functional antigen
LPS	lipopolysaccharide
µCi	microcurie
µg	microgram
µl	microlitres
µm	micrometer
µM	micromoles
M	molar
mAb	monoclonal antibody
MACS	magnet activated cell sorter/sorting
MHC	major histocompatibility complex
mg	milligram
ml	millilitre
ML	mononuclear leukocytes
MLR	mixed lymphocyte reaction
mm	millimetre
mM	millimolar
MMF	mycophenolate mofetile
mmol	millimol
MPT	mitochondrial permeability transition
mRNA	messenger RNA
MTT	3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide
mU	milli-units
N	nucleotide / nucleoside/ base (e.g. in dNTP)
n/a	not applicable
NFAT	nuclear factor of activated T cells
ng	nanogram
NK cells	natural killer cells
nM	nanomoles
NPC	non-parenchymal cell fraction
NS	normal serum
OD	outer diameter
OKT3	monoclonal anti-CD3 antibody
pAb	polyclonal antibody
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PE	R-phycoerythrin conjugate
pH	$-\log_{10}[H^+]$
r	recombinant, e.g. rGM-CSF
R	roentgen
RAI	rejection activity index
RNA	ribo(se)nucleic acid
RT-PCR	reverse transcriptase PCR

SCF	stem cell factor
S DCi	spleen-derived immature DC
S DCm	spleen-derived mature DC
s.e.m.	standard error of the mean
Taq	Thermus aquaticus DNA polymerase
TBS	Tris-buffered saline
TCR	T cell receptor
TGF	transforming growth factor
Th cell	T helper cell
Th0	T helper type 0
Th1	T helper type 1
Th2	T helper type 2
TNF	tumour necrosis factor
TNF-R	TNF-receptor
Tr cell	regulatory T cell
TRADD	TNF-receptor associated death domain
Tris	tris (hydroxymethyl) aminomethane
U	units
UNG	uracil-*N*-glycosylase
UNOS	United Network for Organ Sharing
wt	wild-type
yr	year

CHAPTER ONE – INTRODUCTION

1.1 History

Transplantation as a possible means to replace diseased organs with healthy ones attracted doctors since the antiquity. The first reference to the concept organ transplantation reaches back to ancient China where the surgeon Tsin Yue-Jen (407-310 B.C.) performed an heart transplant between two soldiers (Kuess, 1991a). After a series of experiments in animals in the 1900's, the first serious attempt of organ transplantation in humans was made by the Russian Voronay in 1933. He performed a homologous renal transplantation using a cadaver kidney and noted post-transplant antibody-bound complement leading to his hypothesis that rejection is an immunological event (Kuess, 1991b). The first successful transplantation in man took place in 1954 in Boston between homozygous twins, however success in homologous transplantation was only made possible by the development and discoveries of transplantation immunology, most prominently by the group of Sir Peter Medawar in Britain. In 1956, they reported the use of lymphoid cells to transfer immunity to skin grafts and other tissues in the mouse (Billingham, 1956), while at the same time the work of Dausset let to the discovery of tissue transplantation antigens (Dausset, 1954). The first clinical successes followed in 1958-1960 in France using immunosuppression by total body irradiation. This form of immunosuppression was rapidly replaced by chemotherapy, i.e. methotrexate, cyclophosphamide and finally 6-Mercaptopurin (Küss et al, 1991b).

The ability to suppress rejection in renal transplants led to attempts to transplant technically more difficult organs. In 1963, Thomas Starzl carried out the first orthotopic liver transplantation followed by reports of the first extended survival in 1968 with the emergence of the new immunosuppressive agents antilymphocyte globulin, azathioprine and corticosteroids (Starzl, 1968). The improvement of organ preservation and the introduction of cyclosporin in 1979 (Calne et al , 1979) finally led to the breakthrough of organ transplantation, including liver transplantation, as an non-experimental procedure as stated in 1983 by the National Institute of Health. However, right from the beginning it was noted that liver transplants were less prone to rejection with episodes of rejection resolving spontaneously without the need to

increase immunosuppression. In animals, this phenomenon was even more spectacular as shown by spontaneous acceptance of liver allografts in pigs without immunosuppression (Calne, 1969). Finally, in 1993 Starzl's group reported the first patients who had stopped their immunosuppression following liver transplantation without loosing their graft to chronic rejection (Starzl, 1993).

Today, around 4000 liver transplants are performed each year in the United States (UNOS data source) and around 3500 in Europe (European liver transplant registry). The majority of transplants are in patients with end-stage liver disease, fulminant hepatic failure or intra-hepatic malignancies with chronic Hepatitis C related cirrhosis being the commonest indication worldwide (Terrault, 2000). A variety of new immunosuppressive agents are currently tested, however the goal of transplantation remains to induce tolerance to the graft without the need for toxic long-term medication.

1.2. Rejection – clinical perspective

1.2.1 Definition

Rejection can be broadly defined as the immunological response of the host to the presence of a foreign tissue or organ with the potential to result in graft dysfunction and failure (Int. Working Party, 1995). It is caused by the genetic disparity between donor and recipient, and viewed from the biological perspective, it occurs by default. In clinical practice however, only some recipients manifest symptoms of allograft recognition while on baseline immunosuppression. To facilitate diagnosis and management, hepatic allograft rejection is therefore commonly divided into hyperacute (humoral), acute (cellular) and chronic (ductopenic) rejection (Neuberger, 1995). These three forms of rejection have different clinical and histological patterns and are thought to reflect different mechanisms of graft damage. Of fundamental importance however is the observation that hepatic allograft survival, unlike the survival of other solid organ transplants, is not adversely affected by early acute

rejection (Neuberger, 1998). Moreover, animal models of spontaneous liver allograft acceptance undergo an initial acute rejection crisis (Qian, 1994). Thus, immune activation might be required for the induction of liver transplant tolerance, but could be inhibited by excessive immunosuppressive therapy (Bishop, 2001).

1.2.2 Baseline immunosuppression

Immunosuppressive strategies in transplantation are typically classified into induction regimens, maintenance immunosuppression and treatment of established allograft rejection (Denton, 1999). However, immunosuppressive requirements following hepatic transplantation are usually lower than for other solid organs and a variety of regimens are therefore used and assessed throughout the world (Neuberger, 2000). The risk of allograft rejection is highest immediately post-transplant, and some centres use an induction regimen with high-dose intravenous corticosteroids rapidly tapered to oral steroids by the end of the first week (Keefe, 2001). Corticosteroids have a range of immunosuppressive effects, and probably the most important in transplantation are down-regulation of MHC antigen expression and inhibition of inflammatory cytokine transcription (Denton, 1999). Other strategies for immunosuppressive induction previously consisted of antibody preparations such as antithymocyte globulin (ATG) or the anti-CD3 antibody OKT3, however lack of superior outcome and serious complications with infection and post transplant lymphoproliferative disorder (PTLD) have led to a decline in their use (McDiarmid, 1991). Currently, two anti-IL-2 receptor antibodies are tested in clinical trials and show no major side-effects and a low incidence of acute rejection, but as yet no difference in graft survival (Langrehr, 1997; Nashan, 1996). Further experimental strategies include the blockade of T cell co-stimulation with the fusion protein CTLA-4Ig or of T cell adhesion molecules with antibodies to ICAM-1, LFA-1 or CD45RB (Neuberger, 2000).

The calcineurin inhibitors cyclosporin and tacrolimus are the basis for the majority of maintenance immunosuppression protocols and are usually initiated at the time of transplantation. Both are derived from fungi and inhibit calcineurin thereby

preventing the dephosphorylation of the nuclear factor of activated T cells (NFAT). This nuclear factor is responsible for the transcription of several cytokines, in particular interleukin-2, and its inhibition leads to a reduction in T cell activation and expansion (Denton, 1999). Both agents have substantial side-effects such as hypertension, nephrotoxicity, neurotoxicity and a higher risk of developing malignancies. In 1994, two multicentre trials compared the Sandimmune preparation of cyclosporin with tacrolimus and found a lower incidence of acute rejection, steroid-resistant rejection and chronic rejection (The U.S. multicenter FK506 liver study group, 1994; European FK506 multicentre liver study group, 1994). However, the microemulsion preparation (Neoral) of cyclosporin has now replaced the older version and new trials are currently undertaken. Most centres use one of the calcineurin inhibitors in a triple therapy together with corticosteroids and azathioprine (Neuberger, 2000). Corticosteroids are usually the first agents to be withdrawn, often within the first 6-12 months, because of their major side-effects with obesity, diabetes mellitus, osteoporosis and hypertension (Reding, 2000). Azathioprine, a purine analogue inhibiting DNA synthesis and proliferation of T lymphocytes, is also primarily effective during the first post-operative year and can be withdrawn thereafter (Padbury, 1998). Its major adverse effects are bone marrow suppression and pancreatitis.

Mycophenolate mofetile (MMF) belongs to the newer generation of immunosuppressive drugs and like azathioprine inhibits T cell proliferation. MMF blocks the de novo synthesis of purines by inhibiting the guanosine nucleotide synthesis (Sievers, 1997). Since T and B lymphocytes, unlike other cell types, lack a salvage pathway, MMF provides a more specific immunosuppression. It is used for both maintenance and treatment of acute rejection with diarrhoea and leucopenia being the major side-effects (Hebert, 1999). Sirolimus (rapamycin) finally is a macrocyclic lactone inhibiting not the T cell production of cytokines like the calcineurin inhibitors, but the intracellular signal transduction pathway distal to the interleukin-2 receptor, thereby inhibiting T and B cell proliferation. Since sirolimus also binds to the same immunophillins as tacrolimus, it is designed for combination

with cyclosporin rather than tacrolimus. Its major adverse effects are bone marrow suppression and hyperlipidaemia (Kelly, 1997).

1.2.3 Hyperacute (humoral) rejection

Hyperacute rejection is a rare, early event usually occurring within hours or days of transplantation (Hubscher, 1989). The syndrome is more common in patients transplanted across ABO barriers and is associated with complement activation and detection of antibodies, e.g. anti-major histocompatibility complex or anti-ABO. The antibodies are either preformed or develop post-transplant, and suggest a humoral mechanism (Gugenheim, 1990). The histological findings are those of hepatocyte necrosis, sinusoidal congestion and haemorrhagic necrosis with linear deposits of IgG or IgM antibodies in arteries, veins and sinusoids. Clinically, the patient presents in a similar manner to acute liver failure with encephalopathy and coagulopathy, biochemical characteristics are great elevation of serum aminotransferases, prolongation of prothrombin time and a decreased total serum complement activity. Hyperacute rejection is unresponsive to all forms of immunosuppression and the only effective form of treatment is urgent re-grafting (Adams, 1990).

1.2.4 Acute (cellular) rejection

Acute rejection occurs in between 24-80% of liver transplants depending on the baseline immunosuppression used (Fisher, 1995). It usually becomes clinically apparent between 4 and 14 days, but may recur or present for the first time several months post-transplant in patients with inadequate immunosuppression (Adams, 1990). Acute cellular rejection is thought to be mediated predominantly by $CD4^+$ and $CD8^+$ T lymphocytes. The primary target cells are endothelial and biliary epithelial cells which express the highest levels of self-antigens, i.e. MHC class I and II antigens (Vierling, 1992). Thus, the hallmark and histological pattern of acute rejection is the triad portal inflammation with predominantly ML including lymphoblasts and eosinophils, bile duct inflammation/damage and subendothelial inflammation (Snover, 1984). Clinical findings may include malaise, fever and

jaundice, but are usually unspecific. Laboratory investigations show increased liver enzymes and a rise or cessation in the fall of bilirubin, but no correlation between these tests and the histological severity of acute rejection have been found (Neuberger, 1995). A number of studies have implicated various markers in serum and bile to be associated with rejection including eosinophil count (Dollinger, 1996) or IL-2 and ICAM-1 (Lalli, 1992). However, in view of the more accurate diagnosis by histology with a higher specificity and sensitivity, most transplant centres rely on liver biopsies as the "gold standard".

At least two features of the above triad are required for a histopathological diagnosis of acute rejection (Int. Working Party, 1995). Additional features such as arteritis, perivenular necrosis without inflammation, hepatocyte ballooning or interstitial haemorrhage may be observed, but are less reproducible. The first attempt at establishing a grading system to facilitate comparisons between centres was made by Demetris and colleagues in 1995 using the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK) national liver transplant data base (Demetris, 1995). Several more grading systems followed, and in 1997 a consensus document was published with a system thought to be simple, reproducible and clinical relevant (Demetris, 1997a). The proposed system grades rejection according to both, an overall impression and a semiquantitative rejection activity index (RAI) intended to help in particular academic research. Thus, acute rejection can be either indeterminate (portal infiltrate fails to meet criteria for acute rejection), mild (portal infiltrate affects a minority of triads), moderate (portal infiltrate expands most or all triads) or severe (as moderate with periportal spillover and perivenular inflammation that extends into the hepatic parenchyma and is associated with perivenular hepatocyte necrosis). Additionally, the inflammation or damage occurring within the portal triad, the bile duct epithelium and the venous endothelium is scored each according to its severity (0-3), giving a final RAI score of 0-9 with 0 indicating no rejection and 9 the most severe rejection.

The treatment of established acute rejection is with high dose corticosteroids with regimens varying between oral prednisolone (100-200 mg/day for 3 days),

intravenous methylprednisolone (500-1000 mg/day for 3 days) or intravenous hydrocortisone (up to 1g/day for 3 days) (Neuberger, 2000). There is little evidence to support any particular regimen and the majority of cases of acute rejection respond to the treatment. The rate of reported steroid resistant rejection is between 7-18% and can be treated by a switch of cyclosporin-based immunosuppression to tacrolimus (Klintmalm, 1993), increase of the tacrolimus dose itself (Boillot, 1998) or in some cases by the use of the monoclonal antibody OKT3 directed against T lymphocytes (Keefe, 2001). Acute rejection is not associated with an increased mortality, however a higher morbidity and significant cost increase as a result of hospitalisation have been reported (Fisher, 1995; Martin, 1997). Recent surveys showed that a single episode of rejection has no impact on or indeed might even improve long-term graft outcome (Dousset, 1998; Wiesner, 1998; Avollo, 1998). Severe acute rejection or acute rejection in patients with Hepatitis C-related liver disease however does adversely affect graft outcome (Wiesner, 1998). There is some evidence, that late acute rejection occurring after 30 days post-transplant is less responsive to steroids and concomitant viral infection or low immunosupressant levels were identified as risk factors for its development (Cakaloglu, 1995; Mor, 1992). Both, late acute rejection and steroid resistant rejection also carry an increased risk of development of chronic rejection, however the reasons remain unclear (Neuberger, 1998).

1.2.5 Chronic (ductopenic) rejection

The incidence of chronic rejection is diminishing worldwide with the newest surveys showing graft loss in about 5% of cases (Wiesner, 1999). Improvements in immunosuppressive therapy are the most likely cause for this. Chronic rejection usually does not occur within 60 days of transplantation and risk factors in the primary graft include a positive lymphotoxic cross-match, low immunosuppression in the early post-operative days, the recipients age, the diagnosis of primary sclerosing cholangitis or Hepatitis C, CMV infection and transplantation between ethnic groups (Evans, 2000). Patients undergoing a re-graft for chronic rejection also carry a higher risk of redeveloping chronic rejection (Wiesner, 1999). Chronic rejection can develop following recurrent episodes of acute rejection or indolently

over a period of months or years. It remains still unclear, if acute and chronic rejection share the same pathogenesis, however T lymphocytes are again the predominant infiltrating cell type (Int. Working Party, 1995). The two main histopathological findings of chronic rejection are loss of small bile ducts (< 60 μm) involving more than 50% of portal triads and obliterative arteriopathy with foam cells. Other unspecific features include a chronic inflammatory infiltrate, cholestasis and hepatocyte necrosis. Clinically, chronic rejection is characterised by increasing jaundice with deteriorating cholestatic liver function tests until graft failure with loss of the hepatic synthetic function. Although recent reports have suggested improving graft function with the use of tacrolimus-based immunosuppression in the early phase of chronic rejection (Sher, 1997), advanced chronic rejection is progressive and the only therapy available is re-grafting.

1.2.6 Clinical Tolerance

Development of tolerance is the major goal in transplant immunology and in clinical transplantation, several observations indicate, that liver allografts are more "tolerogenic" than other solid organs. Liver transplants are less prone to rejection with a lower rate of chronic rejection reflected in a greater half-life of the graft (Opelz, 1992). A positive antibody cross-match does usually not affect graft survival (Donaldson, 1995) and MHC matching of donor and recipient is not routinely performed, since the adverse effect on outcome is only marginal (Doran, 2000). Even transplantation in the presence of ABO incompatibility has a 40% probability of long-term graft survival (Neuberger, 2000). Liver allografts have also been reported to protect kidney transplants from the same donor from humoral or cellular rejection, indication for a systemic rather than local effect (Rasmussen, 1995). However, the most compelling observation for tolerance induction has been made in the recent years following an initial report in 1993 about 6 noncompliant liver recipients who discontinued all immunosuppressive medication without losing their graft to rejection (Starzl, 1993). This report led to several formal studies, and it appears now that in contrast to other solid organ transplants, about 20% of patients with long-term liver grafts can be successfully weaned from their immunosuppression (Mazariegos,

1997). Initially, the detection of systemic microchimerism was linked with a successful drug withdrawal, but other studies found chimerism in only 40% of tolerant patients, while a low incidence of acute rejection, transplantation for non-immune-mediated liver disorders and fewer donor-recipient MHC mismatches were positive indicators (Devlin, 1998).

1.3. Rejection – pathogenesis

1.3.1 Definition

Allograft rejection is the universal response of the immune system to the presence of foreign tissue and is based on the body's ability to distinguish between self, i.e. autologous cells, and non-self, i.e. allogeneic cells. This ability is based on the genetic disparity between individuals of the same species, termed allogenicity, and does not apply to syngeneic, i.e. genetically identical, individuals such as monozygotic twins or animals of the same strain. Following the presentation and recognition of the foreign antigen, the immune system mounts an alloresponse with a number of effector cells which cause the eventual damage to the foreign target cells (Hall, 2000).

1.3.2 Antigen-recognition

The ability of the immune system to recognise transplanted tissue as foreign is based on a region of highly polymorphic genes, called the MHC complex (Wilson, 1967). These genes encode cell surface glycoproteins, categorised into class I and class II MHC antigens, which allow the immune system the distinction between autologous and allogeneic cells (Halloran, 1993). MHC class I antigens are constitutively expressed on virtually all nucleated cells and can bind to the T cell receptor (TCR) on CD8+ T lymphocytes with the CD8 molecule acting as a co-receptor. MHC class II antigens bind in a similar way to CD4+ T lymphocytes, but their constitutive expression is usually limited to immune cells and endothelial cells. Inflammatory

cytokines, and in particular interferons, can increase the expression of both MHC antigens and induce MHC class II antigen expression on other cells, e.g. epithelial cells (Wood, 1994). In the normal liver, MHC class I molecules are expressed on biliary epithelium and vascular endothelium, but usually not on hepatocytes (Fleming, 1981). MHC class II expression is confined to antigen-presenting cells (APCs) such as macrophages and dendritic cells (DCs) or endothelial cells (Daar, 1984). During allograft rejection, MHC class I molecules are expressed by hepatocytes, MHC class II antigens by the biliary epithelium and vascular endothelium (Steinhoff, 1988). However, a direct correlation between the extent of MHC antigen expression and the severity of rejection could not be substantiated (Rouger, 1990).

In the 1980's, Lechler and Batchelor suggested two routes of allorecognition, the direct and the indirect pathway. The initiation of allograft rejection is thought to be by direct presentation of MHC antigens on APCs to recipient T lymphocytes, in particular MHC class II-reactive CD4+ T cells (Lechler, 2001). Experiments in animals have shown, that the reduction of donor APCs within grafts, which prevents direct presentation, leads to a marked reduction in acute rejection (Lechler, 1982). The site of the initial stimulation appears to be the recipient's secondary lymphoid tissue, since animals lacking organised lymphoid tissue are unable to reject vascularised grafts (Lakkis, 2000). DCs, the predominant migrating APC population, are most likely the initial APCs (Larsen, 1990; Steinman, 1991). In animal models of spontaneous liver allograft acceptance, pre-treatment of the donor to increase the number of DCs leads to graft rejection (Steptoe, 1997). The importance of direct allorecognition is also supported by the fact, that the precursor frequency of T cells recognising an allogeneic MHC molecule directly is 100 times higher than that of T cells responding to the same MHC molecule indirectly (Liu, 1993). As many as 2% of a host's T lymphocytes are capable of responding to a single allogeneic MHC molecule. This antigenicity probably derives from various amino acid residues of the MHC molecule as well as endogenous peptides bound in the antigen binding groove of the MHC (Matzinger, 1977).

Although direct allorecognition has historically been the focus of research in transplant immunology, newer studies have underlined the importance of the indirect pathway. Indirect allorecognition involves the processing of donor-derived MHC as well as minor histocompatibility complexes by host APCs, which then present the allopeptides in the context of self-MHC to host T lymphocytes (Rudensky, 1993). There is evidence to implicate the indirect pathway already in the initial rejection process following transplantation (Gould, 1999), however the most intriguing hypothesis links indirect allorecognition to the development of recurrent acute rejection and chronic rejection. While allopeptide-reactive T cells are undetectable in the graft and circulation during stable graft function, they are a prominent feature during chronic rejection (Hornick, 2000). Furthermore, initial rejection episodes are associated with T cells against only one immunodominant alloantigen. In contrast, during recurrent acute rejection or chronic rejection, T cells reactive to other allopeptides can also be isolated. This process is called epitope spreading and is one of the explanations for persistent alloreactivity (Suciu-Foca, 1998).

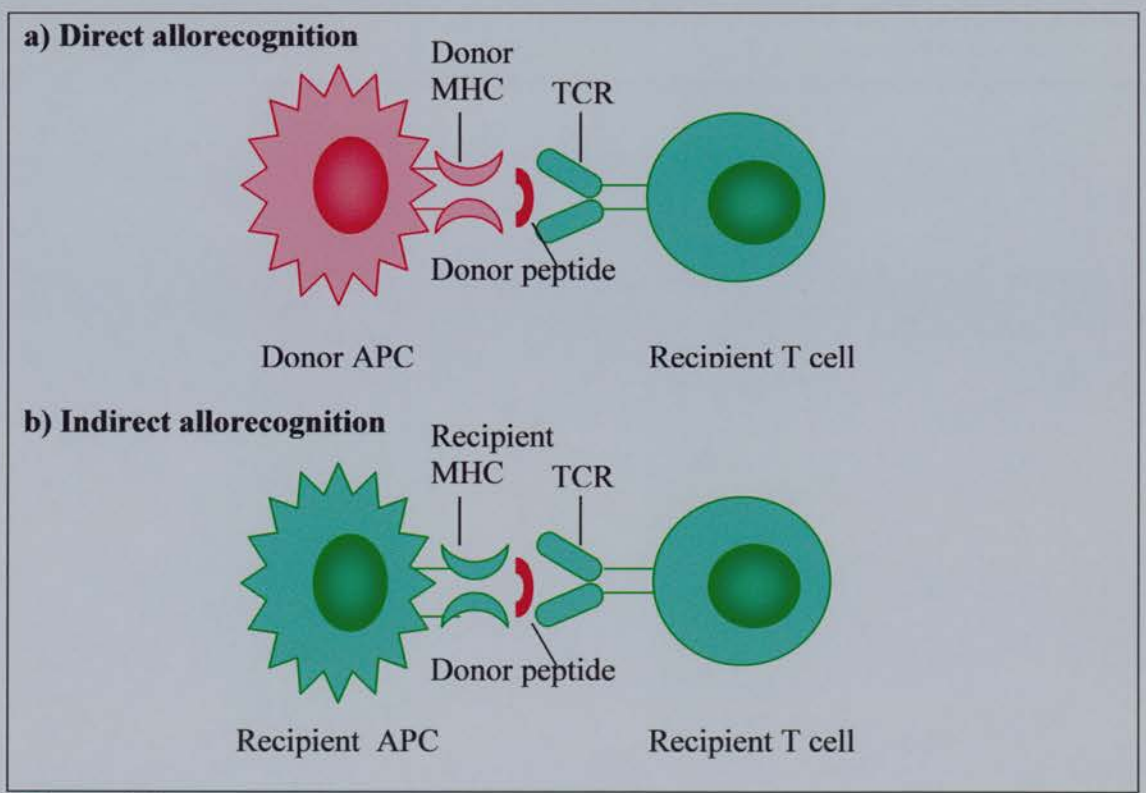


Figure 1.1

Proposed pathways of allorecognition in transplant rejection.

1.3.3 Antigen-presentation

Antigen-recognition via engagement of MHC molecules and the TCR complex alone is insufficient to fully activate naïve T lymphocytes, i.e. lymphocytes which never encountered antigen before. A second, non-antigen specific, signal must be provided by cognate ligands on the APC (Dai, 1999). This co-stimulation induces the T cell to proliferate and differentiate into helper or effector lymphocytes. In the absence of co-stimulation, T lymphocytes become anergic and may undergo apoptosis (Gimmi, 1993; Noel, 1996). Although a number of co-stimulatory proteins have been characterised, three of them are generally believed to be essential for alloimmune responses, B7-1 (CD80), B7-2 (CD86) and CD40 (Dai, 1999).

B7-1 and B7-2 are both ligands for the CD28 receptor on T lymphocytes (Samson, 2000). CD28 ligation facilitates T cell activation by reorganisation of the TCR contact site, resulting in sustained TCR signalling with increased IL-2 production by the T lymphocyte as an autocrine growth factor (Viola, 1999). A competitive receptor for CD28 is CTLA-4, which mediates an inhibitory signal to the T lymphocyte (Walunas, 1996). CTLA-4 has a higher affinity for the two ligands than CD28, but is not expressed on resting T cells and up-regulated only after T cell activation (Alegre, 1996). The expression of B7-1 and B7-2 is also differentially regulated on APCs. In general, B7-2 is constitutively expressed and more rapidly up-regulated, while B7-1 is usually not expressed on resting APCs but the more potent signal (Larsen, 1994). There is speculation, that B7-1 can bias the T cell response towards a Th1-response and B7-2 towards a Th2-response, however studies in knock-out mice rule out an exclusive function for either of the ligands (Schweitzer, 1997).

The second signalling pathway, CD40/CD40L (CD154) appears to have the most pronounced immunostimulatory effect (Dai, 1999). CD40 is expressed on APCs, the ligand CD40L on T lymphocytes. Engagement of CD40 induces a number of pro-inflammatory activities by the APCs including up-regulation of B7-1 and B7-2 expression or the release of IL-12 and adhesion molecules essential for leukocyte

migration (Guo, 1996; Ridge, 1998). CD40 ligation on B cells is also important for the generation of humoral responses with antibody release (Ranheim, 1993).

The central role of co-stimulation in allograft rejection has been demonstrated by multiple animal experiments with blocking antibodies or proteins (Wekerle, 1998; Azuma, 1996). Blockade of CD28 or CD40 signalling has therefore become a focus for new immunosuppressive strategies (Denton, 2000). The discovery of co-stimulatory signals has also shed light on the physiological role of APCs. DCs, monocytes/macrophages and B lymphocytes are thought of as professional APCs (Germain, 1993). However, DCs in particular are equipped to up-take and process foreign antigen, migrate to secondary lymphoid tissue and stimulate primary immune responses (Steinman, 1991). Endothelial cells have also been shown to express co-stimulatory signals, however their exact role in antigen-presentation remains unclear (Rose, 1998; Knolle, 2001).

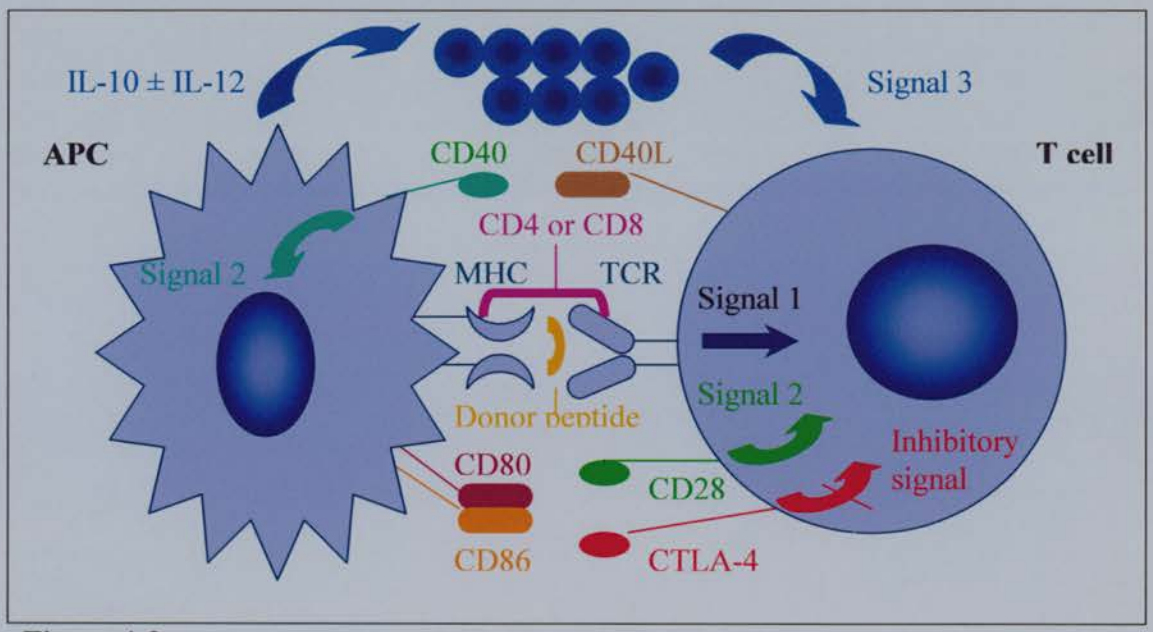


Figure 1.2

Proposed mechanism of antigen-presentation during allograft rejection. The interaction between APCs and T cells involves three signals (Signal 1: ligation between MHC and TCR/CD4 or CD8; Signal 2: ligation between CD80/CD86 and the T cell receptor CD28, ligation between CD40L and the APC receptor CD40; Signal 3: release of cytokines resulting in distinct T cell responses). Engagement of the T cell receptor CTLA-4 by CD80/CD86 leads to T cell inhibition.

1.3.4 Cytokines

Cytokines are specialised proteins and the soluble mediators of immune responses. Their role in allograft rejection has been largely inferred from their *in vitro* functions and from *in vivo* experiments in which cytokine expression has been correlated with rejection. However, due to the complexity of the cytokine cascades triggered after transplantation, many studies have been contradictory and experiments with knock-out mice have indeed proven, that cytokine actions are highly redundant (Dai, 1999). IL-2 for example is an important T cell mitogen, but IL-2-/- mice are still able to reject allografts (Steiger, 1995).

Most of the cytokines, which are important for the development of the alloresponse, are released by APCs and CD4+ Th cells (Hall, 2000; Mosmann, 1991). Th cell responses are classically divided into Th1 and Th2 responses dependent on their cytokine release pattern. Th1 cells release IL-2 and IFN-γ promoting a cellular immune response, while Th2 cells support humoral responses with antibody production by releasing IL-4, IL-5 and IL-10 (Strom, 1996). APCs preferentially induce a Th1 response via secretion of IL-12 or a Th2-response via secretion of IL-10 without IL-12 (Macatonia, 1995; Rissoan, 1996). The cytokine pattern of both Th responses promotes the maturation and proliferation of T lymphocytes, however IFN-γ in particular has been shown to up-regulate the expression of MHC antigens and co-stimulatory signals as well as induce CD8+ CTLs (Hall, 1985; Goes, 1995; Sansom, 2000). In contrast, some of the cytokines released by Th2 cells have immunoregulatory functions, e.g. IL-10, which can down-regulate the expression of MHC antigens and co-stimulatory signals (De Waal Malefyt, 1991; Ding, 1993). These findings have led to the proposal that Th2 responses might be beneficial in achieving graft survival and acceptance. However, recent experiments with knock-out mice have again demonstrated that allograft rejection proceeds in the presence or absence of any of these cytokines (Hall, 2000), and the adoptive transfer of Th2-type lymphocytes induced allograft rejection rather than tolerance (Matesic, 1998). The only exemption appears to be IFN-γ, but only in the context of MHC II-incompatible grafts, which are not rejected by IFN-γ-/- recipient mice (Ring, 1999).

In liver transplantation, several investigators have studied intra-graft cytokine levels during rejection. Some have found an increase of the Th1 cytokines IL-2 and IFN-γ (Bishop, 1990), while others found little IL-2, but an increase of the Th2 cytokines IL-4 and IL-5 (Martinez, 1992). IL-10 was usually not detected during rejection (Conti, 1998), but the pro-inflammatory cytokine TNF-α was shown to be up-regulated within the graft (Teramoto, 1999). This corroborates the findings by Bathgate and colleagues, who demonstrated a correlation between TNF-α but not IL-10 gene polymorphisms with liver allograft rejection (Bathgate, 2000).

1.3.5 Cellular immune response

1.3.5.1 Reperfusion injury

The earliest immune response detectable in organ allografts following transplantation involves the innate rather than adaptive immune system. The period of ischemia necessitated by the explantation, transfer and implantation of the organ followed by the reperfusion with blood results in complement activation, up-regulation of adhesion molecules, inflammatory cell infiltration and cytokine release (Baldwin, 2001). Cells mostly implicated in this immune activation are neutrophils, monocytes/macrophages and NK cells, which can often be found within the graft before T lymphocyte infiltration (Petersson, 1997). Reperfusion injury is not essential for allograft rejection as demonstrated by studies using T cell-deficient mice that show prompt graft rejection after T cell reconstitution even when grafts had recovered for more than 100 days within the recipient (Bingaman, 2000). However, reperfusion injury is thought to augment the severity of rejection and influence graft outcome by direct tissue damage as well as T cell recruitment (Baldwin, 2001). Clinical evidence for this influence comes from transplants from living genetically unrelated donors, which survive as well or better than transplants from genetically matched cadaver donors (D'Alessandro, 1998).

1.3.5.2 Afferent immune response

The earliest presentation of allogeneic antigens is mediated via the direct pathway by haematolymphoid donor cells, as demonstrated by multiple rodent experiments in which these so-called passenger leukocytes were eliminated by in vitro culturing or "parking" in intermediate hosts, which allowed the repopulation of the graft with haematolymphoid cells syngeneic to the recipient (Guttmann, 1969). Using the same experimental model, Lechler and Batchelor showed that DCs were the most immunogenic among the passenger leukocytes implicating them as key players in the initiation of the immune response (Lechler, 1982). DCs are regarded as the sentinels of the immune system and can be found in most tissues and organs, in the liver usually within the portal tracts (Prickett, 1988). In peripheral organs such as the liver, DCs are immature with a low expression of co-stimulatory signals and capable of taking up antigen (Woo, 1994). Subsequently to antigen uptake or maturation signals received during transplantation, DCs mature and migrate to secondary lymphatic tissue where they encounter and stimulate T lymphocytes (Steinman, 1997). Following liver transplantation, efferent lymphatic channels are disrupted for 2-3 weeks (Rabin, 1991), and APCs including DCs as well as other immune cells migrate haematogenously or via intact regional donor lymphatic channels to the recipient spleen or regional donor lymph nodes (Fung, 1989). Donor cells are found already after one day post-transplant in the spleen and after two days in regional lymph nodes, but not in other lymph nodes or the thymus (Demetris, 1991). This is called central sensitisation, however peripheral sensitisation within the allograft might also occur, since clustering between DCs and lymphoblasts can be observed within the portal triads at day 3 post-transplant (Demetris, 1991).

Since donor APCs are short-lived, replacement with recipient cells occurs within the first weeks following transplantation, both peripherally within the graft and centrally in the spleen and lymph nodes (Gouw, 1987). This phenomenon is further accelerated by the severity of rejection with release of immuno-active cytokines and adhesion molecules (Demetris, 1997b). Migration of APCs from the blood to the liver is facilitated by the hepatic sinusoids which form only a discontinuous barrier

lacking tightly regulated junctions (Jaeschke, 1997). In particular DCs utilise this system as their preferred route of translocation from the blood to the lymphatic system (Kudo, 1997), however peripheral sensitisation might also be a significant feature following transplantation. In chronic rejection, recipient APCs and in particular recipient DCs are found within lymphoid aggregates inside the grafts (Oguma, 1988) and the number of recipient DCs correlates directly with the severity of the inflammation, supporting the role of indirect allopresentation in the development of chronic rejection (Demetris, 1997b).

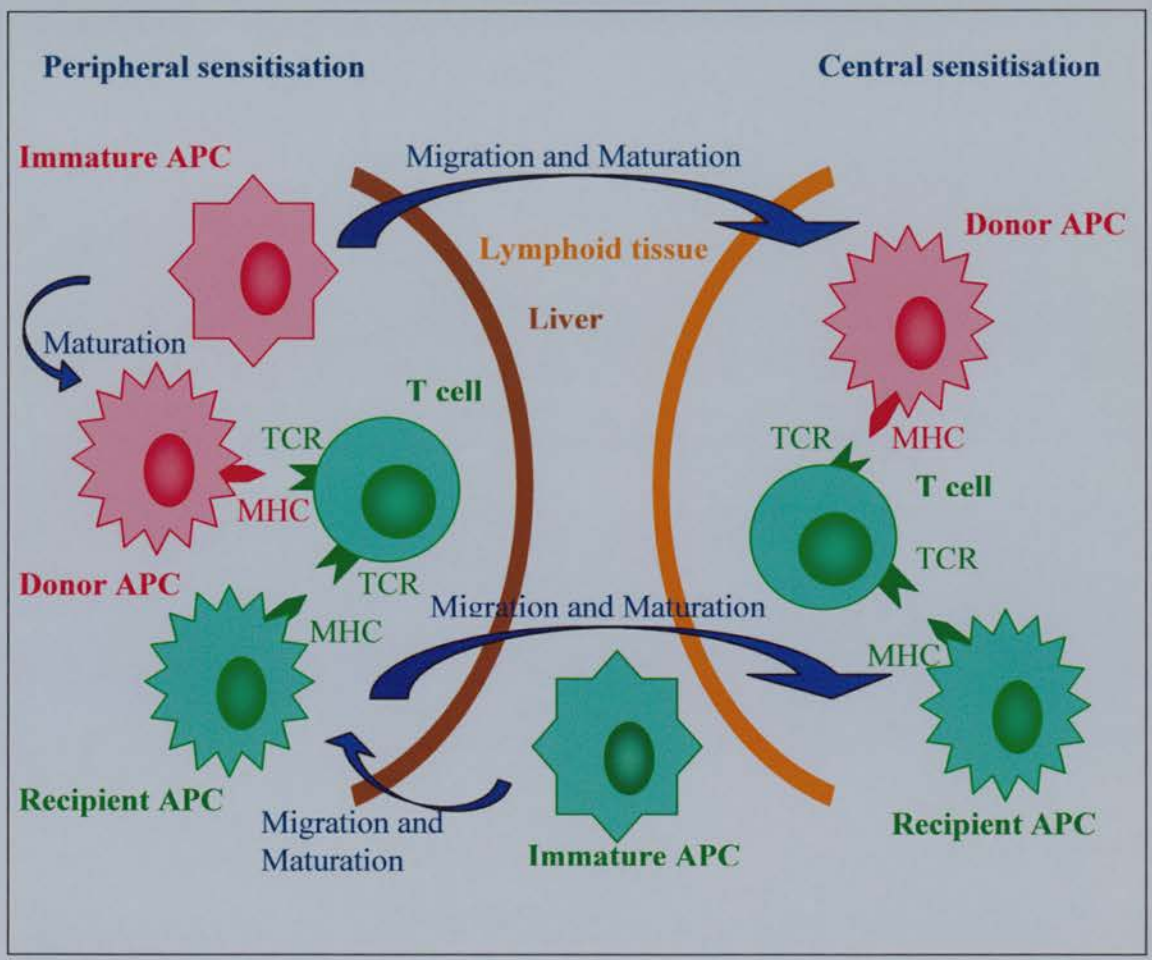


Figure 1.3

Proposed mechanism of central and peripheral sensitisation of recipient T cells following liver transplantation. Donor APCs either mature within the liver or migrate to the secondary lymphatic tissue. Recipient APCs infiltrate the liver transplant and either mature locally or migrate back to the secondary lymphatic tissue.

1.3.5.3 Efferent immune response

T lymphocytes are the essential alloreactive cells during rejection (Hall, 1978) and following liver transplantation, T cell proliferation in the secondary lymphoid tissue as well as T cell infiltration into the graft occurs within 2 days post-transplant (Demetris, 1991). Although this suggests simultaneous sensitisation in the graft and the secondary lymphoid tissue, naïve T lymphocytes are usually found only within lymphatic tissue, since they lack the necessary levels of surface integrins for transendothelial migration in peripheral organs (Brezinschek, 1995). This would suggest, that the initial activation of T lymphocytes occurs within spleen and lymph nodes, and recent experiments have demonstrated the crucial role of secondary lymphoid tissue for the rejection of vascularised organs (Lakkis, 2000). The liver however might be an exemption to this rule, since it allows as the only solid organ direct contact between naïve T cells and parenchymal cells through fenestrated endothelial cells (Bertolino, 2000).

Both, CD4+ and CD8+ T lymphocytes are activated in fully MHC-mismatched transplants, and both T cells are found within the liver allograft following transplantation (Wood, 1994). However, in most experimental models, CD8+ T cell activation is dependent on concurrent activation of CD4+ T cells providing help in the form of IL-2 and IFN-γ (Hall, 1985; Mosmann, 1991). This is corroborated by the fact that MHC class I mismatched grafts are more easily accepted than MHC class II mismatched grafts (Gallico, 1979). CD4+ T lymphocytes are therefore thought to be the dominant cell type mediating rejection, activating the cytotoxic effector cells such as CD8+ T cells, B lymphocytes or macrophages (Hall, 1985; Lanzavecchia, 1985; Hao, 1990). After the initiation of the immune response, both, CD4+ and CD8+ T lymphocytes are predominantly directly stimulated memory CD45RO+ cells, however their frequency diminishes over-time (Baker, 2001). In contrast, during chronic rejection increased frequencies of indirectly stimulated T cells can be observed (Hornick, 2000), implicating this pathway in the development of recurrent acute or chronic allograft rejection.

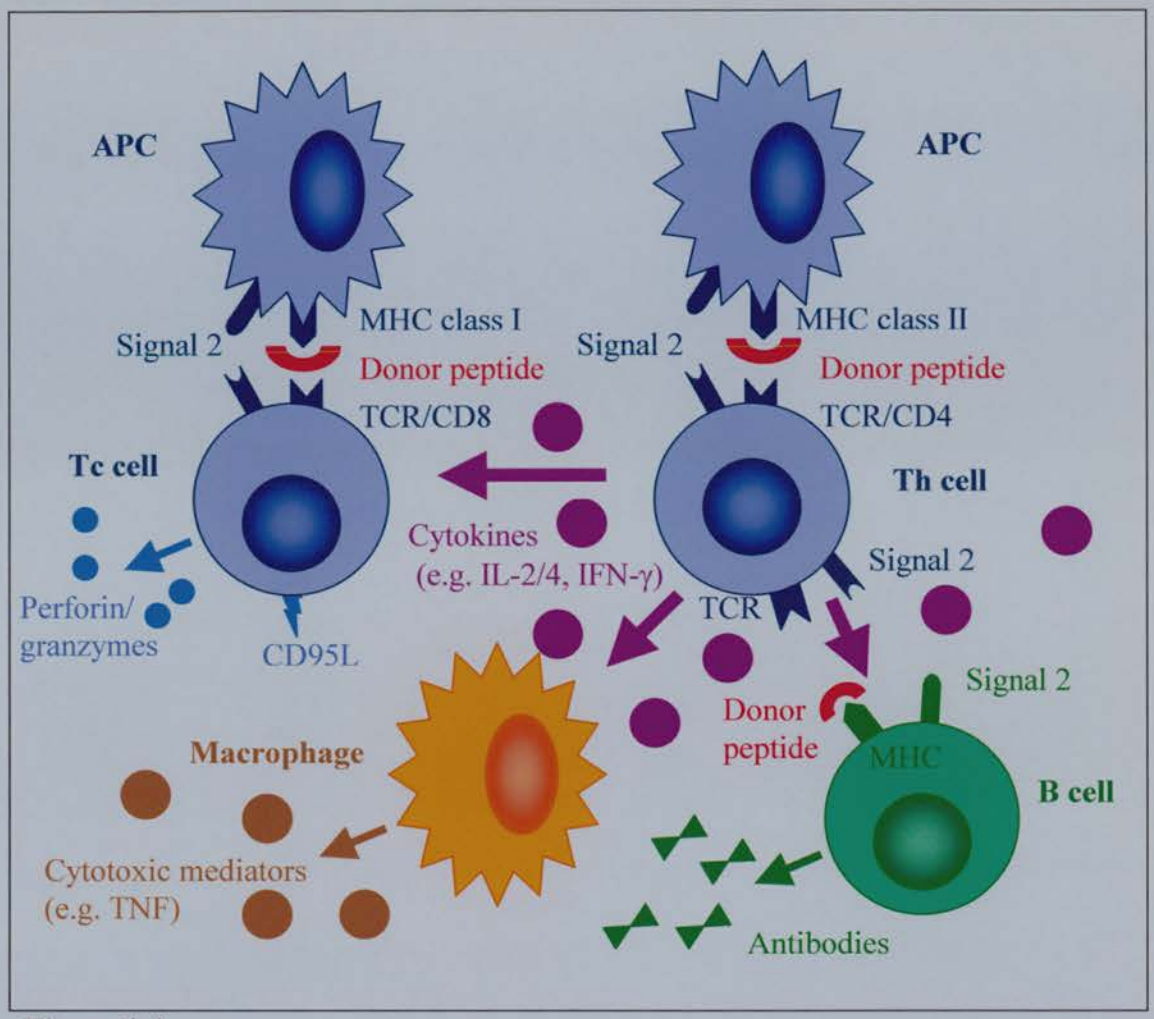


Figure 1.4

Efferent immune response during allograft rejection. CD4+ Th cells are the dominant mediators of rejection, activating cytotoxic effector cells such as CD8+ Tc cells, B cells and macrophages.

1.3.6 Cytotoxicity

1.3.6.1 Mode of cell death

Graft dysfunction and failure is ultimately a consequence of dying parenchymal donor cells killed by alloreactive effector cells of the recipient. There are two distinct modes of cell death, apoptosis and necrosis. Apoptosis describes a co-ordinated sequence of signals leading to cell shrinkage and internal nuclear fragmentation

without loss of the plasma membrane integrity (Wyllie, 1980). In contrast, necrosis involves cell swelling and loss of the plasma membrane integrity leading to lysis (Majno, 1995). Although specific signals are thought to induce predominantly one or the other form of cell death, apoptosis and necrosis can co-exist in diseases depending on factors such as the metabolic status of the target cell, the various effector mechanisms acting on the target cell and external signals which render target cells more susceptible to cell death (Kaplowitz, 2000). Recently, the mitochondrial function has emerged as a key link for apoptosis and necrosis (Lemasters, 1999). As part of the intracellular apoptotic programme, extracellular cytotoxic signals are essentially propagated either mitochondria-independent via direct activation of effector caspases, or mitochondria-dependent via activation of the mitochondrial permeability transition (MPT), a protein megachannel, resulting in loss of the mitochondrial membrane potential and release of cytochrome c (Bernardi, 1996). Depending on the cell type and the external cytotoxic signal, the MPT activation may not be essential for the induction of apoptosis, however the apoptotic signal cascade subsequent to the MPT activation can only proceed in the presence of sufficient APT production (Liu, 1996). In contrast, MPT activation with APT depletion as a result of the rapid disruption of most of the cellular mitochondria will lead to necrosis with cell lysis (Eguchi, 1997).

Apoptosis and necrosis are difficult to detect *in vivo,* since dead cells are rapidly cleared by phagocytes (Wyllie, 1980). However, both necrosis and apoptosis have been described in liver biopsies of patients with hepatic allograft rejection (Snover, 1984). Experimental data suggests that allogeneic tissue damage is predominantly mediated by apoptosis as a consequence of the immune-mediated cytotoxicity. In skin transplants from tetraparental mice only allogeneic, but not syngeneic target cells are affected, indicating a contact-dependent cytotoxicity with selective death signals (Rosenberg, 1988). Using an experimental rat model of liver transplantation with a minimum of reperfusion injury, Krams and colleagues were able to show equivalent numbers of apoptotic hepatocytes in allografts and isografts on day 1 post-transplant, followed by an increase of hepatocyte apoptosis over the next 7 days only in allografts (Krams, 1995a). Other animal models of hepatic allograft rejection

as well as studies in human biopsies post-transplant also found predominantly apoptotic hepatocytes in rejecting grafts, their number usually correlating with the severity of rejection (Afford 1995).

Although apoptotic hepatocytes can be more readily detected during acute and chronic rejection (Krams, 1998a), bile duct epithelial cells and endothelial cells are thought of as the primary targets of hepatic allograft rejection (Demetris, 1997a). Several, but not all studies were able to demonstrate apoptotic biliary epithelial cells during liver rejection, possibly due to the rapid disappearance of the cells (Nawaz 1994; Gapany, 1997). Apoptotic endothelial cells have not been described in rejection of liver allografts, however apoptosis is thought to be the predominant mode of cell death during rejection of allogeneic vessel grafts (Dong, 1996).

1.3.6.2 Cytotoxic mechanisms

CTLs induce target cell-specific cell death via two major mechanisms, the perforin-dependent granule-exocytosis pathway and the CD95-Ligand/CD95 pathway (Kaegi, 1996). In the granule-mediated pathway, TCR engagement leads to the release of lytic granules containing perforin and serine proteases known as granzymes. Granzymes are found exclusively in CTLs, NK cells and lymphocyte activated killer (LAK) cells, and the most important ones expressed by CTLs are Granzyme A and B (Liu, 1996). Initially, perforin was thought to kill the target cells via lysis of the cell membrane, however the perforation of the membrane is now believed to facilitate the influx of granzymes into the target cell. Inside the cell, granzymes then induce cell apoptosis by either cleaving effector caspases directly or by activating bid, a member of the bcl-2 family, which acts on the MPT (Darmon, 1996; Yang, 1998). In allogeneic responses, Granzyme B in particular has been shown to play a dominant role. *In vitro*, Granzyme B is expressed by T cells in response to alloantigen, and CTLs from Granzyme B-/- mice are unable to induce rapid DNA fragmentation of allogeneic target cells (Prendergast, 1992; Heusel, 1994). Increased Granzyme B mRNA expression has also been detected by PCR during acute rejection of human liver, kidney and heart transplants (Krams, 1995b; Legors-Maida, 1994; Sharma,

1996). However, *in vivo* experiments with perforin or granzyme B-/- mice have shown, that solid organ allografts are still rejected, although more slowly than in wild-type mice, indicating the presence of additional mechanisms (Schulz, 1995).

The second target cell-specific pathway of cell death utilised by CTLs is CD95L/CD95. CD95 (Fas/APO-1), a type 1 transmembrane molecule, belongs to the TNF receptor superfamily and is expressed by a variety of tissues including liver heart, lung and kidney (Leithaeuser, 1993). CD95L on the other hand is the physiological ligand of CD95, a type II transmembrane protein belonging to the TNF superfamily (Suda, 1993). The expression of CD95L is constitutively more restricted compared with CD95, however it can be induced on various immune cells such as CD8+ and CD4+ T cells, NK cells, B cells and macrophages (Kaegi, 1996) as well as on endothelial and epithelial cells including hepatocytes (Mueschen, 1998). Apoptosis of the target cell is initiated upon engagement of the CD95 receptor by CD95L expressed on the surface of the cytotoxic effector cell. These individual receptor molecules trimerize and bind to a death domain called FADD (Fas-associated death domain), which activates the caspase cascade, either directly or via induction of members of the bcl-2 family and activation of the MPT (Scaffidi, 1998). CD95-mediated apoptosis has been implicated in several models of allograft rejection (Larsen, 1995; Krams, 1998b) and elevated expression of CD95 and CD95L mRNA has been found in rejecting human liver, kidney and cardiac allografts (Seino, 1996; Krams, 1998a). However, allografts are still rejected in experiments with CD95 or CD95L-deficient mice (Larsen, 1995), and a combined experiment with perforin-deficient recipients and CD95-deficient grafts demonstrated, that both pathways are not exclusively involved in the rejection of solid organs and can be compensated by other mechanisms (Schulz, 1995).

The two cytokines TNF-α and IFN-γ have both been implicated in allograft rejection of solid organs and are known to induce cell death in a variety of cells. As cytokines, they are soluble mediators and do not require contact between the cytotoxic cell and the target cell. TNF-α is thought to be predominantly released by macrophages during rejection (Teramoto, 1999) and engages its receptor TNF-R1 on the surface of

the target cell (Bradham, 1998). Similar to CD95, a death domain called TRADD (TNF-receptor associated death domain), is activated, which in turn binds to FADD, thereby triggering apoptosis. IFN-γ in contrast has been reported to induce both caspases as well as pro-apoptotic members of the bcl-2 family (Pammer, 1999, Takahashi 1999). In addition, it has recently be shown to activate the tumour-suppressor gene p53, which acts via bax on the MPT (Kano, 1997). However, most importantly in the context of a complex inflammatory response, IFN-γ has been demonstrated *in vitro* to increase the susceptibility of target cells to other death signals such as CD95 or TNF-α (Tillman, 1998). Other non-target cell-specific mediators of cell death are reactive oxygen species (ROS), released by a variety of immune cells during inflammation (Kaplowitz, 1996), and specifically during liver allograft rejection bile acids, which act directly on the MPT as well as induce trimerization of the CD95-receptor without prior stimulation by CD95L (Faubion, 1999).

1.4 Allograft acceptance and tolerance

1.4.1 Definition

Tolerance has been defined in clinical and experimental transplantation according to a variety of criteria and is arguably often misused. Immunologically, tolerance should not only be based on prolongation of allograft survival, but on indefinite graft acceptance without immunosuppression, which should also extend to second grafts from the same donor (Hall, 2001). Some authors further require the ability to transfer tolerance with regulatory T cells to naïve secondary recipients (Qin, 1993). However, regulatory or suppressor T cells are usually only detected several weeks after transplantation, in rodent models between 50 to 100 days post-transplant (Gassel, 1992). During this time, the graft has to be protected by other mechanisms such as deletion of alloreactive T cells, inadequate T cell stimulation or the effect of passenger leukocytes including microchimerism (Bishop, 2001). Liver allografts are the most tolerogenic grafts among solid organs, spontaneously accepted without

immunosuppression in all mice strains, many low-responder rat strains, out-bred pigs and primates (Calne, 1969; Qian, 1997). Recipients accepting liver allografts also accept second grafts from the same donor, however within days post-transplant before the presumed establishment of Tr cells (Kamada, 1984). Liver allografts have even be shown to rescue previously transplanted organ grafts from the same donor, which are acutely rejected (Wang, 1997), and they are accepted in some models by recipients, which had been sensitised to the donor antigen by skin grafts (Kamada, 1981). Despite this evidently systemic tolerance induction, Tr cells have only been detected at late stages of liver transplant tolerance (Gassel, 1992). In the early stages, liver allograft acceptance is rather characterised by the co-existence of alloreactive T cells in the periphery with a stable graft function, which led to the term split tolerance (Damen, 1994). Several mechanisms appear therefore to work together in the establishment of tolerance, however observations in animal models and even human transplantation have to be treated cautiously. Some results in rodent models, e.g. cytokine production, depend strongly on the genetic background of the rodent strain (Hall, 2000). Other observations might be rather the consequence than the cause of tolerance induction, best illustrated by the debate about microchimerism following transplantation (Starzl, 1996; Anderson, 2001).

1.4.2 T lymphocyte apoptosis

One of the long-standing explanations for tolerance induction in transplantation has been immunological ignorance, i.e. the non-recognition of foreign antigen by the recipient's immune system (Medawar, 1960). However, many models of transplant tolerance, and in particular models of spontaneous allograft acceptance, are characterised by an initial T cell expansion associated with infiltration of the graft (Sharland, 1998). The difference between rejection and acceptance is the subsequent apoptosis of either the graft cells or the donor-reactive T cells, respectively. Cell death of infiltrating ML has also been described in human liver allografts (Afford, 1995), and a recent study demonstrated that intact T cell-apoptosis pathways are required for tolerance induction across MHC barriers (Wells, 1999). Of particular importance was however the finding, that both IL-2 and IFN-γ were essential

cytokines for tolerance induction (Li, 1999; Mele, 2000), indicating the death of activated rather than resting T cells. Following TCR engagement, activated T cells are liable to die by activation-induced cell death (AICD) through engagement of the CD95 receptor (Alderson, 1995) or by passive cell death, when deprived of survival signals, e.g. growth factors or CD28 signalling (Li, 2000). Interestingly, AICD can be inhibited by glucocorticoids and cyclosporin A (Brunner, 1995), while naïve or resting T cells are resistant due to their inability to recruit caspase 8 (Peter, 1997). A number of mechanisms have been made responsible in liver transplantation for the induction of T cell apoptosis. Soluble MHC class I antigens released by the large pool of donor cells has been detected in the serum of transplanted individuals and induces apoptosis of alloreactive T cells *in vitro* (Davies, 1989; Zavazava, 1996). Both sinusoidal endothelial cells and hepatocytes are able to express CD95L and could kill T cells similar to other immuno-privileged sites such as testis or the eye (Mueschen, 1998; Griffith, 1995). Additionally, both cell types have been proposed to delete CD8+ T cells by inadequate stimulation resulting in passive cell death or fracticide by neighbouring CD95L expressing T lymphocytes (Bertolino, 1999; Limmer, 2000). Finally, Bishop and colleagues recently raised the possibility, that T cell death in the allograft is a result of inappropriate and early over-stimulation of the cells within the secondary lymphoid tissue, which then die by neglect due to insufficient survival signals (Bishop, 2001).

1.4.3 Passenger leukocytes

Despite their role in direct allorecognition and initiation of rejection, donor leukocytes have been found to be essential for spontaneous acceptance of liver allografts. Depletion of passenger leukocytes by irradiation or "parking" experiments in an intermediate host led to abolition of tolerance induction in several studies (Sriwatanawongsa, 1995; Sun, 1995). However, the responsible mechanism for mediating tolerance remains as yet unclear. In most models of spontaneous or drug-induced allograft acceptance, selective reconstitution of irradiated allografts with T lymphocytes restores tolerance induction (Sun 1996). The cells used are usually mixed splenic T lymphocytes rather than hepatic T cells and appear to loose their

tolerogenic effect if not applied at the time of transplantation (Shimizu, 1996). This might indicate, that the effect of the T lymphocytes is based on presentation of donor antigen without co-stimulatory signals similar to models of tolerance induction with pre- or peri-operative transfusion of donor immune cells including erythrocytes, platelets or T cells (El-Malik, 1984). However, the liver is also a site with a unique composition of haematopoietic cells including stem cells, T lymphocyte precursors, γδ T cells and DCs (Jonsson, 1997; Taniguchi, 1996). In particular the presence of stem cells has led to the hypothesis of tolerance induction by chimerism of donor cells into the recipient's bone marrow, which is most prominent in liver transplantation (Starzl, 1996) and regarded by some authors as the cause of tolerance induction, by others as the result (Bonham, 1997). Additionally, hepatic DCs might have a tolerogenic effect, based on a number of mechanisms. Progenitor or immature DCs have been shown to induce Tr cells (Jonuleit, 2000; Gorczynski, 1999) or prolong allograft survival (Fu, 1996) and the liver has been demonstrated to release the cytokines IL-10 and TGF-β, which delay DC maturation (Yamaguchi, 1997; De Smedt, 1997). In mice, the hepatic DC population is further composed not only of myeloid-derived, but also of lymphoid-derived DCs, which might have a regulatory function on immune responses and possibly even induce apoptosis of T lymphocytes via CD95L (Suss, 1996; O'Connell, 2001). Finally, DCs could be tolerogenic after phagocytosis of apoptotic rather than necrotic cells, a concept which has been proposed for the maintenance of peripheral tolerance (Gallucci, 1999).

1.4.4 Regulatory T lymphocytes

Several models of allogeneic transplantation have recently demonstrated tolerance induction by blocking the stimulatory signals for T cell activation, either by using antibodies against CD4 and CD8 for inhibiting the TCR complex or anti-CD40L antibodies and the fusion protein CTLA-4Ig to inhibit co-stimulatory signals (Hall, 2000). Some of these strategies have also been used in tolerance induction to liver allografts (Fu, 1999), and the pathway via CTLA-4 might be of particular physiological importance, since the receptor is up-regulated on activated T lymphocytes and suppresses T cell proliferation following engagement (Linsley,

1991). The most striking feature in these models is the development of Tr lymphocytes, although only after a median time of 50 – 100 days (Qin, 1993). In most models, these Tr cells are CD4+, while the existence of CD8+ Tr cells is debated (Zhai, 1999). These Tr cells usually require the continued presence of alloantigen to maintain their function (Hall, 1990). Spontaneous liver allograft acceptance is also associated with a persistence of donor-reactive host CD4+ T cells (Olver, 1998), while in models of tolerance induction via the portal vein, γδ T cells have been described as the Tr cell population (Gorczynski, 1994). However, Tr cells are only detectable late after tolerance induction, which contrasts with the immediate tolerogenic function of liver allografts for second grafts from the same donor (Wang, 1997). Recently, regulatory CD4+ CD25+ T cells have been described in models of autoimmune diseases, which maintain tolerance to self-antigens by releasing IL-10 and TGF-β (Annacker, 2001). These Tr cells would be capable of suppressing immune responses immediately post-transplant, but their presence in the setting of transplantation remains speculative.

1.5 Conclusions and aims

The improvements in surgical technique and immunosuppressive treatment over the last decades has transformed transplantation of solid organs from an experimental to a routine clinical procedure. However, big discrepancies remain in the long-term survival of the various organs, largely due to the differences in their immunogenicity as a graft and the resulting ease of rejection by the host (Hall, 2000). Among solid organs, liver allografts are the least prone to rejection and can even induce tolerance in many animal models. Since hepatic grafts induce a systemic immune response by the host with donor-reactive T lymphocytes detectable within the circulation similar to other organ grafts (Dahmen, 1994), it could be hypothesised that the liver's inherent tolerogenicity might be mediated within the graft itself.

The aim of this thesis was therefore to examine the intra-hepatic development of the allogeneic immune response following liver transplantation and to analyse the role of

hepatic donor cells in this process. Our initial investigations were based on two observations. Firstly, acute cellular rejection does not appear to affect hepatic allograft outcome adversely (Neuberger, 1998), possibly indicating a reduced susceptibility of liver donor cells to the cytotoxicity of the infiltrating host effector cells. Secondly, liver graft acceptance and tolerance induction requires activation rather than anergy of the host's immune system (Bishop, 2001). The necessary modulation of the immune response towards a tolerogenic response might be mediated within the graft itself, or at least by hepatic donor cells.

Using biopsies of patients following liver transplantation, we first analysed differences in the inflammatory infiltrate between patients with acute or chronic rejection and patients without rejection by immunohistochemistry. Our aims were to:

1. analyse the composition of the infiltrate with respect to cell type
2. assess the proliferation index to examine local expansion of the infiltrate
3. analyse T lymphocytes for naïve and memory phenotype to assess primary and secondary antigen-presentation

The same biopsies were additionally investigated by immunohistochemistry for up-regulation of pro-apoptotic signals in both, donor and recipient cells, in particular for assessment of:

1. the mode of T cell-mediated cytotoxicity by staining for Granzyme B and CD95 expression
2. the susceptibility of donor target cells to undergo apoptosis by staining for intracellular signals of the apoptotic cascade
3. the susceptibility of infiltrating inflammatory cells to undergo apoptosis by staining for the receptor CD95 and intracellular apoptotic signals

Our results indicated, that of the donor target cells, hepatocytes were particular sensitive to CD95-mediated apoptosis. However, expression of the CD95 receptor on hepatocytes was found in biopsies of patients with or without rejection, indicating additional mechanisms of regulation. Previous publications demonstrated amplification of CD95-induced hepatocyte cell death by the chemotherapeutic agent

bleomycin mediated via the tumour-suppressor gene p53 (Mueller, 1998). We hypothesised, that the pro-inflammatory cytokine IFN-γ, which also induces p53 expression in hepatocytes (Kano, 1997), might have a similar effect. We therefore used an *in vitro* model with murine hepatocytes to assess:

1. the effect of IFN-γ on CD95-mediated apoptosis with bleomycin as control
2. the role of p53 in this process by using p53-/- hepatocytes
3. up-regulation of the extracellular apoptotic signals CD95 and CD95L
4. intracellular amplification of the apoptotic signal via mitochondrial depolarisation using the specific inhibitor cyclosporin A

Another important observation during our initial investigations was the detection of proliferating naïve CD4+ T lymphocytes, indicating a primary immune response within the hepatic allograft. The cycling naïve T lymphocytes were found in biopsies of patients with or without rejection within 7 days post-transplant implying donor cells as APCs with a possible modulating function on the immune response. DCs are particular equipped to stimulate naïve T lymphocytes and many recent reports have speculated on a tolerogenic function of hepatic DCs (Thompson, 1999). In the final experiments, we therefore adapted a method of immuno-magnetic separation to isolate DCs from liver, kidney and spleen in a murine model. Our specific aims were to:

1. analyse the phenotype of each DC subpopulation
2. compare the stimulatory capacity of the DC subpopulations
3. assess the effect of the immunoregulatory cytokines IL-10 and TGF-β on the DC function in comparison with the immunosuppressive peptide CTLA-4Ig

Chapter two - Experimental procedures

2.1 Histopathological studies

2.1.1 Background

Specific cellular antigens or proteins can be detected in tissue sections by immunocytochemistry (Leong, 1996). This method was employed in Chapters three and four to assess the intra-hepatic immune response as well as pathways of cytotoxicity during allograft rejection directly in biopsies of patients following liver transplantation. The major advantage of immunocytochemistry lies in its suitability for use in formalin-fixed, paraffin-embedded tissue, which allows good cytomorphological analysis and antigen localisation within the tissue. All antibodies used in the experiments described are commercially available with quality controls performed by each respective company. Since most of the specific antibodies to the relevant antigens in our studies were unconjugated, all antibodies were purchased in this form and an indirect immunoenzyme technique was used. This indirect method has the additional advantage of increasing the sensitivity of the immunoenzyme stain by increasing the amount of reaction product deposited at the antigen-antibody reaction sites.

2.1.2 Biopsies

The Scottish Liver Transplant Unit (SLTU) in the Royal Infirmary of Edinburgh is the regional referral centre for acute and chronic liver failure and all biopsies post-transplant were obtained from patients who underwent liver transplantation in Edinburgh between 1994 and 1998. The biopsies were performed either according to clinical indication or as part of a clinical protocol. Patients with acute or chronic rejection were biopsied as part of the diagnostic procedures, patients without clinical evidence of rejection as per management protocol of the Scottish Liver Transplant Unit on day 7 post-transplant. Control tissue with normal liver histology was derived from biopsies of patients undergoing routine staging for malignancy or before treatment with hepatotoxic medication. All biopsies were reported in the Department of Pathology of the University of Edinburgh and presented at clinico-pathological

meetings. The final diagnosis was made after discussion of the histology and the clinical features of the patient with the referring physician.

2.1.3 Patient characteristics and diagnostic criteria

2.1.3.1 Acute Rejection

Liver tissue was obtained from 10 patients (4 male/6 female; age range 25-60 yr) with acute cellular rejection following orthotopic liver transplantation. The median time post-transplant was 8 days with a range of 6-26 days. Indications for transplantation were primary biliary cirrhosis (4 patients), primary sclerosing cholangitis (2 patients) and chronic active hepatitis, alcoholic liver disease, fulminant hepatic failure due to paracetamol overdose and graft failure due to chronic rejection (one patient each). Post-transplant, patients received standard immunosuppressive therapy with prednisolone (20 mg/day), azathioprine (2 mg/kg/day) and cyclosporin A (10 mg/kg/day). The target trough levels for Cyclosporin A were 175-200 mmol/l. Acute rejection was diagnosed using clinical (malaise, jaundice) and biochemical criteria (abnormal liver function tests) in combination with histological evaluation of the biopsies according to standard scoring systems (Demetris, 1997a). The main histological features of rejection assessed were portal inflammation, bile duct damage and subendothelial inflammation, each scored on a scale of 0 (none) to 3 (severe). The combined rejection scores of the biopsies used in this study were 6/9 or above. All patients with acute rejection were treated with a daily regimen of 1g of methylprednisolone intravenously for three days. Following treatment, a second liver biopsy was taken to confirm the resolution of the rejection episode. In all cases a significant reduction of the total rejection scores was achieved with maximum scores of 4/9. The histological changes were associated in all cases with improvement of the clinical and biochemical parameters.

2.1.3.2 Chronic Rejection

Chronic Rejection. Liver tissue was obtained from 10 patients (1 male/9 female; age range 20-58 yr) with chronic ductopenic rejection following orthotopic liver

transplantation. The median time post-transplant was 6.5 months with a range of 3.5-9.5 months. Indications for transplantation were primary biliary cirrhosis, fulminant hepatic failure due to paracetamol overdose and chronic rejection of the first allograft in 3 patients each and chronic hepatitis B in one patient. All patients received standard immunosuppressive therapy as per management protocol for postoperative care in the Scottish Liver Transplant Unit. Six of the patients were initially treated with prednisolone (20 mg/day), azathioprine (2 mg/kg/day) and cyclosporin A (10 mg/kg/day, target trough levels 175-200 mmol/l). The other four patients received prednisolone (20 mg/day) and tacrolimus (0.1 mg/kg/day, target trough levels 10-15 ng/l). In all patients, prednisolone was reduced and stopped within 6 months post-transplant. The diagnosis of chronic rejection was based on a combination of standard clinical (malaise, jaundice) and biochemical (abnormal liver function tests) features together with histological criteria including bile duct loss and obliterative arteriopathy (Demetris, 1997a). During follow-up 8 out of the 10 patients consequently lost their graft due to chronic rejection, while the remaining two patients recovered after their immunosuppressive regimen was changed from cyclosporin A to tacrolimus.

2.1.3.3 No Rejection

Liver tissue was obtained from 10 patients (2 male/8 female; age range 20-64 yr) with routine biopsies on day seven post-transplant as per management protocol for postoperative care in the Scottish Liver Transplant Unit. Indications for transplantation were primary biliary cirrhosis (4 patients), fulminant hepatic failure due to paracetamol overdose (3 patients) and alcoholic liver disease, cryptogenic cirrhosis and hepatocellular carcinoma (one patient each). The standard immunosuppressive therapy post-transplant consisted of prednisolone (20 mg/day), azathioprine (2 mg/kg/day) and cyclosporin A (10 mg/kg/day, target trough level 175-200 mmol/l) in all cases. None of the patients showed clinico-biochemical signs of acute or chronic rejection and the histological evaluation of the liver biopsies amounted only to mild inflammation with a score of 3/9 or less in each case. During

follow-up none of the patients developed an episode of acute or chronic rejection and no further biopsies were taken.

2.1.3.4 Normal Controls

Control liver tissue was obtained from 15 patients (8 male/7 female; age range 25-67) without liver pathology. Indications for the liver biopsies were routine staging for malignant lymphoma, staging of colon carcinoma at the time of the resection or staging of the liver before therapy with methotrexate for psoriasis. All liver specimens were histologically evaluated and reported as normal. Prior to the biopsy, no clinical signs of liver disease were present and liver function tests were normal in all 15 patients.

2.1.4 Tissue processing

All tissue samples were fixed in 10% buffered formalin (pH 7.4) immediately after the percutaneous biopsy to improve conservation of the liver architecture. The biopsies were then embedded in low-temperature paraffin wax and processed for routine histology. The tissue blocks were stored in the archives of the Department of Pathology.

2.1.5 Antigen-retrieval

Serial sections (3 μm in thickness) of the paraffin-embedded biopsies were mounted on glass slides, air-dried at room temperature, dewaxed in xylene and rehydrated in a graded ethanol series. Since cellular antigens are often masked by cross-linking fixatives such as formaldehyde, all sections were pre-treated for antigen retrieval by irradiation in a microwave oven in the presence of a 10 mM EDTA buffer (pH 7.4; Sigma, UK). The sections were irradiated for 3×5 minutes, evaporated buffer replaced before the second and third irradiation.

2.1.6 Inhibition of endogenous enzymes

The active enzymes of the immunoenzyme technique used in the studies were peroxidase and alkaline-phophatase. Both enzymes exist in the liver as endogenous proteins, which can cause unspecific staining. Endogenous tissue peroxide was therefore blocked by incubating the tissue sections in a hydrogen peroxide solution (0.5% H_2O_2 in methanol, both Sigma, UK) for 10 minutes following antigen-retrieval. Endogenous alkaline-phosphatase was inhibited at the end of the immunoenzyme staining by adding a 1M levamisole solution (Sigma, UK) to the chromogen.

2.1.7 Immunoenzyme staining

Tissue sections were washed twice for 10 minutes in Tris-buffered saline (TBS, pH 7.4; Sigma, UK) in between each step described below. To reduce background staining, the sections were then incubated in a 3% solution of normal serum (NS) derived from the same species as the secondary antibody (SAPU, UK) for 20 minutes. This was followed by incubation with the primary and biotinylated secondary antibodies diluted in NS for 40 minutes each at room temperature. Details on antibodies and dilutions are given in table 1. Finally, sections were incubated with peroxidase-conjugated avidin (in single stains) or alkaline-phosphatase-conjugated streptavidin (in double stains) diluted 1/1000 with NS for 30 minutes at room temperature. The chromogen used with peroxidase was diaminobenzidine tetrahydrochloride (Dako, UK), the chromogen for alkaline-phosphatase Vector Red (Vector Lab., UK). Both solutions were used according to manufacturers recommendations (staining for 10 minutes), however levamisole was added to Vector Red to block endogenous alkaline-phosphatase.

For double-immunostaining, the sections were first stained by the avidin-biotin-peroxidase complex method for the first antigen, followed immediately by incubation with the second primary antibody to the second antigen and visualisation by the

streptavidin-biotin-alkaline phosphatase complex method. At the end of the procedure, all slides were counterstained with haematoxylin (Sigma, UK).

Antibody	Species	Dilution	Source
anti-CD4 mAb	mouse	1/20	Novocastra, UK
anti-CD8 mAb	mouse	1/20	Dako, UK
anti-CD20 mAb	mouse	1/50	Dako, UK
Anti-CD45RA mAb	mouse	1/50	Dako, UK
Anti-CD45RO mAb	mouse	1/50	Dako, UK
Anti-CD57 mAb	mouse	1/50	Zymed, UK
Anti-CD68 mAb	mouse	1/100	Dako, UK
Anti-Ki-67 mAb	mouse	1/100	Dako, UK
Anti-granzyme B mAb	mouse	1/50	Monosan, UK
Anti-CD95 mAb	mouse	1/100	Immunotech, UK
Anti-bcl-2 mAb	mouse	1/50	Dako, UK
Anti-bax pAb	rabbit	1/200	Autogen Bioclear, UK
Anti-bcl-x pAb	rabbit	1/200	Autogen Bioclear, UK
Biotinylated anti-mouse IgG pAb	rabbit	1/2000	Dako, UK
Biotinylated anti-rabbit IgG pAb	goat	1/2000	Dako, UK

Table 2.1

Primary and secondary antibodies used in immunohistochemistry. The antibodies are monoclonal (mAb) or polyclonal (pAb).

2.1.8 Controls

Negative controls for each run were performed by omitting the primary antibody for the single-stain procedures or both primary antibodies for the double-stain procedures. Positive controls were obtained by using tissue from normal human lymph nodes for all primary antibodies against immune cells, or by using carcinoma tissue as recommended by the manufacturer for primary antibodies against apoptotic signals.

2.1.9 Histological analysis

The number of cells staining positively was enumerated by two independent observers (MMD & DJH) using the Zeiss HOME microscope at x40 magnification. This was done "blindly", i.e. without knowledge of the underlying patient's characteristics. According to the liver's architecture, portal tracts and liver lobules were assessed separately. Portal tracts were defined as connective tissue containing branches of the hepatic artery and portal vein surrounded by hepatocytes. Liver lobules were recognised by the central vein (terminal hepatic venule) surrounded by parenchyma in a hexagonal shape with radial orientated hepatocytes. Because of the size of the needle biopsies, five portal tracts and five liver lobules were studied per section with the exception of specimens with chronic rejection, in which all visible portal tracts were examined. Since the absolute cell numbers of the immune cells varied not only with the diagnosis, but also within the diagnostic groups and within the same biopsies, numbers of positive staining cells were expressed as the percentage of all cells of this type. In contrast, staining in hepatocytes and biliary epithelial cells changed both, in numbers of positive cells and in intensity of the staining. Positive staining was therefore assessed semi-quantitatively as either high or low. High was defined as clearly visible granular stain, low as definitive but weak immunopositivity which was consistently greater than in negative control sections. The analysis of each section was repeated twice with an error rate of less than 5%, the degree of concordance between the two observers was greater than 95%.

2.1.10 Statistical analysis

The student's *t*-test for differences between two means was used to compare the mean percentage (± s.e.m.) of the relative number of cells staining positively in biopsies of each subgroup of patients. Results with a p-value less than 0.05 were considered as significant.

2.2 Cell cultures and experiments

2.2.1 Experiments with hepatocytes

2.2.1.1 Background

All cell cultures used in the studies were derived from murine cells. The isolation of hepatocytes, which form the basis of the experiments in chapter 5, is facilitated by the fact, that they make up 80% of the total liver cell mass (Blouin, 1977). In addition, hepatocytes are significantly larger and more dense than other hepatic cells, i.e. non-parenchymal cells (Zahlten, 1981). However, difficulties can arise from the susceptibility of hepatocytes to oxidative stress and their requirement for specific support media, which may affect viability of the cells. In this study, hepatocytes were isolated by a two-step retrograde perfusion of the liver with a collagenase solution for enzymatic digestion of the connective tissue, thereby releasing single cells from the liver matrix (Berry, 1969). The cell suspension was then separated by density centrifugation over percoll into parenchymal and non-parenchymal cell fractions (Pertoft, 1977). Hepatocytes obtained by this protocol are viable and metabolically active with intact membrane surface receptor expression (Steer, 1979).

2.2.1.2 Animals

Male C3H mice (10 weeks of age) were purchased from B&K Universal Ltd. (Grimston Aldbrough Hull, UK) and maintained together with age-matched, male p53-deficient mice which have been previously described (Clarke, 1993). Animals were housed five per cage under appropriate conditions of temperature and humidity and with a 12-hour artificial light cycle. Food and water were available *ad libitum*. All experiments were conducted under local guidelines for the care of animals under the Code of Practice for the Housing and Care of Animals used in Scientific Procedures – HMSO 1989.

2.2.1.3 Cell isolation

Hepatocytes were isolated as described previously (Bellamy, 1997) with minor modifications. Mice were killed by cervical dislocation and dissected to open the thoracic and abdominal cavity. A catheter (ID 0.5 mm, OD 1 mm; Falcon, UK) was inserted via the right chamber of the heart into the thoracic vena cava inferior and the liver perfused *in situ* with 5 ml of Liver Perfusion Medium (37°C, supplemented with penicillin-streptomycin; both Gibco BRL, Life Technologies, UK) to exsanguinate the liver and initiate the loosening of cell to cell contact. Excess perfusion pressure was avoided by opening the portal vein. The perfusion was then continued with 10 ml of Liver Digest Medium (37°C; Gibco BRL, Life Technologies, UK) containing collagenase and dispase for the dissociation of the hepatic cells.

The digested liver was excised, the gallbladder removed and a single cell suspension established by filtration of the loosened cells through a 100 µm mesh (Falcon, UK). The cell suspension was washed three times in ice-cold Williams E medium (Sigma, UK) supplemented with 10% foetal calf serum (FCS; SAPU, UK) and 1 ng/ml epidermal growth factor (EGF; Sigma, UK) by slow centrifugation (2 minutes at 50xg), then fractionated by Percoll density centrifugation (35% Percoll in phosphate-buffered saline (PBS); both Sigma, UK) for 20 minutes at 1000xg and 4°C. Non-parenchymal cells and cell debris were discarded and the cell pellet containing the viable hepatocytes washed and resuspended in Williams E medium supplemented as previously with FCS and EGF. Viability of the hepatocytes was tested by the trypan blue exclusion test using a Neubauer cytometer. Only hepatocyte suspensions with a viability of more than 90% were used for subsequent experiments.

2.2.1.4 Cell culture

0.5 ml of the final hepatocyte suspension (density 10^6 cells per ml) was inoculated on uncoated plastic dishes (30 mm in diameter; Life Technologies, UK) and cells cultured at 37°C (5% CO_2/30% O_2). After 2 hours, the medium was changed to

serum-free culture conditions with Williams E medium supplemented with 1 ng/ml EGF alone. These conditions reduced interference by cytokines or growth factors contained within the FCS on the subsequent experiments. Cultured hepatocytes were then treated with the experimental reagents as outlined below. After 24 and 48 hours, hepatocytes were harvested by incubation with a Cell Dissociation Buffer (Life Technologies, UK). Hepatocytes cultured for the same length of time in medium alone were used as negative control for all experiments.

2.2.1.5 Treatment with experimental reagents

Hepatocyte cultures were treated with bleomycin (Sigma Chemicals, UK), interferon-γ (IFN-γ; R&D Systems, UK) or cyclosporin A (CsA; Sandoz Pharmaceuticals, Frimley Camberley, UK). Reagents were added at the following concentrations: 15 mU/ml of bleomycin; 100 U/ml (ED_{50}) of IFN-γ; 0.15, 1 and 15 μg/ml of CsA. Concentrations for each reagent were chosen according to their biological activity as previously described (Mueller, 1998; Kano, 1997; Yokoyama, 1997). CD95 receptor stimulation and CD95L inhibition were performed using functional anti-CD95 (200 ng/ml, $ED_{50;}$ Jo-2, Pharmingen, USA) and anti-CD95L antibodies (200 ng/ml, ED_{50}; MFL-3, Pharmingen, USA). After 24 and 48 hours hepatocytes were harvested by incubation with a Cell Dissociation Buffer.

2.2.1.6 Cytotoxicity assays

Cell death of hepatocytes as a result of the experimental treatments was assessed by cytotoxicity assays. The MTT assay is a colorimetric assay based on the ability of viable cells to reduce a soluble yellow tetrazolium salt to blue formazan (Green, 1984). 70 μl of cell suspension (10^5 hepatocytes/ml) were distributed into each well of a 96-well microtiter plate (Nalge Nunc International, UK) and incubated for 24 or 48 hours with each combination of reagents. Four hours before the end of the incubation time, plates were washed twice with PBS and 70 μl of Williams E medium containing 0.5 mg/ml MTT dye (Sigma, UK) added into each well. The optical density was determined by eluting the dye with dimethil sulphoxide (DMSO;

Sigma, UK) and reading the absorbance values at 540 nm using an automatic multiwell spectrophotometer (Dynatech MR5000, UK). Each measurement was repeated on eight independent wells from at least three different mice and cell viability calculated as percentage of untreated controls, set arbitrarily at 100%. Results are expressed as mean ± s.e.m.

To distinguish between growth arrest and cell death, both causing a reduction of absorbance in the MTT assay, all results were verified by morphological analysis using acridine orange, a fluorescent dye which binds to DNA (Hayashi, 1990). Apoptotic hepatocytes were recognised by nuclear condensation and fragmentation as well as cell shrinkage and blebbing of the cytoplasm (Wyllie, 1980). 0.5 ml of cell suspension (10^6 hepatocytes/ml) were inoculated on uncoated plastic dishes (30 mm in diameter) and treated as described above. After 24 or 48 hours, the medium was replaced with 1% acridine orange in PBS (both Sigma, UK) and visualised under a fluorescence microscope. For each experiment, at least 200 cells were counted on a standard morphometric grid. Results are expressed as the mean percentage ± s.e.m. of apoptotic cells of all hepatocytes and represent data from three experiments on hepatocytes from at least three different mice.

2.2.1.7 Flow cytometry

Cell surface antigen expression on isolated hepatocytes before and after the experimental treatment was assessed using immunofluorescent staining and analysis by flow cytometry. The hepatocytes (2 x 10^5/sample) were washed twice in PBS (pH 7.4) containing 0.5% bovine serum albumin (BSA) and 2% sodium azide (NaN_3; all Sigma, UK). The cells were then incubated for 1 hour at 4^0 C with 0.3 mg/ml hamster anti-mouse CD95L mAb (MFL3, PE conjugated; Pharmingen, UK) or 0.25 mg/ml hamster anti-mouse CD95 mAb (Jo-2, FITC-conjugated; Pharmingen, UK). After the staining, the hepatocytes were again washed twice in PBS, fixed in 1% paraformaldehyde and analysed using a COULTER®EPICS®XL Flow Cytometer (Beckman-Coulter Electronics, Luton, UK). PE and FITC were both excited at 488nm, the red fluorescence emitted by PE was detected at 620nm and the green

fluorescence emitted by FITC was detected at 525nm. Cells were gated according to size and scatter to eliminate dead cells and debris. Unstained hepatocytes and PE- or FITC-labelled, isotype-matched antibodies were used as negative control. Data from at least 10000 events were acquired. For statistical analysis, results were calculated as the ratio of the mean fluorescence intensity of the experimental samples to the mean fluorescence intensity of the isotype controls and expressed ± s.e.m.

2.2.1.8 Statistical analysis

To test for synergism between the cytotoxic effects of anti-CD95 antibodies and the experimental reagents a balanced two-way ANOVA (model with fixed effects) was performed (Slinker, 1998). For all other experiments a Student's t-test was used to define statistical differences. p values < 0.05 were regarded as significant.

2.2.2 Experiments with dendritic cells

2.2.2.1 Background

Isolation of DCs, which form the basis of the experiments in chapter six, is more difficult, largely due to the fact, that they are a trace cell type making up less than 1% of cells in most lymphoid and non-lymphoid organs (Schuler, 2000). Additionally, DCs exist in a multitude of subsets with distinct developmental pathways, functional properties and maturational stages. In peripheral tissues such as solid organs, DCs are mostly myeloid-related and functionally immature, i.e. highly capable to process new antigen, but unable to stimulate T cells due to low expression of MHC antigens and co-stimulatory signals (Steinman, 1997). Upon antigen-uptake, DCs migrate to secondary lymphoid tissue and start to develop into mature stellate cells, i.e. lose the capacity for up-take of new antigen, but up-regulate MHC antigens and co-stimulatory signals, thereby becoming effective stimulators of T lymphocytes. Under steady state conditions, most DCs in the marginal zones of the lymphatic tissue are immature, myeloid-related DCs. In contrast, DCs in the T cell areas, so-called

interdigitating DCs, are predominantly mature, however they include both, myeloid- and lymphoid-related DCs.

In the mouse model, cell isolation is facilitated by the DC-specific expression of cell surface markers. Most DCs in lymphoid tissue and peripheral organs express the integrin CD11c (Metlay, 1990). Mature DCs in the T cell areas of the spleen also express DEC-205, a member of the C-type lectin receptor family (Kraal, 1986). Originally thought to be specific for interdigitating DCs, DEC-205 has also been found on DCs within the liver, but no other solid organ (Woo, 1994). Lymphoid-related murine DCs additionally express CD8α as a homodimer (Vremec, 2000). Finally, contamination with other leukocyte-subsets is routinely excluded on the basis of absent or low expression of specific markers such as CD3 for T lymphocytes, IgG for B lymphocytes, CD56 for NK cells or F4/80 for monocytes/macrophages (Schuler, 2000).

In this study, DCs were isolated from liver, kidney and spleen based on a positive selection method utilising the MACS system (Miltenyi, 1990). Positive selection methods by flow cytometry (FACS) or magnetic sorting (MACS) use monoclonal antibodies to separate DCs from freshly isolated low-density fractions (Metlay, 1990; Crowley, 1990). Splenic DCs isolated by this method are functionally immature with the phenotype of marginal zone DCs. To include all splenic DCs, mature interdigitating and immature marginal zone DCs, the method of positive selection was further refined by using collagenase digestion and Ca^{2+}- free conditions/EDTA for the establishment of single cell suspension from the spleen (Vremec, 1992). This improves the disintegration of the DC-T cell clusters in the T cell areas, which previously prevented staining of interdigitating DCs with the monoclonal antibodies.

Human blood DCs	**Origin**		
	Myeloid	Myeloid	Lymphoid/ Plasmacytoid
Surface phenotype	**CD1c+**	CD1c-	CD1c-
	CD4+/-	CD4+/-	CD4+
	CD11c+	CD11c+/-	CD11c-
	CD33+	CD33+/-	CD33-
	CD45RA-	CD45RA-	CD45RA+
	CD123-	CD123-	**CD123+**
	BDCA2-	BDCA2-	**BDCA2+**
	BDCA3-	**BDCA3+**	BDCA3-
	BDCA4-	BDCA4-	**BDCA4+**
	CMRF58-	**CMRF58+**	CMRF58-
Proposed/Reported DC function (T cell-response)	**Th1**	**Th0**	**Th2**	**Th0/Tr1**	**Th2**
Murine DCs	**Origin**		
	Myeloid	Myeloid	Lymphoid/ Plasmacytoid
Surface phenotype	CD4-	**CD4+**	CD4-
	CD8α-	CD8α-	**CD8α+**
	CD11b+	CD11b+	CD11b-
	CD11c+	CD11c+/-	CD11c+/-
	DEC-205+/-	DEC-205-	DEC-205+
Proposed/Reported DC function (T cell response)	**Th1**	**Th2**	**Th1**

Table 2.2

Surface phenotype and function of human and murine DC subsets. The most relevant surface markers are highlighted. DC function is classified according to the DC-induced Th cell response as reported in the literature (adapted from Grabbe, 2000).

	DC progenitor	Signal	**Immature DC**	Signal	**Mature DC**	Signal	**Activated DC**
Surface phenotype	MHC+/- CD40- CD80- CD86-	GM-CSF SCF Flt-3L	MHC+ CD40+/- CD80+/- CD86+/-	TNF LPS Apoptoic bodies	MHC+ CD40+ CD80+ CD86+	CD40L TRANCE	MHC+ CD40+ CD80+ CD86+
Function			Antigen-processing		Antigen-presentation		Antigen-presentation
Location	Bone marrow		Tissue		Tissue/ circulation		Lymphoid tissue

Table 2.3

Stages of DC maturation, each with discrete cellular function and location within the body. Transition between the stages of maturation are mediated by specific signals as listed (adapted from Stockwin, 2000).

2.2.2.2 Animals

Male Balb/c (H-2^d) and C3H (H-2^k) mice, 8-12 wk of age, were purchased from B&K Universal Ltd. (Grimston Aldbrough Hull, UK). Animals were housed five per cage under appropriate conditions of temperature and humidity and with a 12-hour artificial light cycle. Food and water were available *ad libitum*. All experiments were conducted under local guidelines for the care of animals under the Code of Practice for the Housing and Care of Animals used in Scientific Procedures – HMSO 1989.

2.2.2.3 Cell isolation

DCs were isolated from liver, kidney and spleen of groups of 5 mice in parallel. Mice were killed by cervical dislocation and dissected to open the abdominal cavity. A catheter (ID 0.5 mm, OD 1 mm; Falcon, UK) was inserted into the vena cava inferior and the liver and kidney perfused *in situ* with 5 ml of PBS containing 5 mM EDTA followed by 5 ml of 0.4% collagenase (type IV; Sigma, UK) in medium (RPMI 1640, 37°C, supplemented with penicillin-streptomycin; Life Technologies, UK). The

spleen was directly injected into the subcapsular space with 1 ml of PBS/EDTA and then 1 ml of collagenase solution. The gallbladder was removed, the organs excised and digested for a further 15 minutes in the collagenase solution at 37°C.

Single cell suspensions were established by passing the cells from liver and kidney through a 100 µm cell strainer (Falcon, UK) followed by a 40 µm strainer, the splenic cells through a 40 µm strainer only. The cells were washed in ice-cold PBS/EDTA, centrifuged for 10 minutes at 300xg and resuspended in ice-cold RPMI 1640 medium. The non-parenchymal cell fraction (NPC) of liver and kidney was purified by density centrifugation using 30% Percoll (Sigma, UK) at 1000xg for 30 minutes at 4°C, which retained the epithelial cells in the top layer. The splenic cell suspension was centrifuged at the same speed and length over Lympholyte M (Cedarlane Lab., Canada) to enrich the mononuclear cell fraction. The separated cell fractions were washed again in ice-cold medium to remove the Percoll solutions.

The recovered cell populations were resuspended in staining buffer (PBS with 2 mM EDTA and 0.5% BSA (fraction V, Sigma, UK), pH 7.2) and incubated with either a mAb against CD11c (N418), which was directly conjugated to magnetic beads (Miltenyi Biotec, Germany), or an unconjugated rat mAb against DEC-205 (NLDC-145; Serotec, UK). Both antibodies were used undiluted at 100 µl/10^8 cells for 30 minutes at 6-12°C. After washing in staining buffer, Dec-205-stained cells were incubated with a secondary goat anti-rat IgG antibody conjugated to magnetic beads (Miltenyi Biotec, Germany). The secondary antibody was used undiluted at 60 µl/10^8 cells for 15 min at 6-12°C. Positively stained cells were isolated on MACS separation columns (Miltenyi Biotec, Germany), placed within the magnet. The cell suspensions were passed through the columns, the effluent discarded, the magnet removed and the cells retained within the column washed out with 1 ml of the final culture medium (Iscove's Modified Dulbecco's Medium supplemented with penicillin/streptomycin (both Life Technologies, UK) and 10% FCS (SAPU, UK)). The isolated cells were tested for their viability by the trypan blue exclusion test and counted using a Neubauer cytometer.

2.2.2.4 Flow cytometry

The isolated DC populations were examined for cell surface antigen expression using immunofluorescent staining suitable for analysis by flow cytometry. DCs (2 x 10^5/sample) were washed twice in PBS (pH 7.4) containing 5% BSA and 0.1% NaN_3 (all Sigma, UK). The cells were then incubated for 30 minutes at 4^0 C with each respective monoclonal antibody. The antibodies and their specific characteristics are listed in table 2.2. After the staining, cells were again washed twice in PBS, fixed in 1% paraformaldehyde and analysed using a COULTER®EPICS®XL Flow Cytometer (Beckman-Coulter Electronics, Luton, UK). PE and FITC were both excited at 488nm, the red fluorescence emitted by PE was detected at 620nm and the green fluorescence emitted by FITC was detected at 525nm. Cells were gated according to size and scatter to eliminate dead cells and debris. Unstained DCs and DCs incubated with PE- or FITC-labelled, isotype-matched antibodies purchased from each respective company were used as negative control. Data from at least 10000 events were acquired. The selected cell populations were negative for CD3, CD19 or the Pan-NK cell-marker and expressed low levels of F4/80 as assessed by flow cytometry (data not shown).

mAb	Species	Fluorogen	Source
anti-MHC II ($I\text{-}A^d/I\text{-}E^d$; 2G9)	rat	FITC	Pharmingen, UK
anti-CD80 (B7-1; 16-10A1)	hamster	PE	Pharmingen, UK
anti-CD86 (B7-2; GL1)	rat	FITC	Pharmingen, UK
anti-CD40 (3/23)	rat	PE	Serotec, UK
anti-CD8α (Ly-2)	rat	FITC	Serotec, UK
anti-CD3ε (145-2C11)	hamster	FITC	Pharmingen, UK
anti-F4/80 (Cl:A3-1)	rat	PE	Serotec, UK
anti-CD19 (1D3)	rat	FITC	Serotec, UK
Anti-Pan-NK cells (DX5)	rat	FITC	Pharmingen, UK
anti-CD95L (FasL; MFL3)	hamster	PE	Pharmingen, UK

Table 2.4

Monoclonal antibodies used for analysis of cell surface antigen expression of DCs by flow cytometry.

2.2.2.5 Mixed leukocyte reaction

Purified DC preparations were resuspended in Iscove's Modified Dulbecco's Medium supplemented with penicillin/streptomycin (both Life Technologies, UK) and 10% FCS (SAPU, UK). The DC subpopulations were then either used immediately or cultured for 48 hours in the presence of GM-CSF (100 U/ml, ED_{50}) with or without dexamethasone (10^{-6} M/ml; Matyszak, 2000), CTLA4-Ig (100 ng/ml; ED_{50}), IL-10 (100 U/ml; ED_{50}) or TGF-β (100 U/ml; ED_{50}). After culture, DCs were rigorously washed in medium to remove surplus cytokines or immunosuppressive reagents and gamma-irradiated (2000 R) to prevent further DNA synthesis. $2x10^4$ cells per well were plated in triplicates in 96-well, round-bottom plates (Falcon, UK).

T lymphocytes from C3H ($H\text{-}2^k$) mice were used as responder cells following enrichment by a single passage through nylon wool columns (Havenith, 1992). Mice were killed by cervical dislocation and dissected to open the abdominal cavity. The spleen was injected into the subcapsular space with 1 ml of PBS/EDTA and excised. A single cell suspension was established by passing the splenic cells through a 40 µm strainer (Falcon, UK). The cells were washed in ice-cold PBS/EDTA, centrifuged for 10 minutes at 300xg and resuspended in RPMI 1640 medium (37°C, supplemented with penicillin-streptomycin; Life Technologies, UK). The cell suspensions were then layered onto nylon wool columns (Polysciences, USA), which had been pre-coated by incubation for one hour at 37°C with RPMI 1640 medium supplemented with 10% FCS. After incubation for another hour at 37°C, the non-adherent cells, i.e. the enriched T lymphocytes, were washed out with medium, tested for their viability by the trypan blue exclusion test and counted using a Neubauer cytometer. $2x10^5$ cells were added as responder cells to each well of the 96-well plates.

The mixed leukocyte cultures were incubated for a maximum of 120 hours in Iscove's Modified Dulbecco's Medium supplemented with penicillin/streptomycin and 10% FCS (37°C, 5% $C0_2$ in air). [^{3}H]thymidine (1 µCi in 10 µl medium) was added to each well for the final 18 hours of each culture. Cells were harvested after 72, 96 and 120 hours and [^{3}H]thymidine incorporation measured in a liquid

scintillation counter. Each experiment was repeated at least three times. Negative controls were performed by using responder cells alone, positive controls by using concanavalin A (Sigma, UK) as mitogen. Results are expressed as the mean counts per minute ± s.e.m. Supernatants of each culture were harvested at 72, 96 and 120 hours for analysis of the cytokine release.

Reagent	Species	Source
rGM-CSF	mouse	R&D Systems, UK
rIL-10	mouse	R&D Systems, UK
rTGF-β	human	R&D Systems, UK
CTLA-4/Fc chimera	mouse	R&D Systems, UK
dexamethasone	n/a	Sigma, UK

Table 2.5

Reagents used as treatment of the DC populations in the MLR or before RT-PCR/Real-time PCR.

2.2.2.6 ELISA

The differentiation of the T lymphocytes in response to the antigen-presentation by the isolated DCs was assessed by measuring IFN-γ and IL-10 in the supernatant of the MLR by ELISA technique. The cytokines were measured using the appropriate murine ELISA kits purchased from R&D Systems (UK) and used according to manufacturer's instructions. 96-well microplates (Falcon, UK) were coated with the capture antibody (R&D Systems, UK) and incubated overnight at room temperature. The plates were then washed with PBS containing 0.05% Tween 20 (Sigma, UK), and this was repeated in between each step described below. The plates were blocked for 1 hour with PBS containing 1% BSA, 5% sucrose and 0.05% NaN_3 (all Sigma, UK), followed by incubation for 2 hours at room temperature with either the undiluted samples or the standard samples (R&D Systems, UK) at 2-fold serial dilutions giving a seven point standard curve. The plates were then incubated with the biotinylated detection antibody (R&D Systems, UK) for 2 hours, streptavidin conjugated to horseradish-peroxidase (R&D Systems, UK) for 20 minutes, and

finally with the substrate solution containing a 1:1 mixture of H_2O_2 and tetramethylbenzidine (R&D Systems, UK) for 20 minutes, all at room temperature. The reaction was stopped by adding a 2 N H_2SO_4 solution (Sigma, UK) to each well. The optical density of each well was determined by reading the absorbance values at 450 nm (wavelength correction set to 540 nm) using an automatic multiwell spectrophotometer (Dynatech MR5000, UK). Each measurement was repeated on three independent wells from at least three different experiments. Results are expressed as pg/ml ± s.e.m.

2.2.2.7 RT-PCR

Induction of IL-10 and IL-12 p40 mRNA expression by the DC subpopulations in response to the experimental treatments were analysed qualitatively by RT-PCR. Total cellular RNA was extracted from isolated DC populations (5x 10^5 cells) before and after stimulation with GM-CSF alone or in combination with dexamethasone, CTLA4-Ig, IL-10 or TGF-β. DCs were analysed after 4, 6 and 24 hours of stimulation. Each experiment was repeated three times. The cells were homogenised with 1 ml of total RNA isolation reagent (TRIR; Life Biotechnologies, UK), followed by RNA extraction using 0.2 ml of chloroform (Sigma, UK) for 5 minutes on ice. The homogenate was centrifuged at 12000xg for 15 minutes and the RNA precipitated using isopropanol (Sigma, UK) and centrifugation at 12000xg for 10 minutes. The RNA pellet was washed twice in 75% ethanol (Sigma, UK) and dissolved in DEPC-treated water.

First-strand cDNA was synthesised from 1μg RNA samples using SuperScript^{TM}II RNase H Reverse Transcriptase (Life Biotechnologies, UK). The RNA was mixed with a 10 mM dNTP Mix (10 mM each dATP, dGTP, dCTP and dTTP at neutral pH), the specific primers for mouse IL-10 (Cat. No. 1101/1102) and IL-12 p40 (Cat. No. ILE-1039/1040; both Maxim Biotech, USA) and sterile, distilled water. The mixture was heated to 65°C for 5 minutes, then chilled on ice. After brief centrifugation, 5X First-strand buffer, 0.1 M DTT and RNaseOUT recombinant ribonuclease inhibitor were added together with the reverse transcriptase and

incubated at 42°C for 50 minutes. The reaction was inactivated by heating to 70°C for 15 minutes.

50 to 100 ng of cDNA were amplified by PCR reaction according to manufacture's specifications (Maxim Biotech). The cDNA was mixed with 10X PCR buffer, 10 mM dNTP Mix, 5U/µl Taq DNA polymerase (all Life Biotechnologies, UK), the gene specific primers (Maxim Biotech, USA), 50 mM $MgCl_2$ (Sigma, UK) and autoclaved, distilled water. After initial denaturation at 94°C for 2 minutes, 30 cycles of PCR were performed using a PTC-100 thermal cycler (Genetic Research Instrumentation, UK). Each cycle consisted of 60 seconds at 94°C and 120 seconds at 60°C with a final extension time of 10 minutes at 70°C. After the PCR reaction, amplified products were separated on 2% agarose gels by electrophoresis and visualised with ethidium bromide (both Sigma, UK). Negative controls were performed for each PCR reaction by omitting the reverse transcriptase, positive controls were provided by the manufacturer (Maxim Biotech, USA) in the from of cytokine cDNA for IL-10 (IL-10 cDNA, 10^7copies/µl) and IL-12 p40 (IL-12 cDNA, 10^7copies/µl).

2.2.2.8 Real Time PCR

The Real Time assay (Heid, 1996) using the ABI PRISM™ 7700 Sequence Detection System (PE Applied Biosystems, USA) is based on the 5'nuclease activity of the Taq DNA polymerase to cleave a TaqMan probe containing reporter and quencher dye during PCR. Amplification in the presence of the target-specific double fluorescent-labeled probe allows increasing numbers of unquenched reporter molecules to be detected, indicating directly the accumulation of the PCR product. IL-10 mRNA expression in DCs stimulated with GM-CSF alone (experimental control) was compared with the expression in DCs stimulated with GM-CSF and IL-10 or TGF-β (target samples). cDNA was synthesised from RNA (200 ng) as described above for RT-PCR. The primer sequences of the forward and reverse primers as well as for the double-labelled fluorogenic probe specific for IL-10 mRNA (kindly donated by Professor Jonathan Lamb, University of Edinburgh) are

given in table 2.3. The PCR reaction contained 300 nM of forward and reverse primers, 200 nM of fluorogenic probes, 3.5 mM MgCl, 200 Um dATP, dCATP, dGTP, 400 Um dUTP, 0.025 U/µl AmpliTaq Gold, 0.01 U/µl AmplErase uracil-*N*-glycosylase (UNG) and 1x PCR buffer A (50 mM KCL, 10 mM Tris-HCl, 0.01 M EDTA and 60 nM dye that serves as an internal control called Passive Reference 1). All reagents were obtained from the TaqMan PCR reagent kit (PE Applied Biosystems). Each PCR reaction was performed in triplicates in 96-well plates. The conditions were: 2 minutes of incubation at 50°C to allow the UNG cleavage, 10 minutes at 95°C to activate the Ampli-Taq Gold, followed by 40 cycles of 15 seconds at 95°C and 1 minute at 60°C. An endogenous control (18s RNA) was used to compensate for different levels of PCR inhibition, bulk splenocytes as positive control for the PCR reaction. The relative gene expression in the samples was calculated by comparing the relative threshold cycles (C_T value; comparative method). All experiments were repeated at least three times and the results expressed as fold-increase ± s.e.m. of the mRNA expression in the target samples compared with the expression in the experimental control.

IL-10	sequence
Forward primer	5'-CCACAAAGCAGCCTTGCA-3'
Reverse primer	5'-GTAAGAGCAGGCAGCATAGCA-3'
Fluorogenic probe	5'-FAM-AGAGCTCCATCATGCCTGGCTCAGC-TAMRA-3'

Table 2.6

Sequences of primers and the double-labelled fluorogenic probe specific for mouse IL-10 mRNA as used for Real-time PCR.

2.2.2.9 Statistical analysis

The student's *t*-test for differences between two means was used to compare the mean percentage (± s.e.m.) of normally distributed results obtained by [^{3}H]thymidine incorporation, ELISA and Real-time PCR. Results with a p-value less than 0.05 were considered as significant.

Chapter Three - Phenotype and proliferation index of the intra-hepatic immune response post-transplant

3.1 Summary

Background: Liver allograft acceptance appears to be dependent on initial immune activation rather than anergy and is associated with continuous detection of systemic donor-reactive T lymphocytes. Since the liver is the only solid organ to allow transendothelial migration of naïve T lymphocytes, hepatic allograft rejection might be modulated within the liver by peripheral sensitisation.

Aims: The inflammatory infiltrate within the liver post-transplant was examined to:

1. analyse the composition of the infiltrate with respect to cell type
2. assess the proliferation index to examine local expansion of the infiltrate
3. analyse T lymphocytes for naïve and memory phenotype to assess primary and secondary antigen-presentation

Methods: Liver biopsies of patients with acute and chronic rejection or without clinical rejection post-transplant were assessed by single and double label immunocytochemistry. T cells were identified by CD4 and CD8, B cells by CD20, NK cells by CD57 and macrophages by CD68. Naïve and memory T lymphocytes were distinguished by their expression of CD45RA or CD45RO, respectively. Proliferating cells were recognised by the expression of the nuclear antigen Ki-67.

Results: Graft rejection was predominantly characterised by an increase of CD4+ T lymphocytes. This was in part due to local expansion as assessed by their proliferation index. However, proliferation of naïve CD4+ T lymphocytes was a prominent feature of both, rejecting and non-rejecting grafts, indicating primary antigen-presentation within the liver post-transplant.

Conclusions: These findings suggest that there may be a primary immune response generated within the allograft as well as in draining lymphatic tissue. This implicates not only intra-hepatic proliferation of T lymphocytes as a prominent feature of rejection, but would also allow local modulation of the donor-reactive immune response independent of lymphatic tissue.

3.2 Introduction

Allograft rejection of solid organs including the liver is thought to be initiated by recipient T lymphocytes in response to alloantigens (Hall, 1978). The primary immune response is dependent on donor APCs activating naïve T lymphocytes which subsequently start to proliferate and differentiate (Lechler, 1982). This primary activation is believed to occur exclusively within secondary lymphatic tissue as central sensitisation, since naïve T cells lack the necessary levels of surface integrins for transendothelial migration outside of the lymphatic tissue (Brezinschek, 1995). In accordance with this hypothesis, donor cells as well as proliferating T cells are found in spleen and regional lymph nodes within 2 days of liver transplantation (Demetris, 1991). Recent experiments in animals lacking secondary lymphatic tissue have additionally demonstrated an inability of these animals to reject allogeneic transplants (Lakkis, 2000). Only after the primary activation of naïve T lymphocytes, grafts are thought to be infiltrated by donor-reactive CD4+ and CD8+ T lymphocytes, the predominant effector cells of allogeneic rejection (Hall 1985, Mosmann 1991). NK cells, macrophages and neutrophils either from the donor or the recipient are also found within the liver, but do not appear to be essential for the development of allograft rejection (Bingamann, 2000).

However, despite the common pathways of rejection in response to solid organs, liver allografts seem to be less immunogenic than other organs after transplantation. In clinical practice, liver grafts are more resistant to rejection than other organs despite lower levels of immunosuppression, and HLA matching between donor and recipient is not required (Opelz, 1992; Doran, 2000). In animal models, transplantation is possible without immunosuppressive agents and tolerance induction is described in both patients and animals (Starzl, 1993; Calne 1969). This tolerance induction is alloantigen-specific for other organs of the same donor and can over-ride priming (Kamada, 1981). Many authors argue that the tolerance is mediated within the recipient's lymphatic tissue in response to migration of donor passenger leukocytes (Bishop, 2001). However, systemic donor-reactive T lymphocytes are detectable even in the circulation of tolerant hosts (Kamada, 1985),

and liver allograft acceptance appears to be dependent upon a minimum amount of rejection and intra-hepatic lymphocyte turn-over (Qian 1997; Zavazava, 2000). Since intra-hepatic clustering between APCs and lymphoblasts can be observed within 2-3 days post-transplant (Demetris, 1991), intra-hepatic immune activation might be an important factor in mediating the host's immune response. Moreover, the liver has been recently shown to be the only solid organ allowing direct contact between parenchymal cells and naïve T lymphocytes through fenestrated endothelial cells (Bertolino, 2000), raising the possibility of peripheral sensitisation and even regulation of the host's immune system.

The aim of this part of the study was to analyse the intra-hepatic immune response following transplantation. Using liver biopsies of patients post-transplant as a model, the phenotype of potentially donor-reactive immune cells was assessed by their expression of the subset specific antigens CD4 and CD8 for T lymphocytes, CD20 for B lymphocytes, CD57 for NK cells and CD68 for macrophages (Abbas, 1994). Comparisons were made between biopsies of patients with acute and chronic rejection or without clinical rejection after transplantation to examine the relevance of the composition of the infiltrate for graft outcome. Local expansion of each cell subset was assessed using the proliferation marker Ki-67, a nuclear antigen specific for the late G1, S, G2 and M phases of the cell cycle (Gerdes, 1984). Finally, cycling T lymphocytes were investigated for their expression of the two isoforms CD45RA and CD45RO of the leukocyte common antigen family (LCA, CD45), which allow the distinction of naive and memory cells and thereby between centrally or peripherally sensitised T lymphocytes (Mackay, 1993).

3.3 Results

3.3.1 Phenotype of infiltrating mononuclear leukocytes post-transplant

Following liver transplantation, inflammatory cells were found in the allograft both within the portal tracts close to the vascular endothelium and biliary epithelium and within the liver parenchyma close to hepatocytes. The absolute cell numbers of the inflammatory infiltrate varied with the diagnosis and were significantly higher in biopsies during acute rejection compared with biopsies during chronic rejection or without rejection (Fig. 3.1). They also varied both within the diagnostic groups and within the same biopsies; to account for these variations, numbers of positive staining cells were expressed as the percentage of all cells of this type.

ML were phenotyped by their expression of subset specific antigens. T lymphocytes were identified by CD4 and CD8, B lymphocytes by CD20, NK cells by CD57 and macrophages by CD68. In all biopsies post-transplant, CD4+ T lymphocytes were predominantly located within the portal tracts, while CD8+ T lymphocytes and NK cells were found both within the portal tracts and the liver parenchyma. Macrophages were mainly situated within the liver parenchyma during all stages of rejection, but in biopsies of patients without rejection they were a prominent feature inside the portal tracts.

Assessment of the percentage of mononuclear cell subsets according to the diagnosis showed significant changes only for CD4+ T lymphocytes and NK cells (Fig. 3.2). During acute rejection, 43.8±2.5% of ML were positive for CD4 compared with 18.9±1.9% ($p = 0.0015$) in biopsies of patients without rejection, 26.8±3.1% ($p = 0.013$) following treatment of acute rejection and 22.9±6.0% ($p = 0.033$) during chronic rejection. NK cells made up 6.7±0.6% of all ML during acute rejection, significantly higher than in patients without rejection (2.1±0.3%, $p = 0.018$) or patients with chronic rejection (2.7±0.4%, $p = 0.011$). B lymphocytes were occasionally present within portal tracts of some but not all of the biopsies, accounting for less than 1% of all ML.

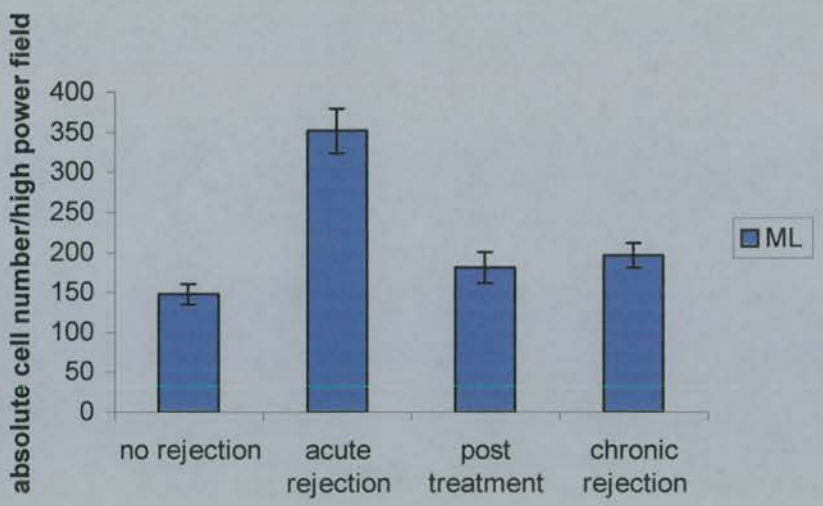


Figure 3.1:

Absolute cell number of ML within portal tracts in liver tissue following orthotopic transplantation. The cell number is expressed as mean percentage ± s.e.m./high power field (x40 magnification; n = 10).

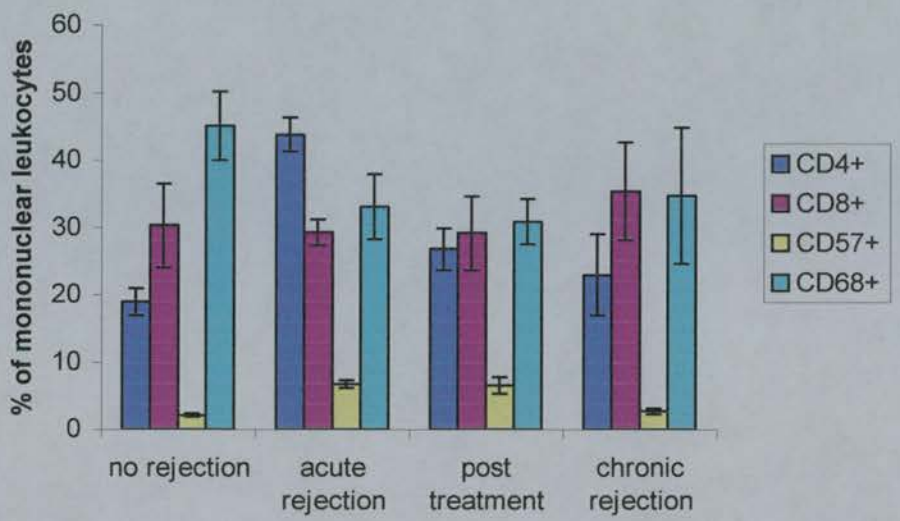


Figure 3.2

Expression of CD4, CD8, CD57 and CD68 in ML in liver tissue following transplantation. The number of CD4+ T lymphocytes, CD8+ T lymphocytes, CD57+ NK cells and CD68+ macrophages is expressed as mean percentage ± s.e.m. of all ML (n = 10).

3.3.2 Proliferation of mononuclear leukocytes post-transplant

Proliferation of intra-hepatic leukocytes was assessed by their expression of the nuclear antigen Ki-67, which is closely associated with the cell cycle. Biopsies from patients without liver disease were used to study the proliferation rate in normal liver tissue. In these, Ki-67+ ML were rarely observed with a mean percentage of 1 ± 0.3% (Fig. 3.3) and were located throughout the liver parenchyma as well as inside the portal tracts.

Biopsies taken on day 7 post-transplant from patients without clinical rejection revealed increased numbers of ML expressing Ki-67 compared with normal liver tissue (15.4±2.4% vs. 1±0.3%, $p = 0.0038$). These were located predominantly inside the portal tracts (Fig. 3.4).

During acute rejection, the percentage of proliferating ML rose to 61.1±1.9%, significantly higher than in patients without rejection ($p < 0.0001$). The distribution of Ki-67+ cells was mainly inside the portal tracts with some present in the adjacent liver parenchyma (Fig. 3.5). Following treatment with corticosteroids, the number of ML expressing Ki-67 fell to 23.2±2.5%. This was significant compared with the previous biopsies during acute rejection ($p < 0.0001$), but not with biopsies from patients without rejection ($p = 0.052$).

During chronic rejection, the number of ML in cell cycle was 24.9±5.9%, significantly different to biopsies from patients without liver disease ($p = 0.004$) or with acute rejection ($p = 0.0004$), but not from patients without rejection ($p = 0.17$). More of the Ki-67+ cells were present within the liver parenchyma, but again the majority was situated inside the portal areas (Fig. 3.4).

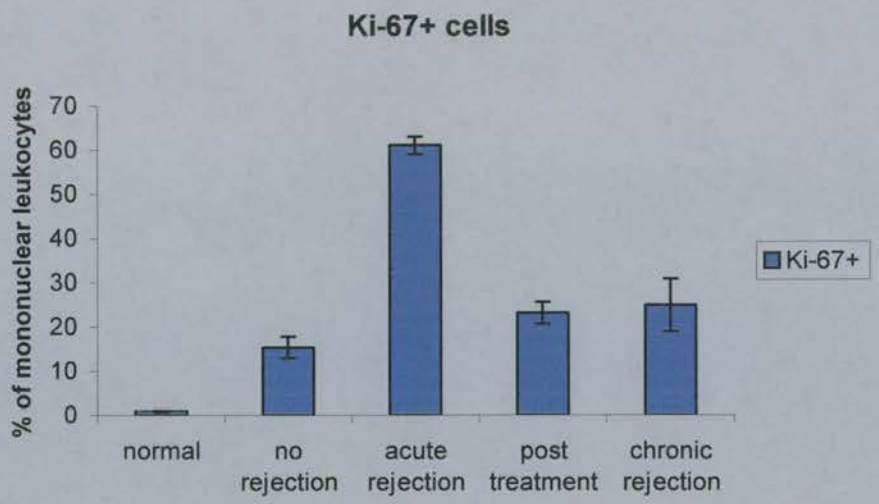
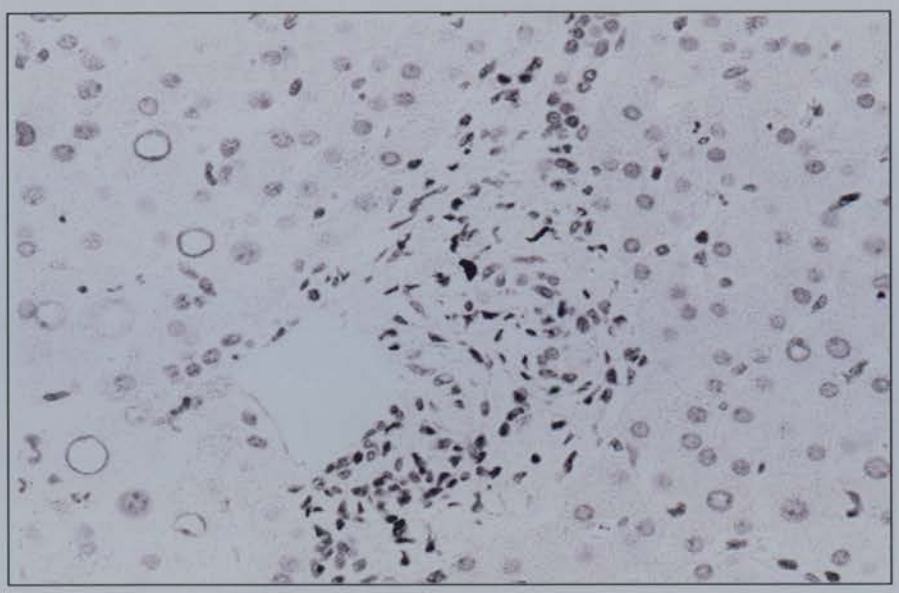


Figure 3.3

Ki-67 expression of ML in normal liver tissue and following transplantation. The number of Ki-67+ cells is expressed as mean percentage ± s.e.m. of all ML (normal tissue: n = 15; all other tissue: n = 10).

A



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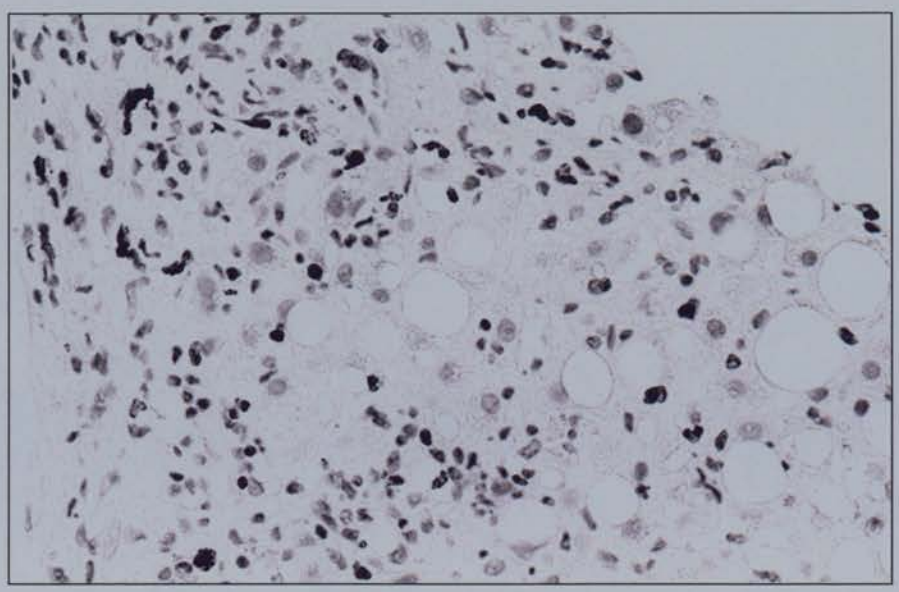
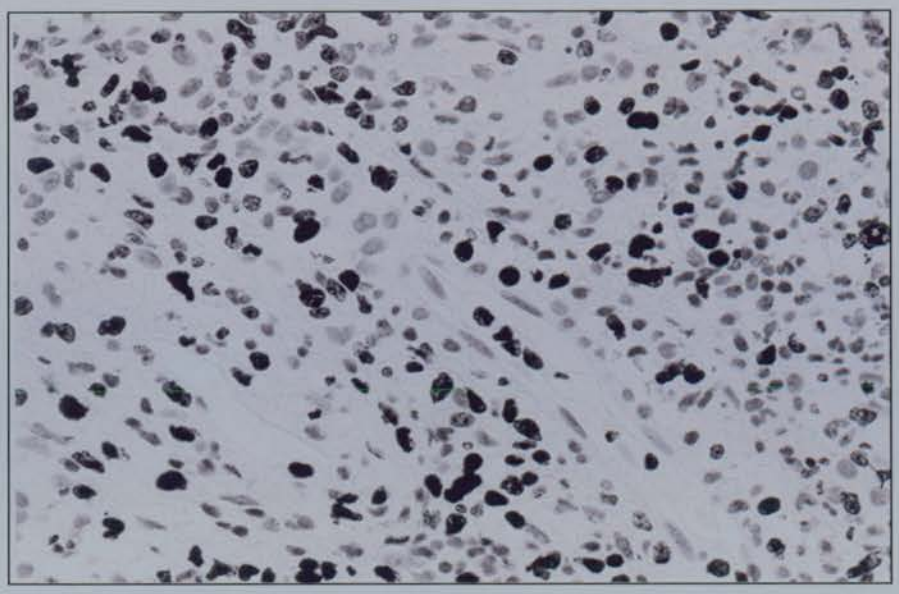


Figure 3.4

Ki-67 expression of ML following transplantation: a) moderate expression by periportal ML in biopsies of patients without significant rejection: b) moderate expression by periportal and intraparenchymal ML during chronic rejection.

A



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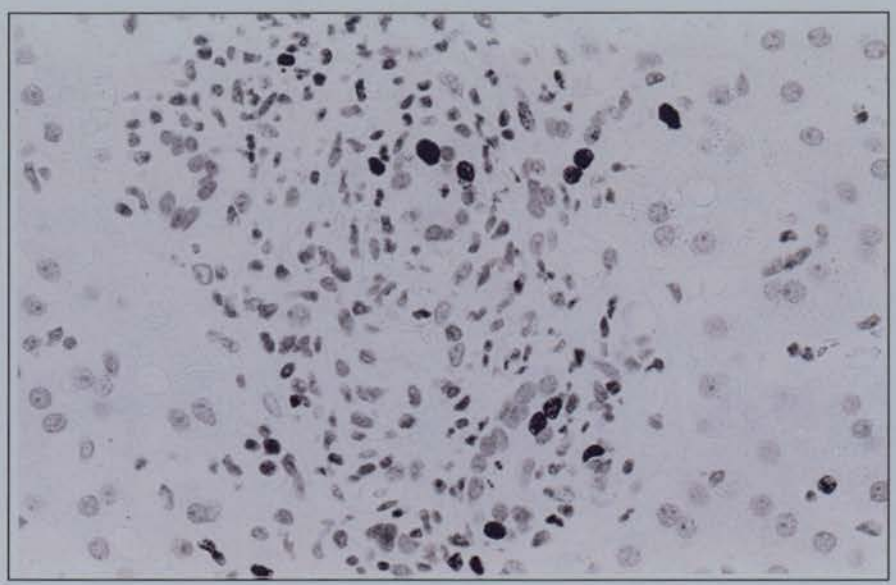


Figure 3.5

Ki-67 expression of ML following transplantation: a) high expression by periportal ML during acute rejection; b) reduced expression following treatment for acute rejection with corticosteroids.

3.3.3 Proliferating leukocytes are predominantly CD4+ T lymphocytes

In order to study the phenotype of ML proliferating after transplantation, we used a double-staining method to assess which subsets of cells were co-expressing the nuclear antigen Ki-67. Positive double-staining of CD20, CD57 or CD68 on Ki-67+ cells was not observed. CD4+ and CD8+ T lymphocytes were therefore reassessed as a percentage of all lymphocytes.

As previously described, CD4+ T lymphocytes were predominantly located inside the portal tracts in all biopsies post-transplant. In biopsies of patients without rejection, CD4+ lymphocytes represented 31.6±3.3% of all lymphocytes (Fig. 3.6). During acute rejection, the number of CD4+ cells was significantly higher with 62.6±3.6% ($p = 0.0031$). Treatment of acute rejection with i.v. corticosteroids resulted in a significant reduction of CD4+ cells to 38.3±4.4% ($p = 0.013$), similar to chronic rejection with a percentage of 32.8±8.6% (62.6±3.6% vs. 32.8±8.6%, $p = 0.033$).

Like CD4+ T lymphocytes, proliferating cells were predominantly located inside the portal tracts in biopsies of patients without rejection. Accordingly, the majority of Ki-67+ cells were CD4+ on double-staining with a mean percentage of 90.0±3.0% (Fig. 3.7 and 3.8). A similar distribution of CD4+ T lymphocytes was observed during acute rejection with 96.0±1.3% of proliferating cells positive for CD4. Treatment of acute rejection with corticosteroids did not change the distribution of cells, but significantly reduced the percentage of CD4+ T lymphocytes in cell cycle to 87.8±1.6% ($p = 0.029$). During chronic rejection, most of the CD4+ T lymphocytes remained located inside the portal tracts, but the percentage of CD4+ proliferating cells (75.5±2.5%) was significantly lower compared with acute rejection ($p = 0.0054$) or no rejection ($p = 0.034$).

In contrast to CD4+ T lymphocytes, CD8+ T cells were predominantly situated within the liver parenchyma with only a minority inside the portal tracts in all tissues post-transplant. In biopsies of patients without rejection, CD8+ lymphocytes represented 50.5±10.4% of all lymphocytes with no significant changes to the

percentage during acute or chronic rejection (Fig. 3.6). In addition to CD4+ T lymphocytes, CD8+ T lymphocytes were only occasionally positive for the nuclear antigen Ki-67 on double-staining (Fig. 3.7 and 3.8). No significant changes were detected in the percentage of CD8+ proliferating lymphocytes between the four groups of biopsies.

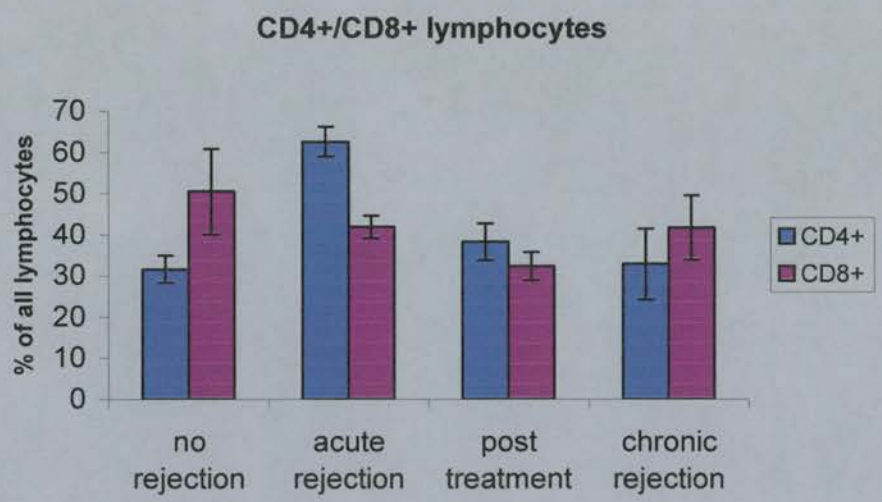


Figure 3.6

Expression of CD4 and CD8 in lymphocytes in liver tissue following transplantation. The number of CD4+ and CD8+ lymphocytes is expressed as mean percentage ± s.e.m. of all lymphocytes (n = 10).

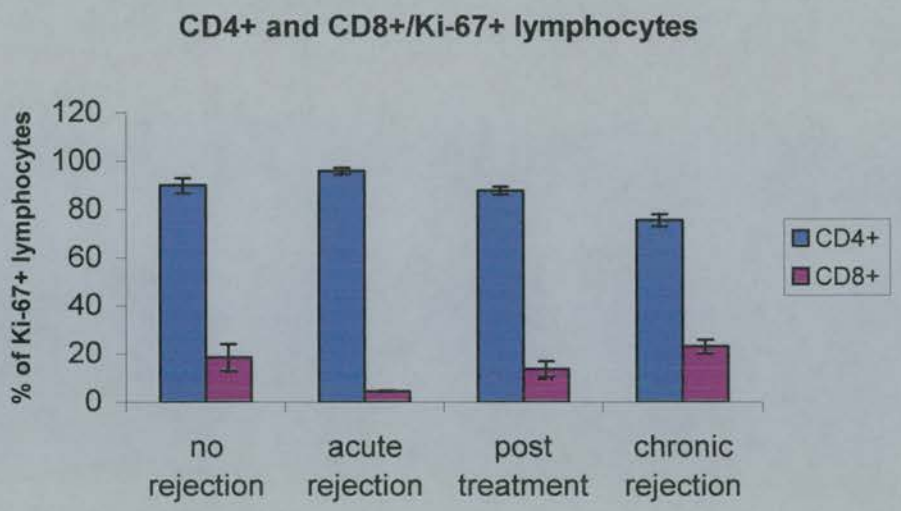
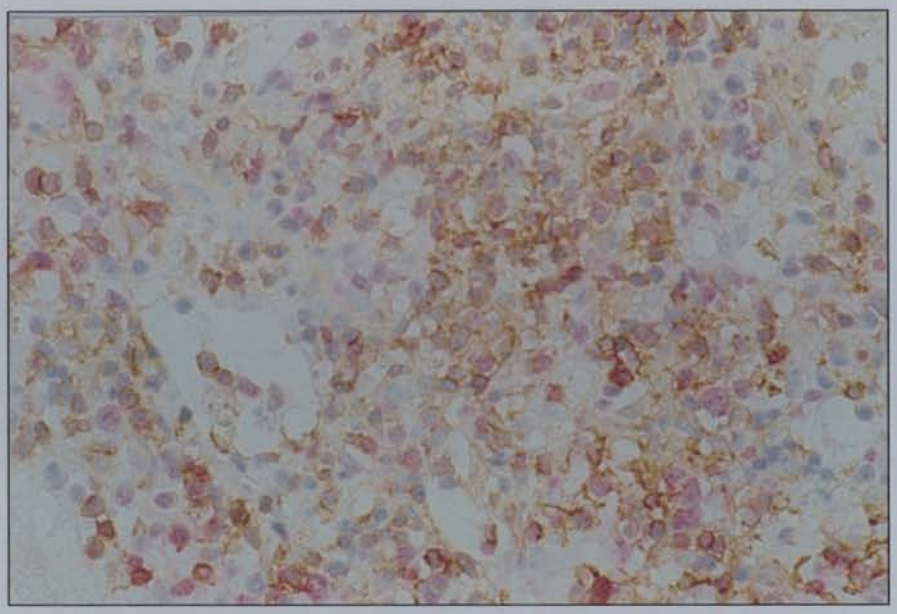


Figure 3.7

Expression of CD4 and CD8 in proliferating lymphocytes in liver tissue following transplantation. The number of CD4+ and CD8+ T lymphocytes is expressed as mean percentage ± s.e.m. of all Ki-67+ cells (n = 10).

A



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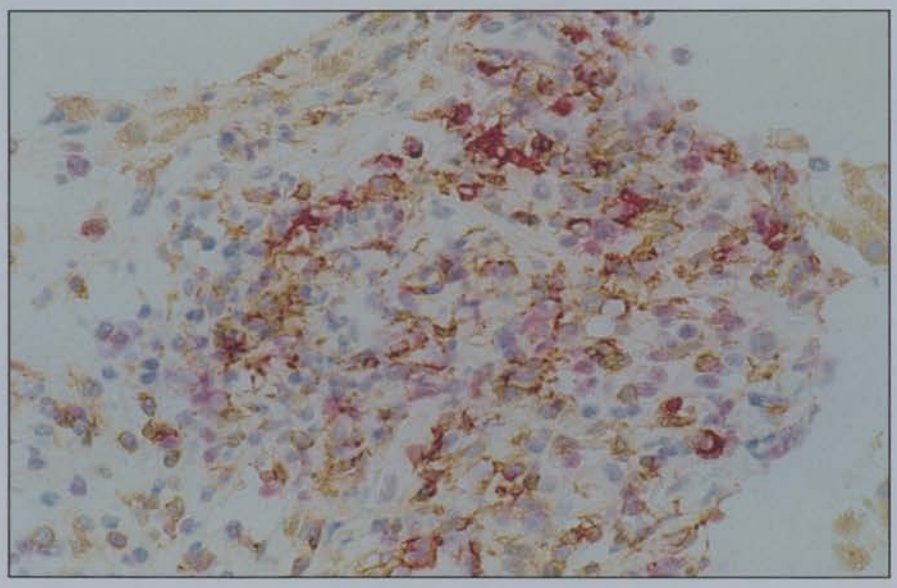


Figure 3.8

Double-staining of CD4+, Ki-67+ and CD8+, Ki-67+ T lymphocytes in liver tissue following transplantation (brown staining: CD4, CD8; red staining: Ki-67; blue staining: haematoxylin): a) predominance of proliferating CD4+ T lymphocytes in portal tracts post-transplant; b) minority of proliferating CD8+ T lymphocytes in portal tracts post-transplant.

3.3.4 Proliferating T lymphocytes are both naive and memory cells

To establish whether the proliferating CD4+ T lymphocytes were of the naive or memory phenotype, we used the double-staining method to examine proliferating lymphocytes for their expression of the two isoforms CD45RA (naive lymphocytes) and CD45RO (memory lymphocytes) of the leukocyte common antigen family (LCA, CD45). The number of naive and memory T cells was expressed as the percentage of all lymphocytes that were either CD45RA+ or CD45RO+.

In all biopsies, CD45RA+ lymphocytes were predominantly located inside the portal tracts. During acute rejection, naive lymphocytes (Fig. 3.9) increased significantly compared with patients without rejection (38.9±2.3% vs. 25.3±1.7%, $p = 0.018$). However, treatment with corticosteroids significantly reduced this number (38.9±2.3% vs. 29.8±0.3, $p = 0.034$). There was no significant difference in the percentage of naive lymphocytes during acute compared with chronic rejection.

Of the proliferating lymphocytes (Fig. 3.10 and 3.11), CD45RA+ cells represented 40.9±3.3% and 47.3±3.3% in tissues of patients without rejection or with acute rejection, respectively. Steroid treatment of acute rejection did not change this percentage, but during chronic rejection (16.7±3.2%) it was significantly lower compared with acute ($p = 0.0069$) and no rejection ($p = 0.013$).

In all biopsies post-transplant, CD45RO+ lymphocytes were present in the parenchyma throughout the liver lobules, but the majority was situated inside the portal areas. No significant difference was observed in the percentage of CD45RO+ cells (Fig. 3.9) between biopsies of patients without rejection and with acute rejection, but the percentage increased significantly following treatment with i.v. corticosteroids (52.3±1.4% vs. 40.7±0.9%, $p = 0.0064$). Additionally, the percentage was significantly higher during chronic rejection compared with acute rejection (55.7±2.7% vs. 40.7±0.9%, $p = 0.034$).

CD45RO+ lymphocytes represented 53.8±0.2% of Ki-67+ proliferating cells (Fig. 3.10 and 3.11) in tissue from patients without rejection. There was no significant change during acute rejection, but during chronic rejection the percentage of memory lymphocytes was 80.3±4.4%, which was significantly higher than in biopsies from patients with acute (55.7±1.5%, $p = 0.033$) and without rejection (53.8±0.2%, $p = 0.026$).

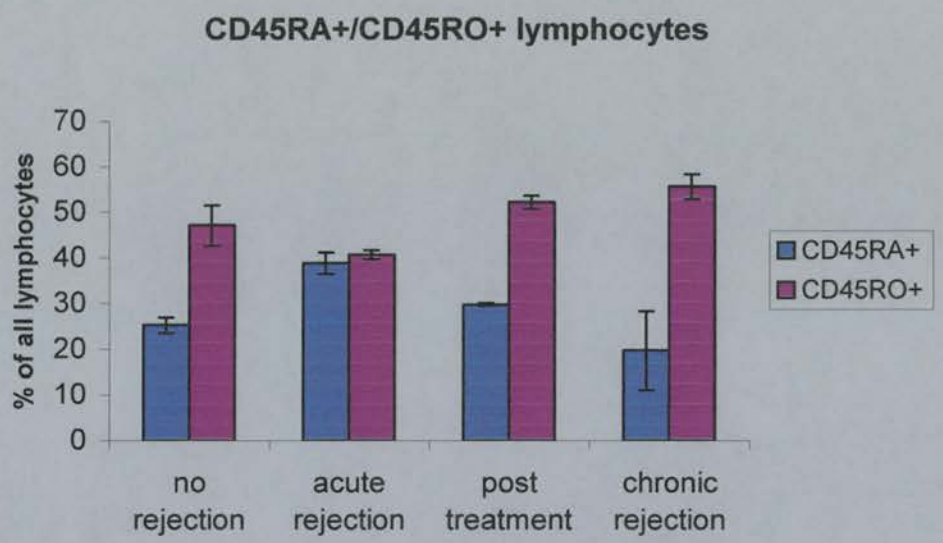


Figure 3.9

Expression of CD45RA and CD45RO in lymphocytes in liver tissue following transplantation. The number of CD45RA+ and CD45RO+ lymphocytes is expressed as mean percentage ± s.e.m. of all lymphocytes (n = 10).

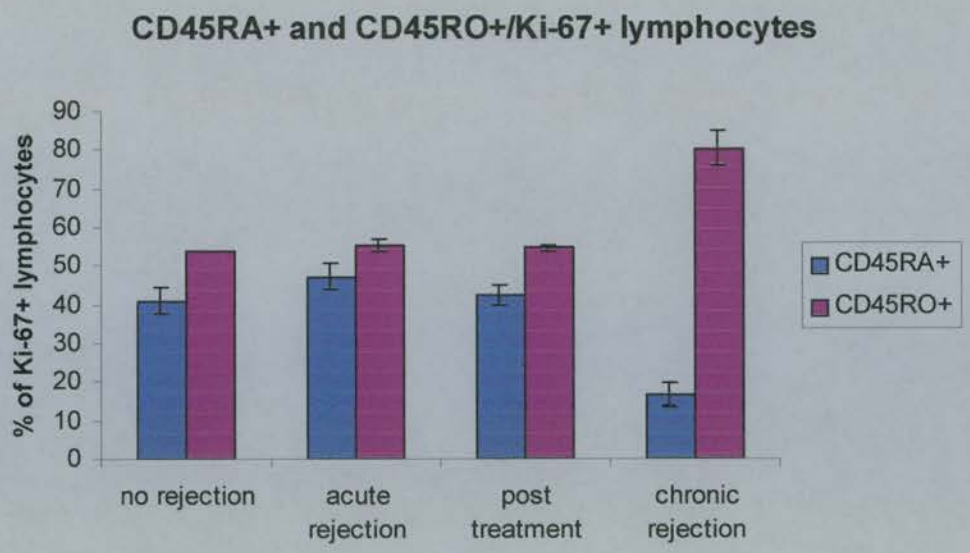
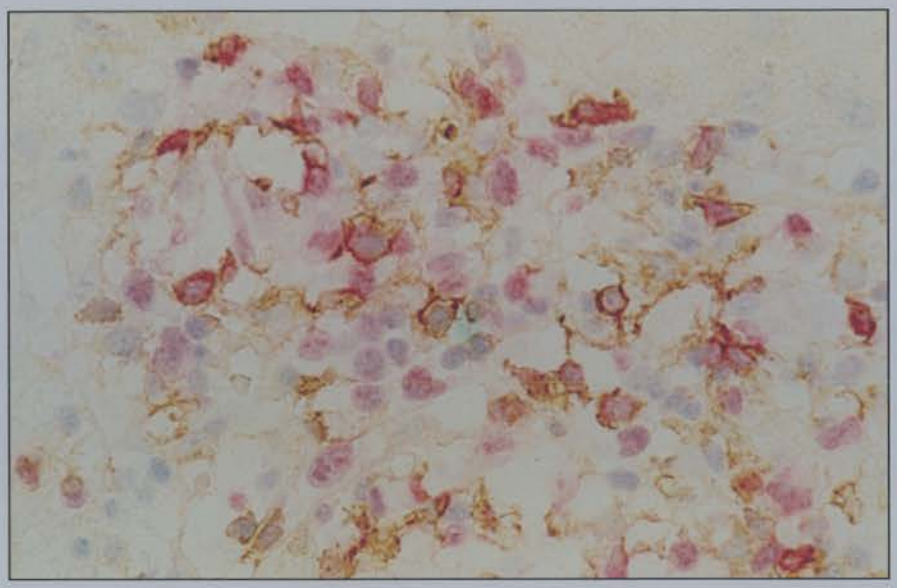


Figure 3.10

Expression of CD45RA and CD45RO in proliferating lymphocytes in liver tissue following transplantation. The number of CD45RA+ and CD45RO+ lymphocytes is expressed as mean percentage ± s.e.m. of all Ki-67+ lymphocytes (n = 10).

A



B

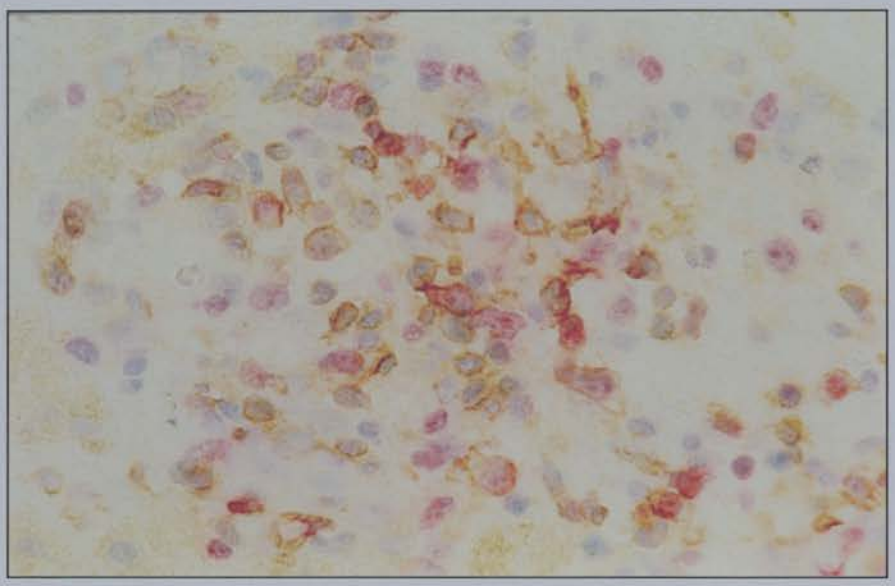


Figure 3.11

Double-staining of CD45RA+, Ki-67+ and CD45RO+, Ki-67+ lymphocytes in liver tissue following transplantation (brown staining: CD45RA, CD45RO; red staining: Ki-67; blue staining: haematoxylin): proliferation of a) naive (CD45RA+) and b) memory (CD45RO+) lymphocytes post-transplant.

3.4 Discussion

The results in this study indicate two major findings. Firstly, proliferation of ML inside the human liver allograft was a prominent feature of rejection, suggesting that a significant proportion of the "inflammatory infiltrate" is due to local expansion rather than migration. Secondly, leukocytes in cell cycle were predominantly CD4+, CD45RA+ and CD4+, CD45RO+ T lymphocytes, that is T lymphocytes of both, naive and memory phenotype. This would implicate that the primary immune response following liver transplantation with proliferation and differentiation of naive T lymphocytes might not be restricted to secondary lymphatic tissue, but could additionally occur within the allograft.

The current concept of homing and migration patterns of lymphocyte subsets (Pape, 1997) suggests that naive T lymphocytes recirculate preferentially through lymphoid tissue which provides the necessary microenvironment for antigen stimulation. Naïve lymphocytes are able to enter lymph nodes through high endothelial venules, distinct from other microvessel endothelia (Szekanecz, 1992). In contrast, T lymphocytes in non-lymphatic tissue, e.g. during inflammation, are predominantly of the memory and effector phenotype, due to their ability of transendothelial migration (Bianchi, 1997). Their rapid increase in numbers during inflammation has been accounted for by migration rather than local expansion (Oppenheimer-Marks, 1997).

The observations in this study appear to contrast significantly with this concept. Not only was a substantial number of naïve T lymphocytes, expressing CD45RA, residing in the peripheral tissue of the graft, but there were also sufficient signals to activate these cells as demonstrated by their expression of the proliferation marker Ki-67. There are however potential limitations for both markers, CD45RA and Ki-67, relating to their specificity. CD45RA is an isoform of CD45, which is expressed by all leukocytes including T and B lymphocytes as well as NK cells (Abbas, 1994). Moreover, recent publications have shown some memory CD8+, CD45RO+ T cells to revert back to a more stable, long-lived CD45RA+ phenotype without losing their antigen-specificity (Appay, 2002). The same study defined naïve CD8+ T cells by

the absence of cytotoxic factors such as granzymes and by the expression of CD45RA together with CD27, CD28 and the chemokine receptor CCR7, a secondary lymphoid organ-homing marker (Campbell, 1998). A correlating CD45RA+ memory phenotype has also been hypothesised for CD4+ T lymphocytes (Bell, 1998), although CD4+ T cells might rather co-express CD45RA and RO than lose the activation marker CD45RO altogether (Arlettaz, 1999). Ki-67 on the other hand is specific for certain phases of the cell cycle (Gerdes, 1984), but does not ultimately prove cell division. Another potential cell fate of cycling lymphocytes is cell death, and hepatocytes were recently demonstrated to induce apoptosis in naïve CD8+ T cells after their initial progression into the cell cycle (Bertolino, 1999).

In the current study, most of the CD45RA+, Ki-67+ cells co-expressed CD4, indicating them to be CD4+ T lymphocytes. The highest number of CD45RA+, Ki67+ T cells was found in biopsies of patients undergoing acute rejection one week post-transplant, when recently activated memory T cells would still express CD45RO (Appay, 2002). Although apoptosis rather than proliferation of the cells cannot be excluded, other publications in kidney transplantation have also demonstrated proliferation of lymphocytes within the allograft, linking it to rejection (Nemlander, 1982; Von Willebrand, 1983). Moreover, aggregates of DCs and lymphoblasts are a common feature of acute and chronic rejection in heart and liver allografts, indicating *in situ* stimulation of the lymphocytes (Forbes, 1986; Demetris, 1991 and 1997b). This has led to the concept of peripheral sensitisation, but only newer studies in kidney and heart allograft rejection were able to describe the ratio between naive and memory T cells using CD45RA and RO as markers (Ibrahim, 1993b and 1995). In both organs CD45RO+ memory T lymphocytes increased during rejection, while data on proliferation was not provided. However, the existence of naïve T cells within the grafts could indicate, that allografts in general become accessible to transendothelial migration of naïve T cells, in contrast to organs during other forms of inflammation.

Recent publications on the other hand have described intra-hepatic subpopulations of naive T lymphocytes capable of proliferation and T cell receptor rearrangement

leading to the hypothesis of extralymphatic T cell development in the liver (Makino, 1993; Sato, 1995; Collins, 1996). This hypothesis is now being supported by the new finding, that the liver is the only peripheral organ to allow migration of naïve T cells through fenestrated endothelial cells (Bertolino, 2000). Additionally, both, hepatocytes and sinusoidal endothelial cells appear to interact with naïve T lymphocytes, inducing either apoptosis or anergy (Bertolino, 1998; Knolle, 2001). Following transplantation however, lymphoblasts are found in close contact with DCs (Demetris, 1991 and 1997b), which are located within the portal tracts (Hart, 1981). In this study, naïve T cells were also predominantly observed within the portal tracts, implicating DCs as the likely APCs.

Hepatic DCs have recently been hypothesised to be tolerogenic (Thonpson, 1999), but the association of T cell proliferation with acute rejection in this study rather suggests a local expansion of the inflammatory infiltrate. The predominance of CD4+ T lymphocytes is compatible with their role as the principle mediators of rejection, which provide the signals for other lymphoid cells to become cytotoxic effector cells (Mosmann, 1991; Hao, 1990). During chronic rejection, CD45RA can not be used as a certain marker of naïve T lymphocytes, but most of the proliferating cells in the respective biopsies of this study expressed CD45RO rather than RA, compatible with the function of CD45RO as an activation marker of memory cells (Bell, 1998).

In contrast to rejection, the role of CD4+ T cells co-expressing CD45RA and Ki-67 in the biopsies of patients without rejection is less clear. Since the biopsies were taken on day 7 post-transplant, CD45RA can be assumed to be a marker of naivety. Ki-67 on the other hand might not necessarily indicate proliferation in the absence of an expanding infiltrate. Indeed, CD8+ T lymphocytes appear to decline in stable grafts (Wong, 1998) and the expression of Ki-67 might occur prior to cell death of the CD4+ T cells in these grafts. Recently however, spontaneous liver allograft acceptance has also been associated with persisting intra-hepatic CD4+ T cells (Olver, 1998). It is therefore tempting to speculate, that some of these CD4+ T

lymphocytes will become Tr cells or have a regulatory function similar to CD4+ CD25+ T cells in models of autoimmune disease (Annacker, 2001).

In conclusion, this study presents data which challenges the view that solid organs and specifically the liver only represent a target for the immune system following transplantation. Recent publications on transplantation in patients and animal models have led to the hypothesis that the migration of donor passenger leukocytes into recipient lymphoid tissue influences transplantation outcome (Starzl, 1996; Bishop, 2001). The present results suggest that the graft itself might be relevant for the development of the immune response. Either all solid organs post-transplant provide a location for allorecognition and the subsequent primary lymphocyte activation, or the liver has specific immunological properties because of its unique vascular architecture.

Chapter Four – T cell-mediated cytotoxicity post-transplant

4.1 Summary

Background: Cell death of donor target cells during liver allograft rejection is thought to be predominantly by apoptosis and caused by cytotoxic T lymphocytes via perforin/granzyme B and CD95L/CD95. However, liver grafts appear to be more resistant to rejection than other solid organs and intra-hepatic depletion of donor-reactive T cells might precede tolerance induction.

Aims: Immune and target cells within the liver post-transplant were assessed for:

1. T cell-mediated cytotoxicity by staining for Granzyme B and CD95 expression
2. the susceptibility of target cells to apoptosis by staining for intracellular signals of the apoptotic cascade
3. the susceptibility of immune cells to apoptosis by staining for the receptor CD95 and intracellular apoptotic signals

Methods: Liver biopsies of patients with acute and chronic rejection or without clinical rejection post-transplant were assessed by single and double label immunocytochemistry. T cells were identified by CD4 and CD8, B cells by CD20, NK cells by CD57 and macrophages by CD68. Proteins related to cytotoxicity and apoptosis were analysed by staining for granzyme B, CD95, bcl-2, bcl-x, bax and p53.

Results: Granzyme B was highly expressed by CD8+ T cells and NK cells during graft rejection, while CD95 expression was a prominent feature in the group without rejection in the periportal infiltrate. CD95 was also constitutively expressed by hepatocytes and up-regulated during rejection, but undetectable in the biliary epithelium or vascular endothelium. The anti-apoptotic protein bcl-2 was expressed in ML during all stages of rejection, while bcl-x and bax were constitutively expressed in hepatocytes and biliary epithelial cells and up-regulated during rejection. p53 was only expressed post-transplant correlating with the severity of rejection.

Conclusions: Both pathways, perforin/granzyme B and CD95L/CD95 appear to be implicated in hepatic allograft rejection. However, CD95-mediated apoptosis seems to be restricted to the secondary targets hepatocytes and might be, together with bcl-2, important in the intra-hepatic regulation of the inflammatory infiltrate.

4.2 Introduction

Included in the minimal histological criteria of acute and chronic rejection (Demetris, 1997a), cell death of biliary epithelial cells and vascular endothelial cells is the most prominent feature of liver allograft rejection. Inflammatory cells are usually found in close proximity to these cells making them the primary targets of the immune-mediated injury. In contrast, hepatocellular damage appears to be rather indicative of severe and prolonged rejection, associated with the spill-over of inflammatory cells into the liver parenchyma (Int. Working Party, 1995).

Target cell death during liver allograft rejection appears to be predominantly by apoptosis, first described in experimental porcine allograft rejection (Searle, 1977) and during rejection of human liver transplants (Snover, 1984). Apoptosis of hepatocytes and biliary epithelial cells correlates well with the degree of rejection using a rat model (Krams, 1995a). However, detection of apoptosis, in particular of biliary epithelial cells and vascular endothelial cells, has remained difficult possibly due to the rapid clearance of the dead cells by phagocytosis (Wyllie, 1980). Other studies have therefore used the detection of intracellular signals involved in the apoptotic cascade to assess the susceptibility of target cells to undergo apoptosis. Down-regulation of the anti-apoptotic protein bcl-2 has been recently associated with loss of bile duct cells during acute rejection (Gapany, 1997).

In addition to cell death of donor cells, DNA fragmentation and apoptosis of periportal ML has been described following liver transplantation (Afford, 1995). This has led to the hypothesis that apoptosis of donor-reactive T cells precedes tolerance induction and a recent study demonstrated that intact T cell-apoptosis pathways are required for tolerance induction across MHC barriers (Wells, 1999). Activated rather than resting T cells appear to be susceptible to cell death which is thought to be mediated by activation-induced cell death following the engagement of the apoptotic receptor CD95 (Alderson, 1995).

In the previous chapter, we analysed the composition of the inflammatory infiltrate post-transplant and found a disproportionate increase of CD4+ T lymphocytes during acute rejection. In contrast, the relative number of CD8+ T lymphocytes, the presumed cytotoxic T cells, did not change significantly in the biopsies of patients with or without rejection, although absolute numbers did increase during acute rejection. CTLs may induce apoptosis and kill their target cells via two distinct pathways (Liu, 1996). Firstly, they are able to secret the pore-forming protein perforin and proteases such as granzyme A and B, which are specific for cleavage and activation of DNA binding proteins and caspases of the apoptotic sequence. Secondly, T cells may express the protein CD95L, which engages its receptor CD95 on the surface of the target cells and triggers the apoptotic sequence on receptor-bearing cells. Both pathways have been implicated in allograft rejection of solid organs using knock-out mice (Kaegi, 1996) or PCR for measurement of mRNA levels (Sharma, 1996), but the distribution and regulation of the signals *in situ* remains unclear.

Using liver biopsies of patients following transplantation, our aim was to examine the two pathways of T cell-mediated cytotoxicity during rejection *in situ* to elucidate their individual significance in the immune response. The expression of granzyme B and the CD95 receptor was therefore assessed according to the cell type by immunocytochemistry. Additionally, the expression of intra-cellular signals related to apoptosis were investigated by staining for three members of the bcl-2 family and p53. bcl-2, bax and bcl-x as well as p53 are able to modulate the initial apoptotic signal (Hale, 1996), and their expression was examined to evaluate the susceptibility of target cells to undergo cell death.

4.3 Results

4.3.1 Increased granzyme B expression during acute and chronic rejection

ML expressing Granzyme B were assessed as the percentage of positive staining cells of all visible ML. No staining was detected in biopsies from patients without liver disease used as normal controls. ML in biopsies taken on day 7 post-transplant from patients without clinical rejection expressed granzyme B occasionally with a mean percentage of 4.6±0.9% (Fig. 4.1). Positive cells were located both in the portal tracts and the liver parenchyma.

During acute rejection, the percentage of granzyme B positive ML was significantly higher than in biopsies of patients without rejection (13.9±0.4% vs. 4.6±0.9%, p = 0.01), but with a similar distribution in both the portal tracts and the liver parenchyma. Following treatment with corticosteroids, the percentage of positive staining cells fell significantly to 5.9±1.2% (p=0.025). The highest percentage of ML expressing granzyme B was found during chronic rejection (Fig. 4.1 and 4.2a) with 26.0±2.9%, significant compared with biopsies of patients without rejection (p=0.019), but not compared with acute rejection (p=0.053). Positive cells were predominantly located within the liver parenchyma rather than in the portal tracts.

Further investigation of the ML subsets expressing granzyme B by double-staining with subset markers revealed, that granzyme B positive cells were mostly CD8+ T lymphocytes (Fig. 4.2b) and occasionally CD57+ NK cells.

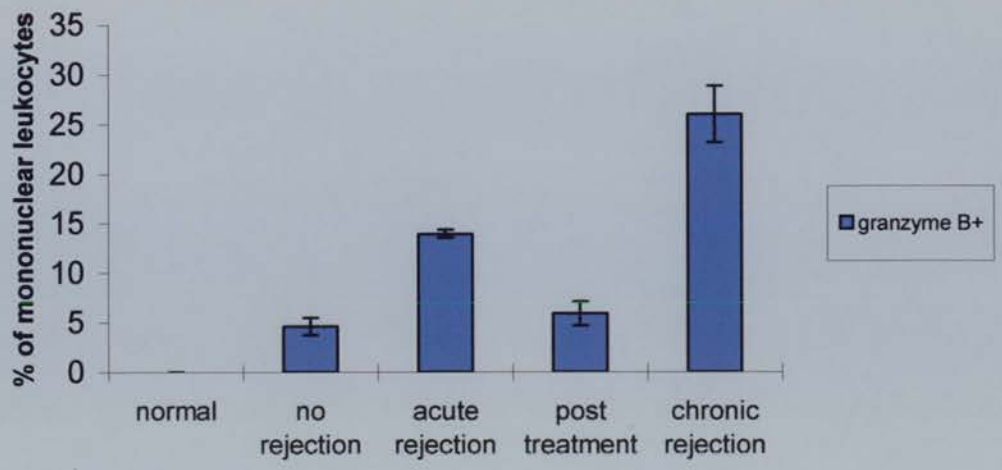


Figure 4.1

Expression of granzyme B in ML in normal liver tissue and following transplantation. The number of granzyme B+ leukocytes is expressed as mean percentage ± s.e.m. of all ML (n = 10).

A B

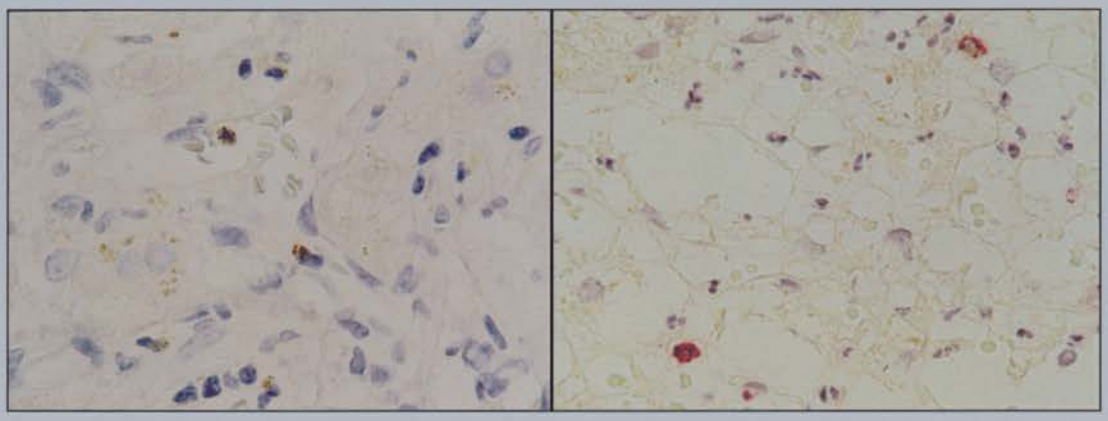


Figure 4.2: Granzyme B expression post-transplant

Expression of granzyme B in liver tissue following transplantation: a) granzyme B expression by intraparenchymal ML during chronic rejection (brown staining: granzyme B; blue staining: haematoxylin); b) double-staining of CD8+ and granzyme B+ T lymphocytes during chronic rejection in an ischaemic perivenular area (brown staining: granzyme B; red staining: CD8; blue staining: haematoxylin).

4.3.2 Cell type-dependent CD95 expression following transplantation

In all biopsies examined, CD95 was only detected in two cell types, ML and hepatocytes. Expression of CD95 in ML was assessed as the percentage of positive staining cells of all ML. In biopsies of patients without liver disease, a mean percentage of 36.0±9.4% of ML expressed CD95 (Fig. 4.3). Positive cells were located both in the portal tracts and within the liver parenchyma.

Following transplantation in biopsies of patients without rejection on day 7, the mean percentage of CD95 positive ML was significantly higher (70.8±4.6% vs. 36.0±9.4%, $p = 0.029$). Positive cells were predominantly situated inside the portal tracts and only occasionally within the liver parenchyma (Fig. 4.3 and 4.4a). In contrast, during both acute and chronic rejection, the percentage of CD95 expressing ML was significantly lower with 16.7±3.4% ($p = 0.0025$) and 9.5±5.1% ($p = 0.003$), respectively. There was no significant difference to the normal controls. Positive cells were again located predominantly within the portal tracts and treatment of acute rejection with corticosteroids neither changed the percentage of positive cells nor their distribution.

CD95 expression in hepatocytes was homogenous but varied in intensity. It was assessed semi-quantitatively according to low or high expression. Hepatocytes in normal control biopsies showed a low, constitutive expression of CD95 with a uniform distribution in the liver parenchyma (Table 4.1). No change of intensity of staining or distribution was detectable in biopsies of patients without rejection on day 7 post-transplant.

During acute and chronic rejection, predominantly perivenular hepatocytes stained highly positive for CD95 (Fig. 4.4b and Table 4.1) compared with both periportal hepatocytes in the same biopsies and all hepatocytes in biopsies of normal controls and patients without rejection. Treatment of acute rejection with corticosteroids had no effect on the CD95 expression of hepatocytes.

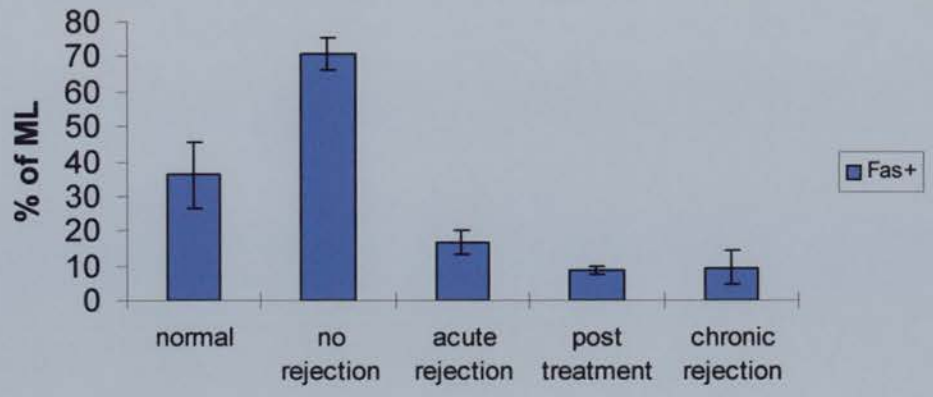


Figure 4.3: CD95+ mononuclear cells

Expression of CD95 in ML in normal liver tissue and following transplantation. The number of CD95+ ML is expressed as mean percentage ± s.e.m. of all ML (n = 10).

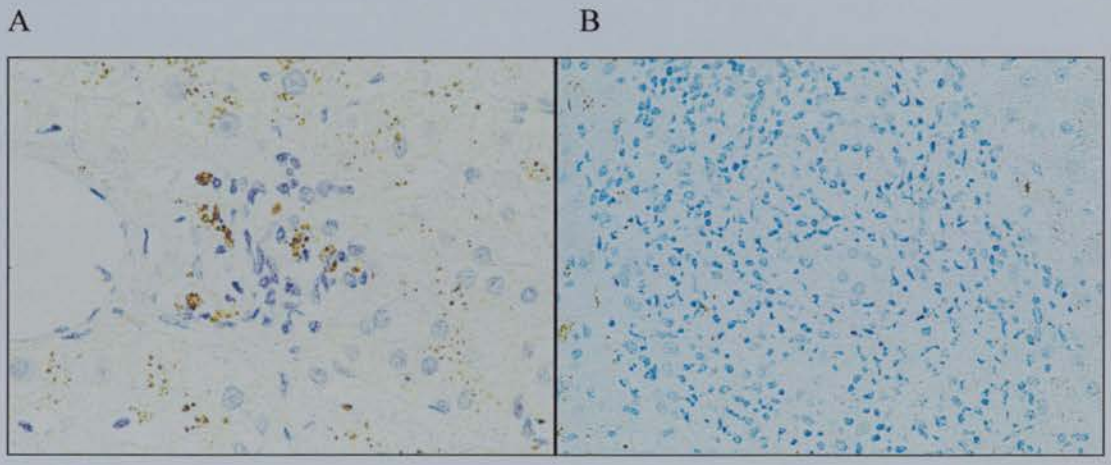


Figure 4.4: CD95 expression by ML post-transplant

Expression of CD95 by periportal ML following transplantation: a) up-regulated expression in the group without rejection; b) low expression during acute rejection (brown staining: CD95; blue staining: haematoxylin).

	CD95 Hepatocytes	
	low	**high**
normal (n = 15)	15	0
no rejection (n = 10)	10	0
acute rejection (n = 10)	0	10
post treatment (n = 10)	1	9
chronic rejection (n = 10)	0	10

Table 4.1: Parenchymal cell expression of CD95 in biopsies post-transplant

Semi-quantitative assessment of the parenchymal cell expression of CD95 in biopsies before and after orthotopic liver transplantation (normal controls: n=15; all other tissues: n=10).

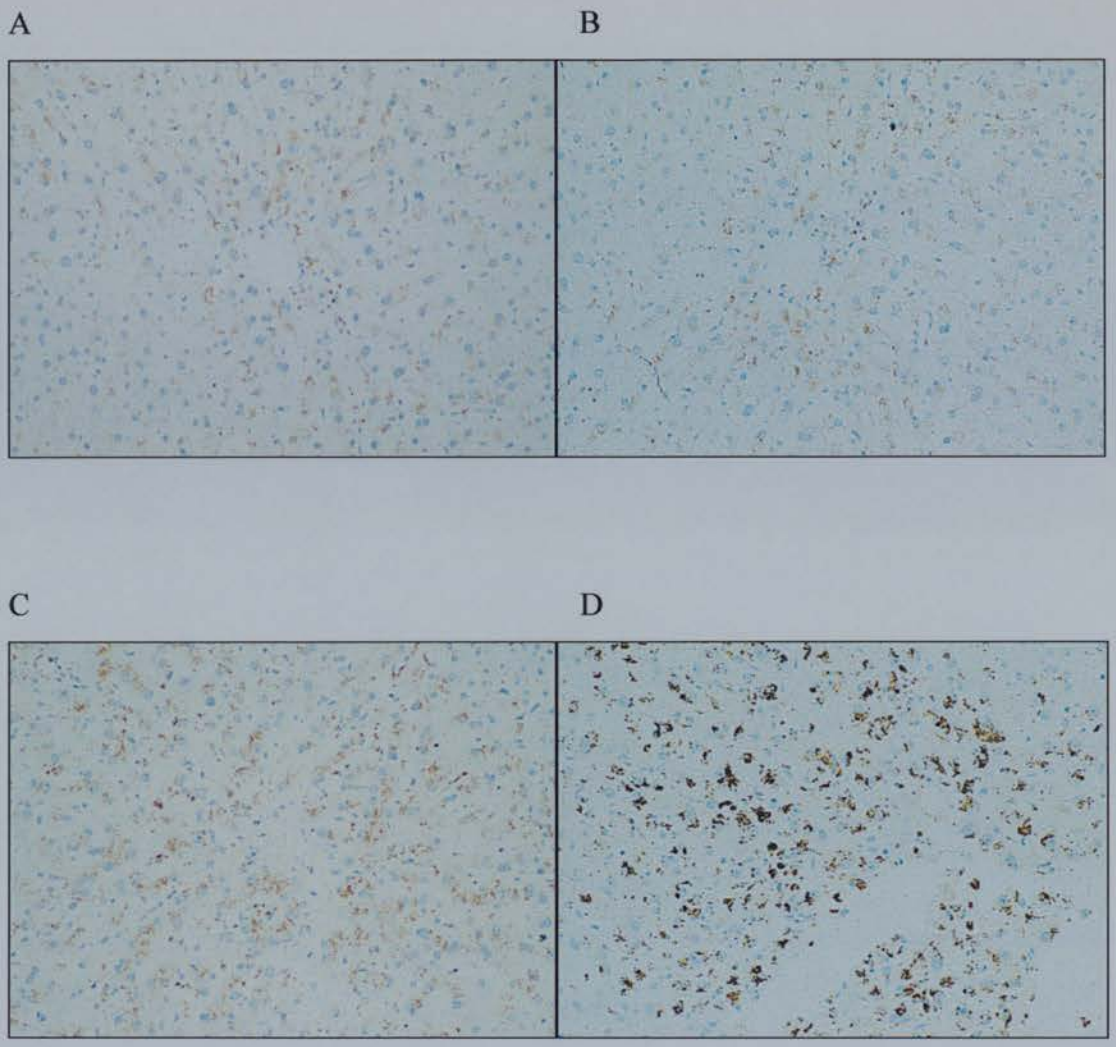


Figure 4.5: CD95 expression by hepatocytes

Expression of CD95 by perivenular hepatocytes in normal liver tissue or following transplantation: a) normal liver tissue; b) post-transplant without rejection; c) during acute rejection; d) during chronic rejection (brown staining: CD95; blue staining: haematoxylin).

4.3.3 Cell type-dependent expression of apoptotic signals post-transplant

The expression of three members of the bcl-2 family, bcl-2, bcl-x and bax was assessed together with the expression of p53. ML and biliary epithelial cells stained positive for bcl-2, while bcl-x, bax and p53 were found in biliary epithelial cells and hepatocytes.

Expression of bcl-2 in ML was assessed as previously as the percentage of positive staining cells of all ML. 28.0±10.4% of ML expressed bcl-2 in biopsies of patients without liver disease used as normal controls (Fig. 4.5). Positive cells were predominantly located within the portal tracts and occasionally within the liver parenchyma. Despite an increase in the absolute number of intra-hepatic ML following transplantation, no significant change of the percentage of cells expressing bcl-2 nor another distribution was detected in biopsies of patients with or without rejection.

Only 3/15 biopsies of patients without liver disease used as normal controls had bile ducts with epithelial cells expressing bcl-2. Following transplantation, bcl-2 expression was undetectable in bile ducts.

The expression of both, bcl-x and bax, was homogenous in biliary epithelial cells and hepatocytes and was assessed semi-quantitatively as low or high expression. Both antigens showed a low constitutive expression in hepatocytes and biliary epithelial cells in the normal control biopsies and similar following transplantation in biopsies of patients without rejection (Table 4.2). The staining was uniform with no dominant pattern.

During both acute and chronic rejection, bcl-x and bax expression was up-regulated in hepatocytes in most biopsies with a homogenous lobular distribution. Expression of both antigens in biliary epithelial cells was very strong in all biopsies during acute rejection and all biopsies with identifiable bile ducts during chronic rejection. Treatment of acute rejection with corticosteroids did not alter the staining.

The expression of p53 in hepatocytes and biliary epithelial cells was perinuclear and varied in intensity between cells within the same biopsies. It was therefore assessed semi-quantitatively as either positive or negative. In biopsies of patients without liver disease, p53 expression was undetectable (Table 4.3).

Following transplantation, p53 expression was found in some, but not all biopsies of patients without rejection (Fig. 4.9). However, all biopsies during acute and chronic rejection had p53 positive hepatocytes or biliary epithelial cells. Treatment with corticosteroids did not change the staining significantly.

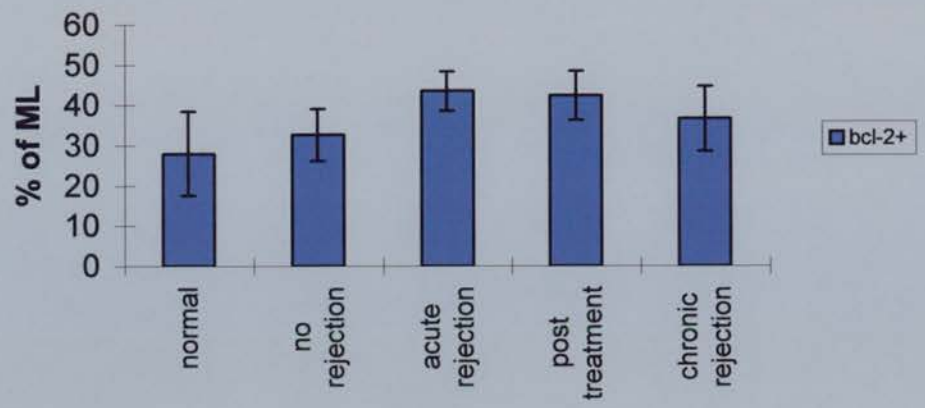


Figure 4.6: bcl-2+ mononuclear leukocytes

Expression of bcl-2 in ML in normal liver tissue and following transplantation. The number of bcl-2+ ML is expressed as mean percentage ± s.e.m. of all ML (n = 10).

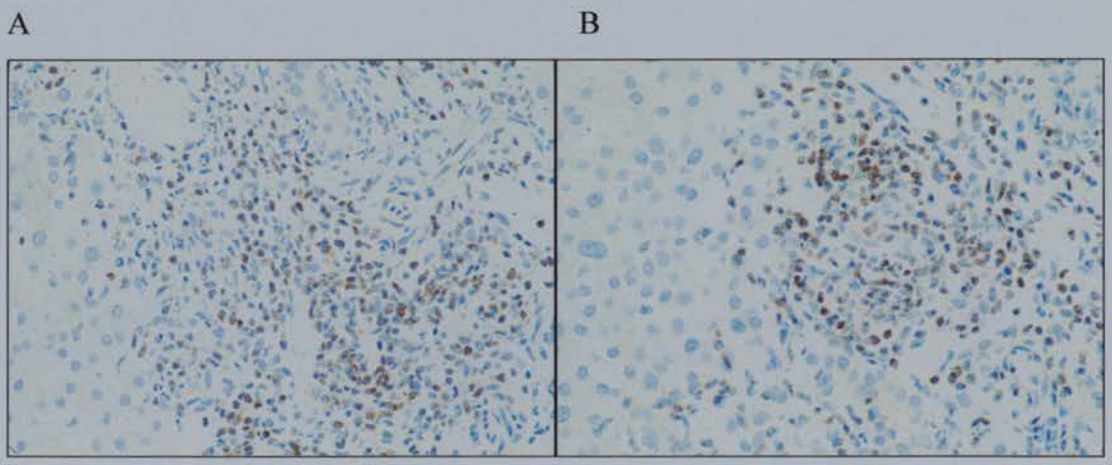


Figure 4.7: bcl-2 expression by ML post-transplant

Expression of bcl-2 by periportal ML following transplantation: a) during acute rejection; b) in the group without acute rejection (brown staining: bcl-2; blue staining: haematoxylin).

	bcl-x				bax			
	Hepatocytes		Bile ducts		Hepatocytes		Bile ducts	
	low	high	low	high	low	high	low	high
normal (n = 15)	15	0	15	0	15	0	15	0
no rejection (n = 10)	10	0	10	0	10	0	10	0
acute rejection (n = 10)	3	7	1	9	1	9	0	10
post treatment (n = 10)	2	8	2	8	3	7	2	8
chronic rejection (n = 10)	3	7	0*	6*	2	8	0*	6*

Table 4.2: Parenchymal cell expression of bcl-x and bax in liver tissue

Semi-quantitative assessment of the parenchymal cell expression of bcl-x and bax in biopsies in normal liver tissue or following transplantation (normal controls: n=15; all other tissues: n=10).

* only 6/10 biopsies of patients with chronic rejection had identifiable bile ducts

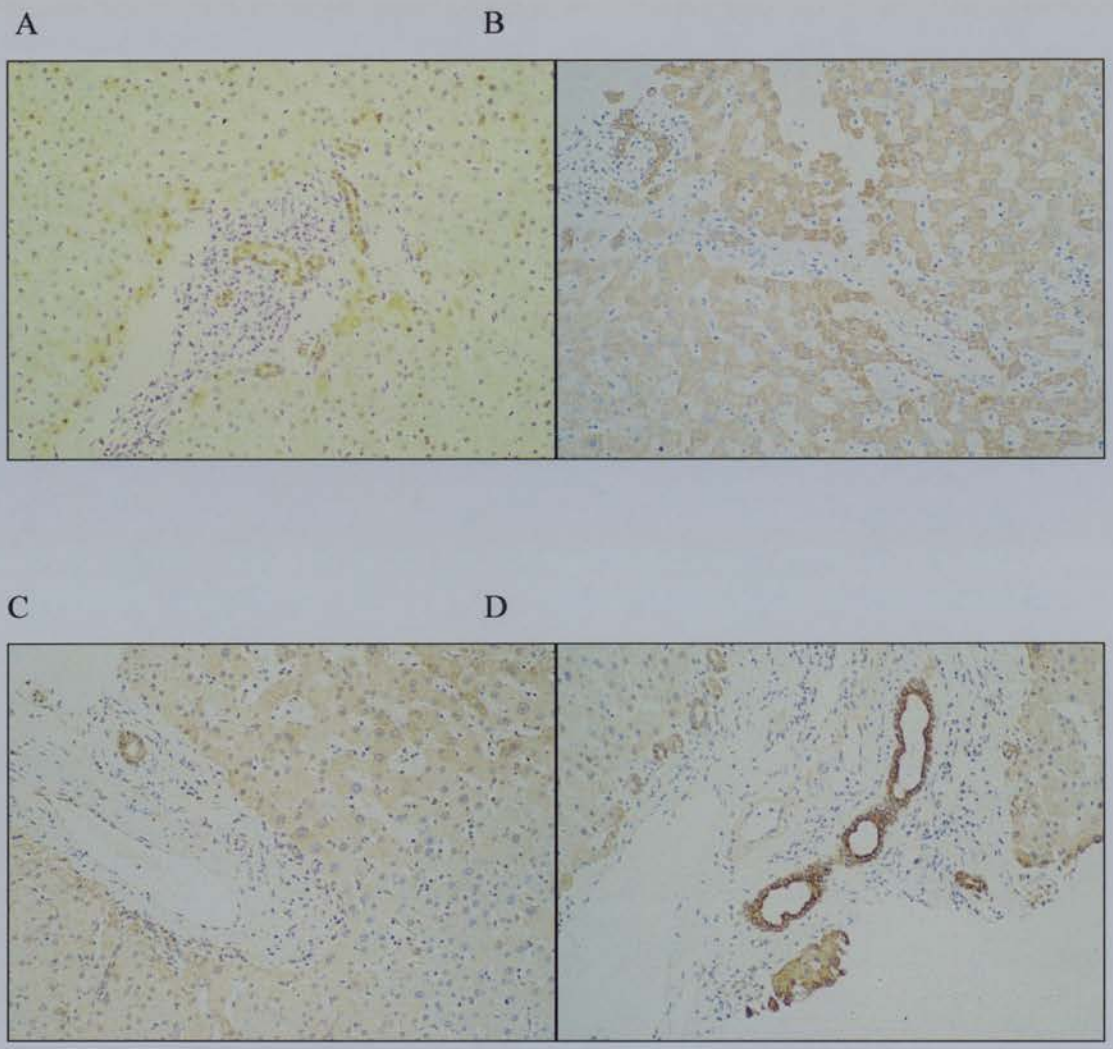


Figure 4.8: bcl-x and bax expression by hepatic parenchymal cells

Expression of bcl-x and bax by hepatocytes and biliary epithelial cells following transplantation: a) and c) expression of bcl-x and bax post-transplant without rejection; b) and d) expression of bcl-x and bax during acute rejection; (brown staining: bcl-x or bax; blue staining: haematoxylin).

	P53			
	Hepatocytes		Bile ducts	
	+	-	+	-
normal (n = 15)	0	15	0	15
no rejection (n = 10)	4	6	2	8
acute rejection (n = 10)	10	0	9	1
post treatment (n = 10)	10	0	8	2
chronic rejection (n = 10)	10	0	5*	1*

Table 4.3: Parenchymal cell expression of p53 in liver tissue

Semi-quantitative assessment of the parenchymal cell expression of p53 in biopsies in normal liver tissue or following transplantation (normal controls: n=15; all other tissues: n=10).

* only 6/10 biopsies of patients with chronic rejection had identifiable bile ducts

A B

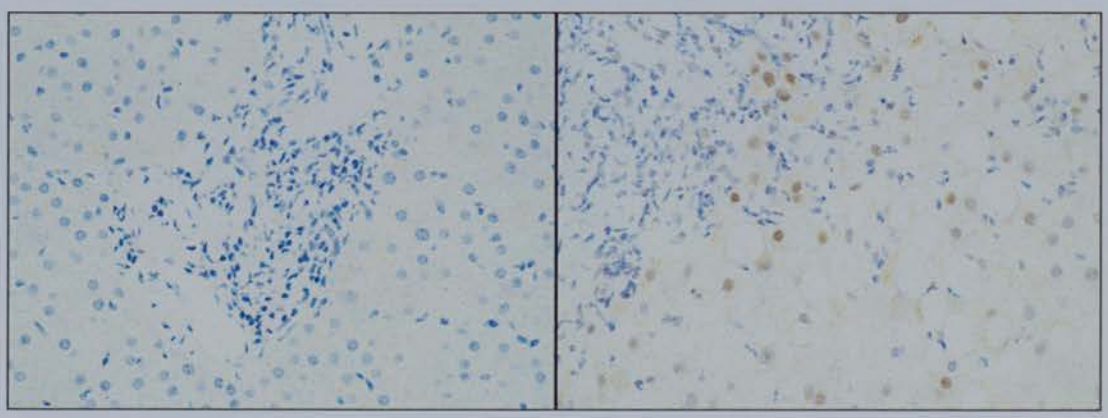


Figure 4.9: p53 expression by hepatic parenchymal cells

Expression of p53 by hepatocytes and biliary epithelial cells following transplantation: a) no expression of p53 post-transplant without significant rejection; b) expression of p53 during chronic rejection; (brown staining: p53; blue staining: haematoxylin).

4.4 Discussion

T cell activation is the prominent feature of hepatic allograft rejection and the present study implicates both major pathways of T cell-mediated cytotoxicity, perforin/granzyme B and CD95L/CD95, in the execution of the immune response. Similar to results obtained by measuring mRNA levels in a rat model (Krams, 1998a), granzyme B and CD95 expression was up-regulated during human allograft rejection. However, of all the hepatic target cells, only hepatocytes expressed CD95, indicating different susceptibilities of the hepatic target cells to the two pathways.

Granzyme B was predominantly expressed in this study by CD8+ T lymphocytes in correlation with the degree of rejection. Granzyme B activates the caspase cascade and triggers apoptosis following direct contact of immune and target cell (Liu, 1996), in the case of CTLs following engagement of the TCR and MHC class I molecules. Cells with high levels of MHC class I antigen expression such as biliary epithelial cells and vascular endothelial cells are therefore the preferential targets for CTLs (Steinhoff, 1988). However, the distribution of the granzyme B+ T cells within both, the liver parenchyma and the portal tracts, suggests their ability to target all hepatic target cells. In addition to cytotoxic T cells, NK cells are also able to kill via perforin/granzyme B, but we only found a low percentage of intra-hepatic NK cells (2-7% of ML) during allograft rejection compared with normal liver tissue (43-56% of ML) or liver tissue following viral infection (39-58% of ML) (Jonsson, 1997; Hata, 1990). NK cells have been implicated in particular in reperfusion injury (Baldwin, 2001), however their role in allograft rejection is less clear, since they can be inhibited by allogeneic MHC class I antigens via killer inhibitory receptors (Manilay, 1998).

In contrast to granzyme B, CD95Ligand has to engage its receptor CD95 on the surface of the target cell before activating caspases via its death domain FADD/Mort1 (Chinnaiyan, 1995). The present results show, that of the possible parenchymal target cells, only hepatocytes expressed the receptor molecule CD95 and were therefore receptive to this mode of cell death. Hepatocytes express CD95

constitutively (Leithaeuser, 1993), and the pattern of predominantly perivenular distribution of hepatocellular CD95 expression in the biopsies post-transplant matched recent reports on the main location of apoptotic hepatocytes during allograft rejection (Afford, 1995). In contrast, biliary epithelial cells and vascular endothelial cells did not bear detectable CD95 before or after transplantation and were therefore unlikely to be affected by this cytotoxic pathway.

ML were also found to express the receptor CD95 in this study, both before and after transplantation. CD95-mediated apoptosis is thought to play a major role in the self-regulation of immune responses via activation-induced cell death (Alderson, 1995) and apoptosis of donor-specific, graft-infiltrating T lymphocytes has been hypothesised as a possible mechanism of liver allograft acceptance (Qian, 1997). The present results suggest, that CD95-induced cell death might be responsible for the apoptosis of donor-reactive ML following transplantation, in particular in patients without rejection. The high level of CD95 expression in the periportal infiltrate in biopsies of these patients could be an indication for down-regulation of at least the early inflammatory infiltrate (Sharland, 1998). Since local expansion of the T lymphocytes was the prominent feature of acute allograft rejection as described in chapter three, it could be speculated, that alloreactive T cells without sufficient intra-hepatic activation become prone to CD95-mediated apoptosis. The cell death could then be induced either by fratricide in response to CD95L-expressing, neighbouring T cells (Piazza, 1997) or in response to CD95L-expressing sinusoidal endothelial cells or hepatocytes (Mueschen, 1998).

One previous study suggested that the down-regulation of the anti-apoptotic protein bcl-2 following transplantation might be implicated in the susceptibility of biliary epithelial cells to apoptosis (Gapany, 1997). Indeed, low levels of bcl-2 expression was found in bile ducts of normal controls in this study, but no expression post-transplant. In contrast, many ML expressed bcl-2, which has been described to prevent apoptosis induced by signals such as CD95L and glucocorticoids (Itoh, 1993; Montague, 1995). Although this suggests a regulatory function of bcl-2 on the survival of lymphocytes within the allograft, significant differences between the

relative number of bcl-2 positive cells in rejecting or non-rejecting grafts were not demonstrated.

In contrast to bcl-2, the other two members of the same family, bcl-x and bax, were found to be constitutively expressed by hepatocytes and biliary epithelial cells confirming previous studies (Krajewski, 1994 a and b). During allograft rejection, both proteins were up-regulated comparable to other liver diseases with bile duct damage such as primary biliary cirrhosis (Graham, 1998). While bax antagonises bcl-2 and promotes apoptosis, the gene for bcl-x encodes two proteins with opposite functions, bcl-x_L preventing and bcl-x_S favouring cell death (Hale, 1996). The findings in this study implicate, that during rejection both proteins are part of the triggered apoptotic sequence within hepatic parenchymal cells and that members of the bcl-2 family might play an important role in the intracellular regulation of external death signals to liver parenchymal cells. This appears also to be the function of p53, which was expressed by hepatocytes and biliary epithelial cells only after transplantation. p53 has been shown to induce CD95 as well as bax expression in hepatocytes, and recently, p53 expression was demonstrated in inflammatory liver diseases in correlation with hepatocyte destruction (Akyol, 1999).

In conclusion, the data in this study implicate both pathways of T cell-mediated cytotoxicity into hepatic allograft rejection. Perforin/granzyme B appears to be involved in killing the primary targets, biliary epithelial cells and vascular endothelial cells. Hepatocytes in contrast could be susceptible to both cytotoxic pathways, but seem particular sensitive to CD95L/CD95 during rejection with up-regulation of the CD95 receptor expression. Together with intra-cellular signals such as bcl-2, CD95-mediated apoptosis appears also to be important in the intra-hepatic regulation of the immune response. Elimination of graft-infiltrating cells might be the first step to acceptance of the liver allograft despite continuing generation of donor-reactive T lymphocytes in the lymphatic tissue (Damen, 1994).

Chapter Five – Amplification of CD95-mediated apoptosis by interferon-γ in primary murine hepatocytes

5.1 Summary

Background: During liver allograft rejection, hepatocytes appear to be in particular receptive to CD95-mediated apoptosis as indicated in chapter four. However, CD95 expression on hepatocytes was found in biopsies of patients with or without rejection, indicating additional regulatory mechanisms. The chemotherapeutic agent bleomycin amplifies CD95-induced hepatocyte cell death by induction of the tumour-suppressor gene p53. The pro-inflammatory cytokine IFN-γ, which also induces p53 expression in hepatocytes, might therefore have a similar effect.

Aims: Murine hepatocytes were isolated to assess:

1. the effect of IFN-γ on CD95-mediated apoptosis with bleomycin as control
2. the role of p53 in this process by using p53-/- hepatocytes
3. up-regulation of the extracellular apoptotic signals CD95 and CD95L
4. intracellular amplification of the apoptotic signal via mitochondrial depolarisation using the specific inhibitor cyclosporin A (CsA)

Methods: Primary hepatocytes were isolated from wild-type and p53 -/- mice and cultured under serum-free conditions. Apoptosis was induced using functional antibodies against CD95 in the presence or absence of IFN-γ, bleomycin and CsA. Cell death was assessed by MTT-test and morphology, cell surface expression of CD95 and CD95L by flow cytometry. The functional role of CD95L expression was examined by blocking antibodies against CD95L.

Results: IFN-γ amplified CD95-induced apoptosis by activation of p53, in contrast to constitutive CD95 function and surface expression, which was p53-independent. This amplification of CD95 function induced by IFN-γ did not require increased CD95 or CD95L surface expression, but was sensitive to CsA, an inhibitor of the mitochondrial permeability transition.

Conclusions: In primary hepatocytes, p53 appears to play an active part in the apoptotic cascade during inflammation by amplifying CD95-mediated cell death in

response to interferon- γ. This implicates the pro-inflammatory cytokine IFN-γ as one of the regulatory mechanisms which increases the susceptibility of hepatocytes to apoptosis during liver allograft rejection. CsA in contrast might have a protective effect on hepatocytes at low concentrations by inhibiting the mitochondrial branch of the apoptotic cascade.

5.2 Introduction

During liver allograft rejection, CD95-mediated apoptosis appears to be one of the two major cytotoxic pathways of recipient T cells in damaging donor target cells (Krams, 1998a). As shown in chapter four of this thesis, only hepatocytes expressed detectable levels of the CD95 receptor following transplantation, indicating their particular sensitivity to this mode of cell death. However, hepatocytes are known to express CD95 constitutively (Leithaeuser, 1993), and positive CD95 staining of the parenchymal cells was found in biopsies of patients with or without rejection. Since CD8+ cytotoxic T lymphocytes were present under both conditions, additional regulatory mechanisms might be required to increase the susceptibility of hepatocytes to undergo CD95-triggered apoptosis.

One of the major changes in the hepatic microenvironment during allograft rejection is the release of inflammatory cytokines such as IFN-γ from infiltrating leukocytes (Ring, 1999). Recently, IFN-γ has been demonstrated to sensitise various epithelial cells to CD95-mediated cytotoxicity (Tillman, 1998; Matsue, 1995) and might therefore have the same effect on hepatocytes. Indeed, in animal models of IFN-γ-mediated hepatitis, apoptosis via activation of CD95 is thought to be the predominant mode of cell death (Okamoto, 1999). The mechanism, by which IFN-γ amplifies CD95-mediated signalling has not been clarified. In primary hepatocytes however, IFN-γ can induce the tumour-suppressor gene p53 (Kano, 1997), which is known to enhance CD95-mediated apoptosis, either by increased cell surface trafficking of the CD95-receptor as demonstrated in a transgenic model (Bennett, 1998) or activation of the CD95 gene and *de novo* synthesis in response to chemotherapeutic drugs such as bleomycin (Mueller, 1998).

However, the sensitivity of hepatocytes to CD95-mediated apoptosis post-transplant might not only change as a result of the immune response, but also as a result of the immunosuppressive treatment. Recently, CsA has been reported to up-regulate surface CD95 expression in primary hepatocytes, implying increased susceptibility to CD95-mediated cell death (Yokoyama, 1997). In contrast, CsA is also commonly

used as an inhibitor of the mitochondrial branch of the apoptotic cascade due to its blocking effect on calcineurin activation and Ca^{2+}-dependent signalling (Bradham, 1998). In addition, CsA prevents CD95L up-regulation through transcriptional inhibition of the CD95L promoter-enhancer region (Mittelstadt, 1998), and both effects would rather reduce hepatocyte apoptosis post-transplant.

The purpose of this study was to investigate the potential of the pro-inflammatory cytokine IFN-γ and the immunosuppressive drug cyclosporin A to modulate CD95-induced apoptosis in primary murine hepatocytes. In order to assess the role of p53 in activating CD95, hepatocytes from wild-type and p53-deficient mice were used and the results compared with the p53-dependent effect of the chemotherapeutic drug bleomycin. Two time points were chosen for the analysis, 24 hours, when most of the hepatocytes retain the original G_0 status, and 48 hours, when hepatocytes are released from G_0, p53 becomes functional and hepatocytes sensitive to regulatory cytokines (Bellamy, 1997).

5.3 Results

5.3.1 IFN-γ amplifies CD95-mediated apoptosis in primary hepatocytes

The effect of IFN-γ and CsA on CD95-mediated apoptosis was studied using serum-free cultures of primary murine hepatocytes treated with anti-CD95 antibodies (200 ng/ml). Stimulation of the CD95 receptor with antibodies alone resulted in rapid appearance of apoptotic hepatocytes within 4 to 6 hours. Quantification of viable cells by MTT staining demonstrated a 20% reduction of staining after 24 hours and of 30% after 48 hours compared with hepatocyte cultures without the antibody (Fig. 5.1). Combined treatment of hepatocytes with IFN-γ (100 U/ml) and antibodies led to a further reduction in staining to 40% after 24 hours and 55% after 48 hours (Fig. 5.2), although IFN-γ alone caused only a minor reduction of the MTT staining indicating a sensitising rather than additive effect of INF-γ ($p<0.01$ and $p<0.05$, two-way ANOVA model with fixed effects). A similar sensitising effect was observed in hepatocytes treated with a combination of bleomycin (15 mU/ml) and anti-CD95 antibodies (Fig. 5.3) with a reduction of the MTT staining by 35% after 24 hours and 65% after 48 hours ($p<0.01$ and $p<0.05$, two-way ANOVA model with fixed effects). CsA (0.015 μg/ml and 15 μg/ml) in contrast caused itself a dose-dependent reduction of the staining, but did not sensitise hepatocytes to the CD95 antibodies as indicated by an additive effect of the combined treatment with CsA and anti-CD95 antibodies (Fig. 5.4).

To verify, that results obtained by the MTT assay were secondary to increased cell death rather than growth arrest, all experiments were repeated with acridine orange staining of cellular DNA for assessment of nuclear morphology. Although bleomycin and IFN-γ alone caused a reduction of positive staining in the MTT assay, this was not reflected by an increased number of apoptotic cells implying growth arrest as cause for the effect (Table 5.1). CsA had dose-dependently an even more pronounced effect on the MTT staining, but again failed to up-regulate the apoptotic count significantly ($p<0.1$). In contrast, combined treatment with anti-CD95 antibodies led

in all experiments to a significant increase of apoptotic hepatocytes (Table 5.1) with nuclear condensation and fragmentation as morphological signs.

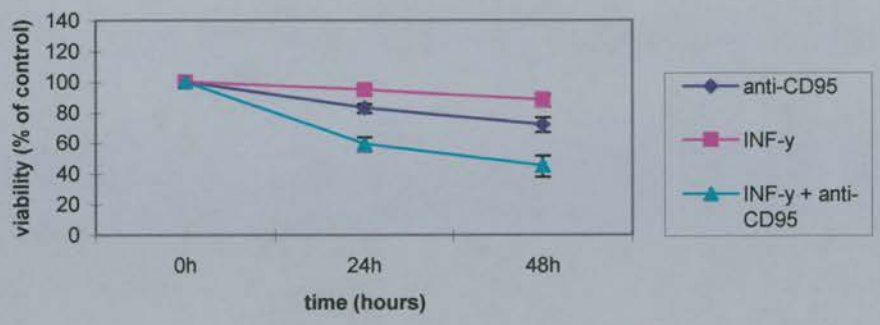
5.3.2 p53 is required for amplification of CD95-mediated apoptosis by IFN-γ

To test if the amplification of CD95-mediated cell death depended on functional p53 in primary hepatocytes, we repeated the MTT assay with anti-CD95 antibodies (200 ng/ml, ED_{50}) in hepatocyte cultures obtained from p53-deficient mice (homozygous p53-/-). Compared with untreated cultures of p53-deficient hepatocytes, CD95 stimulation alone resulted again in a 20% reduction of staining within the first 24 hours, equivalent to results obtained in wild-type hepatocytes and indicating the constitutive CD95 function to be intact. In contrast, no further reduction of the staining was observed after 48 hours (Fig. 5.1). Moreover, both IFN-γ and bleomycin lost the sensitising effect for anti-CD95 antibodies thereby indicating an abrogation of the increased susceptibility of hepatocytes to CD95-mediated cell death (Fig 5.2 and 5.3). The dose-dependent reduction of the MTT staining by CsA was also diminished, but showed no change in the additive effect with anti-CD95 antibodies (Fig. 5.4).

Figure 5.1

Reduction of MTT staining of viable cells over 48 hours induced by anti-CD95 antibodies in wt (♦) and p53-deficient (■) hepatocytes. Data (mean ± s.e.m.) are expressed as fraction of viable cells in culture without treatment (normal control) set arbitrarily at 100%, and represent 3 different experiments repeated on eight independent wells from at least three different mice.

wt



p53 -/-

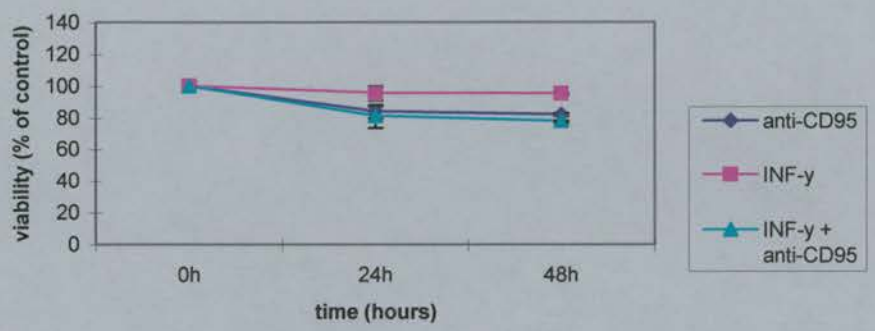
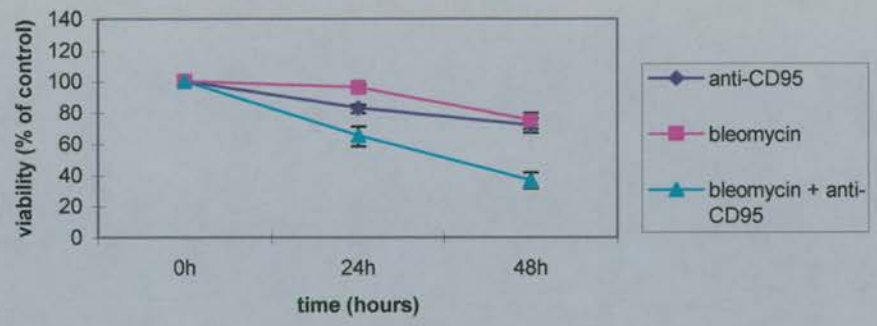


Figure 5.2

IFN-γ (100 U/ml) sensitises wt but not p53-/- hepatocytes to the effect of anti-CD95 antibodies (200 ng/ml) on the MTT staining of viable cells. Hepatocytes were treated with anti-CD95 antibodies (♦), IFN-γ (100 U/ml) (■) or a combination of both (▲) for 24 and 48 hours. Data (mean ± s.e.m.) are expressed as fraction of viable cells in culture without treatment (normal control) set arbitrarily at 100%, and represent 3 different experiments repeated on eight independent wells from at least three different mice. A balanced two-way ANOVA (model with fixed effects) was used to test for an interaction between the two treatments comparing the combined effect to an additive effect as the null-hypothesis (Altman, 1991). $P < 0.05$ was regarded as statistically significant.

wt



p53 -/-

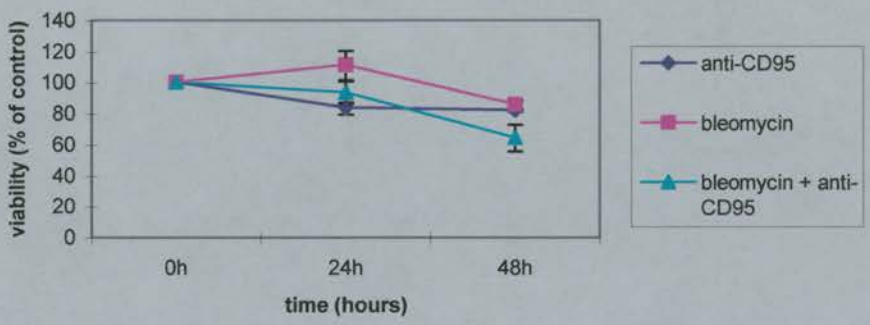
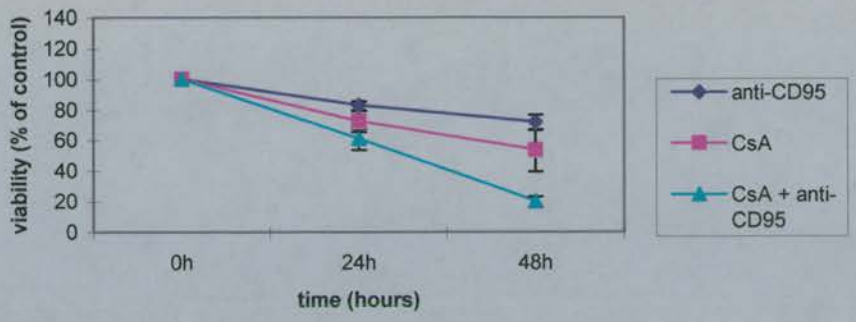


Figure 5.3

Bleomycin (15 mU/ml) sensitises wt but not p53-/- hepatocytes to the effect of anti-CD95 antibodies (200 ng/ml) on the MTT staining of viable cells. Hepatocytes were treated with anti-CD95 antibodies (♦), each respective treatment (■) or a combination of both (▲) for 24 and 48 hours. Data (mean ± s.e.m.) are expressed as fraction of viable cells in culture without treatment (normal control) set arbitrarily at 100%, and represent 3 different experiments repeated on eight independent wells from at least three different mice. A balanced two-way ANOVA (model with fixed effects) was used to test for an interaction between the two treatments comparing the combined effect to an additive effect as the null-hypothesis (Altman, 1991). $P < 0.05$ was regarded as statistically significant.

wt



p53 -/-

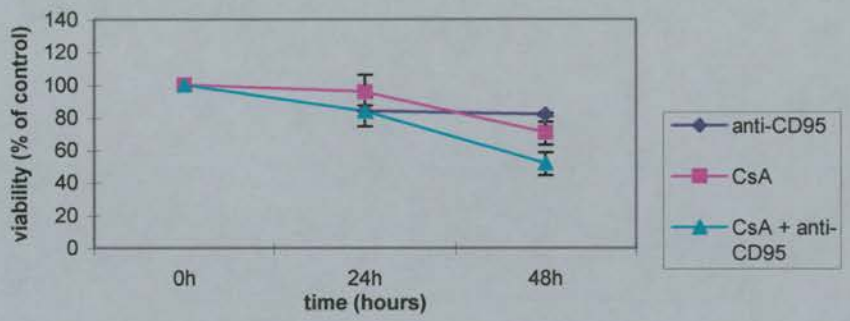


Figure 5.4

Additive effect of cyclosporin A (15 μg/ml) and anti-CD95 antibodies (200 ng/ml) in wt and p53-/- hepatocytes as measured by MTT staining of viable cells. Hepatocytes were treated with anti-CD95 antibodies (♦), each respective treatment (■) or a combination of both (▲) for 24 and 48 hours. Data (mean ± s.e.m.) are expressed as fraction of viable cells in culture without treatment (normal control) set arbitrarily at 100%, and represent 3 different experiments repeated on eight independent wells from at least three different mice. A balanced two-way ANOVA (model with fixed effects) was used to test for an interaction between the two treatments comparing the combined effect to an additive effect as the null-hypothesis (Altman, 1991). $P < 0.05$ was regarded as statistically significant.

	No treatment	*IFN-γ (100 U/ml)*	*Bleomycin (15 mU/ml)*	*CsA (15 μg/ml)*
no treatment	5.5±0.5	7.6±1.1	5.6±0.5	12.8±3.75
anti-CD95 (200U/ml)	18.2±0.2	28.8±0.26	38.5±0.5	60.0±12.2
significance	$p < 0.05$	$p < 0.05$	$p < 0.05$	$p < 0.05$

Table 5.1

Morphological analysis of apoptosis in hepatocyte cultures using nuclear staining with acridine orange after 24 hours. For each experiment, at least 200 cells were counted on a standard morphometric grid. Results are expressed as the mean percentage ± s.e.m. of apoptotic cells of all hepatocytes and represent data from three experiments on hepatocytes from at least three different mice. Statistical differences were estimated using a Student's t-test.

5.3.3 CD95 surface expression is up-regulated by CsA, but not IFN-γ

One explanation for increased susceptibility of cells to CD95-mediated cell death is a higher CD95 receptor surface expression allowing more antibodies to trigger the apoptotic cascade. We therefore assessed the expression of CD95 on hepatocytes following treatment with IFN-γ, bleomycin and CsA using flow cytometry. For statistical purposes, results were calculated as the ratio of the mean fluorescence intensity of the experimental samples to the mean fluorescence intensity of the isotype controls. Both, wild-type and p53-deficient hepatocytes, displayed immediately post-isolation a weak constitutive CD95 expression (mean fluorescence intensity ratio 1.8±0.06 and 1.7±0.1, respectively) (Fig 5.5a). However, after 24 hours of serum-free cell culture, wild-type, but not p53-deficient hepatocytes showed a significantly increased receptor surface expression (mean fluorescence intensity ratio 2.9±0.2 vs. 1.8±0.06, $p<0.05$). Treatment with bleomycin further enhanced this expression in wild-type hepatocytes (mean fluorescence intensity ratio 3.7±0.1 vs. 2.9±0.2, $p<0.01$), but again had no significant effect in p53-deficient cells (Fig. 5.5b). A similar effect was seen following treatment with CsA (Fig 5.5c), although significance was only reached with higher concentrations (CsA 15 μg/ml: mean fluorescence intensity ratio 3.9±0.4 vs. 2.9±0.2, $p<0.05$). In contrast to the two drugs, IFN-γ had no influence on CD95 surface expression of the hepatocytes indicating that its effect on cytotoxicity was not mediated through increased receptor expression (Fig. 5.5d). The flow cytometry was again repeated at 48 hours but no further changes in CD95 expression were noted for any of the different treatments.

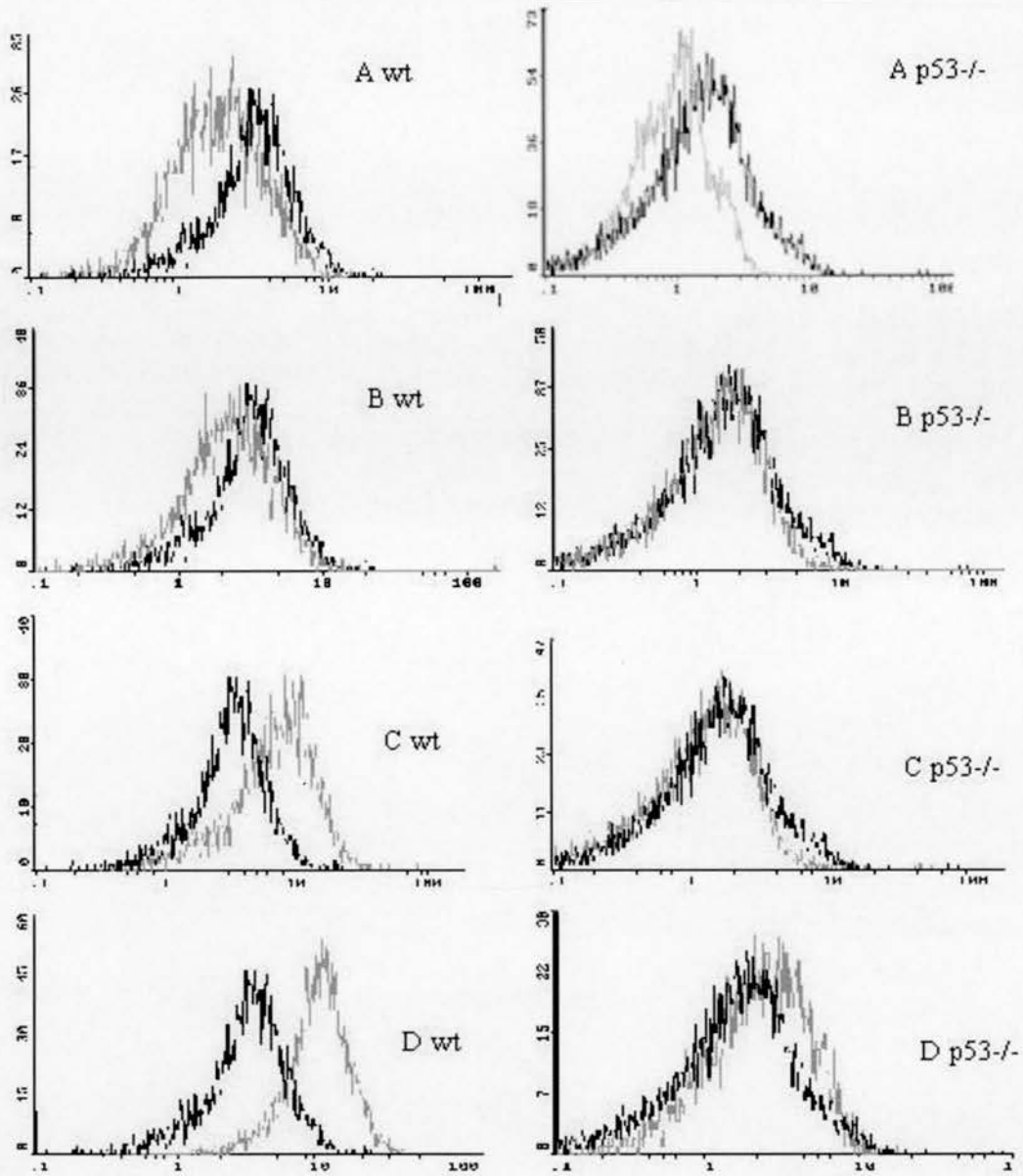


Figure 5.5

CD95 receptor surface expression of wt and p53-/- hepatocytes as analysed by flow cytometry. (A) Constitutive CD95 expression post-isolation (black: CD95; grey: isotype control). (B) CD95 expression after 24 hour treatment with IFN-γ (100 U/ml) (black: constitutive expression; grey: IFN-γ). (C) CD95 expression after 24 hour treatment with bleomycin (15 mU/ml) (black: constitutive expression; grey: bleomycin). (D) CD95 expression after 24 hour treatment with CsA (15 μg/ml) (black: constitutive expression; grey: CsA). Data were acquired from at least 10000 events and representative stainings from at least three different mice are shown.

5.3.4 CD95L expression is delayed by CsA, but not induced by IFN-γ

To exclude increased expression of CD95L on wild-type hepatocytes as a cause for the amplification of CD95-mediated apoptosis, cell surface protein expression of CD95L was assessed by flow cytometry. For statistical analysis, results were again calculated as the ratio of the mean fluorescence intensity of the experimental samples to the mean fluorescence intensity of the isotype controls. Immediately post-isolation, hepatocytes did not express CD95L on their cell surface, but demonstrated induction of CD95L expression after 24 and 48 hours of serum-free culture (mean fluorescence intensity ratio 2.78±0.53 and 2.86±1.08 vs. 1.3±0.4, $p<0.05$) (Fig. 5.6a). Although treatment with bleomycin tended to enhance CD95L expression further, this failed to reach significance ($p<0.2$). In contrast, CsA delayed dose-dependently the onset of CD95L up-regulation for the first 24 hours compared with serum-free culture (mean fluorescence intensity ratio 1.22±0.07 vs. 2.78±0.53, $p<0.05$) (Fig. 5.6b), but this effect was only temporary with equivalent levels of CD95L expression at 48 hours. IFN-γ did not affect the ligand surface expression (Fig. 5.6c), indicating that the amplification of CD95-mediated apoptosis was not caused by increased CD95L expression on hepatocytes. This was also corroborated by functional assessment of CD95L in this model with a MTT-test, which proved that addition of anti-CD95L antibodies in concentrations up to 1 μg/ml had no significant protective effect on hepatocyte survival (data not shown).

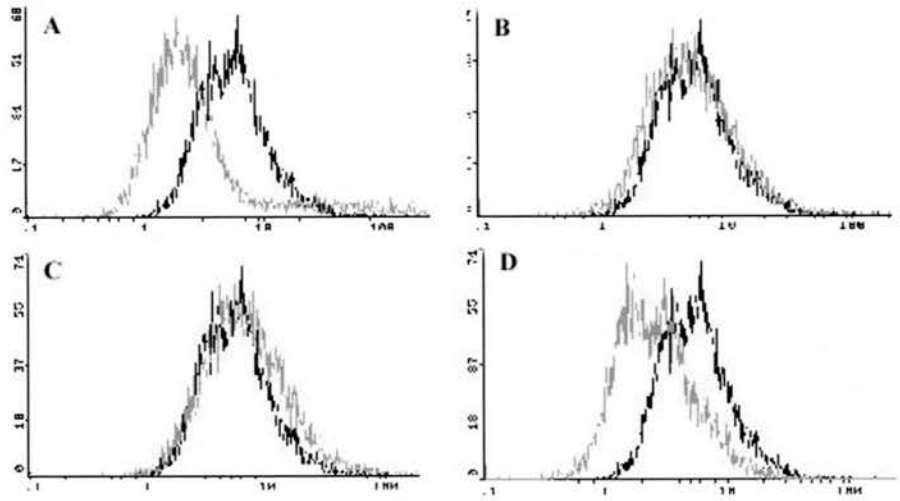


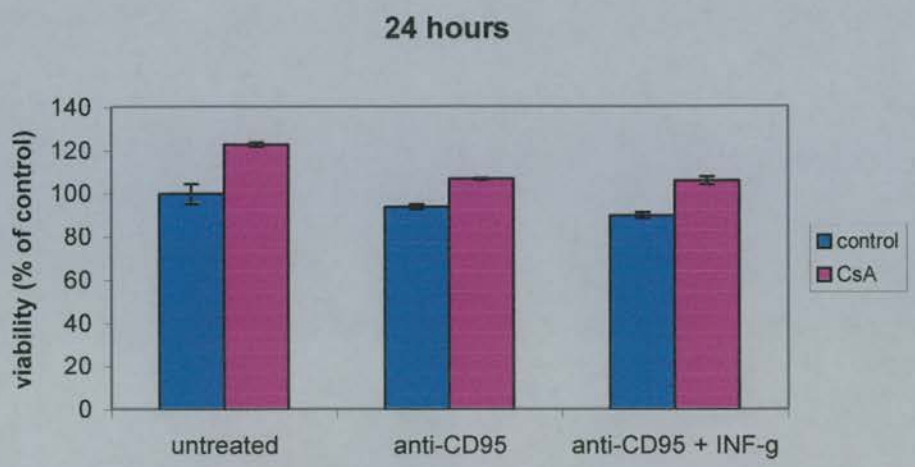
Figure 5.6

CD95 ligand surface expression of wt and p53-/- hepatocytes as analysed by flow cytometry. (A) CD95L expression after 24 hour cell culture (no treatment) (black: CD95L; grey: isotype control). (B) CD95L expression after 24 hour treatment with IFN-γ (100 U/ml) (black: no treatment; grey: IFN-γ). (C) CD95L expression after 24 hour treatment with bleomycin (15 mU/ml) (black: no treatment; grey: bleomycin). (D) CD95L expression after 24 hour treatment with CsA (15 μg/ml) (black: no treatment; grey: CsA. Data were acquired from at least 10000 events and representative stainings from at least three different mice are shown.

5.3.5 Amplification of CD95-mediated apoptosis is sensitive to CsA

These results demonstrated that IFN-γ amplified CD95-mediated hepatotoxicity via induction of p53, but without increasing CD95 or CD95L surface expression, indicating an intracellular mechanism. Recently, CD95 had been demonstrated to signal through two pathways, one dependent, the other independent of mitochondrial depolarisation (Scaffidi, 1998). CsA is a known inhibitor of the mitochondrial permeability transition (Bradham, 1998) and in this study up-regulated CD95 surface expression without amplifying CD95-mediated cell death. To assess, if the effect of IFN-γ on CD95 function was dependent on mitochondrial depolarisation, various concentrations of CsA were tried, and 1 μg/ml was found to have the optimal hepato-protective effect. As previously, hepatocytes were incubated for 48h with anti-CD95 antibodies (200 ng/ml) and IFN-γ (100 U/ml), this time in the presence or absence of CsA. Using the MTT-test, CsA (1 μg/ml) abrogated the reduction in staining caused by anti-CD95 antibodies alone within the first 24 hours (Fig. 5.7a). However, this effect lost its significance after 48 hours (Fig. 5.7b), demonstrating only a transient blocking of CD95 signalling. In contrast, the amplification of CD95-mediated cell death caused by IFN-γ was abrogated both after 24 and 48 hours with no significant difference between samples treated with or without IFN-γ (Fig. 5.7a and b). This complete block indicated that the p53-dependent effect of IFN-γ on CD95 function was dependent on mitochondrial depolarisation and cytochrome c release.

A



B

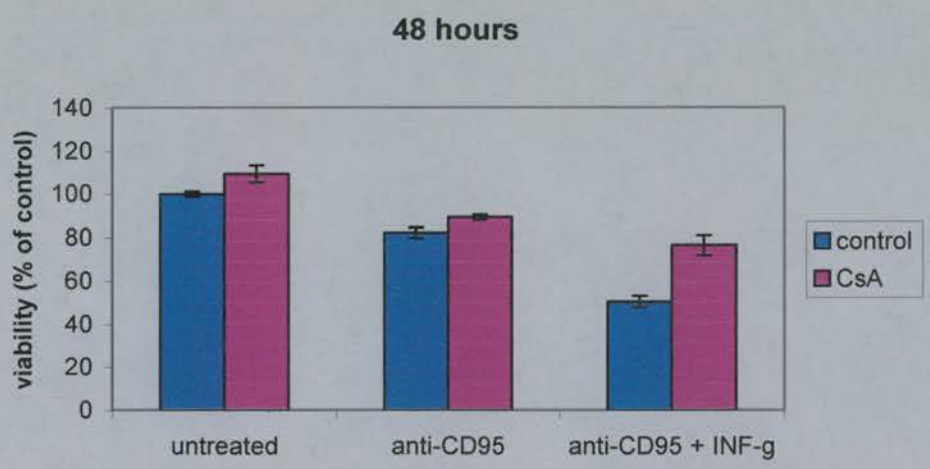


Figure 5.7

Reduction of MTT staining of viable cells induced by anti-CD95 antibodies (200 ng/ml) alone or in combination with IFN-γ (100 U/ml) in the presence or absence of CsA (1 μg/ml). Data (mean ± s.e.m.) are expressed as fraction of viable cells in culture without treatment (normal control) set arbitrarily at 100%, and represent 3 different experiments repeated on eight independent wells from at least three different mice. A Student's t-test was used to define statistical differences and $p<0.05$ was regarded as significant.

5.4 Discussion

Although CD95-mediated apoptosis has been recognised as a key mechanism in hepatocyte death (Krammer, 1999), studies in murine models with agonistic CD95-antibodies revealed a discrepancy between the ready susceptibility of hepatocytes to undergo apoptosis *in vivo* (Ogasawara, 1993) compared with the resistance of hepatocytes *in vitro* requiring additional stimuli such as protein synthesis- or protein kinase-inhibitors to amplify their sensitivity to CD95 (Ni, 1994; Rouquet, 1995). Recently, p53-responsive elements within the CD95 gene were found during studies demonstrating activation of CD95 by p53 in response to DNA damaging chemotherapeutic drugs such as bleomycin (Mueller, 1998). The data in this study provides evidence that p53 might also regulate the induction of CD95-mediated cell death by the inflammatory cytokine IFN-γ, however in contrast to chemotherapeutic drugs without affecting CD95 surface expression.

Wild-type and p53-deficient murine hepatocytes were found to express CD95 constitutively, and in both, CD95 was functional with induction of apoptosis in about 20% of cells 24 hours after stimulation with agonistic CD95-antibodies. The results are comparable with previous studies (Ni, 1994) and demonstrated constitutive CD95 expression and function to be p53-independent (Unger, 1998). In contrast, wild-type, but not p53-deficient hepatocytes continued to undergo apoptosis for another 24 hours, when cultured in serum-free conditions, and this coincided with increased CD95 surface expression. As described for hepatoma cell lines (Mueller, 1997), bleomycin further amplified the CD95 function and expression in wild-type hepatocytes, but failed to do so in p53-deficient hepatocytes. Both observations are compatible with p53 activation in response to oxidative stress and DNA-damage caused by hypoxia during cell isolation (Yu, 1999) or serum-deprivation (Hassan, 1999), which is enhanced by bleomycin. Subsequently, p53-dependent induction of CD95 function might be mediated through receptor up-regulation secondary to increased cell surface trafficking of pre-formed protein (Bennett, 1998) or gene activation and *de novo* synthesis (Mueller, 1998).

Interferon-γ (IFN-γ) however proved also to amplify the effect of CD95-antibodies dependent on functional p53, although without up-regulation of the CD95 receptor surface expression. Augmentation of CD95-dependent apoptosis by IFN-γ has been reported in several different cell types, either with (Matsue, 1995) or without (Owen-Schaub, 1994) increase of receptor surface expression. In these studies, IFN-γ was further shown to cause induction of pro-apoptotic signals such as caspases 1, 3 and 8 or bak and bcl-x_S and down-regulation of anti-apoptotic signals like bcl-2, all intracellular components of the apoptotic cascade (Pammer, 1999; Takahashi, 1999; Ugurel, 1999). p53 was not examined during these investigations, but IFN-γ has been reported to induce p53-expression in murine hepatocytes by signalling through the tumour suppressor gene interferon regulatory factor-1 (IRF-1) (Kano, 1999), which shares a transcriptional regulatory element with p53 (Lallemand, 1997). Moreover, the transcriptional function of p53 can be further enhanced by the IFN-γ-inducible protein kinase PKR (Cuddihy, 1999).

Since the effect of IFN-γ in this study appeared to be mediated through intracellular amplification of CD95-mediated cell death, possible mechanisms were investigated. p53 has been shown to regulate the expression of members of the bcl-2 family, causing in particular down-regulation of bcl-2 and induction of bax (Miyashita 1994 and 1995). Both proteins affect the MPT (Krammer, 1999), and mitochondrial depolarisation is an essential part of the signalling cascade during TNF-α-induced hepatocyte apoptosis (Bradham, 1999). However, recent studies have demonstrated two pathways for CD95-mediated cell death, one dependent, the other independent of the MPT (Scaffidi, 1998). In primary hepatocytes, CD95 appears to signal through both pathways, since inhibition of the MPT pore delays, but does not block the effect of anti-CD95 antibodies (Hatano, 2000). A potential inhibitor of the MPT pore is CsA, which had shown a dose-dependent cytotoxicity in this study (Wolf, 1997), but no induction of CD95-mediated apoptosis despite causing p53-dependent up-regulation of CD95 receptor surface expression similar to bleomycin. By using low-dose CsA in conjunction with IFN-γ and anti-CD95 antibodies, a complete abrogation was demonstrated of the IFN-γ-induced amplification of CD95-mediated

cell death. IFN-γ therefore appears to augment CD95 function by activation of p53 and induction of the signalling through mitochondrial depolarisation.

To exclude a significant contribution of CD95L surface expression on hepatocytes to the results, induction of CD95L was also assessed under the experimental conditions. Similar to CD95, CD95L expression increased over-time in serum-free culture in this study. In hepatoma cells, this increase seems to be in response to oxidative stress (Hug, 1997), which could explain this observation. In contrast to the results in tumour cells however (Hug, 1997; Bernassola, 1999), bleomycin and IFN-γ had no significant additional effect on CD95L, possibly due to lower concentrations used in the present experiments or because of the already existing induction secondary to primary culture. CsA on the other hand prevented up-regulation of CD95L compared with primary culture during the first 24 hours. This effect appears to be mediated through transcriptional inhibition of the CD95L promoter-enhancer region regulated by the CsA-sensitive NF-AT-protein family members (Mittelstadt, 1998). Additionally, there was no protection of hepatocytes against apoptosis when using functional anti-CD95L antibodies, indicating that in this experimental system autocrine or paracrine CD95L-CD95 interactions played no role and confirming that bleomycin and IFN-γ alone had only an anti-proliferative effect.

In conclusion, the results in this study demonstrate for the first time that similar to chemotherapeutic drugs, IFN-γ amplifies CD95-mediated hepatotoxicity via induction of p53. This amplification does not appear to require increased CD95 surface expression, but seems rather to be mediated through depolarisation of the mitochondrial membrane. p53 expression has been described in liver biopsies of patients with inflammatory liver diseases and was linked to the severity of inflammation and hepatocyte destruction (Akyol, 1999). Since p53 expression by hepatocytes was also a prominent feature of allograft rejection in chapter four of this thesis, p53 appears to be an active part of the apoptotic signalling during inflammation. The effect of CsA in contrast seems to be largely dose-dependent. However, CsA has recently been shown to protect mice from fulminant liver

destruction induced by anti-CD95 antibodies alone (Okamoto, 1999), and might have a similar effect during allograft rejection.

Chapter Six – Phenotype and allostimulatory function of murine hepatic, renal and splenic dendritic cells

6.1 Summary

Background: A primary immune response within the liver as indicated in chapter three requires antigen-presentation by donor APCs. DCs are particular equipped to prime naïve T cells and hepatic DCs are thought to be tolerogenic based on their immaturity. However, DCs from other solid organs are also immature, raising the question if hepatic DCs are inherently tolerogenic or react to immunomodulatory signals from their cytokine environment.

Aims: DCs were isolated from murine liver, kidney and spleen to:

1. analyse the phenotype of each DC subpopulation
2. compare the stimulatory capacity of the DC subpopulations
3. assess the effect of the immunoregulatory signals IL-10, TGF-β, dexamethasone (Dex) or CTLA-4Ig on DC function

Methods: Using a mouse model, DCs were isolated by an immuno-magnetic method with antibodies against CD11c and DEC-205. DC expression of MHC II, co-stimulatory signals and the lymphoid marker CD8α was analysed by flow cytometry. DC function, i.e. the ability to prime allogeneic naïve T cells, was assessed before and after exposure to the maturation signal GM-CSF and IL-10, TGF-β, Dex or CTLA-4Ig. Read-outs were T cell proliferation and release of IL-10 or IFN-γ as well as DC synthesis of IL-10 or IL-12 mRNA.

Results: Hepatic (60% $CD8\alpha^+$) and renal (<5% $CD8\alpha^+$) DCs differed in their lineage-related phenotype, but were both functionally and phenotypically immature. In the presence of GM-CSF, they up-regulated IL-10 and IL-12 mRNA synthesis and became as efficient as splenic DCs in priming allogeneic Th1 responses characterised by IFN-γ release. Dex, CTLA4-Ig or TGF-β inhibited the ability of all DCs to induce a Th1 response, IL-10 only the ability of mature splenic DCs. Dex, TGF-β or IL-10 abrogated DC synthesis of IL-12 mRNA, however TGF-β additionally up-regulated DC synthesis of IL-10 mRNA.

Conclusions: Functionally, murine hepatic DCs do not behave differently in allogeneic DC-T cell interactions when compared with DCs from other solid organs or lymphoid tissue. However, environmental signals can influence the DC function, suggesting that their allostimulatory capability may be modulated following transplantation, irrespective of their origin.

6.2 Introduction

The results in chapter three, the intra-hepatic proliferation of naïve T lymphocytes in patients following transplantation, indicated a primary immune response within the liver. Activation of naïve T cells requires the presence of APCs such as macrophages or DCs (Germain, 1993). In chapter three, proliferating T cells were found predominantly within the portal tracts of the liver. In contrast to macrophages, which are scattered throughout the liver parenchyma, hepatic DCs are located close to these portal tracts (Hart, 1981), leading to the hypothesis that they are involved in oral tolerance induction for antigens transported from the gut to the liver via the portal vein (Thomson, 1999). Indeed, tolerance induction by portal vein infusion of the antigen is well described (Yang, 1994) and has been used in a variety of transplant models (Kenick, 1987).

In mice, hepatic DCs display a different phenotype than DCs from other solid organs. All DCs in the liver express the multi-lectin receptor DEC-205, and about half of them CD8α, a marker of lymphoid-derived DCs (Woo, 1994; O'Connell, 2000). Both surface antigens are usually expressed by mature, interdigitating DCs within T cell areas of lymphatic tissue, which might have a regulatory function on T cell responses (Steinman, 1997). In contrast, liver-derived DCs appear to be immature following isolation, lacking co-stimulatory molecules such as CD80, CD86 and CD40 and acquire a stimulatory capacity for T cells only after appropriate activation by maturation signals such as GM-CSF (O'Connell, 2000). It has been therefore hypothesised, that hepatic DCs are tolerogenic as a result of their immaturity and inadequate stimulation of T lymphocytes (Thomson, 1999). Immaturity, however, is also characteristic for DCs from organs such as the kidney, which do not have the tolerogenic properties of the liver (Austyn, 1994). This raises the question whether each respective microenvironment determines the way DCs activate T cells or if hepatic DCs are inherently more tolerogenic or more susceptible to tolerogenic signals.

To assess this question, a method of immuno-magnetic DC separation was adapted to isolate organ-specific DCs from liver and kidney (Miltenyi, 1990). Immature, predominantly $CD8\alpha^-$ and mature, predominantly $CD8\alpha^+$ DCs from the spleen were also isolated as control populations from a lymphatic organ. The DC populations were activated with GM-CSF and exposed to different stimuli before assessing their ability to prime naïve allogeneic T cells. The tolerogenic stimuli tested were dexamethasone and CTLA4-Ig, both used as immunosuppressive agents in clinical and experimental transplantation (Denton, 1999), and the cytokines IL-10 and TGF-β, which have been found within the hepatic microenvironment (Ishizaka, 1996; Tox, 2001; Narumoto, 2000).

6.3 Results

6.3.1 Heterogeneity of hepatic and renal DCs

As a consequence of the low tissue numbers of DCs in organs, a positive selection method via immuno-magnetic cell sorting was used to freshly isolate DCs from the non-parenchymal cell fraction (NPC) of each organ. Hepatic DCs were isolated using the rat antibody NLDC-145 against DEC-205, which returned reliable yields of 1.1 x 10^6 ± 0.1 cells per liver (n = 15). Phenotypic analysis by flow cytometry showed an intermediate cell surface expression of MHC class II and CD86 with a low expression of CD80 and CD40. Around 60% of cells were positive for CD8α (Fig. 1). Renal DCs in contrast were sorted using the N418 antibody against CD11c resulting in yields of 0.4 x 10^6 ± 0.1 cells per kidney (n = 15). Selection with the anti-DEC-205 antibody returned only yields of < 0.5 x 10^5, below the sensitivity of the method. Similar to hepatic DCs, renal DCs expressed intermediate levels of MHC class II and CD86 and low levels of CD80 or CD40. However, they lacked expression of CD8α with a percentage of < 5% (Fig. 6.1).

To obtain positive controls for our experiments, the same method and antibodies were used to isolate DCs from the spleen. Sorting with the anti-CD11c antibody returned an average number of 2.1 x 10^6 ± 0.3 cells per spleen (n = 15). On phenotypic analysis, these cells expressed intermediate levels of MHC class II and CD86 with a low expression of CD80 and CD40, comparable with the expression by hepatic and renal DCs. About 20% of the DCs were positive for CD8α (Fig. 6.1). In contrast, selection using the anti-DEC-205 antibody resulted in yields of 1.3 x 10^6 ± 0.2 cells per spleen (n = 15). Flow cytometry demonstrated the cells to be highly positive for MHC class II and CD86 with an intermediate expression of CD80 and CD40. Around 70% of the DCs were positive for CD8α (Fig. 6.1).

All DC populations were also assessed for their surface expression of the death-inducing ligand CD95L, however this was not detected on any of the DC populations (data not shown).

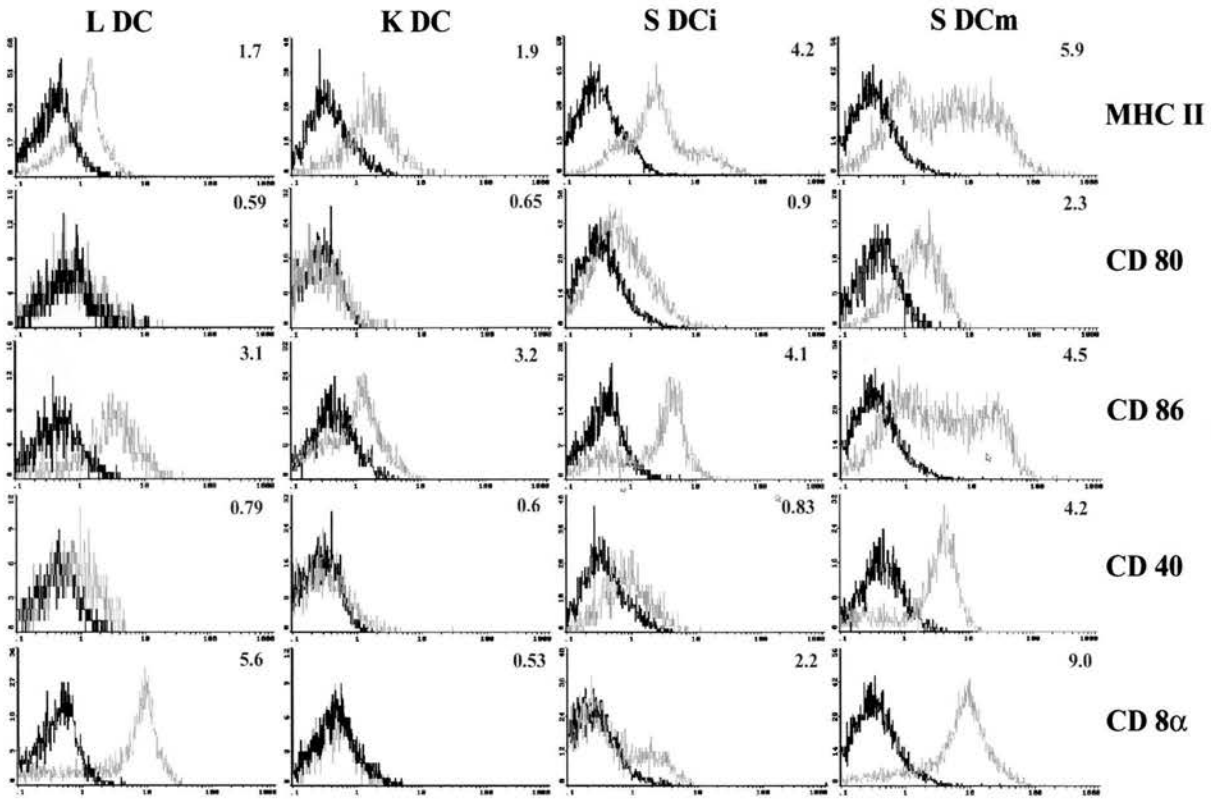


Figure 6.1

Flow cytometric analysis of freshly isolated DCs from liver (L DC), kidney (K DC) and spleen. Splenic DCs were isolated with anti-CD11c mAb (immature phenotype: S DCi) or anti-DEC-205 mAb (mature phenotype: S DCm). The surface expression of MHC class II and the co-stimulatory signals CD80, CD86 and CD40 indicates an immature phenotype for L DC, K DC and S DCi. S DCm in contrast display a mature phenotype. CD8α is highly expressed by L DC (60% positive cells) and S DCm (70% positive cells). Grey profiles indicate the specific staining of DCs, black profiles the isotype controls. The mean fluorescence intensity of the isotype control is 0.3 - 0.5.

6.3.2 Allostimulatory capability of organ-specific DCs

The ability of hepatic and renal DCs (Balb/c (H-2^d) mice) to stimulate primary T cell responses *in vitro* was assessed by allogeneic MLR assays with naïve T lymphocytes isolated from C3H (H-2^k) mice. When hepatic and renal DCs were used immediately following isolation, they were found to be poor stimulators of T lymphocyte proliferation as determined by ^{3}[H]thymidine incorporation (Fig. 6.2). Both DC populations required 48 hours of culture in the presence of GM-CSF for optimal induction of T cell proliferation. Thereafter, they induced equal levels of T cell proliferation (Fig. 6.3a), comparable with the proliferation induced by the two splenic DC populations cultured under the same conditions. Splenic DCs however were able to induce optimal T cell proliferation after only 72h, while both hepatic and renal DCs required 24h longer.

To determine the type of T cell response induced by the DC populations, the cytokine levels of IFN-γ and IL-10 were measured in the supernatant of the MLR. While IL-10 was not detected in any of the supernatants, IFN-γ was up-regulated over-time with the highest levels at day 5 of the MLR (Fig. 6.3b). Although this indicated a Th1-response to all allogeneic DC populations, hepatic and in particular renal DCs induced significantly higher levels of IFN-γ. To exclude that the allostimulatory capability of the hepatic and renal DCs was restricted to a particular MHC background of the stimulating or responding populations, the MLR was repeated with different mouse strain combinations. In all combinations tested to date (C3H (H-2^k) DCs + Balb/c (H-2^d) T cells; Balb/c (H-2^d) DCs + C57/6 (H-2^b) T cells; C57/6 (H-2^b) DCs + C3H (H-2^k) T cells), no change was found in the ability of DCs to induce T cell proliferation nor in the type of the T cell response (data not shown).

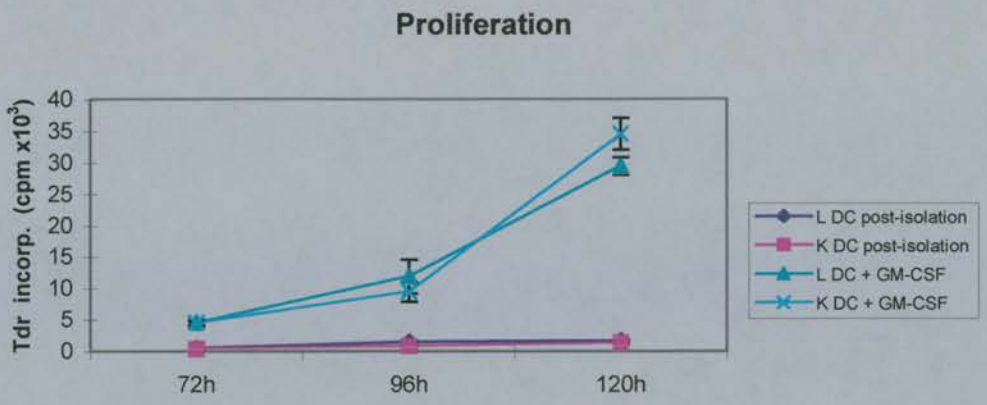
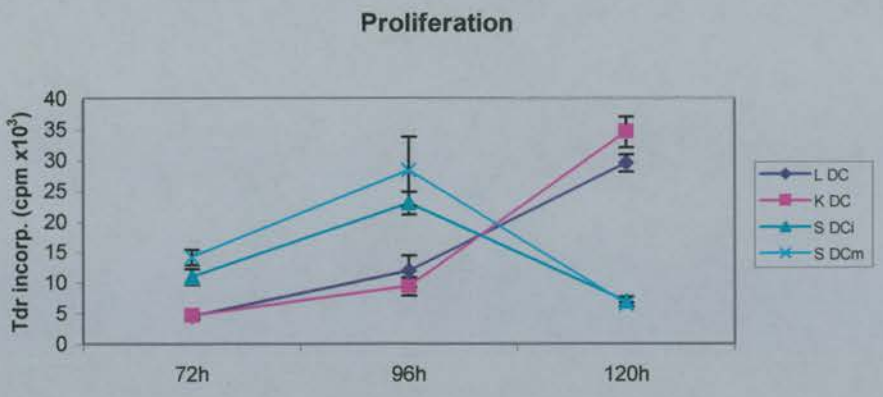


Figure 6.2

Priming of naïve T cells (C3H) as measured by their proliferation in a MLR with allogeneic DCs (Balb/c) isolated from the liver (L DC) or kidney (K DC). DCs were used freshly isolated or after 48 hours of culture with GM-CSF. DCs post-isolation from both organs (L DC ◆; K DC ■) induced a weak T cell response as compared with GM-CSF-treated DCs (L DC ▲; K DC ✱). T cell proliferation was assessed by [3H]thymidine incorporation after 72, 96 and 120 hours (mean counts per minute ± s.e.m.).

A)



B)

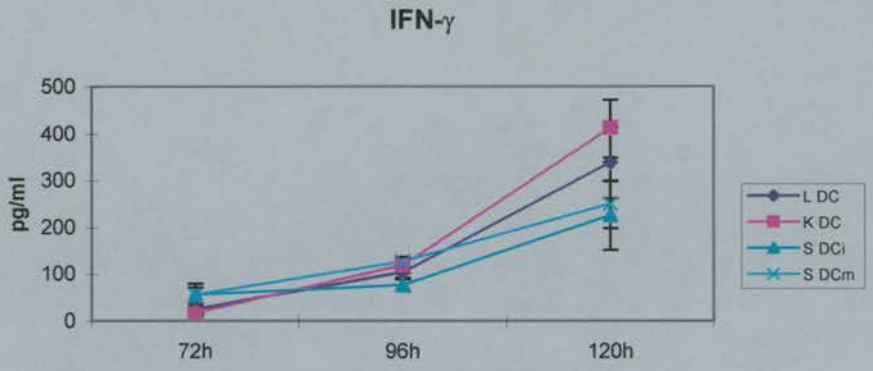


Figure 6.3

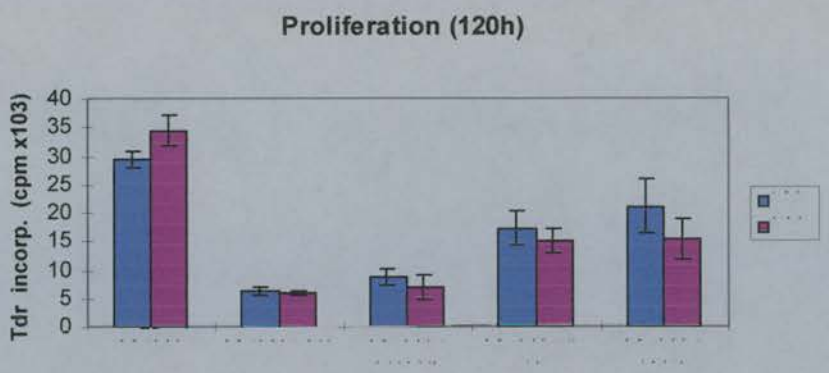
Priming of naïve T cells (C3H) in a MLR with allogeneic DCs (Balb/c) isolated from liver (L DC ◆), kidney (K DC ■) or spleen. Splenic DCs were selected with anti-CD11c mAb (immature phenotype: S DCi ▲) or anti-DEC-205 mAb (mature phenotype: S DCm ✱). Following isolation, all DCs were cultured for 48 hours in the presence of GM-CSF. A) L DC and K DC require 24 hours longer than S DC to induce similar levels of T cell proliferation ([3H]thymidine incorporation; mean counts per minute ± s.e.m.). B) All DC populations induce high levels of IFN-γ release from T cells after 120h (ELISA; pg/ml ± s.e.m.).

6.3.3 Modulation of Th1-response by DC exposure to environmental stimuli

Typical protocols to avoid rejection following transplantation employ immunosuppressive agents such as corticosteroids or the blockade of co-stimulatory signals. To assess the effect of immunosuppression on the allostimulatory capability of the organ-specific DC populations, DCs post-isolation were incubated with GM-CSF for 48h in the presence of dexamethasone or CTLA4-Ig. After rigorous washing to prevent exposure of T cells to the immunosuppressive agents, DCs were used in the allogeneic MLR. Following treatment, all four DC populations had a reduced capability to induce T cell proliferation compared with GM-CSF-treated DC ($p<0.05$, Fig. 6.4a/6.5a). Moreover, IFN-γ production by the T cells was significantly impaired (Fig. 6.4b/6.5b), while IL-10 was again not detectable in any of the supernatants.

In contrast to pharmacological immunosuppression, cytokines such as IL-10 and TGF-β are thought to be involved in the physiological regulation of immune responses. The different DC populations were therefore activated with GM-CSF in the presence of the two cytokines. After washing, DCs were exposed to allogeneic T lymphocytes as previously. Following treatment with the cytokines, all four DC populations had a significantly reduced capability to induce T cell proliferation compared with GM-CSF treatment alone ($p<0.05$, Fig. 6.4a/6.5a). However, the DCs induced significantly higher levels of T cell proliferation than DCs treated with dexamethasone or CTLA4-Ig ($p<0.05$). In contrast, the release of IFN-γ by the T cells was only consistently impaired by pre-treatment of DCs with TGF-β ($p<0.05$, Fig. 6.4b/6.5b). Pre-treatment of immature DCs from liver, kidney and spleen with IL-10 delayed the IFN-γ release from the T cells, but by day 5, the levels failed to show a significant difference (Fig. 6.4b/6.5b). Only treatment of the mature DEC-205-selected DCs from the spleen with IL-10 resulted in a reduction of both, T cell proliferation and IFN-γ release ($p<0.05$, Fig. 6.4b/6.5b). IL-10 release in the supernatant of the MLR was not detected.

A)



B)

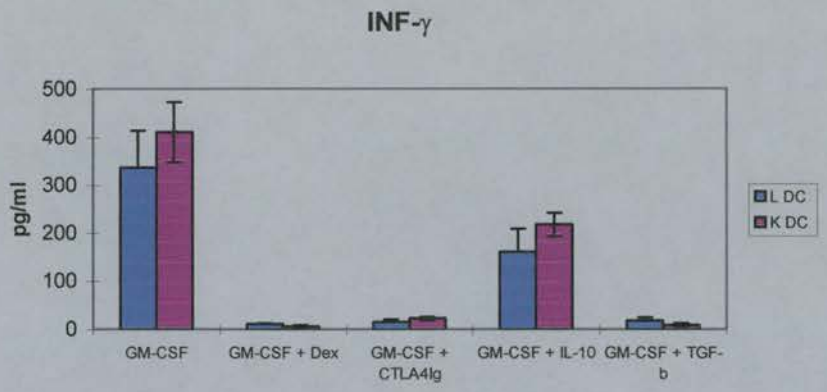
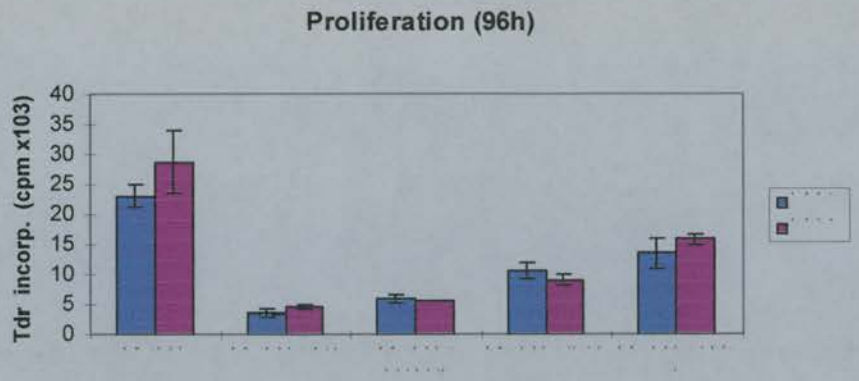


Figure 6.4

Priming of naïve T cells (C3H) in a MLR with allogeneic DCs (Balb/c) isolated from the liver (L DC) or kidney (K DC). Following isolation, DCs were cultured for 48 hours in the presence of GM-CSF alone or in combination with Dex, CTLA-4Ig, IL-10 or TGF-β. A) DCs treated with GM-CSF and any of the four agents induce significantly lower T cell proliferation than DCs treated with GM-CSF alone ([3H]thymidine incorporation after 120 hours; mean counts per minute ± s.e.m.). B) DCs treated with GM-CSF and Dex, CTLA-4Ig or TGF-β induce a significantly lower IFN-γ release by T cells than DCs treated with GM-CSF alone. In contrast, following treatment with GM-CSF and IL-10, LDC and KDC do not induce a significantly lower IFN-γ release. (ELISA after 120 hours; pg/ml ± s.e.m.).

A)



B)

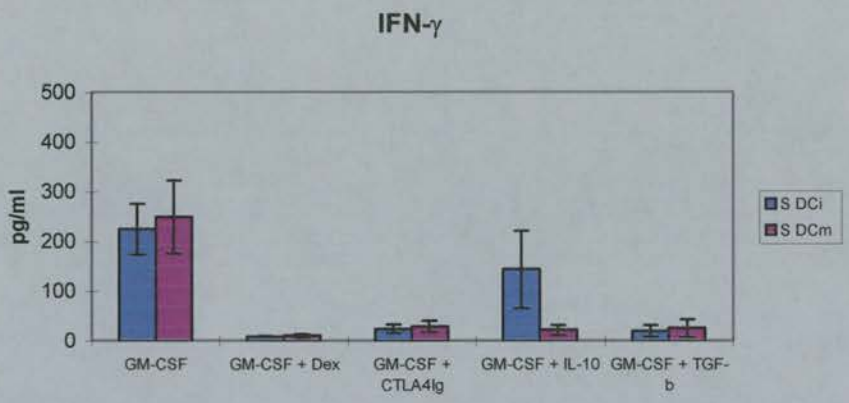


Figure 6.5

Priming of naïve T cells (C3H) in a MLR with allogeneic DCs (Balb/c) isolated from the spleen. Splenic DCs were selected with anti-CD11c mAb (immature phenotype: S DCi) or anti-DEC-205 mAb (mature phenotype: S DCm). Following isolation, DCs were cultured for 48 hours in the presence of GM-CSF alone or in combination with Dex, CTLA-4Ig, IL-10 or TGF-β. A) DCs treated with GM-CSF and any of the four agents induce significantly lower T cell proliferation than DCs treated with GM-CSF alone ([3H]thymidine incorporation after 96 hours; mean counts per minute ± s.e.m.). B) DCs treated with GM-CSF and Dex, CTLA-4Ig or TGF-β induce a significantly lower IFN-γ release by T cells than DCs treated with GM-CSF alone. In contrast, following treatment with GM-CSF and IL-10, only mature splenic DCs (S DCm) induce a significantly lower IFN-γ release. (ELISA after 120 hours; pg/ml ± s.e.m.).

6.3.4 Modulation of IL-10 and IL-12 mRNA synthesis in DCs

To determine the balance of IL-10 and IL-12 production by the DCs, the effect of each pre-treatment on the IL-10 and IL-12 mRNA synthesis was tested in the four DC populations. RNA was extracted from each population at 4, 6 and 24 hours of culture with GM-CSF alone or in combination with the immunosuppressive agents and cytokines. Freshly isolated DCs did not express detectable levels of either mRNA, but treatment with GM-CSF alone induced the expression of both, IL-10 and IL-12 mRNA within 6 hours, irrespective of the phenotype and stage of maturation of the DCs (Fig. 6.6 and 6.7). While concomitant exposure to CTLA4-Ig had no further effect, dexamethasone and the two cytokines IL-10 and TGF-β all abrogated IL-12 mRNA expression by the DCs, which continued to express IL-10 mRNA (Fig. 6.7). However, since TGF-β, but not IL-10 affected the allostimulatory capacity of both, immature and mature DCs, Real-time PCR was used to assess IL-10 mRNA production quantitatively. Using this method, a significant increase of IL-10 mRNA was detectable, with the highest levels at 6 hours post-stimulation, after treatment with TGF-β but not IL-10 as compared with levels from GM-CSF-treated DCs ($p<0.05$, Fig. 6.8). This effect was independent of the phenotype or stage of maturation of the DCs.

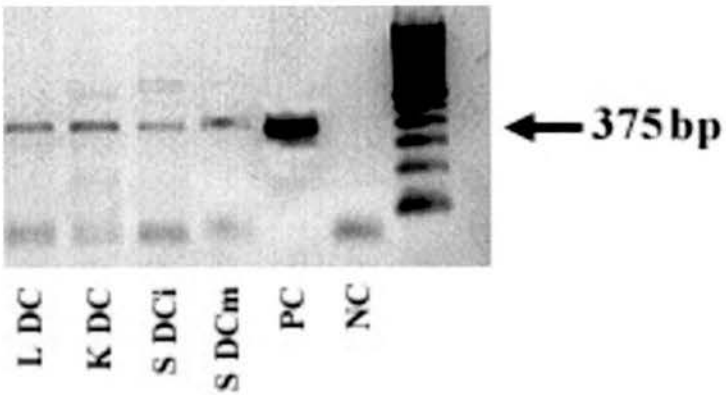


Figure 6.6

RT-PCR analysis of IL-12 mRNA expression in DCs isolated from the liver (L DC), kidney (K DC) or spleen. Splenic DCs were selected with anti-CD11c mAb (immature phenotype: S DCi) or anti-DEC-205 mAb (mature phenotype: S DCm). All DC populations expressed IL-12 mRNA following stimulation with GM-CSF for 6 hours. The primers amplified a 375-bp segment of IL-12 mRNA, a positive control (PC, IL-12 cDNA (10^7copies/μl)) was provided by the manufacturer. A template-free PCR reaction was used as negative control (NC).

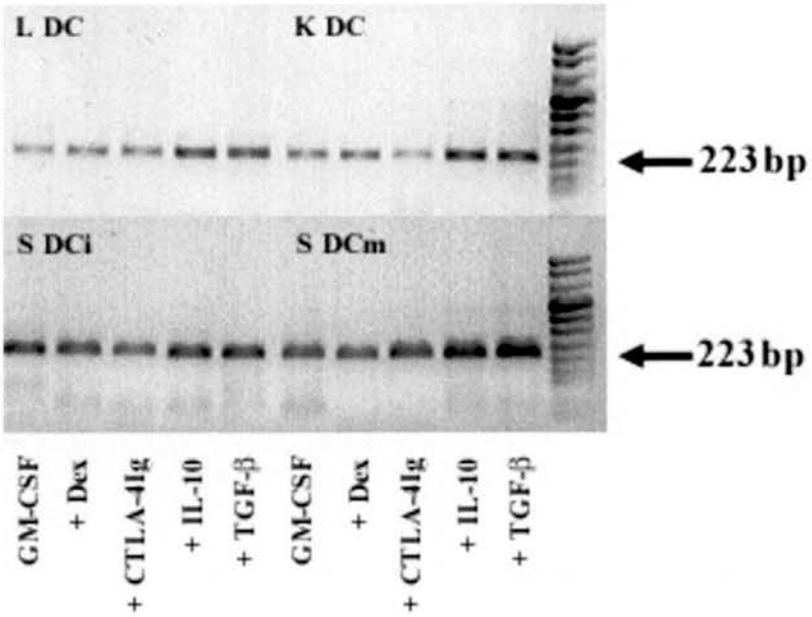


Figure 6.7

RT-PCR analysis of IL-10 mRNA expression in DCs isolated from liver (L DC), kidney (K DC) or spleen. Splenic DCs were selected with anti-CD11c mAb (immature phenotype: S DCi) or anti-DEC-205 mAb (mature phenotype: S DCm). All DC populations expressed IL-10 mRNA following stimulation for 6 hours with GM-CSF alone or in combination with Dex, CTLA-4Ig, IL-10 and TGF-β (n = 3). The primers amplified a 223-bp segment of IL-10 mRNA, a positive control was provided by the manufacturer (IL-10 cDNA, 10^7copies/μl).

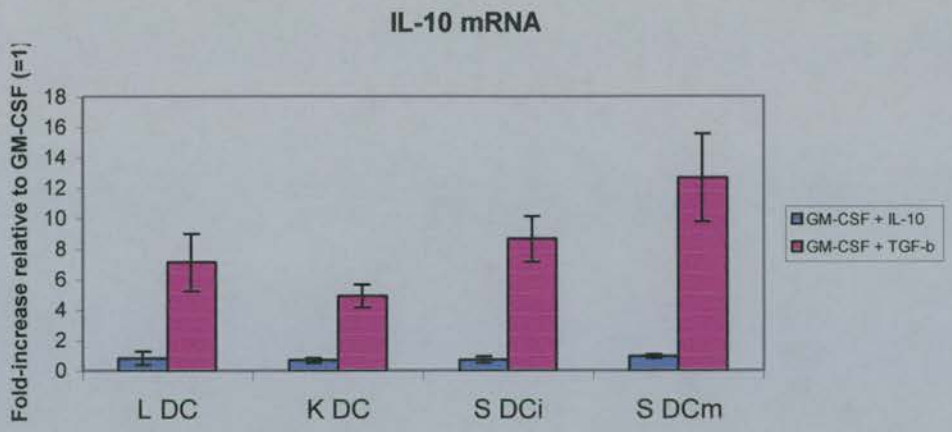


Figure 6.8

Quantitative Real-time PCR for the expression of IL-10 mRNA in DCs isolated from liver (L DC), kidney (K DC) or spleen. Splenic DCs were selected with anti-CD11c mAb (immature phenotype: S DCi) or anti-DEC-205 mAb (mature phenotype: S DCm). IL-10 mRNA expression was assessed after stimulation of the DCs for 6 hours with either GM-CSF alone or in combination with IL-10 and TGF-β. The results are expressed as fold-increase ± s.e.m. of IL-10 mRNA expression relative to the expression after stimulation with GM-CSF alone (= 1).

6.4 Discussion

Although peripheral organs such as the liver or the kidney induce markedly different immune responses following transplantation, few studies have investigated the functional properties of the antigen-presenting DC populations from these organs. This reflects the difficulties in isolating these cells which are only present in limited numbers in peripheral tissue (Steptoe, 2000). In this study, an immuno-magnetic method was used to isolate DCs from murine organs on the basis of their expression of surface markers. Hepatic DCs have been reported to express DEC-205 (Woo, 1994), a multi-lectin receptor, and the antibody NLDC-145 against DEC-205 proved to be particularly good in isolating the hepatic DC population. The purified DCs displayed an immature phenotype as determined by their low to moderate expression of co-stimulatory signals. 60% of the cells co-expressed CD8α suggesting that they were predominantly lymphoid-related DCs. These observations correspond to a recent report by O'Connell and colleagues on the phenotype of hepatic DCs (O'Connell, 2000), and in both studies, hepatic DCs had a poor capacity to prime naïve allogeneic T lymphocytes immediately post-isolation. However, DCs acquired this capacity after culture in the presence of GM-CSF, which increases both viability and maturation of the cells (Vremec, 1997). In the present experiments, DCs required 48 hours of culture rather than the previously described overnight period, which might reflect differences in the isolation technique or DC maturation induced by the Flt3L-treatment of mice used in O'Connell's study to increase DC numbers.

DCs in the kidney do not express DEC-205, but adequate cell numbers were obtained by using the N418 antibody against the DC-associated surface marker CD11c for immuno-magnetic isolation (Metlay, 1990). As previously described (Austyn, 1994), freshly isolated renal DCs had an immature phenotype characterised by low expression of co-stimulatory signals, and they were negative for CD8α. Similar to hepatic DCs, renal DCs were poor stimulators of naïve allogeneic T lymphocytes immediately post-isolation, but acquired a good stimulatory capacity after 48 hours of culture in the presence of GM-CSF. The antibodies against CD11c and DEC-205 were also used to isolate DCs from the spleen as control populations of lymphatic

origin. Since the two surface markers are expressed at different levels by most of the splenic DCs, populations selected by this method were not pure lymphoid-derived ($CD8\alpha^+$) or myeloid-derived DCs ($CD8\alpha^-$) as defined by Vremec et. al. using extensive purification steps (Vremec, 2000). However, based on their expression of MHC II antigen and co-stimulatory signals, DEC-205-selected cells displayed the "mature" phenotype described for interdigitating DCs located within the T cell areas of lymphoid tissue (Shortman, 1997) and were predominantly positive for CD8α. CD11c-selected cells on the other hand showed the "immature" phenotype associated with DCs located in the marginal zone of the spleen (Metlay, 1990) and were mostly negative for CD8α. Both splenic populations primed allogeneic T lymphocytes more quickly than hepatic or renal DCs when activated in the same way with GM-CSF. However, this result differs from earlier reports demonstrating a reduced ability of $CD8\alpha^+$ splenic DCs to induce proliferation of allogeneic T cells, partially as a consequence of their expression of the death-inducing molecule CD95L (Suss, 1996). In the present study, surface expression of CD95L was not detected on any of the DC populations.

As determined by the IFN-γ-release of the allogeneic T lymphocytes, all activated DC populations irrespective of their origin stimulated a Th1 response which has been implicated in allograft rejection (Wood, 1994). In this study, the T cell response was associated with the induction of IL-10 and IL-12 mRNA expression by the DCs and specifically IL-12 release by DCs has been demonstrated to regulate Th1 responses (Moser, 2000). One of the possible mechanisms suggested for the induction of transplant tolerance by hepatic DCs has been based on their immature phenotype with weak allostimulatory capacity and subsequent development of T cell anergy (Thomson, 1999). However, when comparing the two immature DC populations from the liver and kidney, obvious functional differences were not detected despite their phenotypic variability with differential expression of DEC-205 and CD8α. This could suggest, that DCs either react differently to environmental signals or that DCs receive different signals in each respective tissue following transplantation. To investigate this further, DC populations were initially exposed during their activation to the immunosuppressive agents dexamethasone and CTLA-4Ig, both used in

clinical and experimental transplantation to prevent allograft rejection (Hall, 2000). The two agents profoundly reduced the capability of the DCs to stimulate allogeneic T cell proliferation or IFN-γ-release, although complete anergy was not observed. In bone marrow-derived DCs, dexamethasone has been previously reported to inhibit DC function by arresting their maturation, while mature DCs remained unaffected (Matyszak, 2000). However, in this study, dexamethasone had an inhibitory effect on all DC populations including mature splenic DCs, and the DCs were therefore tested for IL-10 and IL-12 mRNA synthesis. Similar to recent studies in human monocyte-derived DCs (Rea, 2000), an abrogation of the GM-CSF-induced expression of IL-12 mRNA was found in all DC populations, which might be the explanation for the glucocorticoid-induced suppression of the DC function independently of the stage of maturation. In contrast, CTLA-4Ig is known to block the co-stimulatory signals CD80 and CD86 on DCs thereby interfering with T cell activation (Denton, 1999). Although there was no effect on the synthesis of IL-10 or IL-12 mRNA, incubation of the DCs with CTLA-4Ig during their activation with GM-CSF was sufficient to inhibit their capacity for T cell stimulation. However, significant differences between the DC populations could not be observed, either with respect to their stage of maturation or their phenotype.

In contrast to the pharmacological agents dexamethasone and CTLA4-Ig, IL-10 and TGF-β are thought to be physiological mediators of tolerance induction (Hall, 2000). Both cytokines are synthesised by hepatic parenchymal or stromal cells (Tox, 2001; Narumoto, 2000), and in particular the release of IL-10 has been associated with stable graft function following liver transplantation (Cosenza, 1995). In this study however, pre-treatment of the immature DCs from liver, kidney and spleen with IL-10 only delayed the development of a Th1 response as characterised by IFN-γ production. Only the mature splenic Dec-205-selected DCs were incapable of inducing a significant IFN-γ release by the allogeneic T cells. Similar to dexamethasone, IL-10 is thought to affect DCs by inhibiting both maturation and IL-12 synthesis (De Smedt, 1997). Since IL-10 was also found to inhibit IL-12 mRNA production in all DC populations, the results suggest, that this effect is sufficient to interfere with the allostimulatory capacity of resident mature DCs. However in

immature DCs, counter-regulatory signals for maturation and activation provided by T lymphocytes, e.g. through CD40 ligation (Cella, 1996), appear to restore their stimulatory function, indicating that IL-10 has to be present during the process of antigen-presentation to prevent the development of a Th1 response. In contrast, all DC populations in the present study were unable to prime a Th1 response when pre-treated with TGF-β. Previous reports already implicated TGF-β as a key environmental factor in sites of immunological privilege such as the anterior chamber of the eye (Streilein, 1992). The tolerogenic effect of TGF-β is thought to be mediated through inhibition of DC maturation (Yamaguchi, 1997). However, similar to dexamethasone, TGF-β affected mature and immature DCs and the cells were therefore tested for synthesis of IL-10 and IL-12 mRNA following GM-CSF activation in the presence of TGF-β. The results indicate, that TGF-β not only inhibits the production of IL-12 mRNA, but also induces a significant up-regulation of IL-10 mRNA synthesis as measured by Real-time PCR, an effect which has also been described in other cell types, notably hepatocytes (Ishizaka, 1996).

In conclusion, the present study demonstrates, that in the murine model freshly isolated DCs from liver and kidney do not exhibit significant functional differences in priming naïve allogeneic T lymphocytes *in vitro*, despite the variation in their lineage-related phenotype. This functional equivalence extends to their response to tolerogenic stimuli which might be present before or during transplantation. A potential tolerogenic role of hepatic DCs would therefore rely on environmental signals from the hepatic tissue. According to this study, TGF-β might be in particular suited to induce this effect, since it sufficiently inhibits the immunostimulatory function of DCs with respect to a Th1 response even when removed during the actual DC-T cell interaction. In the liver, TGF-β has indeed be shown to be released by a variety of cells including hepatocytes and stellate cells, in particular in response to tissue damage (Nakatasukasa, 1990). However, this hypothesis would also suggest, that the immune response following transplantation of other solid organs than the liver could be manipulated by environmental signals to the resident DCs.

Chapter Seven - Discussion

7.1 Intra-hepatic expansion versus depletion of infiltrating T lymphocytes

This thesis examined the hypothesis that the allogeneic immune response following hepatic transplantation is modified within the liver affecting the cell fate of donor and recipient cells, and thereby transplant outcome. The first important observation was that intra-hepatic proliferation of T lymphocytes, and in particular of CD4+ T cells, was a prominent feature of allograft rejection. Since the relative increase of CD4+ T cells was also the predominant difference between the cellular infiltrate during acute rejection compared with non-rejecting liver tissue, it is tempting to speculate, that acute rejection following liver transplantation is a result of local expansion of CD4+ T lymphocytes. As the principle source of cytokines such as IFN-γ and IL-2 (Mosmann, 1991), the cells would then be responsible for activating effector cells such as CD8+ T lymphocytes. However, other groups have found significant changes in the number of CD8+ T cells or no changes for both, CD4+ and CD8+ T cells, between rejecting and non-rejecting liver grafts (Ibrahim, 1993a; Wong 1998). Care has therefore to be taken with the interpretation of descriptive studies such as these. While the criteria for acute and chronic rejection are at least defined (Demetris, 1997a), "no rejection" is rather a diagnosis of exclusion. In this study, all non-rejecting tissue samples were taken shortly after transplant as protocol biopsies without any clinical indication such as rising liver function tests. Additionally, all patients with this diagnosis never developed any episodes of rejection during their follow-up. However, it might be possible, that CD8+ T cells diminish over time in stable grafts (Wong, 1998; Baker, 2001), which could explain the different observations and might be associated with the low proliferation index of intra-hepatic CD8+ T lymphocytes described in this study. In contrast, during chronic rejection, both CD4+ and CD8+ T lymphocytes appear to receive enough signals to sustain proliferation within the graft (Hornick, 2000).

Proliferating CD4+ T lymphocytes were however also found in non-rejecting grafts, raising the question of the lymphocyte function. It could be argued, that these grafts were rather mildly rejected than truly not rejected, thereby giving an explanation for the low level of T cell proliferation. Another observation in this study was however, that most of the intra-hepatic ML additionally expressed the death receptor CD95 in non-

rejecting grafts, but not in rejecting livers. Although CD95 expression in itself does not prove, that the cells actually undergo apoptosis, it is an indication for their susceptibility to CD95 ligation. In both, humans and animal models, acceptance of the liver allograft is usually associated with apoptosis of the infiltrating leukocytes (Sharland, 1998), leading to the hypothesis of tolerance induction by lymphocyte depletion (Wells, 1999). Due to technical difficulties with the staining pattern of the antibody used in this study, it was not possible to assess the phenotype of leukocytes expressing CD95. However, activated T lymphocytes in particular are liable to die by CD95 ligation (Alderson, 1995), and apoptosis of donor-reactive T lymphocytes is the predominant feature in models of allograft acceptance (Bishop, 2001). Several mechanisms for the deletion of alloreactive T lymphocytes have been proposed, and hepatocytes in particular have been described to induce T cell proliferation prior to T cell death (Bertolino, 1999). This could also apply to the cells in cycle found in this study, however most mechanisms including depletion by hepatocytes appear to affect CD8+ T lymphocytes. In contrast, persistence of intra-hepatic CD4+ T cells has been associated with liver allograft acceptance (Olver, 1998) and might be secondary to continuous intra-hepatic proliferation. It remains however unclear, if these CD4+ T cells play an active role in the process of allograft acceptance.

7.2 Peripheral sensitisation within the liver allograft

Further assessment of the proliferating T lymphocytes revealed, that they were of both, naïve and memory phenotype. Clusters of lymphoblasts in rejecting liver allografts have been previously described and hypothesised to reflect sites of peripheral sensitisation of T lymphocytes (Demetris, 1991). However, naïve T lymphocytes preferentially recirculate through lymphoid tissue and lack the necessary adhesion molecules to home to peripheral organs (Brezinschek, 1995). The results presented confirm for the first time that naïve T lymphocytes proliferate within the liver allograft, indicating an intra-hepatic primary immune response. This could simply reflect, that solid organs following transplantation become accessible for naïve T cells and low numbers of CD45RA+ cells have been described in heart and kidney allografts, but appeared not to expand locally (Ibrahim, 1993b and 1995). In contrast, the normal liver has very recently been

recognised to allow as the only solid organ homing of naïve T cells, which enter the organ through fenestrated endothelial cells and the space of Disse (Bertolino, 2000). It remains therefore to be seen, if the primary immune response with proliferation of naïve T lymphocytes within the allograft is unique to the liver.

Stimulation of naïve T cells requires the presence of professional APCs, in particular DCs (Steinman, 1991). While proliferating naïve T cells were more prominent in acute rejection and non-rejecting grafts, chronic rejection was characterised by proliferation of memory T lymphocytes. Since DCs of recipient origin have been found within lymphoid aggregates during chronic rejection (Oguma, 1988), it is likely, that the T cell activation is mediated by indirect antigen-presentation (Demetris, 1997b). In contrast, acute rejection in the early stages appears to be initiated by donor DCs via direct antigen-presentation (Lechler, 1982). Donor DCs are also the most likely APCs in non-rejecting grafts in this study, as all the protocol biopsies were taken at day seven, prior to the replacement of donor leukocytes by recipient cells (Gouw, 1987). Since hepatic DCs have been hypothesised to be tolerogenic rather than immunogenic (Thomson, 1999), the potential role of liver-derived DCs in the stimulation of naïve T lymphocytes was examined *in vitro* as described in chapter six. Because of ease of access and the better characterisation of murine DCs (Schuler, 2000), a mouse model was used for these investigations. To address the problem, that murine DCs might have different allostimulatory capabilities than human DCs, control DC populations were also isolated from kidney and spleen, validating the conclusions at least within the species.

In correlation with previous reports (O'Connell, 2000), freshly isolated hepatic DCs were immature and poor stimulators of naïve T cells. These qualities of liver-derived DCs have been used to explain their tolerogenic function, and recently, immature DCs have indeed been shown to induce non-proliferating CD4+ Tr lymphocytes after repetitive stimulation (Jonuleit, 2000). However, T cell proliferation was a prominent feature in this study, and renal DCs, which derive from an immunogenic organ, were equally poor in activating naïve T cells. Since the immune response following transplantation is associated with the release of a variety of cytokines, it was hypothesised, that external stimuli might play a role. The DC subpopulations were

therefore exposed to the maturation factor GM-CSF (Vremec, 1997) and the tolerogenic cytokines IL-10 and TGF-β, which have both been found within the hepatic microenvironment following transplantation (Ishizaka, 1996; Tox, 2001). The results presented indicate, that following maturation, hepatic DCs become as efficient as renal and splenic DCs in priming an allogeneic Th1-response characterised by the release of IFN-γ. However, priming of the DCs with GM-CSF and in particular TGF-β inhibited the induction of a Th1-response without prohibiting T cell proliferation. This was associated with an abrogation of the DC synthesis of IL-12 mRNA, and an up-regulation of IL-10 mRNA synthesis. Although the preferential release of IL-10 by DCs would favour the induction of either a Th2-response or even of regulatory T lymphocytes, this was not verified in this study, since the T cells did not produce IL-10 as previously described (Jonuleit, 2000). The function of these T lymphocytes remains therefore speculative, however, DCs propagated from murine bone marrow in the presence of GM-CSF and TGF-β have been shown to prolong the survival of heart allografts (Lu, 1997). As an environmental factor, TGF-β is additionally thought to act on APCs in the anterior chamber of the eye, which is characterised by immune deviation and privilege (Streilein, 1992). Since TGF-β is also produced within the liver, in particular by stellate cells in response to tissue damage, it could be hypothesised, that it might be able to render hepatic DCs tolerogenic. However, the results in this thesis indicate, that hepatic donor DCs can be equally immunogenic, probably depending on the balance of stimuli from the microenvironment.

7.3 T cell-mediated cytotoxicity in liver allografts

The results in chapter four of this thesis implicate both major pathways of T cell-mediated cytotoxicity, perforin/granzyme B and CD95L/CD95, as effector mechanisms during liver allograft rejection. However, of all donor target cells, only hepatocytes were receptive to CD95 ligation. A dominant role for both pathways in allograft rejection had already been established in mouse models deficient of one or both mechanisms (Schulz, 1995), although the existence of compensatory modes of cytotoxicity was also evident. In this study using immunohistochemistry, it was

therefore rather intended to investigate the specific contribution of perforin/granzyme B and CD95L/CD95 to the target cell damage during liver allograft rejection.

Granzyme B was found within the cytoplasm of CD8+ T lymphocytes and NK cells, underlining their significance as effector cells, but not within donor target cells. A cytotoxic effect of granzyme B can therefore only be assumed, however the correlation between the number of positively staining cells and the severity of rejection would support a mechanistic role for perforin/granzyme B in graft damage. The distribution of Granzyme B expressing cells, both within the parenchyma and the portal tracts, suggests their ability to target all hepatic donor cells. Since Granzyme B induces apoptosis via both, direct cleavage of effector caspases and activation of the bcl-2 family member bid (Darmon, 1996; Yang, 1998), a conclusion about an increased susceptibility of the target cells specifically to perforin/granzyme B could not be drawn from this study. It is however of note, that only effector cells recognising MHC I antigens expressed granzyme B, which correlates with the observation that only MHC I-mismatched grafts are significantly later rejected in perforin-deficient mice (Schulz, 1995). The role of this pathway appears therefore predominantly limited by the expression of MHC I antigens on donor cells, which is constitutive on biliary epithelial cells and vascular endothelial cells (Fleming, 1981), the primary target cells of liver allograft rejection, but has to be induced on hepatocytes by pro-inflammatory cytokines such as IFN-γ (Steinhoff, 1988).

In contrast to granzyme B, the susceptibility of donor cells to CD95-mediated apoptosis could be examined by staining for the receptor on the target cells. Only hepatocytes expressed CD95, in normal liver tissue as constitutive expression (Leithaeuser, 1993) and up-regulated during rejection. Hepatocytes are not regarded as the primary target cells during liver allograft rejection and their death is recognised as a sign of severe rejection (Demetris, 1997a). Since IFN-γ has been reported to sensitise epithelial cells to CD95 ligation, its effect on primary hepatocytes was examined using a mouse model which allowed the use of p53-deficient hepatocytes. The results as described in chapter five indicate that IFN-γ indeed amplifies CD95-mediated apoptosis of hepatocytes. However, this effect is dependent on functional p53 and can be blocked by cyclosporin

A, which inhibits the MPT, one of the intracellular pathways of CD95-induced apoptosis (Scaffidi, 1998).

7.4 Clinical perspective

Orthotopic liver transplantation has established itself as the treatment of choice for end-stage liver disease, mainly because of its success rates with 1-year patient survival reaching 85-90% (Keefe, 2001). However, as a consequence of this success, indications for transplantation have increased at the same time as the cadaveric donor pool is falling, resulting in a rising length of wait and mortality on the waiting list world-wide (Gilbert, 1999). This has led to a change of focus to the causes of late loss of grafts or patients. Although the incidence of chronic rejection, 5% graft loss after 5 years (Wiesner, 1999), is low compared to other solid organs, it still indicates that some patients are treated inadequately with current immunosuppressive regimens. In contrast, other causes of late graft loss are rather associated with adverse effects of the immunosuppressive therapy. Infections and new malignancies as a possible consequence of the impaired immune system account each for 19% of late deaths after 5 years (Neuberger, 2000). Cardiovascular complications cause 23% of deaths in this study and can be in part related to the development of diabetes mellitus and hyperlipidaemia, known side-effects of the immunosuppressive drugs (Denton, 1999). The drugs are also responsible for renal impairment, which affects up to 80% of liver transplant patients after 5 years (Fisher, 1998). Finally, recurrence of the original liver disease in the graft has now emerged as a further long-term problem and in viral diseases such as Hepatitis B and C, now the major indication for transplantation world-wide (Terrault, 2000), immunosuppression might have a significant impact on rate and severity or at least complicate anti-viral treatment.

Tolerance induction remains therefore a major goal in clinical transplantation of the liver and the ease of acceptance of liver allografts by recipients infers, that tolerance might be a realistic goal even in the clinical setting (Neuberger, 2000). Several recent studies have now focused on the local interaction of the liver with the immune system, and these findings might not only be relevant to transplantation, but also to the

phenomenon of oral and peripheral tolerance induction. Hepatocytes *in vitro* appear to be able to induce proliferation in naïve CD8+ T lymphocytes, which die rapidly thereafter (Bertolino, 1999). Hepatic endothelial cells can present antigen to both, naïve CD4+ and CD8+ T lymphocytes, resulting in anergy or deletion of the CD8+ T cells and a Th2-type response of the CD4+ T cells (Knolle, 2001). Finally, hepatic DCs in their immature state or DC progenitors from haematopoietic stem cells in the liver induce T cell anergy or significantly prolong allograft survival of other tissues (Jonuleit, 2000; Fu, 1996). However, all of these observations only become clinically relevant, since naïve T cells are able to enter the organ and interact directly with the hepatic cells (Bertolino, 2000).

A tolerogenic effect of the hepatic cells is easy to conceptualise under the non-inflammatory conditions of oral tolerance induction. In contrast, following transplantation the liver is exposed to a significant immune response initiated in the secondary lymphoid tissue (Lakkis, 2000). During allograft rejection, naïve T lymphocytes are probably rather immunised within the liver, most likely by donor or recipient DCs (Demetris, 1991 and 1997b). If however the graft is only mildly rejected, donor-reactive T cells might be deleted and regulatory T lymphocytes induced (Sharland, 1998; Gassel, 1992). The ability of the liver to protect other organs alloantigen-specifically (Kamada, 1984) would certainly support the existence of Tr cells, although they remain elusive. Since this tolerance induction represents an active immune response, too much immunosuppression might be contra-productive (Bishop, 2001). This however requires the liver cells to be resistant to the first immune attack and at least the largest subpopulation, hepatocytes, appear to require significant inflammatory stimulation to become susceptible to T cell-mediated cytotoxicity.

7.5 Future Studies

The role of persisting CD4+ T lymphocytes in patients with stable graft function requires further study. Although their functional assessment is difficult in human biopsies, *in situ* hybridisation or new technologies such as laser-guided microdissection might allow the identification of specific mRNA products, in particular of inflammatory

or tolerogenic cytokines. Additionally, CD8+ T lymphocytes were recently demonstrated to revert from a CD45RO+ state to more stable CD45RA+ cells, which have a distinct cytokine release pattern to naïve CD8+ T lymphocytes (Faint, 2001). It might therefore be conceivable, that CD4+ T cells could also revert to a CD45RA+ state after priming, accounting in part for the persistent finding of CD45RA+ cells in the liver post-transplant. Assessment in particular of biopsies from patients with stable graft function might therefore allow the characterisation of T lymphocytes associated with the development of clinical tolerance.

Further study is also required to assess the role of DCs, both in biopsies of patients post-transplant and *in vitro*. The present study did not include the specific differentiation between lymphoid-related and myeloid-related DCs. Recently, both subsets were isolated from the liver in a mouse model and differentially regulated organ allograft survival (O'Connell, 2001). In humans, specific markers for the two subsets have now been described and it might be possible to characterise the phenotype *in situ* (Pulendran, 2001). Additionally, the investigation of hepatic DC function following co-culture with other hepatic cells merits further consideration. In particular hepatocytes and stellate cells might have a tolerogenic influence via cytokines such as IL-10 or TGF-β. This would help to understand, if hepatic DCs are indeed exposed to different stimuli within the liver, resulting in a tolerogenic function. Finally, results from this unit have recently demonstrated, that the immune competence of each individual patient pre-transplant can predict the occurrence of allograft rejection following liver transplantation (Bathgate, 2001). This might indicate differences in DC function between patients, and is currently assessed by isolating DCs from the peripheral blood and correlating their function *in vitro* with transplantation outcome.

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APPENDIX

Intrahepatic proliferation of 'naive' and 'memory' T cells during liver allograft rejection: primary immune response within the allograft

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ABSTRACT **Liver allograft rejection is mediated by a primary response of T lymphocytes, followed by infiltration of the graft with a mixed inflammatory reaction. Using single and double label immunocytochemistry, we examined the proliferation index and the phenotype of leukocytes on liver biopsies from 10 patients with acute rejection before and after treatment with i.v. steroids, 10 patients with chronic rejection, 10 patients without rejection posttransplant, and 15 nongrafted, nonimmunosuppressed patients. Proliferation of mononuclear leukocytes (assessed by expression of Ki-67, a nuclear antigen associated with the cell cycle) inside the allograft was a prominent feature of acute and chronic rejection and was down-regulated by steroid treatment. Leukocytes in cell cycle were located predominantly in the portal tracts at the site of the inflammatory infiltrate. The majority of 'naive' (CD45RA+) and 'memory' (CD45RO+) CD4+ T lymphocytes were also periportally distributed. In contrast, CD8+ T lymphocytes, CD57+ natural killer cells, and CD68+ macrophages were located intraparenchymally throughout the liver lobules, whereas CD20+ B lymphocytes were only present in some of the portal tracts. Predominantly CD4+ and occasionally CD8+ lymphocytes were proliferating (assessed by double staining). The proliferating CD4+ cells were of both naive (CD4+, CD45RA+) and memory (CD4+, CD45RO+) phenotypes. To our knowledge, this is the first description of proliferating naive T lymphocytes in situ in liver allografts. These findings suggest that there may be a primary immune response generated within the allograft as well as in draining lymphatic tissue. This implicates not only intrahepatic proliferation of T lymphocytes as a prominent feature of rejection, but also suggests that the liver has a special immunological status comparable to that of lymphatic tissue.—Dollinger, M. M., Howie, S. E. M., Plevris, J. N., Graham, A. M., Hayes, P. C., Harrison, D. J. Intrahepatic proliferation of naive and memory T cells during liver allograft rejection: primary immune response within the allograft. *FASEB J.* 12, 939–947 (1998)**

Key Words: T lymphocytes · liver allograft rejection · transplantation · leukocyte

DESPITE IMPROVEMENTS in immunosuppressive therapy, allograft rejection after organ transplantation remains a major clinical challenge. In liver transplant recipients, acute cellular rejection occurs in up to 80% of patients and is associated with increased morbidity and length of hospitalization (1). Chronic rejection is commonly resistant to therapy and leads to the loss of up to 15% of grafts (2). In common with the concept of allograft rejection of other solid organs, rejection of the liver seems to be mediated by T lymphocytes in response to alloantigens (3). The primary immune response involves antigen-presenting cells activating 'naive' T lymphocytes, which subsequently start to proliferate and differentiate. According to new research into T cell migration, this primary activation should evolve within lymphatic tissue, since naive T cells appear to recirculate only through lymphatic tissue (4) and lack sufficient levels of surface integrins for transendothelial migration (5). In contrast, the effector mechanism of the response consists of infiltration of the graft, with a mixed inflammatory reaction including predominantly CD4+ and CD8+ lymphocytes together with NK (natural killer)[2] cells, macrophages, and neutrophils (6).

Compared with other solid organs, however, the liver appears to be immunologically privileged after transplantation. In clinical practice, liver allografts are more resistant to rejection than other organs de-

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[2] Abbreviations: i.v., intravenous; NK cells, natural killer cells.

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spite lower levels of immunosuppression, and human leukocyte antigen matching between donor and recipient is not required (7). In animal models, transplantation is possible without immunosuppressive agents and tolerance induction is described in both patients and animals (6, 8). This tolerance induction is alloantigen-specific for other organs of the same donor and can even override priming (9, 10). Many authors argue that this is dependent on a minimum amount of rejection and intrahepatic lymphocyte turnover (6, 11). Possible explanations for the tolerance induction include apoptosis of the reactive lymphocytes (12) or migration of donor passenger leukocytes into recipient lymphoid tissue (8). On the other hand, the fact that the fetal liver is a site of hematopoiesis, together with the immature phenotypic characteristics of many intrahepatic lymphocytes, has led to the hypothesis that the liver is an extralymphatic site of T cell development (11).

The apparent difference of liver allografts to other solid organs despite similarities in the immune response led us to focus our study on the graft itself. Using the actual clinical situation with liver biopsies of patients after transplantation as a model, we intended to examine the proliferation rate and corresponding phenotype of mononuclear leukocytes during allograft rejection and the relevance for transplantation outcome. The percentage of proliferating cells was assessed by their expression of the nuclear antigen Ki-67, which is specific for the late G1, S, G2, and M phases of the cell cycle (13). Ki-67+ cells were then further characterized for their expression of the subset specific antigens (14) CD4 and CD8 (T lymphocytes), CD20 (B lymphocytes), CD57 (NK cells), and CD68 (macrophages). Naive and 'memory' T lymphocytes were distinguished by the two isoforms CD45RA and CD45RO of the leukocyte common antigen family (LCA, CD45).

EXPERIMENTAL PROCEDURES

Subjects

Acute rejection

Liver specimens, obtained by percutaneous needle biopsy from 10 patients (4 male/6 female; age range 25–60 years) with acute cellular rejection after orthotopic liver transplantation, were studied (median time posttransplant 8 days; range 6–26 days). All biopsies were placed into 10% buffered formalin (pH 7.4), embedded in low-temperature paraffin wax, and stored until use. Indications for transplantation were primary biliary cirrhosis (four patients), primary sclerosing cholangitis (four patients), and chronic active hepatitis, alcoholic liver disease, fulminant hepatic failure due to acetaminophen overdose, and graft failure due to chronic rejection (one patient each). After the transplantation, patients received standard immunosuppressive therapy with prednisolone, azathioprine, and cyclosporin A. Acute rejection was diagnosed using clinical and biochemical criteria in combination with histological evaluation of the biopsies according to standard scoring systems (6). The main histological features of rejection assessed were portal inflammation, bile duct damage, and subendothelial inflammation, each scored on a scale of 0 (none) to 3 (severe). The combined rejection scores of the biopsies used in this study were 6/9 or above. All patients with acute rejection were treated with a daily regimen of 1 g of methylprednisolone intravenously (i.v.) for 3 days. After treatment, a second liver biopsy was taken to confirm the resolution of the rejection episode. In all cases, a significant reduction of the total rejection scores was achieved, with maximum scores of 4/9.

Chronic rejection

Liver biopsies from 10 patients (1 male/9 female; age range 20–58 years) with chronic ductopenic rejection after transplantation were studied (median time posttransplant 6.5 months; range 3.5–9.5 months). The specimens were obtained and processed as described above. Indications for transplantation were primary biliary cirrhosis, fulminant hepatic failure due to acetaminophen overdose, and chronic rejection of the first allograft in three patients each and chronic hepatitis B in one patient. The immunosuppressive treatment of patients before the biopsy included either prednisolone, azathioprine, and cyclosporin A (six patients) or prednisolone and tacrolimus (four patients). The diagnosis of chronic rejection was based on a combination of standard clinico-biochemical features and histological criteria (6), including bile duct loss and obliterative arteriopathy. During follow-up, 8 of the 10 patients consequently lost their graft due to chronic rejection, whereas the other two patients recovered after their immunosuppressive regimen was changed from cyclosporin A to tacrolimus.

No rejection

Specimens of liver tissue were obtained from 10 patients (2 male/8 female; age range 20–64 years) and processed as described above, with routine biopsies on day 7 posttransplant, as per management protocol for postoperative care in the Scottish Liver Transplant Unit. Indications for transplantation were primary biliary cirrhosis (four patients), fulminant hepatic failure due to acetaminophen overdose (three patients), and alcoholic liver disease, cryptogenic cirrhosis, and hepatocellular carcinoma (one patient each). The standard immunosuppressive posttransplant therapy consisted of prednisolone, azathioprine, and cyclosporin A in all cases. None of the patients showed clinico-biochemical signs of acute or chronic rejection; histological evaluation of the liver biopsies amounted only to mild inflammation, with a score of 3/9 or less in each case. During follow-up, none of the patients developed an episode of acute or chronic rejection and no further biopsies were taken.

Controls

Control liver tissue was obtained from biopsies of 15 patients (8 male/7 female; age range 25–67) undergoing routine staging for malignant lymphoma and staging of colon carcinoma at the time of the resection or were taken before therapy with methotrexate for psoriasis. All liver specimens were histologically evaluated and reported as normal. Prior to biopsy, no clinical signs of liver disease were present, and liver function

tests were normal in all 15 patients. The biopsies were processed as described above.

Immunocytochemistry

Serial sections (3 μm in thickness) of the paraffin-embedded biopsies were mounted on glass slides, air-dried at room temperature, dewaxed in xylene, and rehydrated in a graded ethanol series. They were pretreated for antigen retrieval by microwaving in 10 mM EDTA buffer (Sigma, St. Louis, Mo.) for 3 × 5 min (mAb CD4) or in 10 mM citrate buffer for 2 × 5 min (all other antibodies), washed, and incubated for 40 min at room temperature with the monoclonal antibodies anti-Ki-67 (MIB-1, Coulter-Immunotech, U.K., Ltd.); anti-CD4 (Novocastra Lab., U.K., Ltd.); anti-CD8, anti-CD20, anti-CD45RA, anti-CD45RO, anti-CD68 (Dako, U.K., Ltd.); and anti-CD57 (Zymed Lab. Inc., U.K., Ltd.). Positive staining was visualized by the standard avidin-biotin-peroxidase complex method, with diaminobenzidine as chromogen. For double immunostaining, the sections were then incubated with the second primary antibody, followed by streptavidin-biotin-alkaline phosphatase with Vector Red (Vector Lab., U.K.) as chromogen. All slides were counterstained with hematoxylin. Negative controls for each run were performed without primary antibody. The number of cells staining positively was counted 'blindly' by two independent observers (M.M.D. and D.J.H.) by using the Zeiss HOME microscope at ×40 magnification. At least five portal tracts were studied per section with the exception of specimens with chronic rejection, in which all visible portal tracts were examined. The degree of concordance between the two observers was >95%.

Statistical analysis

Student's *t* test was used to compare the mean percentage (± SEM) of cells staining positively in the biopsies of each subgroup of patients. Results with a *P* value of less than 0.05 were considered significant.

RESULTS

Intrahepatic proliferation of mononuclear leukocytes after liver transplantation

Proliferation of intrahepatic leukocytes was assessed by their expression of the nuclear antigen Ki-67, which is closely associated with the cell cycle (13). Biopsies from patients without liver disease were used to study the proliferation rate in normal liver tissue. In these, Ki-67+ mononuclear leukocytes were rarely observed, with a mean percentage of 1 ± 0.3% (**Fig. 1**), and were located throughout the liver parenchyma and inside the portal tracts (**Fig. 2**).

Biopsies taken on day 7 posttransplant from patients without clinical rejection revealed increased numbers of mononuclear leukocytes expressing Ki-67 compared with normal liver tissue (15.4±2.4% vs. 1±0.3%, $P=0.0038$). These were located predominantly inside the portal tracts.

During acute rejection, the percentage of proliferating mononuclear leukocytes rose to 61.1 ± 1.9%, significantly higher than in patients without rejection ($P<0.0001$). The distribution of Ki-67+ cells was mainly inside the portal tracts, with some present in the adjacent liver parenchyma. After treatment with corticosteroids, the number of mononuclear leukocytes expressing Ki-67 fell to 23.2 ± 2.5%. This was significant compared with the previous biopsies during acute rejection ($P<0.0001$), but not when compared with biopsies from patients without rejection ($P=0.052$).

During chronic rejection, the number of mononuclear leukocytes in cell cycle was 24.9 ± 5.9%, signifi-

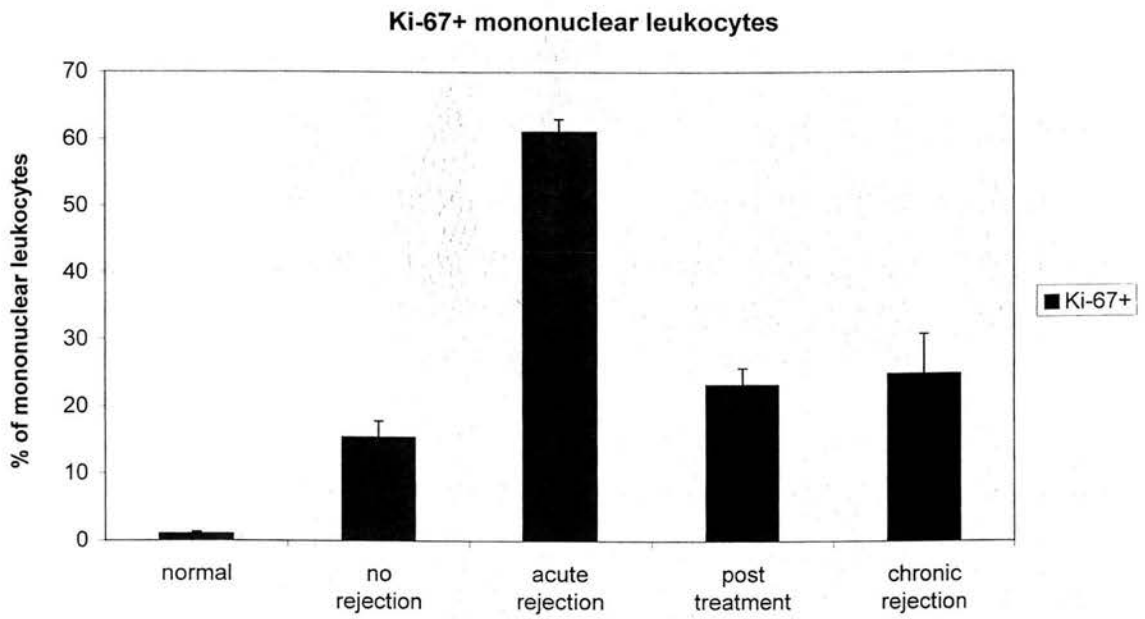

Figure 1. Ki-67 expression of mononuclear leukocytes in normal liver tissue and after transplantation. The number of Ki-67+ cells is expressed as mean percentage ± SEM of all mononuclear leukocytes (normal tissue: $n=15$; all other tissue: $n=10$).

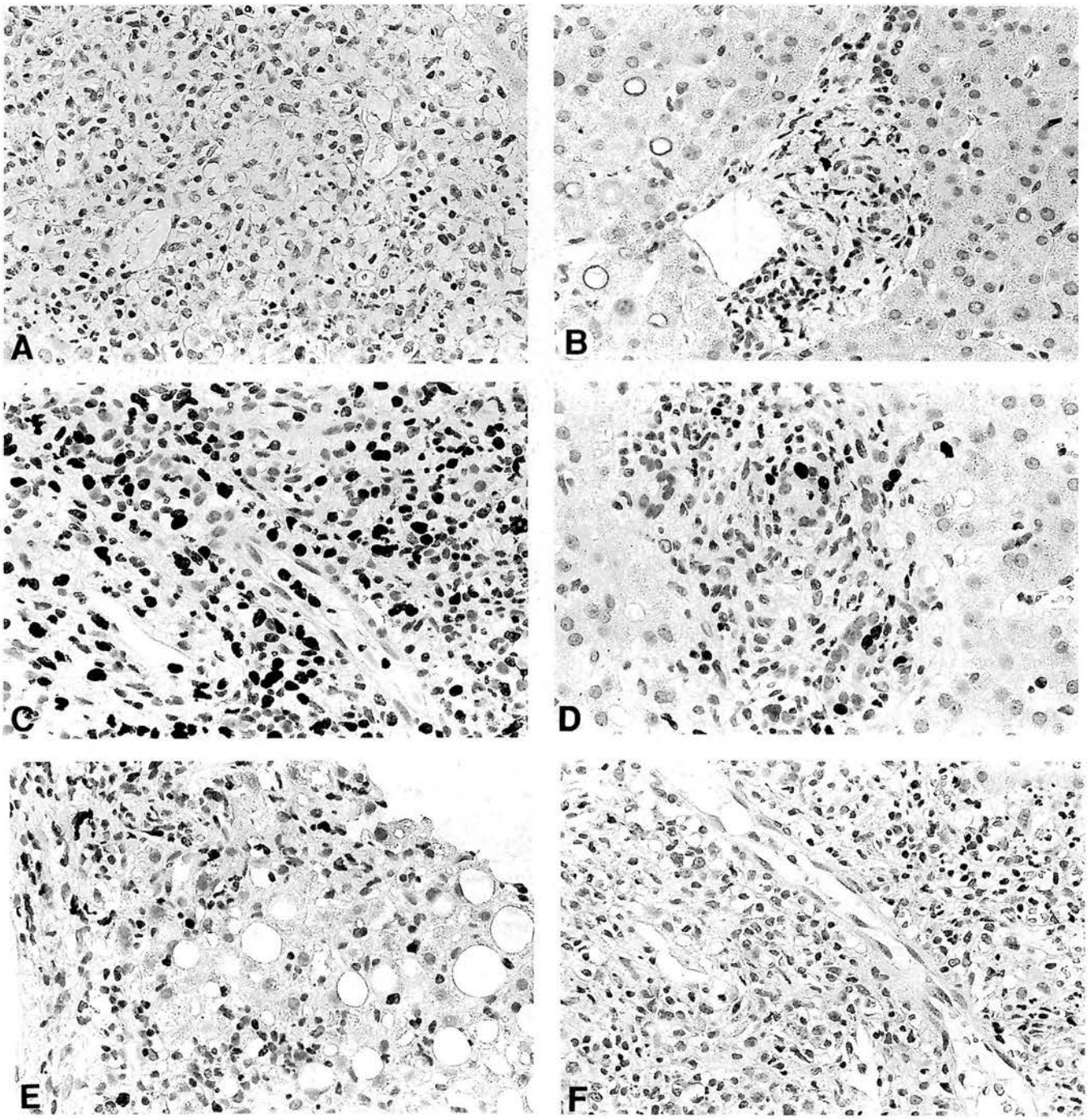

Figure 2. Ki-67 expression of mononuclear leukocytes in normal liver tissue and after transplantation. *A)* Occasional expression in mononuclear leukocytes in normal tissue; *B)* increased expression in periportal mononuclear leukocytes posttransplant without rejection; *C)* highly increased expression in periportal mononuclear leukocytes during acute rejection; *D)* reduced expression after steroid treatment for acute rejection; *E)* increased expression in periportal and intraparenchymal mononuclear leukocytes during chronic rejection; *F)* negative control (no primary antibody) during acute rejection.

cantly different from biopsies from patients without liver disease (P=0.004) or with acute rejection (P=0.0004), but not significantly different from the biopsies of patients without rejection (P=0.17). More of the Ki-67+ cells were present within the liver parenchyma, but, again, the majority were situated inside the portal areas.

Proliferating leukocytes are predominantly CD4+ T lymphocytes

To study the phenotype of mononuclear leukocytes proliferating after transplantation, we used a double staining method to assess which subsets of cells were also ex-

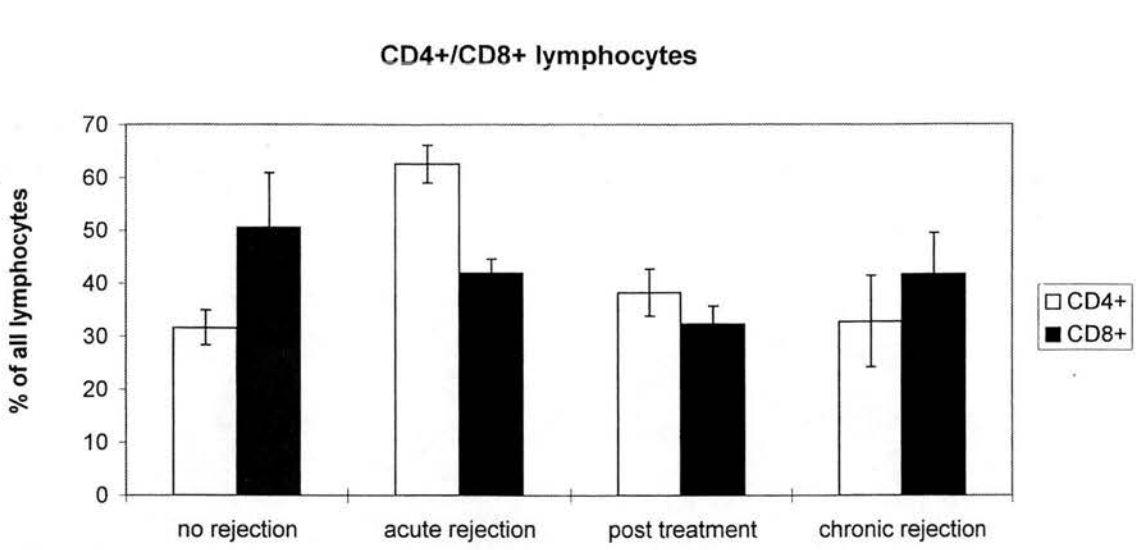

Figure 3. Expression of CD4 and CD8 in lymphocytes in liver tissue after transplantation. The number of CD4+ and CD8+ lymphocytes is expressed as mean percentage ± SEM of all lymphocytes (n=10).

pressing the nuclear antigen Ki-67. T lymphocytes were identified by CD4 and CD8, B lymphocytes by CD20, NK cells by CD57, and macrophages by CD68 (14).

CD4+ T lymphocytes were located predominantly inside the portal tracts in all biopsies posttransplant. In biopsies of patients without rejection, CD4+ lymphocytes represented 31.6 ± 3.3% of all lymphocytes (**Fig. 3**). During acute rejection, the number of CD4+ cells was significantly higher with 62.6 ± 3.6% (P=0.0031). Treatment of acute rejection with i.v. corticosteroids resulted in a significant reduction of CD4+ cells to 38.3 ± 4.4% (P=0.013), similar to chronic rejection with a percentage of 32.8 ± 8.6% (62.6±3.6% vs. 32.8±8.6%, P=0.033).

Like CD4+ T lymphocytes, proliferating cells were located predominantly inside the portal tracts in biopsies of patients without rejection. Accordingly, the majority of Ki-67+ cells were CD4+ on double staining, with a mean percentage of 90.0 ± 3.0% (**Fig. 4**). A similar distribution of CD4+ T lymphocytes was observed during acute rejection, with 96.0 ± 1.3% of proliferating cells positive for CD4 (**Fig. 5**). Treatment of acute rejection with corticosteroids did not change the distribution of cells, but significantly reduced the percentage of CD4+ T lymphocytes in cell cycle to 87.8 ± 1.6% (P=0.029). During chronic rejection, most of the CD4+ T lymphocytes remained located inside the portal tracts, but the percentage of CD4+ proliferating cells (75.5±2.5%) was significantly lower compared with acute rejection (P=0.0054) or no rejection (P=0.034).

In contrast to CD4+ T lymphocytes, CD8+ T cells were situated predominantly within the liver parenchyma, with only a minority inside the portal tracts

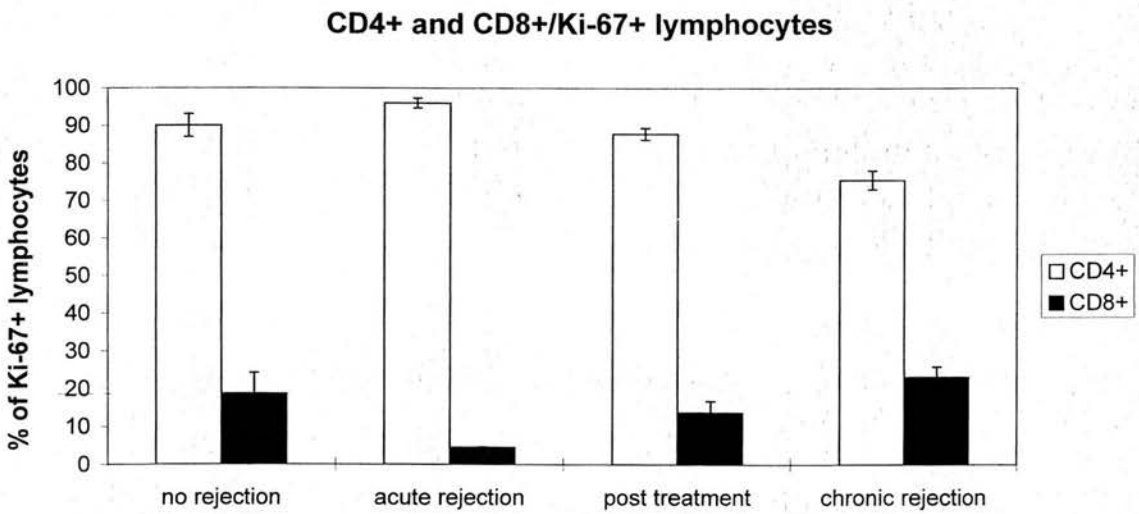

Figure 4. Expression of CD4 and CD8 in proliferating lymphocytes in liver tissue after transplantation. The number of CD4+ and CD8+ T lymphocytes is expressed as mean percentage ± SEM of all Ki-67+ cells (n=10).

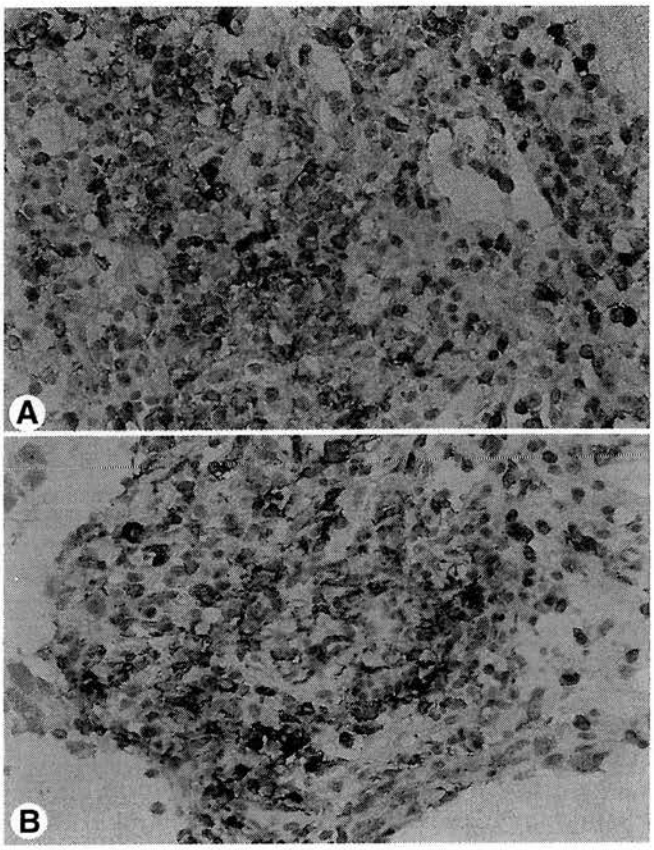

Figure 5. Double staining of CD4+, Ki-67+ and CD8+, Ki-67+ T lymphocytes in liver tissue after transplantation (brown staining: CD4, CD8; red staining: Ki-67; blue staining: hematoxylin). *A)* Predominance of proliferating CD4+ T lymphocytes in portal tracts posttransplant; *B)* minority of proliferating CD8+ T lymphocytes in portal tracts posttransplant.

in all tissues posttransplant. In biopsies of patients without rejection, CD8+ lymphocytes represented 50.5 ± 10.4% of all lymphocytes, with no significant changes to the percentage during acute or chronic rejection (Fig. 3). In addition to CD4+ T lymphocytes, only CD8+ T lymphocytes were occasionally positive for the nuclear antigen Ki-67 on double staining (Fig. 4 and Fig. 5). No significant changes were detected in the percentage of CD8+ proliferating lymphocytes between the four groups of biopsies.

CD20+ B lymphocytes were occasionally present within some but not all of the portal tracts, accounting for less than 5% of all lymphocytes. Positive double staining of CD20, CD57, or CD68 on Ki-67+ cells was not observed.

Proliferating T lymphocytes are both naive and memory cells

To establish whether the proliferating CD4+ T lymphocytes were of the naive or memory phenotype, we used the double staining method to examine proliferating lymphocytes for their expression of the two isoforms, CD45RA (naive lymphocytes) and CD45RO (memory lymphocytes), of the leukocyte common antigen family (LCA, CD45). The number of naive and memory T cells was expressed as the percentage of all lymphocytes that were either CD45RA+ or CD45RO+.

In all biopsies, CD45RA+ lymphocytes were predominantly located inside the portal tracts. During acute rejection, naive lymphocytes (**Fig. 6**) increased significantly compared with patients without rejection (38.9±2.3% vs. 25.3±1.7%, P=0.018). However, treatment with corticosteroids significantly reduced this number (38.9±2.3% vs. 29.8±0.3, P=0.034). There was no significant difference in the percentage of naive lymphocytes during acute compared with chronic rejection.

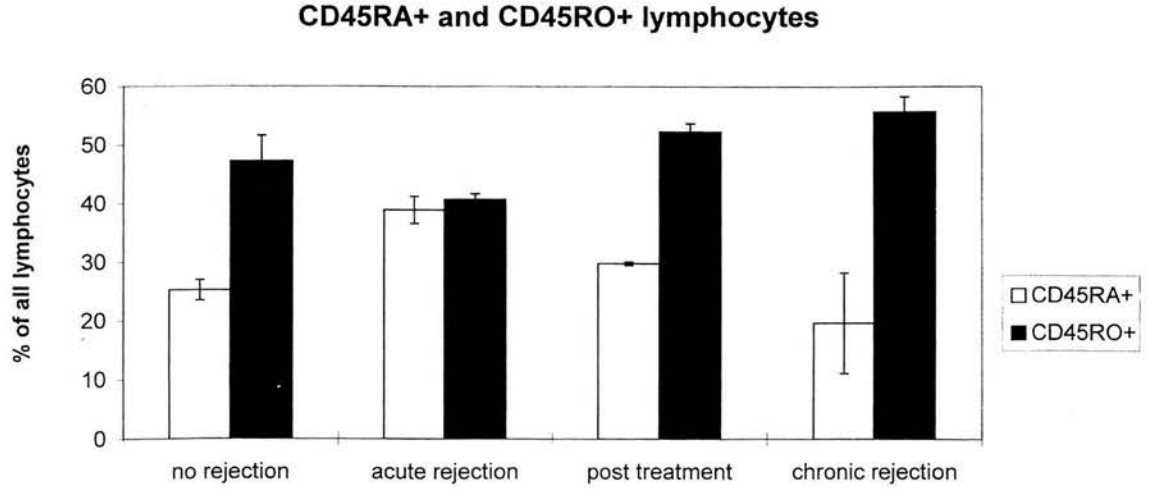

Figure 6. Expression of CD45RA and CD45RO in lymphocytes in liver tissue after transplantation. The number of CD45RA+ and CD45RO+ lymphocytes is expressed as mean percentage ± SEM of all lymphocytes (n=10).

Of the proliferating lymphocytes (**Fig. 7** and **Fig. 8**), CD45RA+ cells represented 40.9 ± 3.3% and 47.3 ± 3.3% in tissues of patients without rejection or with acute rejection, respectively. Steroid treatment of acute rejection did not change this percentage, but during chronic rejection (16.7±3.2%) it was significantly lower compared with acute (P=0.0069) and no rejection (P=0.013).

In all biopsies posttransplant, CD45RO+ lymphocytes were present in the parenchyma throughout the liver lobules, but the majority were situated inside the portal areas. No significant difference was observed in the percentage of CD45RO+ cells (Fig. 6) from biopsies of patients without rejection and those with acute rejection, but the percentage increased significantly after i.v. treatment with corticosteroids (52.3±1.4% vs. 40.7±0.9%, P=0.0064). The percentage was significantly higher during chronic rejection compared with acute rejection (55.7±2.7% vs. 40.7±0.9%, P=0.034).

CD45RO+ lymphocytes represented 53.8 ± 0.2% of Ki-67+ proliferating cells (Fig. 7 and Fig. 8) in tissue from patients without rejection. There was no significant change during acute rejection; during chronic rejection, the percentage of memory lymphocytes were 80.3 ± 4.4%, which was significantly higher than in biopsies from patients with acute rejection (55.7±1.5%, P=0.033) and without rejection (53.8±0.2%, P=0.026).

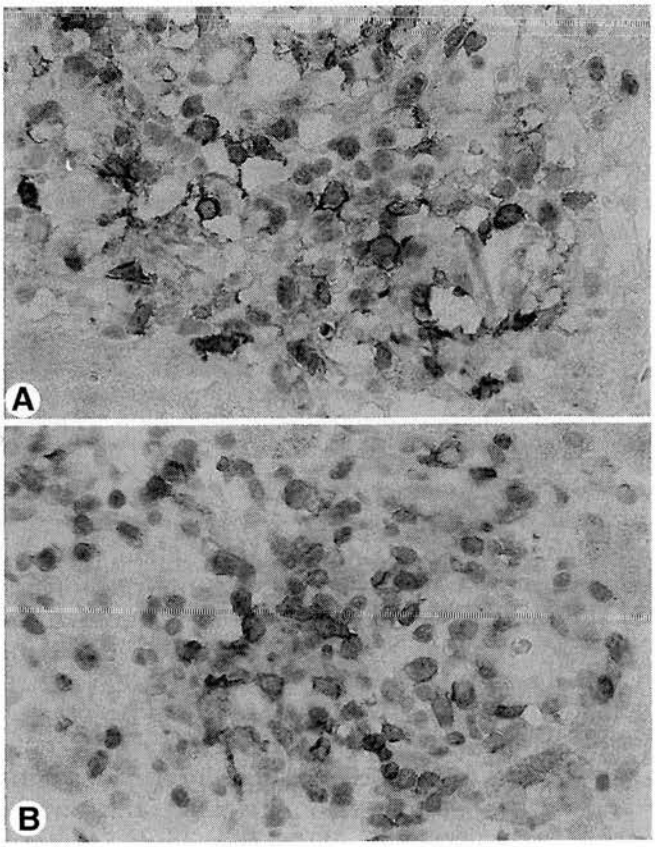

Figure 8. Double staining of CD45RA+, Ki-67+ and CD45RO+, Ki-67+ lymphocytes in liver tissue after transplantation (brown staining: CD45RA, CD45RO; red staining: Ki-67; blue staining: hematoxylin): proliferation of *A)* naive (CD45RA+) and *B)* memory (CD45RO+) lymphocytes posttransplant.

DISCUSSION

Our results indicate two findings that might be important for the current view of the mechanism of allograft rejection. First, we were able to show that proliferation (assessed by Ki-67) of mononuclear leukocytes inside the human liver allograft was a prominent feature of rejection, suggesting that a significant proportion of the 'inflammatory infiltrate' is due to

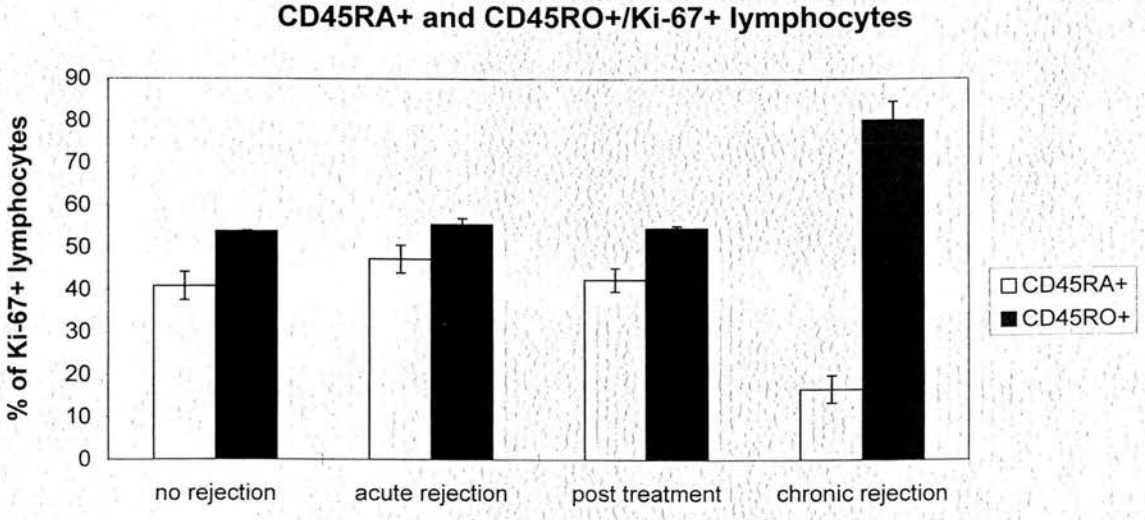

Figure 7. Expression of CD45RA and CD45RO in proliferating lymphocytes in liver tissue after transplantation. The number of CD45RA+ and CD45RO+ lymphocytes is expressed as mean percentage ± SEM of all Ki-67+ lymphocytes (n=10).

local expansion rather than migration. Second, leukocytes in cell cycle (as assessed by double staining) were predominantly CD4+, CD45RA+, and CD4+, CD45RO+ T lymphocytes—both naive and memory T lymphocytes. This would implicate that the primary immune response with proliferation and differentiation of naive T lymphocytes might not be restricted to lymphatic tissue, but could also occur within the allograft, at least during liver graft rejection.

The current concept of homing and migration patterns of lymphocyte subsets (4, 15) suggests that naive T lymphocytes recirculate preferentially through lymphoid tissue, which provides the necessary microenvironment for antigen stimulation. Naive lymphocytes are able to enter lymph nodes through high endothelial venules, distinct from other microvessel endothelia (16). In contrast, T lymphocytes in nonlymphatic tissue, e.g., during inflammation, are predominantly of the memory and 'effector' phenotype, due to their ability of transendothelial migration (5, 17). Their rapid increase in number during inflammation has been accounted for by migration rather than local expansion.

Our findings indicate exactly the opposite occurrence during hepatic allograft rejection, with a substantial number of naive lymphocytes residing in the graft after transplantation and proliferation of both naive and memory T lymphocytes within the graft during the immune response. This could suggest a general difference in allograft rejection from other forms of inflammation, because naive T lymphocytes are able to migrate into the graft and there are sufficient signals to activate them. Indeed, results of our own group show proliferation of less than 10% of mononuclear leukocytes in liver biopsies of patients with primary biliary cirrhosis (unpublished data). Moreover, early reports in kidney transplantation have already demonstrated proliferation of lymphocytes and, occasionally, other lymphoid cells such as monocytes within the allograft (18–20), linking specifically the lymphocyte proliferation to rejection. In other models of heart and liver rejection, aggregates of dendritic cells and lymphoblasts were shown within the graft (21, 22), indicating in situ stimulation of the lymphocytes. In contrast, only newer studies in kidney and heart allograft rejection were able to describe the ratio between naive and memory T cells. In both organs, memory T lymphocytes increased during rejection and data on proliferation were not provided (23, 24).

On the other hand, recent publications have described intrahepatic subpopulations of naive T lymphocytes capable of proliferation and T cell receptor rearrangement (25–27) leading to the hypothesis of extralymphatic T cell development in the liver. Naive T cells seem to be able to migrate into the liver, probably through sinusoidal fenestrations comparable to splenic sinusoidal pores (28). Additional support for intrahepatic T cell development comes from reports linking the liver to oral tolerance, e.g., tolerance induction by injecting antigen-presenting cells into the portal vein in contrast to systemic veins or abrogation of oral tolerance induction by short circuiting the liver (11). These observations attribute properties to the liver usually associated with lymphatic tissue. Intrahepatic T cell depletion might be mediated by apoptosis induced by Fas (CD95, APO-1) (11, 29) or galactin-1, which preferentially causes cell death in CD45RO+ T lymphocytes (30). In contrast, proliferation, which is closely linked to apoptosis in early stages with occasional expression of similar signals including Ki-67 (31), could be stimulated by intrahepatic dendritic cells (32) or endothelial cells (33). The final decision to undergo apoptosis or proliferation would be influenced by additional signals such as the anti-apoptotic protein bcl-2 (34). We recently found high levels of bcl-2 in intrahepatic lymphocytes of patients with liver allograft rejection (35), whereas lymphocytes in liver biopsies of patients without rejection expressed high levels of Fas (36). Intrahepatic T cell development might explain the unique immunological properties of the liver as an allograft, such as inducing tolerance for other subsequently and previously transplanted solid organs comparable to the thymus (37), as well as transferring specific immune memories such as allergies and autoimmune disorders of the donor to the recipient comparable to bone marrow transplantation (38).

We present data that challenge the view that solid organs, and specifically the liver, only represent a target for the immune system after transplantation. Recent publications on transplantation in patients and animal models (including after thymectomy) have led to the hypothesis that the migration of donor passenger leukocytes into recipient lymphoid tissue influences transplantation outcome (8). Our results suggest that the graft itself might be relevant for the development of the immune response. Either all solid organs are partly involved in the immune reaction, providing a location for allorecognition and the subsequent primary lymphocyte activation, or the liver specifically has immunological properties comparable to lymphatic tissue. These hypotheses would add to the current perspective not only on transplantation, but of basic immunology with respect to T lymphocyte-mediated immune responses and tolerance induction. FJ

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