Dynamics of Regulatory T cells in Liver Transplantation

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Dynamiek van Regulatoire T cellen in Levertransplantatie

Proefschrift

ter verkrijging van de graad van doctor aan de
Erasmus Universiteit Rotterdam
op gezag van de
rector magnificus

Prof.dr. S.W.J. Lamberts

en volgens besluit van de College voor Promoties

De openbare verdediging zal plaatsvinden op
woensdag 28 november 2007 om 13:45 uur

door

Ahmet Demirkiran

geboren te Rotterdam

PROMOTIECOMMISSIE

Promotor: Prof.dr. H.W. Tilanus

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General introduction

Chapter 1

Introduction and outline

LIVER TRANSPLANTATION AND TOLERANCE

Liver transplantation has become the only definitive therapy for end stage liver disease. In the early days of liver transplantation, short term patient and graft survival was strongly depending on the surgical technique and the immune system of the recipient. The first successful liver transplantation was performed by Dr. Thomas Starzl in 1967 (1). After the enhancement of the surgical technique with improvement of preservation solutions and operative strategy, adequate anti-rejection treatment was the key factor to further progress. A major step forward was set after the discovery of calcineurin inhibiting immunosuppressive agents: cyclosporine A and later tacrolimus. Cyclosporine was first introduced by Sir Roy Calne and colleagues in 1979 (2), causing a prominent improvement of patient and graft survival. Since then calcineurin inhibitors are universally applied in the immunosuppressive treatment following liver transplantation. Presently, the success of solid-organ transplantation depends on the continuous administration of immunosuppressive agents, which unfortunately are toxic and cause severe complications. These complications include increased risks for opportunistic infections, malignancy, cardiovascular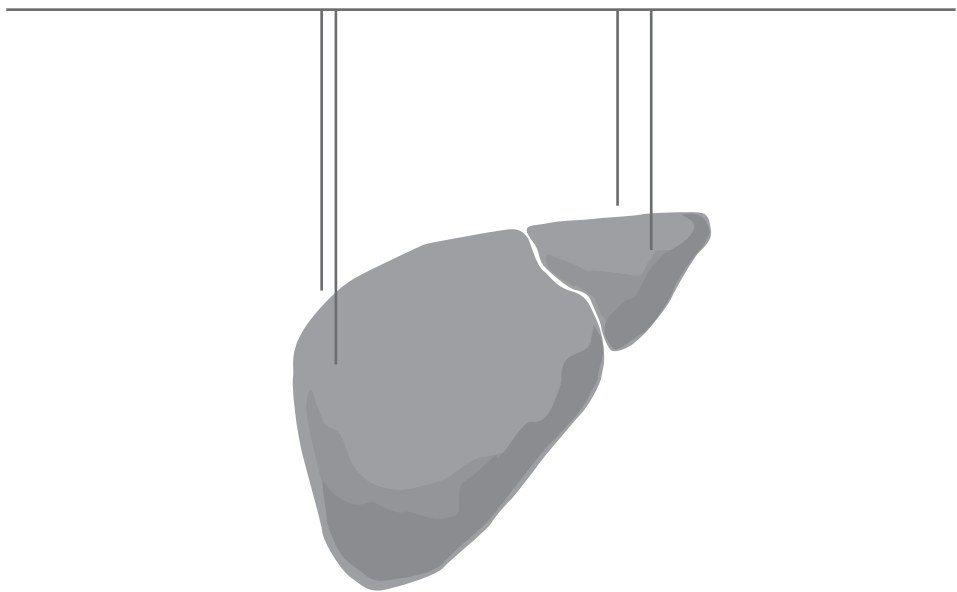 and renal complications as well as a variety of agent-specific side effects. Immunosuppression-related complications are nowadays the most important determinants of long-term patient survival and quality of life (3, 4).

Since the early days of organ transplantation, the immune response against the donor organ has been a major topic of transplantation research. The ultimate goal is the achievement of a state of immunological non-responsiveness against the donor antigens in recipients. This state, also referred to as transplant tolerance, has been reported in a number of transplant patients, who did not comply and stopped using the immunosuppressive drugs. Several studies showed that a subset of patients is tolerant for the graft and theoretically does not require any immunosuppression. In liver transplantation 10 to 30 percent of the recipient have been shown to be tolerant and showed no signs of rejection after immunosuppression withdrawal (5-8). In addition, four out of ten transplant recipients are thought to be over-immunosuppressed, causing unnecessary exposure and severe complications due to side effects. Safe adjustments and weaning of immunosuppression without rejection requires prognostic tests that identify patients who are hypo-responsive or even non-responsive (tolerant) for their graft. So far, no such test exists and therefore all transplant recipients are treated with immunosuppressive agents.

Although the exact mechanisms of transplant tolerance are still not fully understood, several pathways have been described which can contribute to immunological non-responsiveness. These include active immune regulation, chimerism, T-cell and B-cell deletion, anergy and clonal exhaustion. The variety of the named mechanisms illustrates the complexity of understanding transplant tolerance. In the past decade active immune regulation has emerged as an exciting possible candidate to help understand immunological unresponsiveness against

self- and non-self antigens. This thesis focuses on regulatory T cells that have become known as key players in immune regulation and are characterised by their immunosuppressive activity (9, 10). By improving the understanding of active immune regulation, patients who do not mount an immune response against the donor organ may be identified. Subsequently, these patients may be withdrawn from immunosuppressive treatment, as immunosuppressive treatment becomes redundant.

Immune activation and regulation

The immune reaction against donor tissue (antigens), also called the allo-response, is the driving force of graft rejection. Rejection is an inflammatory reaction in the donor organ that involves different cell types. Allo-reactive T cells play a key role in the immune reaction and can be subdivided into two lineages; the helper T cells (CD4^{+}) and the cytotoxic T cells (CD8^{+}). CD4^{+} T cells are known for their central role in the initiation of an immune response and their ability to coordinate the differentiation and effector function of other immune cells, while CD8^{+} T cells are characterized by their cytotoxic activity.

The activation of T cells depends on a cascade of signals. First the interaction of the T cell and the antigen presenting cell: the T-cell receptor (TCR) binds to the major histocompatibility complex (MHC) presenting the antigen. When TCR triggering is accompanied by a co-stimulatory signal, cytokine genes are activated causing production and release of cytokines, an essential element in the course of immune activation. IL-2 is one of the most important activating cytokines causing activation, proliferation and differentiation of T cells. The critical role of IL-2 in mediating an immune response against the donor organ is well demonstrated by the action of immunosuppressive drugs. Cyclosporin A and tacrolimus suppress the allo-response by inhibiting calcineurin signalling, which is required for IL-2 production by T cells. Anti-IL2 receptor blockers, which have helped to further reduce the incidence of acute rejection, prevent binding of IL-2 to its receptor thereby prohibiting downstream signalling. Steroids interfere with cytokine gene activation, including IL-2, and rapamycin inhibits the IL-2 mediated signal transduction pathway. Once adequate activation has occurred, T cells migrate to the donor organ and mediate several effector mechanisms, such as cytotoxicity, delayed-type hypersensitivity and antibody production. These effector mechanisms damage the donor organ and are recognized as acute rejection (11).

Identification of the counterparts of effector T cells, the suppressor or also called regulatory T cells, has inspired transplantation biologists to exploit the role of these cells in controlling allo-responses. Experimental transplantation models have shown an important role for regulatory T cells in the induction and maintenance of transplant tolerance by suppressing allo-reactive T cells (12). Extensive research revealed several subsets of regulatory T cells including CD4^{+} T cells, CD8^{+} T cells (13, 14), CD8^{+}CD28^{-} T cells (15) , NKT cells (16, 17) and CD4^{-} CD8^{-} double

negative T cells (18). Accumulating evidence, however, suggests that immune regulation of allo-antigen-driven immune responses is enriched in the CD4$^+$ T-cell population. As mentioned, CD4$^+$ T cells are crucial in directing and initiating an immune response and therefore this thesis aimed to study CD4$^+$ regulatory T cells in clinical liver transplant recipients, in particular the IL-2 receptor α-chain expressing (CD25$^+$) CD4$^+$ T cells.

Although multiple CD4$^+$ regulatory T cells have been described, including IL-10 producing Tr1 cells and TGF-β producing Th3 cells, the CD4$^+$CD25$^+$ regulatory T cells have emerged as the most potent suppressors of allo-responses. This subset of regulatory T cells is characterized by the expression of CTLA-4 (a co-inhibitory molecule), CD45RO, CD27, CD62L, CD122 and GITR (19-22). All of these markers, however, are not specific for CD4$^+$CD25$^+$ regulatory T cells as they are also expressed on activated or resting T cells. The identification of the nuclear transcription factor Foxp3 in CD4$^+$CD25$^+$ regulatory T cells has facilitated the differentiation of these cells from recently activated or resting T cells (23, 24). Functional studies have shown that CD4$^+$CD25$^+$ regulatory T cells can inhibit CD4$^+$ and CD8$^+$ T cell alloresponses and in experimental models infusion of these cells have been shown to prevent graft rejection (12).

AIM AND OUTLINE OF THE THESIS

Even though an important role of CD4$^+$CD25$^+$ regulatory T cells have been described in experimental models of transplant tolerance, evidence in clinical transplantation is still limited. This thesis aimed to identify and characterize CD4$^+$CD25$^+$ regulatory T cells in circulation and in the liver graft after clinical liver transplantation. Better understanding of the function of this cell subset in relation to allo-reactive T cells may be used as a prognostic tool to identify transplant tolerance and provide tailor-made immunosuppression to individual transplant recipients.

PART I

In chapter two the *in vitro* and *in vivo* effects of immunosuppressive therapy used in solid organ transplantation on the function and survival of CD4$^+$CD25$^+$Foxp3$^+$ regulatory T cells are reviewed. Recent evidence suggests that immunosuppressive therapy not only inhibits effector T cells but also affect regulatory T cells, which may have negative consequences for the induction of transplant tolerance.

PART II

Part two includes three studies involving the hemodynamics of regulatory T cells. In chapter three and four the proportion of CD4$^+$CD25$^+$ regulatory T cells within the helper T cell population was determined in relation to immunosuppressive therapy, to the occurrence of acute rejection and to time after liver transplantation. In chapter five we studied the effect of conversion from a calcineurin inhibitor based immunosuppressive treatment to a mycophenolate mofetil based treatment on regulatory T cells and on calcineurin inhibitor associated side effects.

PART III

In the third part of this thesis the presence of regulatory T cells in the donor liver was studied. In chapter six the phenotype and suppressive capacity of hepatic CD4$^+$CD25$^+$ regulatory T cells, migrated from the liver graft into the perfusate solution at time of transplantation, were investigated. Furthermore, the presence of these donor-derived regulatory T cells in recipient blood was monitored. In chapter seven and eight, intrahepatic regulatory T cells are investigated using liver aspiration biopsy samples taken early and late after transplantation.

PART IV

In chapter nine and ten the results of this thesis are summarized and discussed. The potential use of regulatory T cells and future perspectives are denoted.

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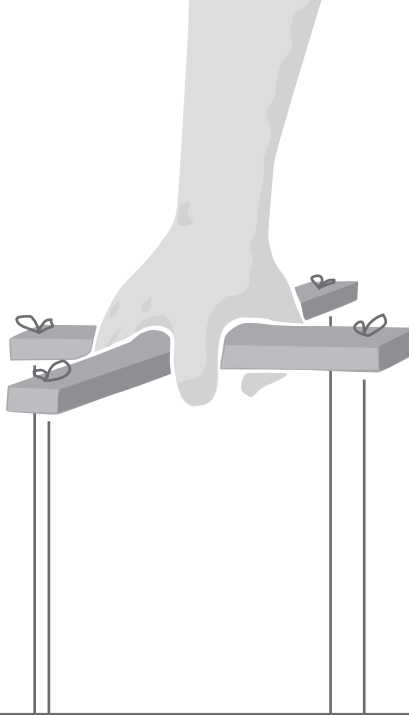
Chapter 2

Impact of immunosuppressive drugs on
CD4$^+$CD25$^+$Foxp3$^+$ regulatory T cells:
Does in vitro evidence translate to the clinical setting?

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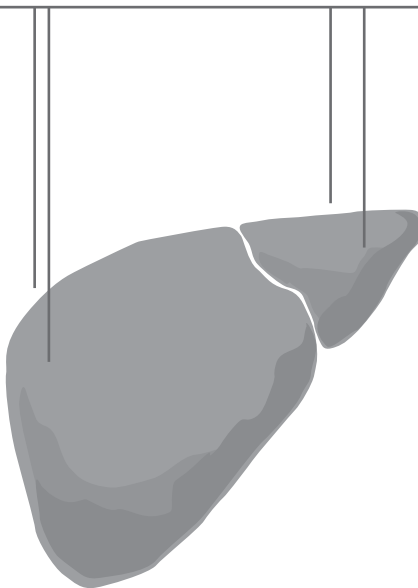
ABSTRACT

The success of solid-organ transplantation mainly depends on the continuous administration of immunosuppressive drugs to prevent rejection. The currently prescribed immunosuppressive medication targets the immune system in a nonspecific fashion, which leads to many debilitating side effects influencing patient- and graft survival. Therefore, it is essential to minimize immunosuppressive therapy. To accomplish this, therapeutic strategies to induce and maintain transplant tolerance have to be developed. One such strategy would be to facilitate the induction of alloantigen specific immune regulation by regulatory T cells (Treg). Results from recent experimental studies indicate that several commonly used immunosuppressive drugs have detrimental effects on the induction and function of Treg while other drugs seem to spare these cells or may even be beneficial. These differential effects may be explained by differences in signaling pathways between Treg and effector T cells. In this review we will discuss whether the effects of immunosuppressive drugs on CD4$^+$CD25$^+$Foxp3$^+$ Treg in vitro translate to the clinical setting. A greater understanding of the impact of immunosuppression on Treg may help to create future opportunities to manipulate the host immune response against alloantigens in order to achieve operational tolerance in transplantation.

INTRODUCTION

Without immunosuppression, transplanted organs are rapidly rejected by the recipients immune system. The current immunosuppressive drugs, effectively reduce the immune response to alloantigens, resulting in a relatively low incidence of acute rejection. Most immunosuppressive drugs target the intracellular signals involved in T-cell activation following antigen presentation (1, 2) as shown in Figure 1. Conventional immunosuppressive drugs include corticosteroids, calcineurin inhibitors (CNI), IL-2 receptor-blocking antibodies, rapamycin and mycophenolate mofetil (MMF) . Characteristics of these drugs are summarized in Table 1. The long-term administration of immunosuppressants leads to many debilitating side effects influencing patient- and graft survival (3). Therefore, it is essential to minimize the use of immunosuppression. To accomplish this, alternative therapeutic strategies to induce and maintain transplant tolerance have to be developed. A possible strategy that is now extensively investigated is the induction and maintenance of transplant tolerance by regulatory T cells (Treg), in particular the CD4$^+$CD25$^+$Foxp3$^+$ Treg. As immunosuppressive drugs affect conventional effector T cells (Teff), they may also affect Treg. This notion has raised concerns about the influence of immunosuppressive drugs on the induction and maintenance of transplant tolerance by Treg and sparked intensive research on this issue.
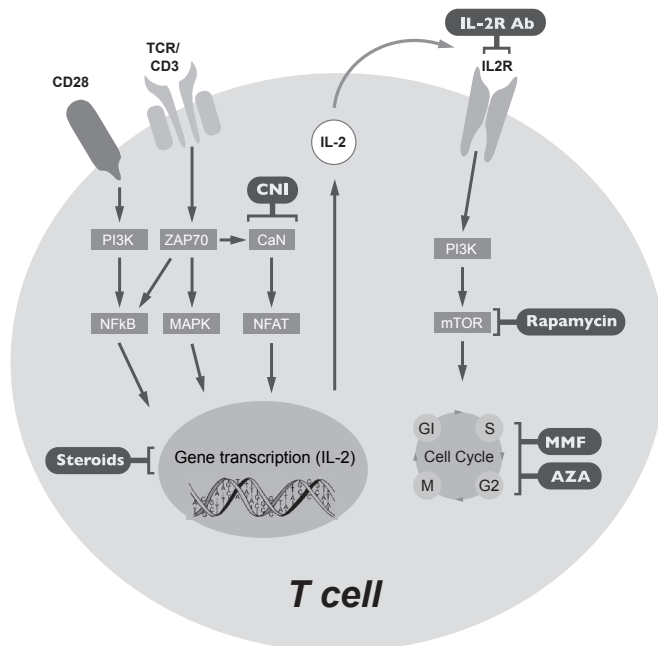
Figure 1. After organ transplantation, the allo-activation of T cells can be suppressed using different immunosuppressive drugs. These drugs intervene with different signaling pathways involved in T-cell activation and proliferation, e.g. by interaction with the IL-2 pathway. Differential effects of immunosuppressive drugs on regulatory T cells (Treg) and effector T cells (Teff) may be explained by differences in signal transduction pathways. For example IL-2 does not primarily activate PI3K in Treg as in Teff.

There is now convincing evidence that activation of Treg is governed by distinct signaling pathways as compared to Teff. These differences may explain how immunosuppressants have distinct effects on Treg. In the following paragraphs we will discuss experimental as well as clinical studies that investigated the impact of immunosuppression on the development, proliferation, survival and function of Treg, and how this may affect tolerance after organ transplantation.

Drug	Mechanism of action	Specific effects on effector T cells	Specific effects on regulatory T cells
Corticosteroids	Cytosolic receptors, Heat shock proteins, interfere with transcription factors	Induces apoptosis, Inhibit cytokine production, interfere with migration	Stimulate Foxp3 expression, preserve suppressive activity and cell survival
Tacrolimus (FK506)	Binds FKBP-12 Inhibits calcineurin	Inhibits cytokine production, inhibits T-cell activation and proliferation	Inhibit Foxp3 expression and possible suppressor function
Cyclosporin A (CsA)	Binds cyclophilins Inhibits calcineurin	Inhibits cytokine production, inhibits T-cell activation and proliferation	Inhibit Foxp3 expression and possible suppressor function
CD25 mAbs (daclizumab/ basiliximab)	Binds CD25 as target epitope	Inhibits IL-2 dependent proliferation	Insufficient data
Rapamycin and derivates (sirolimus & everolimus)	Binds FKBP-12 Inhibits mammalian target of rapamycin (mTOR) Blocks p70 S6 kinase	Blocks T-cell proliferation	Does not appear to effect survival and suppressor function
Mycophenolate mofetil (MMF)	Inosine monophosphate dehydrogenase inhibitor (with high affinity for type 2)	Selective blockade of lymphocyte proliferation	Does not appear to effect survival and suppressor function

Table 1. Immunosuppressive drugs and their effect on T(reg) cells.

Distinct signaling pathways in Treg and Teff
To understand the differential effects of immunosuppression on Treg it is important to know what distinguishes these cells from Teff. It has been established that the X chromosome-encoded transcription factor Foxp3 is highly expressed by Treg and essential for their development in the thymus. Furthermore, transgenic Foxp3 can convey suppressive function to naïve T cells (4, 5). Though Foxp3 possitivity can be observed in activated human Teff, this expression is transient and does not seem to induce a Treg phenotype (6). Mutations in the Foxp3 gene in humans and mice results in an aggressive multi-organ autoimmune disease (7, 8).

Recent transcriptional profiling studies of mouse Treg showed that at least 700 genes are up or down regulated by Foxp3 (9, 10). The IL-2 receptor α-chain (CD25) is one of the upregulated genes, which results in the characteristic high expression of CD25 on Treg. Genes that are downregulated by Foxp3 include IL-2 and T-cell receptor (TCR) signaling molecules Zap70 and ITK. Indeed, it has been shown that Foxp3+ Treg and Teff have a distinct response to TCR triggering (11, 12) e.g., Treg isolated from human or mice do not proliferate in vitro upon appropriate activation nor produce IL-2, IL-4 and Interferon (IFN)-γ cytokines (11). Moreover, signaling pathways involved in reorganization of the actin cytoskeleton (e.g. VAV), which is important for sustained signaling by the TCR, are shutdown in Treg (13). As a consequence of the suppression of IL-2 production in Treg, their development and survival strongly depends on the presence of exogenous IL-2 (14, 15).

It has been suggested that specifically in Treg, Foxp3 inhibits expression of IL-2 and other genes by interfering with transcriptional activation of Nuclear Factor of Activated T cells (NFAT). Indeed, in vitro experiments revealed that genes that were activated upon stimulation of Foxp3 negative cells and suppressed in Foxp3 positive cells were activated in a calcineurin dependent manner (i.e. inhibited by CNI) (9). This is consistent with the notion that NFAT is involved in the activation of these genes. Therefore, CNI may profoundly affect Treg cell programming by direct interference with NFAT:Foxp3 interactions (16, 17).

Another relevant difference in signal transduction between Treg and Teff is the finding that IL-2 primarily activates JAK/STAT signaling in Treg rather than the PhosphoInositide 3-Kinase (PI3K) signaling pathway (18-20). Interestingly, activation of the signaling pathway leading from PI3K to mammalian target of rapamycin (mTOR) was almost completely absent in both primary Treg and Foxp3-transgenic CD4+ T cells upon IL-2 receptor signaling (11). As some immunosuppressants interfere with IL-2 signaling in Teff, they will not prevent PI3K-signaling in Treg.

Effects of immunosuppressive drugs on Treg: Experimental evidence

The possibility to culture and expand (alloantigen-specific) Treg (21-27), has opened up the possibility to study the effect of immunosuppressive drugs on these cells in vitro. Although not all CD4+CD25+Foxp3+ Treg have the same origin, they may all be important in transplantation tolerance. Most experiments with Treg and immunosuppressive medication focused on the natural occurring Treg, which is generated in the thymus. The adaptive or inducible Treg are thought to be either continuously generated from responding memory Teff in the periphery or to originate directly from the thymus with an naïve phenotype (CD45RA+) (28, 29).

Corticosteroids

Corticoteroids are pleiotropic hormones that are used for their potent anti-inflammatory and immunomodulatory action. The most commonly used corticosteroids in transplantation are prednisone, prednisolone and methylprednisolone. Corticoteroids inhibit the action of trans-

cription factors, like NF-κB and AP-1 that are involved in transcription of many cytokine and chemokine genes including IL-2, TNF-α and IFN-γ. It is known that CD4+CD25+Foxp3+ T cells highly express the glucocorticoid receptor as well as glucocorticoid-induced TNF receptor (GITR), a potent T-cell co-stimulatory receptor and regulator of Treg function (30). The activity of GITR has been implicated in peripheral tolerance since inhibition or a deficiency of GITR increases T-cell proliferation by abrogating the suppressive function of Treg (31-34). The first direct evidence that steroids affect Treg came from the observation that the female sex hormone, estrogen, upregulates Foxp3 expression in mice, both in vitro and in vivo (35). Similar results were reported for the synthetic corticosteroid, dexamethasone, which induced Foxp3 expression in short and long-term T-cell cultures, while preserving the suppressive capacity of Treg (36, 37). Furthermore, CD4+CD25+ T cells seemed resistant to dexamethasone-induced T-cell apoptosis (30). In mice, short-term simultaneous administration of dexamethasone and IL-2 expanded Foxp3+ Treg in peripheral lymphoid tissues (38). This treatment improved the suppressive capacity of splenic Treg in such a way that they were able to prevent the onset of autoimmune disease. Taken together, experimental evidence does not indicate that corticosteroids have a negative impact on Treg but rather improve the survival and function of Treg. One could even speculate whether this effect on Treg may account for some of the anti-inflammatory and immunosuppressive efficacy of steroids.

Calcineurin inhibitors

The CNI, Cyclosporin A (CsA) and Tacrolimus (Tacro), are potent inhibitors of the phosphatase calcineurin, which is essential for T-cell activation. By inhibiting calcineurin these drugs suppress the production of IL-2 and related cytokines through prevention of downstream activation of the transcription factor, NFAT. As discussed earlier, NFAT cooperates with Foxp3 to control Treg activation (17, 39). There are a few in vitro studies describing the effect of CNI on Treg. Baan et al. showed that in a Mixed Leukocyte Reaction (MLR) the induction of Foxp3 mRNA was inhibited by both Tacro and CsA (40). This was confirmed by other studies which observed decreased Foxp3 mRNA and protein (41, 42) and a loss of the highly suppressive CD27+ Treg subset in cultures containing CsA (43). These later studies report contradicting effects of CsA on the suppressive function of Treg, one observing no effect with human Treg (43) and the other finding less suppression with mouse Treg (41).

There are now recent in vivo data that show a negative effect of CNI on Treg. Treatment of mice with CsA compromised not only the thymic generation of Treg but also resulted in a sharp reduction of Treg in peripheral immune compartments (44). In a mouse bone marrow transplantation model, CsA administration inhibited Treg mediated suppression which was associated with reduced IL-2 production (41). Exogenous IL-2 was shown to overturn the reduced suppressive function of Treg by CsA. Together, these data suggest that CNI are not beneficial for Treg, but rather seem detrimental to survival and function of these cell. This further underlines the importance of IL-2 for Treg homeostasis.

IL-2 Receptor-blocking antibodies

The chimeric monoclonal antibody (mAb) basiliximab and humanized mAb daclizumab are therapeutic antibodies directed against the α-chain of the IL-2 receptor (CD25). They directly interfere with receptor signaling by inhibiting the association and subsequent phosphorylation of the IL-2 receptor beta- and gamma-chains, induced by binding of IL-2. Few in vitro studies investigated the effect of CD25 mAb on Treg. One study showed inhibition of Foxp3 mRNA induction by CD25 mAb daclizumab in allostimulated PBMC (40) and another study showed downregulated Foxp3 staining after culture with daclizumab (45). However, in direct co-incubations of Treg and Teff, CD25 antibodies did not interfere in the suppressive activities of CD4$^+$CD25$^+$ Treg (46). One recent study in mice did observe that particular CD25 antibodies reduced the percentage of Foxp3$^+$ T cells within the CD4 fraction in vivo (47), whereas one earlier study suggest that anti-CD25 treatment does not reduce or deplete Treg but rather inactivate their suppressive function (48). Despite the convincing evidence that IL-2 is critical for Treg survival and function, no firm conclusions can be drawn from present studies on the effect of CD25 neutralizing antibodies on these cells.

Rapamycin

Rapamycin-derivatives, sirolimus and everolimus, are potent inhibitors of the IL-2 signaling pathway. They exert their effect at the level of mTOR, thereby preventing the transgression from G1 to S-phase (49). There are many studies that have investigated the impact of rapamycin on Treg. Initial in vitro studies did not observe an effect of rapamycin on Foxp3 expression during allogenic stimulation, both for human (40) and mouse (41). This latter study also showed no effect of rapamycin on the suppressive capacity of Treg. Indeed two other studies confirmed that rapamycin does not interfere with the suppressive activity of CD4$^+$CD25$^+$ Treg on allogenic or polyclonal stimulated Teff (43, 46). In this setting, CD4$^+$CD25$^+$ Treg appeared more resistant to the pro-apoptotic effect of rapamycin than CD25$^-$ T cells (46). This observation was confirmed by culture experiments with rapamycin, were selective expansion of murine and human CD4$^+$CD25$^+$Foxp3$^+$ Treg was observed, while at the same time killing or at least preventing expansion of Teff (43, 50-52). These differential effects of rapamycin on Treg and Teff seem paradoxical, given the critical dependency of Treg on IL-2 signals for survival. However, the fact that in Treg IL-2 primarily signals via the JAK/STAT pathway, rather than via the PI3K/mTOR pathway that is dominate in Teff, may help to explain these differences.

Also in vivo, there is evidence that rapamycin treatment has favorable effects on Treg. In a study with mice by Battaglia et al. CD4$^+$CD25$^+$Foxp3$^+$ Treg expanded ex vivo in the presence of rapamycin, prevented rejection of β-islet transplants in vivo (53). Furthermore, a recent study showed that rapamycin induces de novo expression of Foxp3 in murine alloantigen specific T cells dose dependently which appeared to be TGF-β1 dependent (42). Since rapamycin can induce the expression of TGF-β1, this may be an important mechanism contributing to the development of antigen-specific Treg (54). Interestingly a recent study suggests that rapamycin can induce regulatory functions in conventional CD4$^+$ T cells in culture (55). Furthermore,

evidence suggests that rapamycin conditioned dendritic cells are poor stimulators of allogenic T cells, but enrich for antigen specific Treg that can prolong cardiac graft survival in mice (56). In conclusion, there is substantial evidence that rapamycin favors Treg survival and function and, by suppressing Teff cells, tipping the balance from an aggressive towards a more protective type of alloimmune response.

Mycophenolate mofetil

MMF is a pro-drug of mycophenolic acid (MPA), an inhibitor of inosine monophosphate dehydrogenase. This is the rate-limiting enzyme in de novo synthesis of guanosine nucleotides and T cells in particular are dependent on this pathway for cell division. MMF suppresses T-lymphocytic responses to allogeneic cells and other antigens (57). MMF has been shown to decrease the expression of CD25 on stimulated T cells in a dose dependent fashion (58, 59). There is only one study that specifically described the effect of MMF on Treg in vitro (41). MPA did not alter the expression of Foxp3 or affect the suppressive capacity of Treg. Therefore Treg may still be capable to function normally in the presence of MMF. Unpublished results from our laboratory support this, since MMF did not alter Treg function and their expression of Foxp3 in MLR.

Effects of immunosuppressive drugs on Treg: The clinical evidence

As discussed in previous paragraphs, there is evidence that some of the most widely used immunosuppressive drugs in contemporary transplantation medicine have clear and distinct effects on the survival and function of CD4$^+$CD25$^+$Foxp3$^+$ Treg. These effects are now supported by the knowledge that Treg differ in their TCR and cytokine signaling response from Teff. However, what remains to be demonstrated is how immunosuppression affects the balance between immunoreactive and immunoregulatory cells in transplant recipients. In vitro studies and experimental transplant models showed that immunosuppression can affect Treg by interfering with their function, survival and expansion. In the next paragraphs we will review the literature on Treg homeostasis and allosuppressive activity in the clinical setting and discuss the limitation of our current knowledge.

There is a general observation that the percentage of circulating CD4$^+$CD25$^+$ and CD4$^+$Foxp3$^+$ cells drops significantly in the months and years following transplantation (60, 61). Not only peripheral blood levels of Treg are reduced, as indicated in a recent study, also lower Foxp3 mRNA and positive T cells were found in non-inflamed colon tissue from liver transplant recipients on triple therapy compared to healthy controls (62). These effects cannot only be attributed to the effect of immunosuppression, since many clinical parameters change after transplantation as well. However, the observation that operationally tolerant liver transplant recipients display a significantly increased proportion of CD4$^+$CD25^{high} cells compared to recipients on immunosuppression, may support this suggestion (63, 64). On the contrary, the increased Treg proportions may also reflect the operational tolerant state of these patients and not result from the absence of immunosuppression.

One of the first clinical studies that addressed the effect of specific immunosuppressive drugs on Treg was done by Salama et al., identifying CD4$^+$CD25$^+$ Treg from kidney transplant recipients that can suppress alloantigen-specific responses (65). Despite induction therapy with an anti-IL-2 receptor antibody, CD4$^+$CD25$^+$ Treg could still be generated, suggesting no long-term effect of anti-IL-2 receptor antibody on Treg induction. Also, more recent studies suggest that anti-IL-2 receptor antibody treatment does not affect the number or function of Treg in renal transplant recipients (45). During the first three months after transplantation, when the level of antibody is still high, a decreased percentage of CD4$^+$CD25$^+$Foxp3$^+$ cells was observed without an equivalent increase in CD4$^+$Foxp3$^+$ cells. In contrast, in liver transplantation recipients the CD4$^+$Foxp3$^+$ T-cell fraction was not affected after conversion from CNI monotherapy to MMF with a single dose of daclizumab, while expression of CD25 was significantly decreased (66). These differences may be due to the early intensive immunosuppressive treatment following kidney Tx (quadruple treatment) versus the relative low immunosuppression in liver Tx (monotherapy). Hence, the effect of combination therapies, frequently applied in the clinic, on the development of tolerance should be explored.

CNI versus rapamycine or MMF

Also in the clinical setting there is some evidence that IL-2 is a critical regulator of the homeostasis of Treg. In cancer patients treated with exogenous recombinant IL-2, the frequency of CD4$^+$Foxp3$^+$ Treg increases during therapy (67, 68). Now several retrospective studies compared patients receiving CNI-based immunosuppression with those receiving rapamycin or MMF. In a study by Segundo et al., kidney transplant patients receiving CNI maintenance treatment had a significantly lower percentage of peripheral blood CD4$^+$CD25$^+$ and CD4$^+$CD25^{high} T cells compared to patients receiving rapamycin. Functionally, CD4$^+$CD25$^+$ cells from rapamycin treated recipients were shown to suppress the direct immune response, but were not compared to Treg from CNI treated recipients (69). Although in this study the percentage of CD4$^+$Foxp3$^+$ cells was not shown, these findings may suggest a shifted balance between immune reactive and regulatory cells by either CNI or rapamycin. Notwithstanding that, CD4$^+$CD25$^+$ cells from CNI treated kidney transplant patients have suppressive capacities (70).

Following Campath-1H induction therapy sirolimus treated recipients had higher proportions of CD4$^+$CD25^{high} cells compared to patients on CsA (71), confirming the findings of Segundo et al. The increased proportions reported by Noris et al., however, seem overstated, as CD25 expression on all CD4$^+$ cells is increased and the determination of CD25 high, intermediate and low positive cells by subjective gating differed between CsA and sirolimus treated recipients. In vitro depletion of CD4$^+$CD25^{high} cells showed an increase in IFN-γ production by T cells of sirolimus-treated recipients while no changes were seen in the CsA group. This suggests active regulation by Treg in the sirolimus group whereas the IFN-γ production by Teff from CsA treated recipients remained unchanged. Whether the increased proportion of CD4$^+$CD25^{high} T cells in rapamycin treated recipients contributes to a more tolerogenic immune response towards the graft remains to be determined.

In lung transplantation, in contrast to the above mentioned studies, Meloni et al reported that the absolute number of CD4$^+$CD25^{bright} Treg, rather then the percentage of Treg within the T-cell fraction, was not affected by CNI therapy (72). In stable MMF treated kidney transplant patients the measured donor-specific non-responsiveness was mediated by CD4$^+$CD25$^+$ cells (73). However, we have to be aware that almost all transplant patients included in these studies were on double or triple immunosuppression therapy, including corticosteroids in all patients, which complicates the interpretation of their data.

Conclusion: Does in vitro evidence translate to the clinical setting?

Increasing evidence show that immunosuppressive drugs have a differential effect on Teff and Treg. In this context, several immunosuppressive agents have an effect on the number and/or function of Treg. Furthermore, experimental data suggest that the use of specific immuno-suppression can be an essential component of strategies to induce and maintain transplant tolerance by Treg. Indeed, the few results from clinical studies support the distinct effects of various immunosuppressive regimens on Treg, but so far the consequence on clinical tolerance remains unknown. Therefore, the effects of immunosuppressive drugs on Teff and Treg should be further analyzed to extent the limited knowledge we have now.

ACKNOWLEDGEMENTS

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Part II

Regulatory T cells in peripheral blood
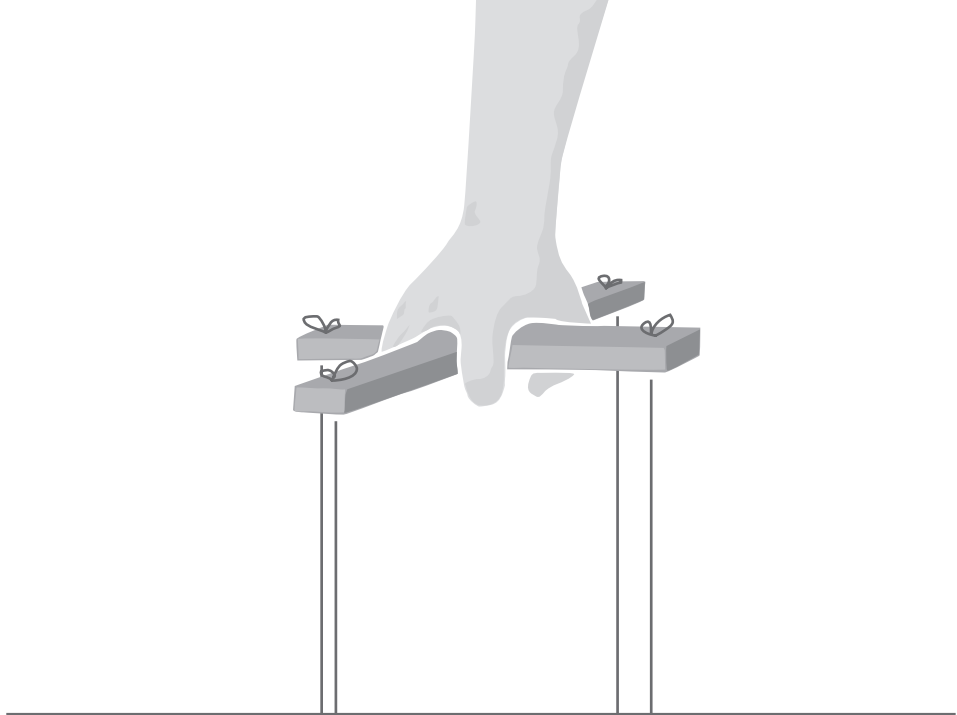
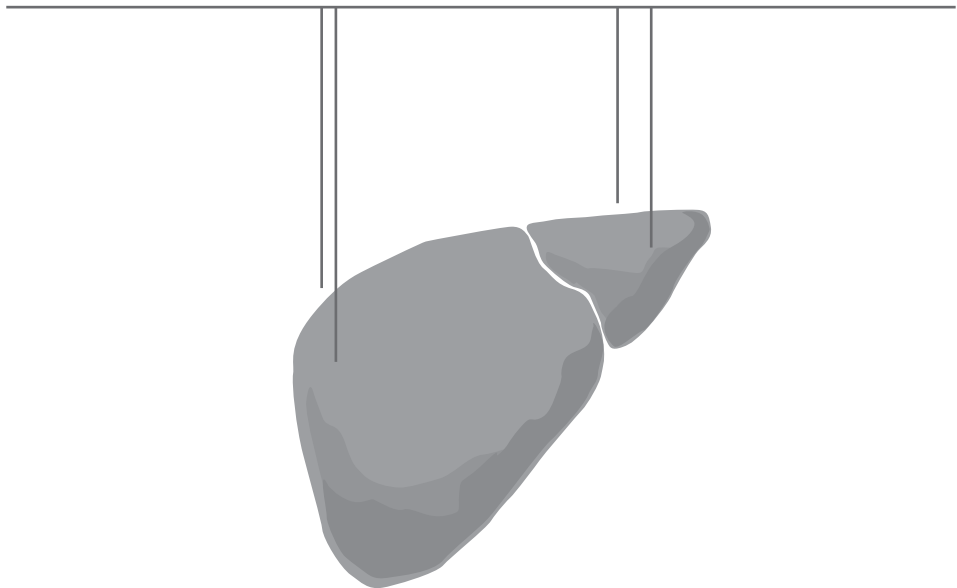# Chapter 3

Decrease of CD4$^+$CD25$^+$ T cells in peripheral blood after liver transplantation: association with immunosuppression

Ahmet Demirkiran, Alice Kok, Jaap Kwekkeboom, Herold J. Metselaar,
Hugo W. Tilanus and Luc J.W. van der Laan

ABSTRACT

CD25 (IL-2 receptor α-chain) marks a population of CD4 positive T cells with a suppressor phenotype. These CD4+CD25+ regulatory T cells can suppress both effector T cells and antigen-presenting cells and have been identified as a principle regulator of tolerance in experimental transplantation models. In the setting of human liver transplantation, however, little is known about the dynamics of these cells in relation to rejection, tolerance and immunosuppression. In the current study we determined CD4+CD25+ T cell in blood of liver transplant recipients using flow cytometry and investigated a possible link with immunosuppressive therapy. Peripheral blood mononuclear cells (PBMC) of 27 liver transplant patients (pre-and 12 months post-transplantation) and 16 healthy controls included. We found that the percentages of CD25+ cells within the CD4+ T-cell population was significantly reduced in patients at one year after transplantation. Also the total percentage of CD4 positive T cells declined significantly within this period, making the absolute reduction of regulatory T cells after transplantation even more profound. Comparing PBMC samples of patients and healthy controls revealed an increased percentage of CD4+ T cells in the patients before transplantation, probably related to the chronic liver illness. The reduction in CD4+CD25+ T cells after transplantation was similar for different immunosuppression regimens. All patients, however, received calcineurin inhibitors suggesting a possible suppressive effect of this therapy on regulatory T cell levels in peripheral blood. Currently, assays for regulatory T cell activity are used to further support this hypothesis.

INTRODUCTION

The IL-2 receptor α chain, CD25, which is expressed on T cells signals proliferation, differentiation, and survival upon ligation with IL-2. Furthermore, extensive evidence suggests that CD25 marks a population of CD4$^+$ regulatory T cells co-expressing Foxp3, CTLA4 and GITR [1-4]. These CD4$^+$ CD25$^+$ regulatory T cells have been shown to be critically involved in experimental models for transplantation tolerance by actively suppressing the allo-reactive T cell response [5-7]. Even though CD4$^+$CD25$^+$ regulatory T cells have been identified in humans, so far no data have been published on the dynamics and function of these cells in the context of liver transplantation and the role of immunosuppressive medication herein. In the current study we determined the levels of CD4$^+$CD25$^+$ T cells in peripheral blood of liver transplant recipients and a possible link with immunosuppressive regimens was investigated.

MATERIALS AND METHODS

After receiving an appropriate informed consent, peripheral blood was obtained from 27 liver transplant recipients within a month prior to and 12 months after liver transplantation. The induction therapy of immunosuppressive medication included cyclosporine A (CsA) or tacrolimus (TAC) and prednisolon, with some patients (n= 15) receiving IL-2 receptor blocking antibody (Basiliximab). The maintenance therapy consisted of CsA or TAC with or without prednisolon (Pred). Control peripheral blood samples were obtained from 16 healthy volunteers. Peripheral Blood Mononuclear Cells (PBMC) were isolated from heparinized blood by density gradient centrifugation using Ficoll Paque Plus (Amersham Biosciences, Upsala, Sweden). Monoclonal antibodies (Abs) used for flow cytometric analysis were: anti-CD4 PerCp-Cy5.5 (SK3) and -CD25 FITC (2A3), isotype controls IgG-1 PE and IgG-2A FITC Abs, all from Becton Dickinson (BD, San Jose, USA) and anti-CD3-PE (UCTH1) Ab from Immunotech (Marseille, France). After thawing, PBMC were washed twice with PBS containing 0.5% BSA. Cells were incubated for 30 minutes at 4° C with conjugated Abs, washed two times and fixed with 2% paraformaldehyde before flowcytometric analysis on the FACS Calibur (BD, Erembodegem, Belgium). All statistical analyses were performed using SPSS Inc. software (Chicago, USA). Significant differences were tested using either the Mann-Whitney U test or the Wilcoxon paired test.

RESULTS AND DISCUSSION

Before examining the percentage of CD25 positive CD4 T cells, the fraction of CD4 positive T helper cells within the total T cell population in peripheral blood was determined. As shown in Figure 1A, the percentage of CD4$^+$ cells of total CD3$^+$ lymphocytes was found to be elevated (p= 0.015) in patients waiting to receive a liver transplant (average 67.5 ± 3.9 SEM) as compared to healthy controls (56.0 ± 2.6 SEM). This increased percentage of CD4$^+$ T cells may be due to the chronic liver illness. When PBMC samples were examined one year post-transplantation, a significant decrease of the CD4$^+$ T cell frequency was found (average 67.5 vs 50.2, p=0.002).

Next we looked at the percentage of CD25$^+$ T cells within the CD4$^+$ population. There was no significant difference in the percentage of CD4$^+$CD25$^+$ T cells between transplantation patients and healthy controls (not shown). However, in patients a significant reduction of the CD4$^+$CD25$^+$ T cell frequency was observed one year after transplantation (Figure 1B). The drop in CD25 positive cells was observed in more than two-third of all patients. Also a reduction in expression of CTLA4, an additional marker expressed on CD25$^+$ regulatory T cells, was observed after transplantation. Furthermore, using a T cell proliferation assay we confirmed that CD4$^+$CD25$^+$ T cells from patients (both pre- and post-transplantation) are hyporesponsive and can suppress the response of CD4$^+$CD25$^-$ T cells in a dose dependent manner (data not shown).

The reduction in CD4$^+$ T cells, and the even further reduction of CD25 positive cells within this T cell fraction, could be a consequence of immunosuppressive medication. Therefore, different induction and maintenance therapies were compared. As shown in Figure 1C, patients receiving anti-IL2 receptor antibody, Basiliximab, had a similar reduction of CD4$^+$CD25$^+$ cells as compared to patients without Basiliximab-treatment. Also, no significant differences were observed between patients with or without steroid treatment as maintenance therapy. It is important to note, however, that all transplant patients received calcineurin inhibitors CsA or TAC. Calcineurin inhibitors selectively block the expression of the IL2 gene. Evidence suggests that regulatory T cells require low dose IL2 signals for their survival and function (8, 9). Our data suggest that immunosuppressive therapies, in particular the use of calcineurin inhibitors, reduce the numbers of CD4$^+$CD25$^+$ regulatory T cells, at least in peripheral blood. This illustrates the dilemma of immunosuppression: preventing acute rejection by blocking the activation of effector T cells, but also inhibiting the development of donor-specific tolerance by suppressing regulatory T cells.
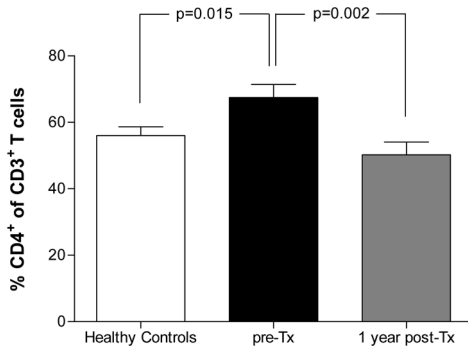
Figure 1A. Percentage of CD4$^+$ T cells in peripheral blood is increased before transplantation and decreases significantly after transplantation (shown is the mean ± SEM).
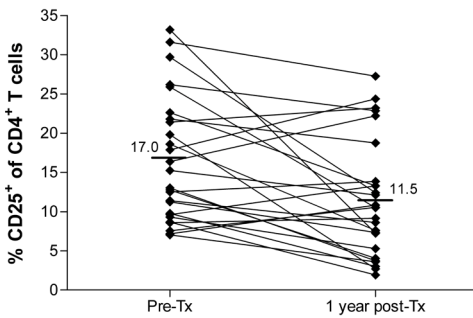
Figure 1B. After liver transplantation CD4$^+$CD25$^+$ T cell frequency significantly reduces (p=0.01, shown are individual CD4$^+$CD25$^+$ T cell frequencies). Bar indicates median.

Figure 1C. Different immune suppressive regimens showed comparable reduction of CD4$^+$CD25$^+$ T cells. Shown is the mean percentage of decrease one year after transplantation.

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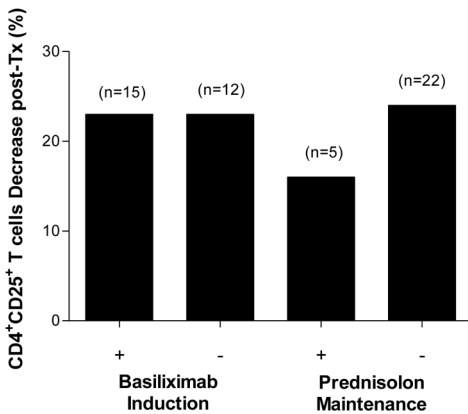
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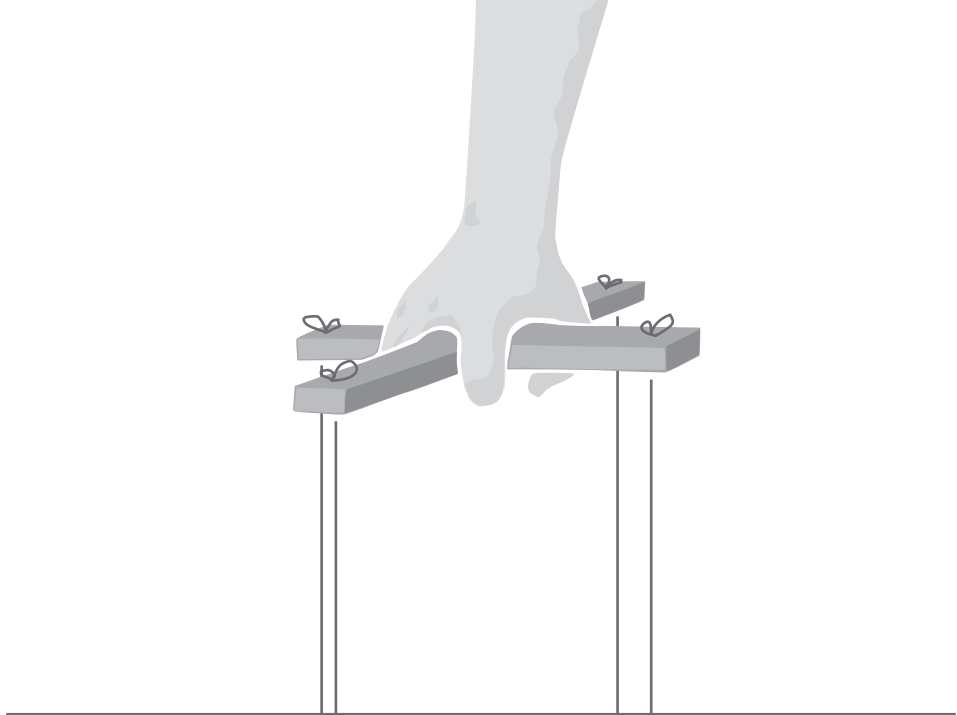
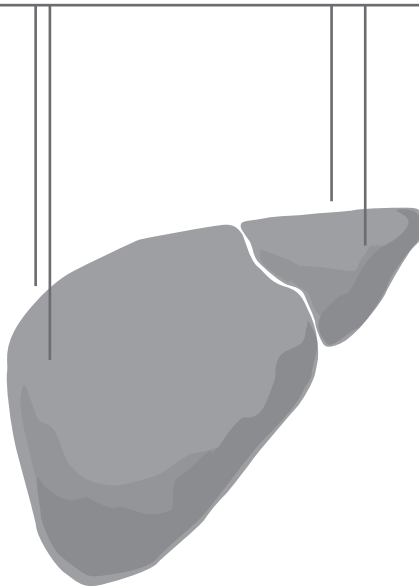# Chapter 4

Low circulating regulatory T cell levels after
acute rejection in liver transplantation

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ABSTRACT

Immune regulatory $CD4^+CD25^+$ T cells play a crucial role in inducing and maintaining allograft tolerance in experimental models of transplantation (Tx). In humans, the effect of Tx and immunosuppression on the function and homeostasis of $CD4^+CD25^+$ regulatory T cells (Treg) is not well characterized. In this study, the frequency of Treg in liver transplant recipients was determined based on flowcytometric analysis of CD4, CD25, CD45RO and CTLA-4 markers and the suppressor activity of Treg was assessed in a mixed-leukocyte-reaction. A link between Treg, acute rejection and immune suppressive treatment was investigated. Liver transplant recipients had significantly higher Treg levels in peripheral blood pre-Tx compared to healthy controls. After Tx, a significant drop in the Treg fraction was observed. This reduction of circulating Treg was transient and was associated with immunosuppression. In recipients who did not develop rejection, a relative recovery of Treg levels was seen within the first year after Tx. Recipients who experienced an episode of steroid-treated acute rejection, however, had sustained low Treg levels. The suppressive activity of $CD4^+CD25^+$ Treg from rejectors, non-rejectors and healthy controls on proliferation and IFN-γ production were indistinguishable. In conclusion, the percentage of $CD4^+CD25^+CD45RO^+CTLA$-4^+ quadruple positive Treg in peripheral blood decrease significantly after liver Tx. Treatment with methylprednisolon during Tx and for acute rejection is associated with low circulating Treg. Despite these quantitative differences between rejectors and non-rejectors, the suppressive quality of $CD4^+CD25^+$ Treg is identical in both groups.

INTRODUCTION

Transplant tolerance, the ultimate goal in solid organ transplantation, occurs more often after transplantation (Tx) of the liver compared to other organs. Cessation of immune suppressive therapy without allograft rejection has been reported to be successful in a considerable proportion of liver transplant recipients (1, 2). The exact mechanisms involved in achieving transplant tolerance remain unknown, although animal models suggest a possible role for regulatory T cells (Treg) (3, 4). In vitro studies of a distinct subset of Treg expressing CD4, the α-chain of the IL-2 receptor (CD25) and the transcription factor Foxp3 showed that these cells do not proliferate upon stimulation, but instead, suppress activation of effector T cells in a cell-contact dependent manner (5-7). Transfer of CD4$^+$CD25$^+$ Treg from animals with long-term surviving allografts to naïve recipients prevents the development of allograft rejection (8). The suppressive capacity of CD4$^+$CD25$^+$ Treg is not only restricted to foreign antigen-driven T-cell responses, but also entails auto-reactive T-cell responses, thereby preventing the development of auto-immune diseases and maintaining peripheral tolerance to self-antigens (9, 10).

In vivo studies on the mechanism of Treg-mediated suppression have shown a functional role for cytotoxic T lymphocyte antigen 4 (CTLA4, CD152), which is constitutively expressed by CD4$^+$CD25$^+$ Treg (11, 12). Interestingly, the majority of suppressive CD4$^+$CD25$^+$ T cells are found in the memory T-cell population, characterized by the expression of CD45RO (13). Jonuleit et al have demonstrated that within the CD4$^+$CD25$^+$ fraction the CD45RO positive cells, and not the CD45RO negative cells, are anergic and have suppressive activity (14). These observations suggest that these cells have already encountered antigens and have acquired the phenotype of highly differentiated CD4$^+$ T cells, distinguishing them from recently activated CD4$^+$ T cells, which do not express CD45RO.

In the context of human liver Tx, the function and dynamics of Treg have not been extensively studied. The aim of this study was to reveal the effect of liver Tx and immune suppression on Treg in peripheral blood of liver transplant recipients. A relationship between the frequency of Treg and the development of acute rejection was investigated.

MATERIALS AND METHODS

Patients and healthy controls

After receiving an informed consent, heparinized peripheral blood was obtained from forty liver transplant recipients before and multiple time points after Tx. Ten patients developed acute rejection within three months after Tx and were treated with intravenous high doses of methylprednisolon (Solumedrol, 3x1000mg). Acute rejection was confirmed by histological examination of liver biopsies using the Banff classification (1997). Rejection activity index of six or more defined rejection, as assessed by an experienced pathologist. Steroids (Prednison) were given to all patients starting with 100 mg per day and were weaned during the first six months after Tx. Some patients

remained on maintenance steroids (n=14). Blood samples obtained from sixteen healthy volunteers (6 males, 10 females) were used as control. The mean age of the controls was 31 years, not significantly different from the patient group. General characteristics and immune suppressive treatment of patients are summarized in Table 1.

Flow Cytometric Analysis

Mononuclear cells were obtained from heparinized blood by density gradient centrifugation over Ficoll-Paque plus (Amersham Biosciences, Buckinghamshire, UK). After isolation, cells were stored in 10% DMSO-containing medium at –180ºC. For flow cytometric analysis of Treg, the following fluorescent monoclonal antibodies were used: anti-CD4 (SK3)-PerCp-Cy5.5, -CD25 (2A3)-FITC, -CD45RO (UCHL-1)-APC purchased from BD Pharmingen (San Diego, CA). PE-conjugated anti-CTLA-4 (BN13) was purchased from Immunotech (Marseille, France). Fluorescent mouse IgG-1 and –2A (BD Pharmingen) antibodies were used as isotype controls.

After thawing, cells were washed twice with phosphate-buffered saline (PBS) containing 0.3% bovine serum albumin (BSA) and incubated for 30 minutes with CD4, CD25 and CD45RO mAbs in PBS/0.3% BSA at 4ºC. Following primary incubation, cells were washed and for staining of intracellular CTLA-4, the cells were fixed and permeabilized using the IntraPrep Reagents (Immunotech, Marseille, France). Subsequently, the cells were washed and analysed by flowcytometry using FACS Calibur and CELLQuest Pro software (Becton Dickinson, San Jose, CA). The percentage of Treg was calculated as quadruple positive cells (CD4$^+$, CD25$^+$, CD45RO$^+$ and CTLA-4$^+$) and expressed as a percentage of CD4 positive cells.

Mixed leukocyte reaction and IFN-γ production

RPMI 1640 medium with L-Glutamine (Bio Whittaker, Verviers, Belgium) supplemented with 10% pooled human serum (Dept. of Immunohematology and Bloodbank, Leiden University Medical Center, Leiden, The Netherlands), 100 μg/ml penicillin and 100 μg/ml streptomycin (Gibco, Paisley, UK) was used for T-cell culture. After isolation of PBMCs from fresh heparinized blood obtained one year after Tx CD4$^+$ cells were purified using the untouched CD4$^+$ T-cell isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany). After washing with PBS/0.3% BSA, CD4$^+$ T cells were incubated with anti-CD25 microbeads (Miltenyi Biotec) followed by a positive selection of CD4$^+$CD25$^+$ T cells according to manufacturer's instructions. The CD4$^+$CD25$^-$ fraction was used as responder cells. The purified Treg fraction contained more than 90% pure CD4$^+$CD25$^+$ T cells. Donor or third-party allogeneic spleen cells were irradiated (5000 rads) and mixed (ratio 1:1) with CD4$^+$CD25$^-$ responder T cells (5x10^4) in 96-well round-bottom plates. Suppression of proliferation was determined by adding increasing numbers (5 x 10^3, 1 x 10^4 or 1.5 x 10^4 cells) of CD4$^+$CD25$^+$ T cells to the mixed leukocyte reaction. At day 4 of culture, supernatants were collected and the concentration of IFN-γ was measured by ELISA (U-CyTech, Utrecht, The Netherlands). At day 5, cultures were pulsed with 1 μCi per well of [3H] thymidine (Amersham, Little Chalfont, UK) for 16 hours. Cells were harvested and proliferation was assessed by measuring radioactivity with a liquid scintillation counter. Cultures were performed in triplicate and the mean counts per minute were calculated.

				Induction Therapy			Maintenance Therapy		
Patient	Sex	Age (y) at Tx	Disease	IL-2R Blocker	Steroids (Prednison)	I.V. MP (500,g)	CNI	Steroids (Prednison)	Acute Rejection
1	M	49	AC	-	+	+	Tac	+	+
2	M	40	AC	-	+	+	Tac	-	-
3	M	46	AC	-	+	+	CsA	+	-
4	M	45	AC	-	+	+	CsA	-	+
5	M	58	AC	+	+	+	Tac	-	+
6	F	56	AC	+	+	+	Tac	-	-
7	M	52	AC	+	+	+	Tac	-	-
8	F	54	AC	-	+	+	Tac	+	+
9	M	47	AC	+	+	+	Tac	+	-
10	M	61	AC	+	+	+	Tac	-	-
11	F	49	PBC	-	+	+	CsA	+	+
12	M	37	PSC	+	+	+	Tac	+	+
13	F	44	PSC	-	+	+	Tac	+	+
14	F	44	PBC	-	+	+	CsA	-	-
15	M	34	PSC	-	+	+	Tac	-	-
16	F	46	PBC	+	+	+	Tac	-	+
17	M	38	PSC	+	+	+	Tac	-	-
18	M	53	PBC	+	+	+	CsA	+	-
19	F	50	PSC	+	+	+	CsA	+	-
20	F	41	PBC	+	+	+	Tac	-	-
21	M	43	HBV	-	+	+	CsA	-	-
22	M	34	HBV	-	+	+	Tac	-	-
23	M	45	HBV	+	+	+	CsA	-	-
24	M	38	HBV	+	+	+	CsA	-	-
25	M	59	HBV	+	+	+	CsA	-	-
26	M	54	HBV	+	+	+	Tac	-	-
27	M	59	HCV	+	+	+	Tac	-	-
28	M	61	HCV	-	+	+	Tac	+	-
29	M	55	HBV	-	+	+	Tac	+	-
30	F	52	HBV	-	+	+	Tac	-	-
31	F	40	APUDoma	-	+	+	Tac	+	-
32	M	43	Wilson	+	+	+	CsA	-	-
33	M	59	AIH	-	+	+	Tac	+	-
34	F	40	CC	+	+	+	Tac	-	+
35	M	37	Byler	+	+	+	Tac	-	+
36	M	58	CC	+	+	+	Tac	-	-
37	M	35	PLD	+	+	+	Tac	-	-
38	M	49	CC	+	+	+	Tac	-	-
39	M	47	AIH	+	+	+	Tac	+	-
40	F	43	PLD	+	+	+	Tac	-	-

Tabel 1. Abbreviations: AC, alcoholic cirrhosis; PBC, primary biliary cirrhosis; PSC, primary sclerosing cholangitis; HBV, chronic hepatitis B virus infection; HCV, chronic hepatitis C virus infection; Wilson, Wilson's disease; AIH, auto-immune hepatitis; CC, cryptogenic cirrhosis; Byler, Byler's disease; PLD, polycystic liver disease. I.V. MP, intravenous methylpred-nisolon; Tac, tacrolimus; CsA, cyclosporin A.

Statistical Analysis

Statistical analysis of the flowcytometry data was performed using software package SPSS version 11.5 (SPSS, Chicago, IL). Significance was tested using the Mann-Whitney U test, the Wilcoxon paired test and the Spearman's two-tailed correlation test. P-values less than 0.05 were considered significant. For the MLR and IFN-γ production, statistical analysis was performed by analysis of the logarithmic transformation of the dependent variable with random intercept and random slope using PROC Mixed in SAS version 8.2. (SAS Institute Inc, Cary, NC).

RESULTS

Increased Treg in patients with end-stage liver disease

Treg were identified in peripheral blood by flowcytometry based on the expression of CD4, CD25, CD45RO and intracellular CTLA-4. Hereafter quadruple positive cells (expressing CD4, CD25, CD45RO and CTLA-4) are referred to as Treg in the remainder of the manuscript. Representative dot plots of the flowcytometric analysis from a liver transplant recipient are shown in Figure 1A. Approximately 20% of the CD4$^+$CD25$^+$ cells express both CD45RO and CTLA-4, whereas 2% of the CD4$^+$CD25$^-$ fraction was positive for these markers (not shown). The majority of CTLA-4 expressing CD4$^+$CD25$^+$ cells had the memory phenotype (CD45RO$^+$).
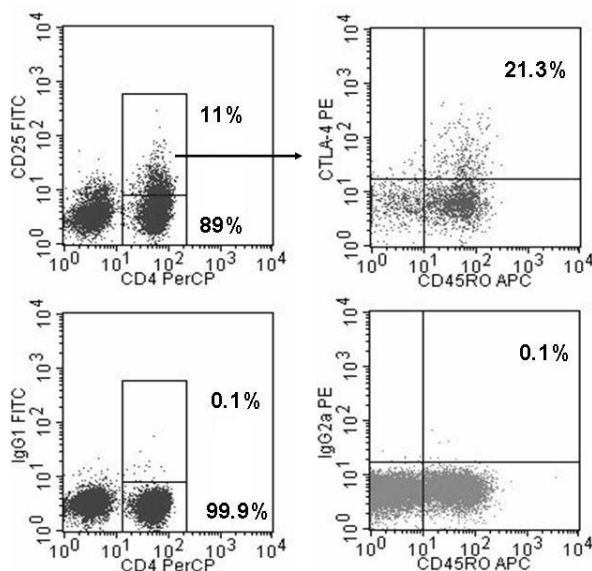

Figure 1A. Assessment of Treg in peripheral blood of liver transplant patients and healthy controls on basis of CD4, CD25, CD45RO and CTLA-4 (CD152) co-expression. Representative dot plots are shown from a liver transplant patient. The right panels were gated on the CD4$^+$CD25$^+$ cells in the upper left plot. The majority of CD4$^+$CD25$^+$ cells have a memory phenotype (CD45RO$^+$) and approximately 20% of these cells co-express CTLA4.

Patients with end-stage liver disease had significantly higher Treg percentages compared to healthy controls (Figure 1B). Highest levels were seen in patients with bile duct diseases and viral hepatitis, whereas modest elevations were found in alcoholic cirrhosis and for other liver diseases.
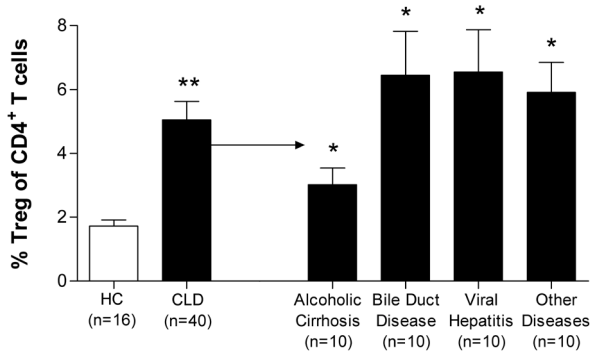

Figure 1B. Elevated levels of Tregs in patients with end-stage liver disease. The percentage of Tregs is calculated as quadruple positive cells (CD4$^+$, CD25$^+$, CD45RO$^+$ and CTLA-4$^+$) and expressed as the percentage of CD4 positive cells. Patients with chronic liver disease (CLD) had increased Tregs in peripheral blood compared to healthy controls (HC, **p<0.001). Treg levels in the four main types of CLD were all significantly different from healthy controls, with highest levels of Tregs in bile duct diseases and viral hepatitis (* p< 0.05). Shown is the mean ±SEM.

Decrease of Treg after liver transplantation

Recently, we have shown that CD4$^+$ and CD4$^+$CD25$^+$ T lymphocytes within the CD3$^+$ population were significantly reduced in peripheral blood one year after liver Tx (15). Assessment of Treg, expressing CD4, CD25, CD45RO and CTLA-4, showed a similar reduction (Figure 2A). Lowest levels were observed at three months after Tx, followed by a relative increase at twelve months and at later time points. A significant correlation was found between Treg levels within the CD4$^+$ population before and one year after Tx (Figure 2B, p<0.001).
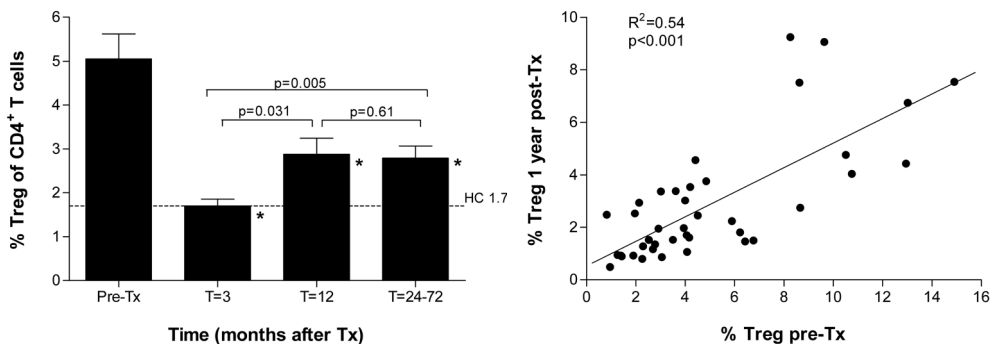

Figure 2A. Decrease of Treg in peripheral blood after liver transplantation. Shown are the mean percentages of CD4$^+$CD25$^+$CD45RO$^+$CTLA-4$^+$ cells within the CD4$^+$ population of liver transplant recipients (n=40). At all post-Tx time points Tregs were significantly decreased (* pre vs. post, p<0.001). Treg decreased dramatically in the early months after liver Tx, but showed a relative increase at one year. (B) Pre-Tx Treg levels correlate significantly with levels one year Tx.

Acute rejection is associated with reduced Treg levels

A link between the level of Treg and the occurrence of a rejection episode in the first three months after liver Tx was investigated. Figure 3A shows Treg levels for rejectors and non-rejectors. One year after Tx, a significantly lower percentage of Treg within the CD4$^+$ T-cell population was observed in patients who experienced an episode of acute rejection compared to patients who did not develop allograft rejection (p=0.005). In Figure 3B representative dot plots of the flowcytometric analysis for a rejector and a non-rejector are shown. Within the CD4$^+$CD25$^+$ fraction, there was an increased proportion of CD45RO$^+$CTLA-4$^+$ cells in non-rejectors compared to rejectors treated with intravenous methylprednisolon. A link between Treg levels and acute rejection was confirmed in an univariate analysis (ANOVA, p=0.003). After the first year, Treg levels showed a relative increase in the rejection group (p<0.05), while remaining stable in non-rejectors. These results suggest a transient reduction of Treg with different kinetics between rejectors and non-rejectors.
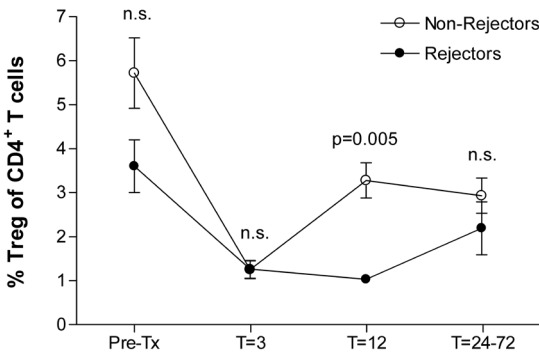
Figure 3A. Acute rejection after Tx was associated with reduced Treg fractions. The mean percentages of CD4$^+$CD25$^+$CD45RO$^+$CTLA-4$^+$ cells (± SEM) are shown. Patients, who experienced an episode of (methylprednisolon-treated) acute rejection (n=10) within three months after Tx, had significantly lower Tregs one year after Tx compared to non-rejectors (n=30). No significant differences were seen at other time points. T= months after Tx.
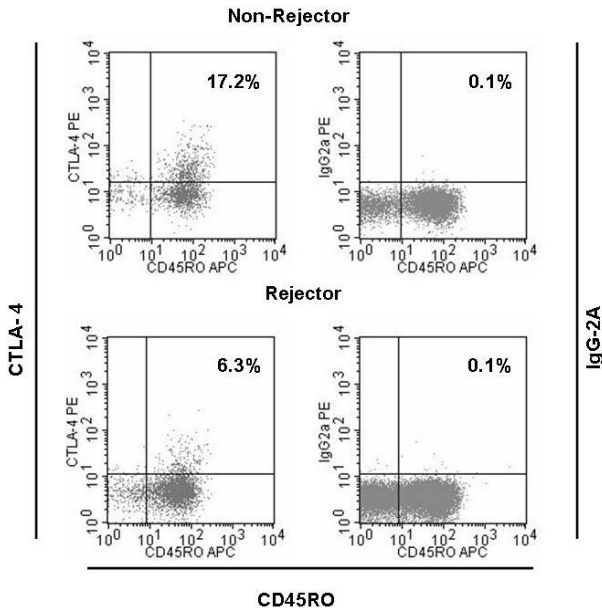

Figure 3B. Representative dot plots are shown of CD45RO and CTLA-4 expression within the gated population of CD4$^+$CD25$^+$ cells for a rejector and a non-rejector at one year post-Tx. Rejectors had significantly lower CD45RO$^+$CTLA-4$^+$ double positive CD4$^+$CD25$^+$ cells compared to non-rejectors. All gates were based on isotype matched control stainings.

Differential effects of immune suppressive regimens on Treg

To determine whether there was a correlation between immune suppressive regimens and Treg in peripheral blood, the relative reduction at one year post-Tx in relation to individual immune suppressants was assessed (Figure 4). All regimens included calcineurin inhibition, either cyclosporine A or FK506 (tacrolimus). Equal Treg reduction was observed in the cyclosporine or the tacrolimus groups at all time points after Tx. IL2-receptor blockade, Basiliximab, given as induction immune suppressive therapy, also had no impact on Treg fractions. Patients receiving steroids (Prednison) as maintenance therapy showed a trend towards a stronger decrease at one year. Both rejectors and non-rejectors received methylprednisolon (1 x 500mg) during Tx, which may explain the reduction of Treg seen in all patients at three months (Figure 3A). Additional treatment with methylprednisolon (3 x 1000mg) for acute rejection resulted in a significantly higher decrease compared to non-rejectors (Figure 4).
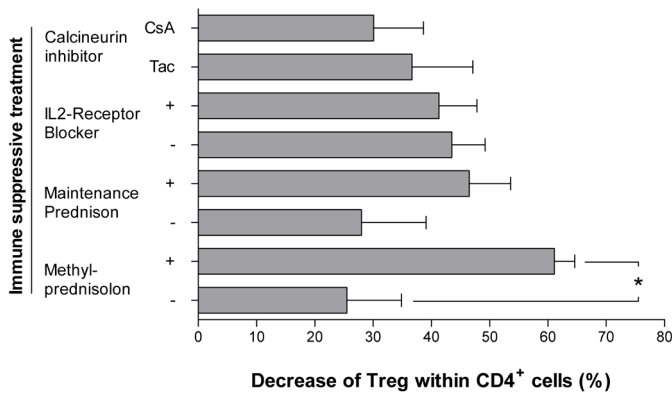

Figure 4. Comparison of different immune suppressive regimens with the percentage decrease of Treg one year after Tx. All patients were on treatment with a calcineurin inhibitor, either cyclosporin A (CsA, n=12) or tacrolimus (Tac, n=28). Steroids (Prednison) as additional maintenance therapy were applied to 14 of 40 patients. IL2-receptor blocker, Basiliximab, was given to 24 patients. Ten patients with acute rejection were treated with high doses of intravenous methylprednisolon (Solumedrol, 3 x 1000mg). Patients treated for acute rejection with methylprednisolon showed a more profound decrease of Treg (*p=0.005). Maintenance steroid treatment also showed a stronger decrease, but did not reach statistical significance. No difference was seen between CsA or Tac use, nor between patients receiving an IL2-receptor blocker and patients who did not.

Treg-mediated suppressive activity is not different between rejectors and non-rejectors

The suppressive activity of Treg one year after Tx was determined from rejectors and non-rejectors in a mixed-leukocyte-reaction using donor and third-party allogeneic spleen cells. Recipient T cells showed a hyporesponsiveness against donor spleen cells compared to third party, even after depletion of CD4$^+$CD25$^+$ T cells (data not shown). Because of this low anti-donor response, further suppression of this response by regulatory CD4$^+$CD25$^+$ T cells could not be determined. Figure 5 shows suppression of the third-party immune response. Suppression of proliferation (Figure 5A) and cytokine production (Figure 5B) of effector CD4$^+$CD25$^-$ T cells from rejectors, non-rejectors and healthy controls was comparable on a cell-for-cell basis, indicating that the suppressive activity of Treg is not affected by continuous immune suppression.
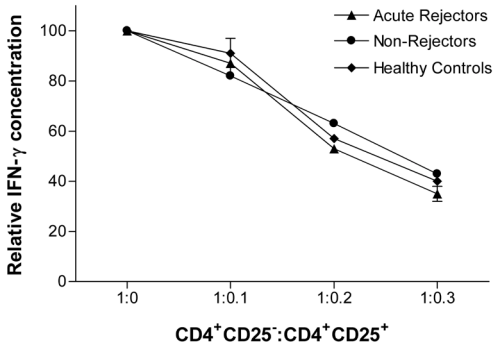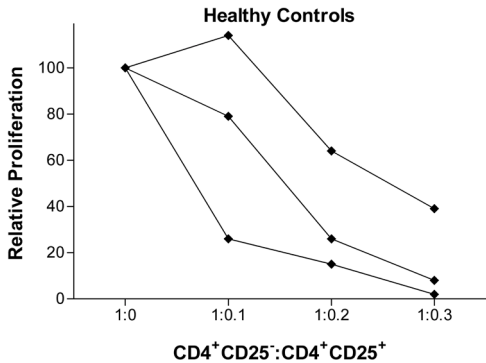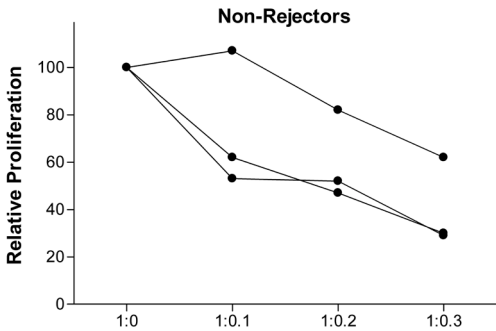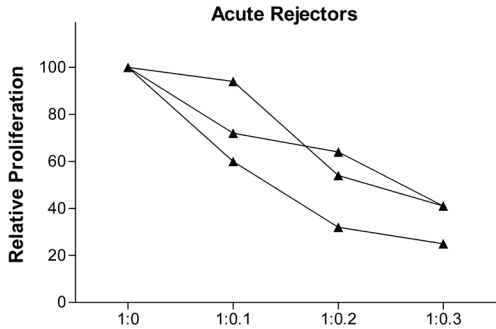
Figure 5. Comparable suppressor activity by Treg from rejectors, non-rejectors and healthy controls. One year after Tx, CD4$^+$CD25$^+$ T-cell mediated suppression was determined in a mixed-leukocyte-reaction for patients who experienced acute rejection (n=3), patients who did not reject (n=3) and healthy controls (n=3). Irradiated third-party spleen cells (5x10^4) were used for stimulation and mixed with responder CD4$^+$CD25$^-$ T cells (5x10^4). Subsequently, purified CD4$^+$CD25$^+$ T cells were added in increasing numbers: 5x10^3 (0.1), 1x10^4 (0.2) and 1.5x10^4 (0.3).

Figure 5A. CD4$^+$CD25$^+$ T cells inhibit the allogeneic T cell response mediated by CD4$^+$CD25$^-$ T cells (p<0.001). After 5 days of culture, T cell proliferation was determined. Relative proliferation was calculated with the means of triplicate determinations. In rejectors, non-rejectors and healthy controls inhibition of proliferation was similar (p= not significant).

Figure 5B. Treg inhibit IFN-γ production by effector CD4$^+$CD25$^-$ T cells. Shown are the relative mean IFNγ concentrations for three individuals in each group, as determined by ELISA.

DISCUSSION

The role of Treg in experimental models of transplant tolerance is well established. In the current study we found that after human liver Tx the percentage of Treg expressing CD4, CD25, CD45RO and CTLA-4, changes dramatically. Levels of these Treg in peripheral blood significantly decreased after Tx. In a previous study we found that also the total CD4^{+} fraction within the T cell population was reduced in the first year after Tx (15). Taken this finding into consideration, the absolute reduction of Treg was even more profound than shown in Figure 2A. Significant differences in Treg levels were found between patients who experienced acute rejection versus non-rejectors (Figure 3A). Accordingly, acute rejection was associated with significantly lower Foxp3 mRNA levels one year after Tx (data not shown). Despite the quantitative changes, the suppressive activity of isolated CD4^{+}CD25^{+} T cells from liver transplant recipients, both rejectors and non-rejectors, was comparable to that of healthy controls (Figure 5). On a cell-for-cell basis the inhibition of T-cell proliferation and IFN-γ production upon allogeneic stimulation was similar for rejectors and non-rejectors, however the levels of Treg in blood were three times lower in rejectors (Figure 3A) indicating a higher overall suppression in non-rejectors.

The direct T-cell response to donor antigens after Tx, as determined by a mixed-leukocyte reaction, was low or undetectable in most recipients. There are a number of non-mutually exclusive mechanisms that can be responsible for this state of donor-specific hyporesponsiveness. These mechanisms include T cell anergy, mixed chimerism, deletion of reactive T cells or active immune regulation by donor specific Treg. The hyporesponsiveness is more frequent in liver Tx compared to heart Tx (16). In our experience, depletion of CD4^{+}CD25^{+} cells did not overcome the hyporesponsiveness in the mixed-leukocyte reaction (data not shown), suggesting that other CD25^{-} Treg populations (17, 18) or other mechanisms than immune regulation might be in play. To be able to determine the suppressive capacity of Treg after Tx, we used third-party cells as stimulus in the mixed-leukocyte reaction. It is well established that the response to unrelated, third-party, allo-antigens is maintained after Tx. This way we were able to demonstrate that the suppressive activity of CD4^{+}CD25^{+} regulatory T cells was maintained after Tx and comparable between rejectors and non-rejectors (Figure 5). This, however, does not exclude a possible role for other significant regulatory populations, like CD8^{+}CD28^{-} suppressor T cells, in clinical transplant tolerance (19-21).

The quantitative changes seen in Treg after Tx might be a result of immune suppressive therapy. A recent study showed reduced levels of CD4^{+}CD25^{high} cells in immune suppressed liver Tx recipients compared to recipients who were free of immune suppression (22). All patients in our study were treated with calcineurin inhibitors (cyclosporin A or tacrolimus). Calcineurin inhibitors are potent inhibitors of T-cell activation by blocking IL-2 production. As IL-2 is essential for Treg function and survival (23), blocking IL-2 production by calcineurin inhibitors may thereby negatively effect the Treg homeostasis and therefore hamper tolerance induction (24-29). In addition, a recent study by Baan et al. showed reduced induction of Foxp3 in the presence of calcineurin

inhibitors (both cyclosporin A and tacrolimus) in the MLR (30). On the other hand, in our study group treatment with anti-IL-2 receptor antibody (Basiliximab) had no clear effect on Treg levels at three months or one year after Tx (Figure 5).

In the first three months after Tx the intensive immune suppressive treatment directly following Tx may have caused the strong drop in Treg percentages. All patients received methylpredniso-lon (Solumedrol) and high doses of steroids (Prednison). It is well known that steroids like me-thylprednisolon induce apoptosis of lymphocytes. Apoptosis of Treg could explain the significant reduction of circulating Treg in the first months after transplantation and the sustained reduction in methylprednisolon-treated rejectors. At later time points after Tx (1-4 years), the percentage of Treg showed a relative increase in patients who were free of acute rejection suggesting that the reduction of Treg is transient. In patients who experienced an episode of rejection, however, Treg levels remained low throughout the first year and only showed a relative increase at two years or later. Additionally, patients receiving steroid maintenance therapy also showed reduced Treg levels after Tx (Figure 4), though this did not reach statistical significance due to small sample size. Taken together, these findings further support the negative and transient effect of steroid treatment on circulating Treg. This is consistent with an earlier report that methylprednisolon inhi-bits spontaneous acceptance of the liver in a rat allo-Tx model, that might be linked to Treg (31). Moreover, in two other models of allograft tolerance methylprednisolon also inhibited acceptance of the transplanted organ (27, 32).

Interestingly, patients with end-stage liver disease have an increased fraction of circulating Treg compared to healthy controls. Recent studies have shown that Hepatitis B and Hepatitis C virus infection is associated with an increase of Treg in peripheral blood (33, 34). Also in our study group patients with viral hepatitis had three times higher Treg levels than controls. Furthermore, we found that high pre-Tx Treg levels correlated with relatively high Treg levels post-Tx (Figure 2B). This correlation suggests that high levels of Treg, linked to the underlying disease, determine the level of Treg at one year post-Tx. Consistently, patients with viral hepatitis did not develop acute rejection (Table 1), a result that may involve the increased Treg levels seen in these patients. It is well documented that patients with hepatitis B infection have a lower incidence of acute rejection (35). On the contrary, patients with PBC or PSC also showed increased Treg, yet rejection was seen in four out of ten patients. This could be a consequence of an impaired Treg function as seen in some autoimmune diseases (36).

Understanding the role of Treg in the immune response after allo-transplantation may contribute to the identification of patients who acquired operational tolerance and therefore would not need immune suppressive treatment. Indeed, there is some evidence that pediatric patients who ac-quired operational tolerance after liver Tx have increased Treg levels compared to patients on im-munosuppression (22). Our study demonstrates an association between the homeostasis of Treg after Tx and acute rejection, illustrating the current dilemma of immune suppressive treatment:

on the one hand preventing rejection, but on the other hand inhibiting Treg and thereby possibly interfering with the development of transplant tolerance.

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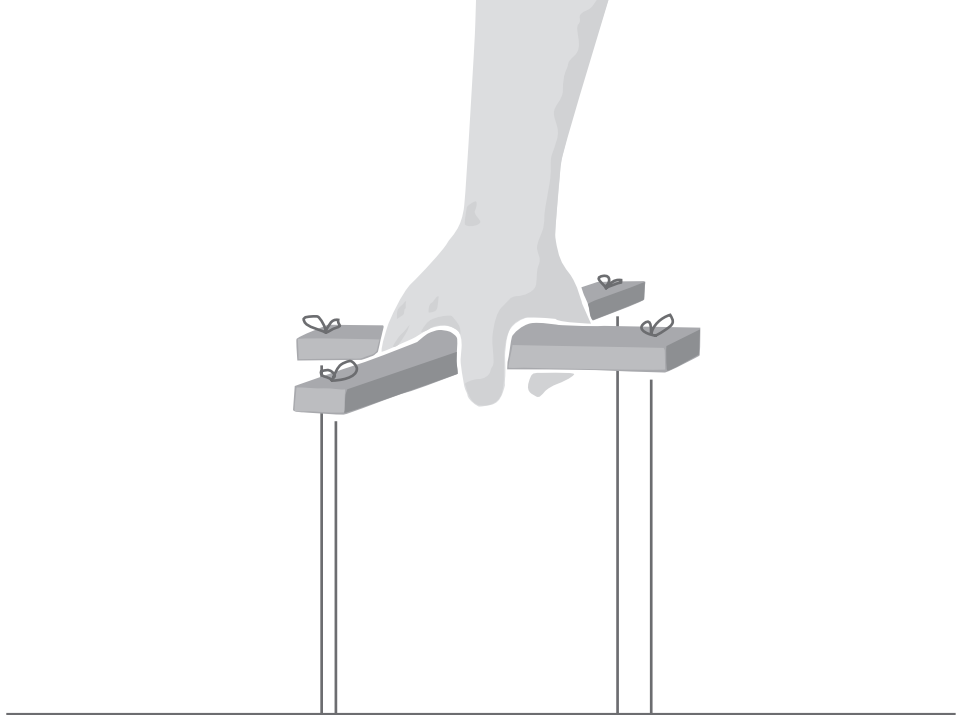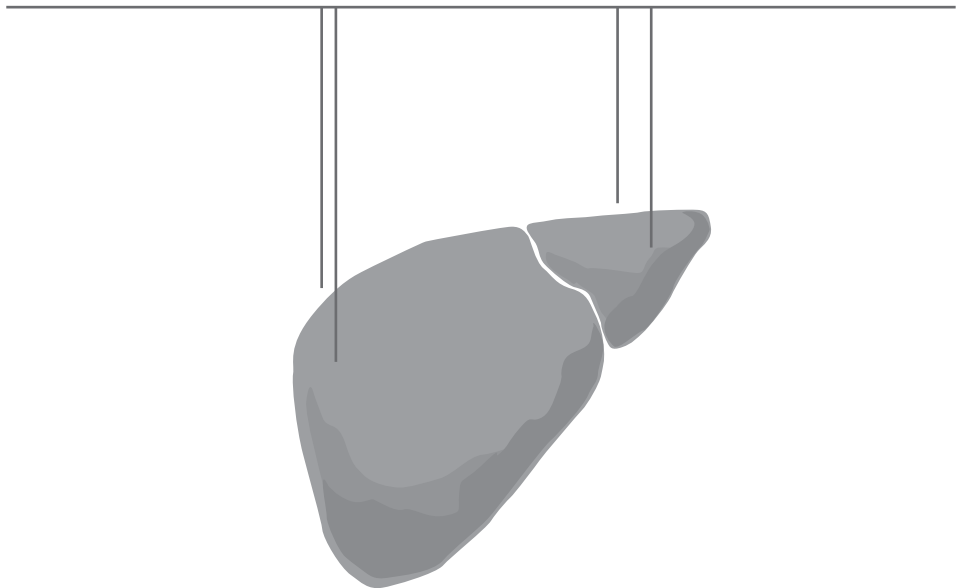
Chapter 5

Changes of circulating CD4$^+$Foxp3$^+$ regulatory T cells
after conversion from calcineurin inhibitor to
mycophenolate mofetil in liver transplantation

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ABSTRACT

CD4$^+$Foxp3$^+$ regulatory T cells (Treg) depend on IL-2 for their function and survival. By interfering with the IL-2 production, calcineurin inhibitors (CNI) may negatively effect Treg. Here, we describe the effects of conversion from CNI to mycophenolate mofetil (MMF) on renal function, Treg frequency and phenotype in liver transplant recipients. Patients (n=16) with renal impairment on CNI were converted to MMF and received a single dose of IL-2-receptor blocking antibody (Daclizumab). Control patients (n=8) continued CNI treatment. Six months after conversion to MMF the percentage of CD4$^+$Foxp3$^+$ and CD4$^+$CD25^{bright} cells within CD3$^+$ cells increased both by 2.2 fold. Foxp3 mRNA analysis of peripheral blood mononuclear cells confirmed the enrichment of Foxp3. CD25 expression on CD4$^+$Foxp3$^+$, but not Foxp3$^-$, cells significantly increased at six months compared to pre-conversion. Daclizumab treatment resulted in a 75 percent blocking of CD25 at one month, but this did not affect Treg levels. Renal function improved and acute rejection occurred in two patients (13%) after conversion. Conversion to MMF significantly improves renal function and increases the percentage and CD25 expression of circulating Treg. This shows that MMF therapy can overturn the repressive effect of CNI on circulating Treg and therefore may promote Treg mediated tolerance.

INTRODUCTION

The checks and balances of immune reactive and immune suppressive cells likely determine whether there is immune reactivity to donor antigens or non-responsiveness (tolerance). Tolerance to self and foreign antigens involves the action of regulatory T cells (Treg) (1, 2). These T cells with suppressive capacities were first identified for their ability to prevent organ specific autoimmune diseases in mice (1). In experimental transplant models these cells can transfer tolerance to donor antigens (2). Recent studies suggest that calcineurin inhibitors (CNI, cyclosporin A or tacrolimus), belonging to current standard immunosuppressive therapy after organ transplantation (Tx), interfere with the expansion, survival and possibly function of Treg in vitro (3, 4) and in vivo (5, 6). CNI are known as potent blockers of IL-2 production and it is established that IL-2 is critically important for the survival of Treg (7). This could explain why CNI have been reported to be detrimental to both spontaneous experimental transplant tolerance in vivo and tolerance induced by co-stimulatory blockade (8, 9). The clinical use of CNI is associated with considerable non-immunological side effects, most importantly renal dysfunction due to nephrotoxicity (10) but also diabetes mellitus, hyperlipidemia and hypertension.

Clinical studies have shown that conversion of CNI to immunosuppressants like rapamycin or mycophenolate mofetil (MMF) can halt or even reverse CNI associated side effects. In liver Tx, CNI conversion to MMF based immune suppression in particular improved renal function but was also associated with a considerable risk of acute rejection (11, 12). Recent studies suggest that rapamycin, unlike CNI, does not interfere with the survival and alloantigen-driven expansion of Treg in vitro (3, 4) and in vivo (6). There is now accumulating evidence that the inosine monophosphate inhibitor MMF, an IL-2 independent immunosuppressant, has similar effects on Treg as rapamycin. It was shown in different experimental transplant models that MMF does not interfere with Treg function and positively effect tolerance induction (5, 9, 13).

The aim of the current study is to determine the effect of conversion from CNI to MMF monotherapy on frequency and phenotype of circulating CD4$^+$Foxp3$^+$ Treg. To minimize the risk of conversion-associated acute rejection recipients were treated with additional induction IL-2 receptor blockade. After conversion to MMF, a rapid improvement in renal function was observed as well as elevated percentages of circulating CD4$^+$Foxp3$^+$ Treg with increased IL-2 receptor alpha chain (CD25) expression. Possible implications in these changes with respect to transplant tolerance will be discussed.

PATIENTS AND METHODS

Patients and study design

Liver transplant recipients transplanted at the Erasmus MC (University Medical Center Rotterdam) on CNI maintenance monotherapy and at least 12 months after Tx, were screened for renal dysfunction. Renal dysfunction was defined based on increased serum creatinin and blood urea

nitrogen (BUN) levels and a calculated creatinin clearance less than 60 ml/min. To rule out other causes of renal dysfunction ultrasound of kidneys and aorta were performed. Only patients older than 18 years, who provided written informed consent, were consecutively enrolled. Exclusion criteria included abnormal graft function, active infection, malignancy, a history of severe (steroid-resistant or repeated) rejection and other causes of renal dysfunction.

Initially this was an open, randomized study comparing MMF-Daclizumab based regimen with standard CNI based drug regimen. At time-point zero one gift of 2 mg/kg bodyweight of Dacluzimab was given intravenously and oral MMF was started at a dose of 2 gram a day. CNI was stopped completely at the same day. MMF dose was not adapted to trough levels. After the study period the majority of the patients in the CNI group were also converted to the MMF-Daclizumab based regimen (Figure 1). The composite primary endpoint of this study was the improvement of renal function: decrease in serum creatinin, BUN and increase in creatinin clearance during the first 6 months after randomization. Creatinin clearance was calculated with the Cockroft-Gault equation and in a subset of patients glomerular filtration rate (GFR) was measured. Secondary endpoints included biopsy proven rejection, allograft dysfunction, patient death at 6 months and graft loss at 6 months. During the follow up, liver and renal functions were assessed on regular basis in the outpatient clinic. When acute rejection was diagnosed intravenous methylprednisolon (Solumed-rol, 3x1000mg) was administered together with re-introduction of CNI and discontinuation of MMF. Acute rejection was confirmed by histological examination of liver biopsies using the Banff classifi-cation. General characteristics of the study group are summarized in Table 1. In the analysis, three patients were excluded: one in the control group due to deterioration of renal function requiring dialysis and three in the conversion group due to acute rejection (n=2) and allograft dysfunction without clear cause (n=1). For flow cytometric and RT-PCR analysis heparinized peripheral blood was obtained from all patients before and at multiple time points after randomization.

Flow Cytometric Analysis

Peripheral Blood Mononuclear Cells (PBMC) were obtained by density gradient centrifugation over Ficoll-Paque plus (Amersham Biosciences, Buckinghamshire, UK) and stored frozen at –135ºC. After thawing, PBMC were washed twice with phosphate-buffered saline (PBS) containing 0.3% bovine serum albumin (BSA) followed by staining with primary monoclonal antibodies in PBS/ 0.3%BSA (30 minutes at 4ºC). The following fluorochrome-conjugated monoclonal antibodies were used: CD25 (clone 2A3)-FITC, CD25 (clone M-A251)-PE, CD4-PerCP-Cy5.5 from Becton Dickinson (San Jose, USA), CTLA-4-APC, CD3-FITC and IgG2a-PE from Immunotech (Marseille, France), Foxp3-APC and isotype IgG2a-APC from eBiosciences (San Diego, USA). To determine the level of CD25 blockade by Daclizumab, we used two different clones of anti-CD25 antibody, which bind different epitopes. Antibody concentrations were titrated to obtain the same mean flu-orescence intensity for both CD25 antibodies. In this manner the the extent of CD25 blocking by cell-bound Daclizumab was calculated. To define maximal receptor blocking, 5×10^6 PBMC were incubated with 25 µg/ml Daclizumab (30 minutes at 4ºC) in vitro prior to CD25 staining.

	Study patients (n=16)	Controls (n=8)	p-value
Mean age ± SD (yrs)	60±11	66±5	NS
M:F	8:8	3:5	NS
Mean time after Tx (yrs)	9±4	7±2	NS
Indication LTx			
Sclerosing Cholangitis	3	2	NS
Alcoholic Cirrhosis	2	2	NS
Cryptogeneic Cirrhosis	3	1	NS
Biliary Cirrhosis	3	1	NS
Viral Hepatitis	2	0	NS
Acute Liver Failure	3	1	NS
Haemangioendothelioma	0	1	NS
Maintenance Immunosuppression			
Cyclosporin A monotherapy	11	5	NS
Tacrolimus monotherapy	5	3	NS

Table 1. Baseline patient characteristics

Following primary incubation cells were washed, and for staining of intracellular CTLA-4 or Foxp3, the cells were fixed and permeabilized using the IntraPrep Reagents (Immunotech, Marseille, France) or fixation/permeabilization agents supplied by eBiosciences, respectively. Analysis by flowcytometry was performed using FACS Calibur and CELLQuest Pro software (Becton Dickinson, San Jose, CA). The percentages of Treg were calculated as a percentage of total CD3$^+$CD4$^+$ T cells.

Quantitative RT-PCR

From PBMC (1 x 10^6 cells) erythrocytes were lysed with a buffer containing ammoniumchloride, sodium bicarbonate and EDTA by incubation for 7 min on ice. The remaining leukocyte fraction was pelleted and resuspended in 0.5 ml TRIzol reagent (Life Technologies, GmbH, Karlsruhe, Germany) at 4ºC. After homogenization, 20 µg poly A (Boehringer, Mannheim, Germany) was added and the TRIzol lysates were directly stored at -80ºC. Total RNA was extracted with 160 µl of cold chloroform-isoamylalcohol and subsequently precipitated with ice-cold 80% ethanol (350 µl). The precipitated solution was then loaded on a RNA-isolation column from the Qiagen RNeasy isolation kit (Qiagen, Venlo, The Netherlands) and RNA was isolated according to the manufacturer's instructions. Total RNA was denatured for 10 min at 80°C and then chilled to 4°C. First-strand cDNA synthesis was performed as previously described (14). Real-time RT-PCR was used to quantify Foxp3 mRNA. The constant region of the T cell receptor α chain (TCR-Cα) RNA was quantified to use as reference gene. The primers and probes for Foxp3 were obtained from Assays-on-Demand Gene Expression Product and pre-developed Taqman PDAR assays (Applied Biosystems). Each Taqman probe was labeled at the 5′end with the reporter dye molecule 6-carboxyfluorescein. 5 µl cDNA was added to 20 µl PCR mixture containing 12,5 µl Universal

PCR Master Mix (Applied Biosystems), 0.625 μl primer/probe mix and 6.875 μl H2O. The choice of primer and probe for the measurement of TCR-Cα transcripts was as previously described (14). Amplifications were performed using the ABI 7700 sequence detector system (Applied Biosystems) under the following conditions: a first step of 2-min 50°C and 10-min 95°C followed by 40 cycles of 15 seconds at 95°C and 1 min at 60°C (for TCR-Cα 1 min at 58°C). The target message was quantified by measuring threshold cycle (Ct). Two negative controls (no template), and two positive reference samples were included for each determination. Samples negative for the TCR-Cα genes were excluded from further analysis. The relative Foxp3 expression based on TCR-Cα was calculated using the comparative ΔΔCt formula: $\Delta\Delta CT$ (Ct Target – Ct TCR-Cα) $_{sample}$ - (Ct Target – Ct TCR-Cα) $_{control}$. The fold change was calculated using $2^{-\Delta\Delta Ct}$.

Statistical Analysis

Statistical analysis was performed using SPSS Inc. software version 11.0 (Chicago, Ill, USA). Comparisons of clinical parameters and Treg levels were performed with Wilcoxon matched paired test or the Mann-Whitney test. P-value less than 0.05 was considered to be significant.

RESULTS

Clinical Outcomes

Eighteen patients fulfilled the entry criteria and were included for the study (Figure 1).
As early as one month after conversion to MMF an improvement of renal function was observed.
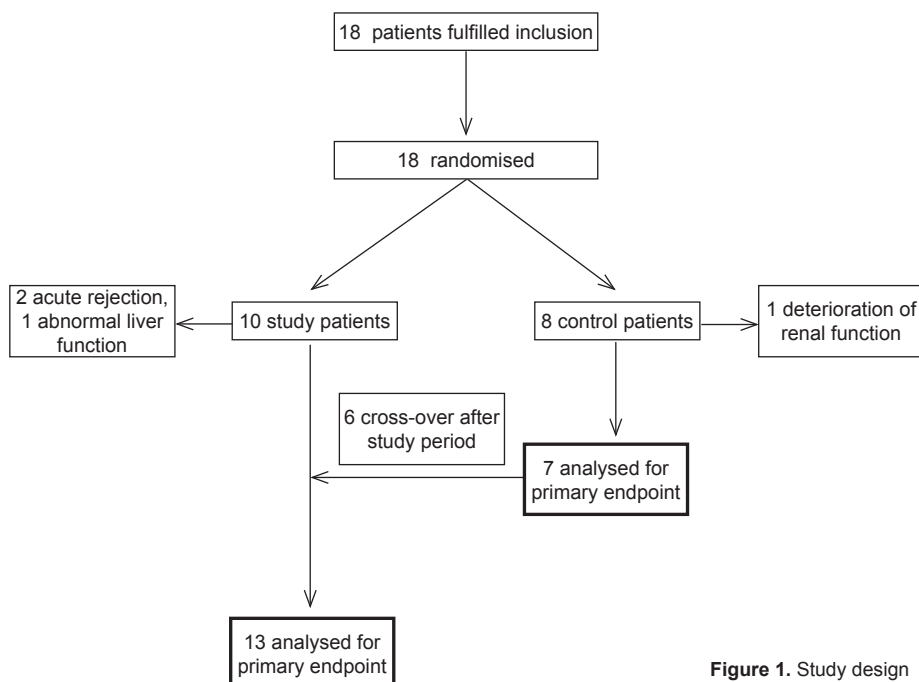
Figure 1. Study design

Relative to baseline values, serum creatinin decreased with a mean of 16% ± 9% (SD) at six months after conversion (Figure 2A). BUN levels also significantly decreased (Figure 2B). In the control group serum creatinin and BUN remained unchanged. The calculated creatinin clearance increased significantly in the conversion group (p=0.001), while remaining stable in the control group (Table 2). In seven patients in the conversion group the GFR was measured and showed a significant increase at six months confirming the improvement of renal function (p=0.018), while no changes were seen in the control group (n=4; Table 2). Conversion patients at 6 months (n=13) had a significant lower serum bilirubin level (p=0.034), while this remained unchanged in control patients (n=7; Table 2). Furthermore, after conversion the systolic blood pressure was significantly lower (p=0.001, data not shown). In these patients also the diastolic pressure seemed lower, but this did not reached significance (p=0.08). Analysis of serum ALT, AST, AP and γGT and lipids showed no significant differences (data not shown).

Two cases of acute rejection occurred in the conversion group: one at one month and the other at six months after conversion. Rejection was confirmed with histologically and successfully treated with intravenous methylprednisolon and re-introduction of CNI.
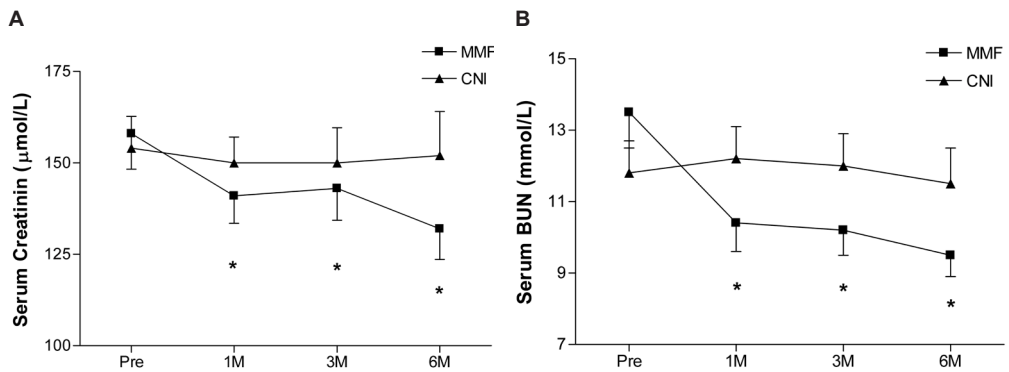
Figure 2. Improved renal function after conversion from CNI to MMF monotherapy. **(A)** Serum creatinin levels were measured at baseline, one, three and six months after conversion (n=13). Significant decrease of serum creatinin levels was observed after conversion at all time points (*p<0.01). **(B)** BUN levels significantly decrease in the conversion group (*p<0.01), but remain unchanged in the control group (n=7). Shown are the means ± SD. Asterisks indicate statistical significance in comparison to baseline.

	Conversion patients			Control patients		
	Baseline	6 months	N	Baseline	6 months	N
Mean serum creatinin (µmol/L)	158±35	132±30 *	13	154±23	152±32	7
Mean serum BUN (mmol/L)	13±4	9±2 *	13	12±2	11±3	7
Creatinin Clearence (ml/min)	45±14	56±19 *	13	38±16	42±14	7
GFR (ml/min)	39±7	46±11 *	7	44±13	44±12	4
Serum Bilirubin	10±3	8±2 *	13	10±2	9±2	7

Table 2. Clinical parameters at baseline and 6 months, * p< 0.05

Increase of CD4⁺Foxp3⁺ T cells after conversion to MMF

To assess the effect of immunosuppression conversion on Treg, flowcytometric analysis of Foxp3 expression on PBMC was performed. A representative dot plot of Foxp3 expression within the $CD4^+$ T cells is shown in Figure 3A. The total proportion of $Foxp3^+$ within the $CD4^+$ T cells significantly increased after conversion (Figure 3B). At six months after conversion the mean increase over baseline levels was 120% ± 37% SEM (p=0.008) and at 9/12 months 99% ± 33% SEM (p=0.015). No significant changes were seen in the control group.
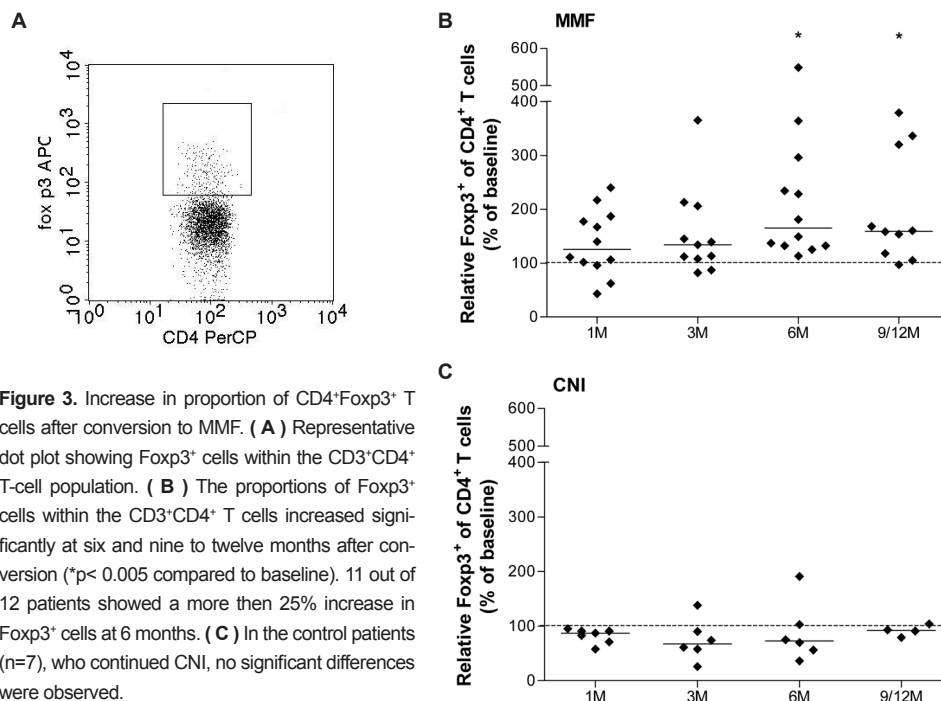
Figure 3. Increase in proportion of CD4⁺Foxp3⁺ T cells after conversion to MMF. **(A)** Representative dot plot showing Foxp3⁺ cells within the CD3⁺CD4⁺ T-cell population. **(B)** The proportions of Foxp3⁺ cells within the CD3⁺CD4⁺ T cells increased significantly at six and nine to twelve months after conversion (*p< 0.005 compared to baseline). 11 out of 12 patients showed a more then 25% increase in Foxp3⁺ cells at 6 months. **(C)** In the control patients (n=7), who continued CNI, no significant differences were observed.

RT-PCR analysis confirmed the increase of Foxp3⁺ at transcript level at six months after conversion (Figure 3C), while no changes were observed in the control group. In two patients who were withdrawn from MMF and reconverted to CNI monotherapy, the proportion of Foxp3⁺ cells and Foxp3 mRNA expression decreased after re-introduction of CNI (Figure 3D). No association was observed between the percentage of Foxp3⁺ cells and the development of acute rejection. These findings suggests that CNI negatively affect the numbers of circulating Foxp3⁺ cells and that MMF does not have this negative effect.

To confirm the increase of Treg after conversion the proportions of CD4⁺CD25ᵇʳⁱᵍʰᵗ cells within total CD3⁺ T cells was determined. In Figure 4A and representative dot plots are shown for CD4⁺CD25ᵇʳⁱᵍʰᵗ cells. The mean proportion of CD4⁺CD25ᵇʳⁱᵍʰᵗ T cells increased significantly at six months after conversion from 1.2 ± 0.4 SEM at baseline to 2.6 ± 0.7 SEM at six months (Figure 4B). At nine to twelve months after conversion, the proportions of CD4⁺CD25ᵇʳⁱᵍʰᵗ T cells remained

significantly elevated. Also the percentage of CD4+CD25+CTLA-4+ within total CD3+ T cells was increased at six months after conversion from 2.0 ± 0.8 SEM at baseline to 4.3 ± 2.4 SEM ($p<0.05$, data not shown). None of these changes were seen in control group (Figure 4B). In sum, these data indicate that conversion from CNI to MMF increases the proportion of circulating T cells with a regulatory phenotype.
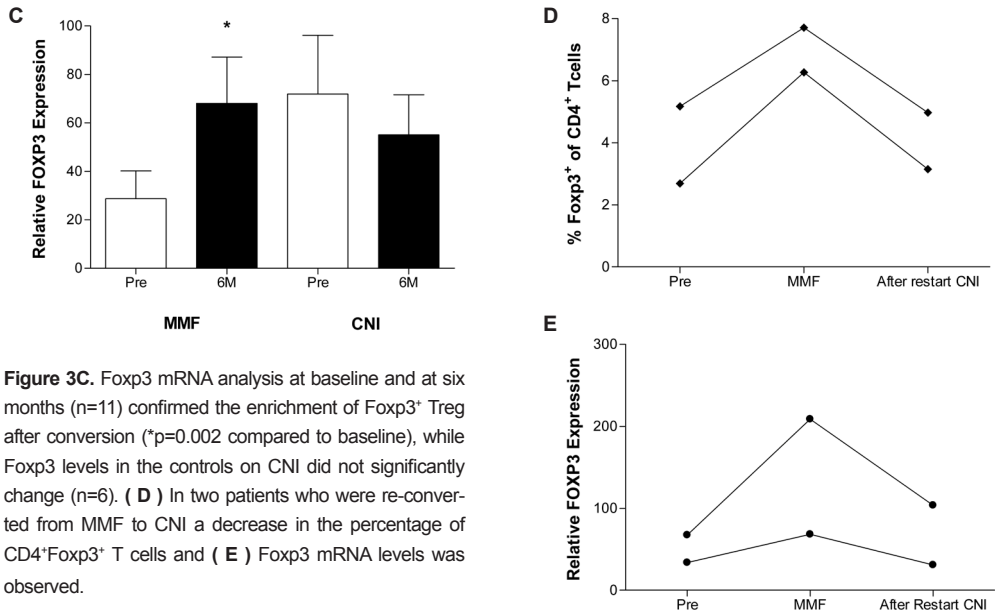

C

Figure 3C. Foxp3 mRNA analysis at baseline and at six months (n=11) confirmed the enrichment of Foxp3+ Treg after conversion (*p=0.002 compared to baseline), while Foxp3 levels in the controls on CNI did not significantly change (n=6). **(D)** In two patients who were re-converted from MMF to CNI a decrease in the percentage of CD4+Foxp3+ T cells and **(E)** Foxp3 mRNA levels was observed.
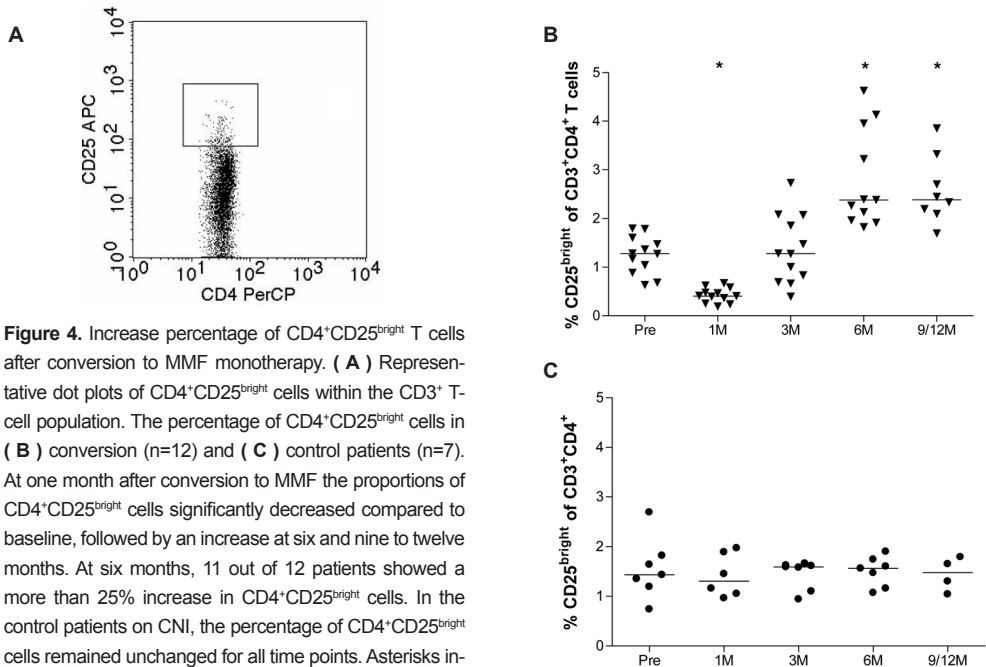
Figure 4. Increase percentage of CD4+CD25bright T cells after conversion to MMF monotherapy. **(A)** Representative dot plots of CD4+CD25bright cells within the CD3+ T-cell population. The percentage of CD4+CD25bright cells in **(B)** conversion (n=12) and **(C)** control patients (n=7). At one month after conversion to MMF the proportions of CD4+CD25bright cells significantly decreased compared to baseline, followed by an increase at six and nine to twelve months. At six months, 11 out of 12 patients showed a more than 25% increase in CD4+CD25bright cells. In the control patients on CNI, the percentage of CD4+CD25bright cells remained unchanged for all time points. Asterisks indicates $p<0.01$ and bar indicates median.

Phenotypical changes of Treg after conversion to MMF

One month after conversion to MMF, CD25 expression on CD4^{+} T cells was significantly decreased (Figure 4B). At this time point the percentage of Foxp3^{+} cells remained unchanged (Figure 3B). The CD25 down regulation was not due to epitope blocking because the antibody used (clone M-A251) for detection recognizes a different (independent) epitope than Daclizumab. Contrary, loss of CD25 expression is probably due to Daclizumab-associated receptor internalization or shedding. The increased CD25 expression on CD4^{+} T cells observed at six months after conversion was predominantly confined to CD4^{+}Foxp3^{+} cells (Figure 5A). A slight increase of CD25 expression was observed on Foxp3^{-} cells, but this did not reach statistical significance.

In order to determine the kinetics of CD25 blocking by Daclizumab, double immuno stainings were performed using a dependent and independent CD25 detection antibody. Results are shown in Figure 5B. Highest CD25 blocking was observed at one month after conversion (75%) and reduced to baseline levels by six months. No significant difference in CD25 blocking was observed between CD4^{+}Foxp3^{+} and CD4^{+}Foxp3^{-} cells (data not shown).
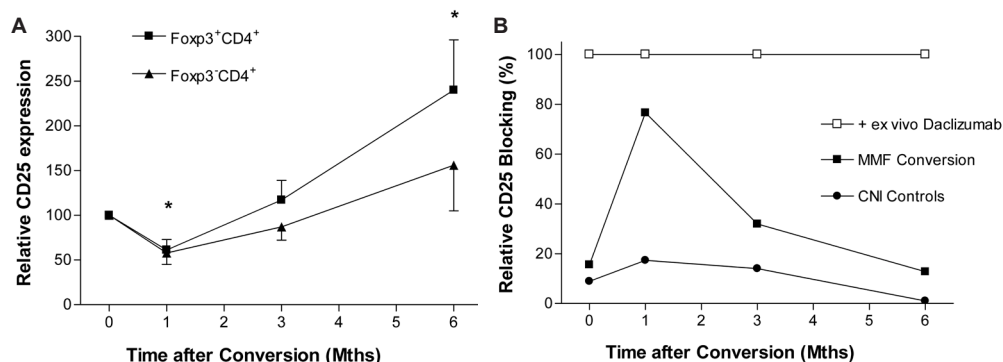
Figure 5. Changes in CD25 expression on Foxp3^{+} cells after conversion. **(A)** Relative to baseline, six months after conversion CD25 expression significantly increased on Foxp3^{+} cells. On Foxp3^{-} cells, CD25 expression also increased though to a lesser extent than Foxp3^{+} cells and not significantly different from baseline. At one month after conversion CD25 expression was significantly decreased on Foxp3^{+} and Foxp3^{-} CD4^{+} T cells (* $p<0.05$). **(B)** Extent of CD25 blocking by Daclizumab of CD4^{+} T cells. Blocking was calculated based on differential staining of two CD25 detecting antibodies, one binding to the same epitope as Daclizumab and one binding a distinct epitope. Highest level of CD25 blocking (>75%) was observed at one month and returned to baseline at six months after conversion (n=9). In the control group (n=5) approximately 15 percent CD25 blocking was observed which was unrelated to Daclizumab but could be related to bound IL-2.

DISCUSSION

The success of solid-organ transplantation depends on the continuous administration of toxic and non-specific immunosuppressive drugs to prevent rejection. Currently, maintenance immunosuppressive therapy in most liver transplant recipients includes a CNI. This study shows that the percentage of Foxp3^{+} cells within the CD3^{+}CD4^{+} T cells significantly increase after conversion from CNI to MMF and decreases again after reconversion from MMF to CNI (Figure 3). Conversion to MMF also resulted in phenotypical changes within the Treg population. After conversion an increase of CD25 expression level (i.e. mean fluorescence intensity) on Foxp3^{+} cells was observed suggesting that these cells may have become more susceptible to IL-2 signals.

IL-2 has been shown to be of critical importance for the function and survival of Treg (7, 15). Immunosuppression interfering with the IL-2 pathway may therefore affect the suppressive capacity and homeostasis of Treg. CNI interfere with the NFAT signaling pathway which is an important regulator of IL-2 production. Recently, a close relationship between NFAT and Foxp3 was shown to be critical for Treg function (16). By interfering with this interaction, CNI are thought to inhibit Treg (17). In experimental bone marrow transplantation CsA administration inhibited Treg function in vivo by reducing IL-2 production (5). Exogenous IL-2 was shown to overturn the suppressed Foxp3 expression and the suppressive function of Treg induced by cyclosporine A, underlining the importance of IL-2 for Treg. In this same study it was shown that rapamycin and MMF had no detrimental effect on the suppressive function of Treg, both in vitro and in vivo (5). There is now accumulating evidence that MMF like rapamycin spares Treg in their survival and function. It was shown in different experimental transplant models that MMF does not interfere with Treg function and positively effect tolerance induction (5, 9, 13). In the clinical setting, Segundo and coworkers recently demonstrated that the use of CNI, and not rapamycin, is associated with a reduced percentage of circulating Treg in renal transplant recipients (18). To our knowledge for the first time our study shows that conversion to MMF therapy can actually reverse the suppressive effect of CNI on the percentage of Treg in circulation.

In our study we showed that the percentage of CD4^{+}Foxp3^{+} Treg increased after conversion from CNI to MMF. Interestingly, this increase was accompanied by a significant increase of CD25 expression on CD4^{+}Foxp3^{+} Treg (Figure 5). However, one month after conversion, when over 75 percent of CD25 is effectively blocked by Daclizumab, CD25 expression was significantly decreased, both on CD4^{+}Foxp3^{+} and CD4^{+}Foxp3^{-} cells. Kreijveld et al. (19) reported that during Daclizumab treatment in kidney transplant recipients levels of Foxp3^{+} Treg decreased. In contrast, Kohm et al showed a functional inactivation, rather than depletion, of Treg by CD25 antibodies (20). These contradicting findings may be due to the intensive immunosuppressive therapy following kidney transplantation, which may act synergistically on Treg. In our study monotherapy with MMF in combination with one gift of Daclizumab did not result in decreased levels of CD4^{+}Foxp3^{+} Treg one month after conversion, but in an increase at six months and beyond and was supported by

analysis of CD25^{bright} (Figure 4) and CD25$^+$CTLA-4$^+$ T cells (not shown).

The possible negative effect of CNI on tolerance by affecting Treg is accompanied with non-immunological side effects, in particular nephrotoxicity, causing significant morbidity. This initially randomized study shows that conversion from a CNI based immunosuppressive regimen to MMF monotherapy improves renal function with a low risk of acute rejection, supporting previous conversion studies in liver transplant recipients (11, 12). Two patients developed an episode of acute rejection, one at one month and the other at six months after conversion. The patient who developed acute rejection at six months after conversion had a MMF blood trough level of 1.8 mg/l at the time of rejection, while being approximately 7 mg/l in the months before. After initial improvement with steroids, biliary obstruction was diagnosed, which may have contributed to the portal cellular infiltrate seen in the liver biopsy. No difference in MMF trough levels were seen in the other patient, who developed acute rejection, and graft function normalized after treatment.

In conclusion, we show that CNI withdrawal in liver transplant recipients increases the proportions of circulating CD4$^+$Foxp$^+$ Treg. Whether this has important implications on the balance between alloreactivity and tolerance remains to be determined. Clinically, conversion from CNI to MMF clearly results in an improvement of renal function and a decrease of blood pressure.

ACKNOWLEDGMENTS

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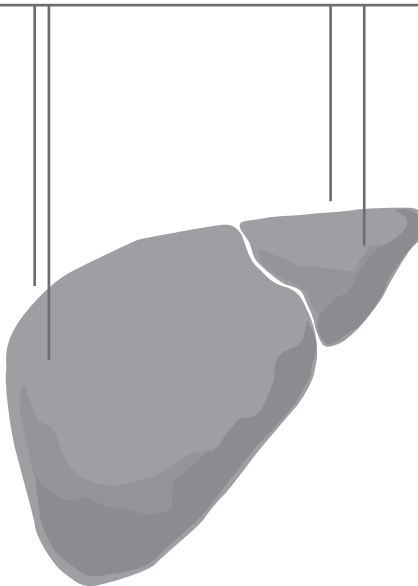
Part III

Regulatory T cells in the liver
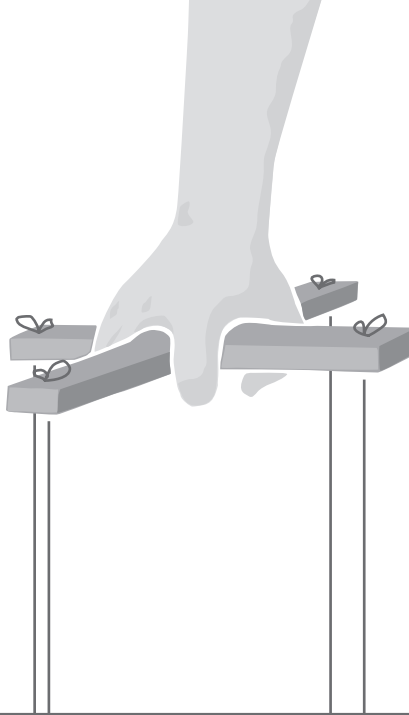
CHAPTER 6

Allo-suppressive donor CD4$^+$CD25$^+$ regulatory T cells detach from the liver graft and circulate in recipients after liver transplantation

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ABSTRACT

Organ transplantation (Tx) results in a transfer of donor leukocytes from the graft to the recipient, which can lead to chimerism and may promote tolerance. It remains unclear whether this tolerance involves donor-derived regulatory T cells (Treg). Here, we examined the presence and the allo-suppressive activity of CD4$^+$CD25$^+$Foxp3$^+$ Treg in perfusates of human liver grafts and monitored their presence in the circulation of recipients after liver Tx. Vascular perfusions of 22 liver grafts were performed with University of Wisconsin preservation and albumin solutions. Flowcytometric analysis revealed that perfusate T cells had high LFA-1 integrin expression and had a reversed CD4:CD8 ratio compared to control blood of healthy individuals. This indicates that perfusate cells are of liver origin and not derived from residual donor blood. Further characterization of perfusate mononuclear cells showed an increased proportion of CD4$^+$CD25$^+$CTLA-4$^+$ T cells compared to healthy control blood. Increased percentages of Foxp3$^+$ cells, which were negative for CD127, confirmed the enrichment of Treg in perfusates. In a mixed-leukocyte reaction, CD4$^+$CD25$^+$ T cells from perfusates suppressed proliferation and IFN-γ production of donor and recipient T cells. In vivo, within the first weeks after Tx up to 5% of CD4$^+$CD25$^+$CTLA4$^+$ T cells in recipient blood were derived from the donor liver. In conclusion, substantial numbers of donor Treg detach from the liver graft during perfusion and continue to migrate into the recipient after Tx. These donor Treg suppress the direct pathway allo-responses and may in vivo contribute to chimerism-associated tolerance early after liver Tx.

INTRODUCTION

Organ transplantation (Tx) results in a transfer of leukocytes present in the graft into the recipient. The migration of donor cells to host tissues results in chimerism and this has been proposed to be associated with donor-specific tolerance (1, 2). The relevance of these passenger leukocytes to Tx outcome has been demonstrated in several models showing significant roles in regulation of immune reactions after organ Tx. In a heart Tx model, selective antibody-mediated depletion of donor leukocytes leads to the prevention of tolerance induction and is associated with severe acute or chronic graft rejection (3). Similar results have been found in experimental liver Tx, in which depletion of passenger leukocytes leads to rejection of the graft in spontaneously tolerant recipients (4, 5). Reconstitution of donor leukocytes by intravenous injection recovers graft acceptance. Part of this recovery has been shown to be dependant on T cells, but not B cells or monocytes/macrophages, as shown by selective deletion from the reconstituting donor leukocytes (6, 7). However, the exact mechanism of this so-called chimerism-associated tolerance remains unclear. It has been suggested that high levels of chimerism, like for instance following bone marrow Tx after myeloablation, are associated with tolerance through clonal deletion or anergy induction of host T cells. Low levels of chimerism, like for instance following solid organ Tx, are associated with tolerance through regulatory mechanisms, which may involve active suppression of allo-responses by regulatory T cells (Treg) (8, 9). Both in clinical and experimental liver Tx, chimerism and chimerism-associated tolerance is more frequently seen compared to other organ Tx (10, 11). Higher levels of chimerism in liver Tx are associated with reduced incidence of acute rejection and better initial graft acceptance (12).

Within the regulatory cell populations, $CD4^+CD25^+$ Treg play a critical role in various models of transplant tolerance (13-16). Regulation of allo-responses seems to depend in part on cytotoxic T lymphocyte antigen 4 (CTLA-4), which is constitutively expressed by Treg (17, 18). Bigenzahn et al showed that depletion of $CD25^+$ cells shortly after non-myeloablative bone marrow Tx and co-stimulation blockade significantly reduced tolerance induction. This finding suggests that $CD4^+CD25^+$ Treg may be actively involved in chimerism-associated tolerance, in particular early after Tx (19). However, this remains controversial since other studies did not confirm a role for $CD4^+CD25^+$ Treg after bone marrow Tx (20, 21) .

To determine the specific role of donor leukocytes in the outcome of solid organ Tx it is important to phenotypically (22) and functionally characterize donor-derived leukocytes. In clinical liver Tx, the effluent solution passing through the graft livers during perfusion prior to Tx has been shown to be useful for this purpose. The leukocytes isolated from this perfusate solution represent detached liver-associated leukocytes, as shown by increased proportion of $CD8^+$ cells that outnumber $CD4^+$ cells and high natural killer cell numbers comparable with numbers in liver tissue (23, 24). Furthermore, the myeloid dendritic cell (DC) population present in the perfusates has an immature phenotype identical to DC isolated from liver tissue and producing higher amounts of IL-10 compared to blood DC (24).

In the current study we determined the migration and suppressive capacity of donor CD4$^+$CD25$^+$ Treg from the liver graft into the perfusate and the recipient after Tx. We show that the lymphocyte population that migrates from the human liver is enriched for CD4$^+$CD25$^+$Foxp3$^+$ cells and that these cells suppress proliferation and IFN-γ production of recipient T cells in vitro. In addition, we demonstrate that donor-derived cells with a regulatory phenotype can be detected in substantial numbers in circulation of recipients after liver Tx.

MATERIAL AND METHODS

Perfusate and peripheral blood collection

Liver perfusates were collected from 22 human liver grafts. During the back table procedure the grafts were perfused through the portal vein with 1 to 2 L of University of Wisconson solution to remove residual blood from the vasculature. Immediately before Tx, the donor liver was perfused with 200 up to 500 ml of human albumin-solution under hydrostatic pressure and the perfusate was collected from the vena cava. Mononuclear cells from perfusate were isolated within 12 hours by density gradient centrifugation using Ficoll Paque Plus (Amersham Biosciences, Upsala, Sweden). Peripheral blood mononuclear cells (PBMC) were obtained from fourteen healthy volunteers, which served as control. After isolation, cells were stored in 10% DMSO-containing medium at –180ºC. From six HLA-A2 negative liver recipients who received an HLA-A2 positive graft, blood samples were taken prior to and in the first weeks after Tx. From five liver transplant recipients peripheral blood was collected pre-Tx for functional assays. This study was approved by the Medical Ethical Committee of the Erasmus MC and informed consent was obtained from all participants.

Monoclonal antibodies

The following fluorochrome-conjugated monoclonal antibodies were used: CD25-APC, CD4-PerCP-Cy5.5, IgG1-FITC and IgG1-APC from Becton Dickinson (San Jose, USA), CTLA-4-PE, CD3-FITC, CD3-PE and IgG2a-PE from Immunotech (Marseille, France), Foxp3-APC, CD127-FITC and isotype IgG2a-APC from eBiosciences (San Diego, USA), CD11a from Biosource (Etten-Leur, the Netherlands), secondary antibody (goat-anti-mouse) FITC, CD8-APC and CD4-APC from DAKO (Glostrup, Denmark). HLA-A2 staining was performed with an anti-HLA-A2 antibody derived from a hybridoma (clone BB7.2, ATCC HB-82) followed by an FITC-conjugated rabbit anti-mouse IgG secondary antibody (DAKO).

Flow cytometric analysis

After thawing, liver perfusate mononuclear cells (LPMC) and PBMC were washed twice with phosphate-buffered saline (PBS) containing 0.3% bovine serum albumin (BSA) followed by staining with primary monoclonal antibodies CD3, CD4, CD8, CD25 and CD127 in PBS/0.3%BSA (30 minutes at 4ºC). Following primary incubation cells were washed, and for staining of intracellular

CTLA-4 or Foxp3, the cells were fixed and permeabilized using the IntraPrep Reagents (Immunotech, Marseille, France) or fixation/permeabilization agents supplied by eBiosciences, respectively. HLA-A2 positive cells were determined by incubation with anti-HLA-A2 antibody followed by staining with FITC conjugated anti-mouse IgG. Expression of leukocyte function-associated antigen-1 (LFA-1, CD11a) was determined in a similar manner by primary staining for CD11a followed by secondary staining with FITC conjugated anti-mouse IgG. Flowcytomteric analysis was performed using FACS Calibur and CELLQuest Pro software (Becton Dickinson, San Jose, CA).

CD4$^+$CD25$^+$ T cell isolation

CD4$^+$ cells were purified from fresh LPMC and PBMC using the untouched CD4$^+$ T-cell isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany). After washing with PBS/0.3% BSA, CD4$^+$ T cells were incubated with anti-CD25 microbeads (Miltenyi Biotec) followed by a positive selection of CD4$^+$CD25$^+$ T cells according to manufacturer's instructions. The CD4$^+$CD25$^-$ fraction was used as responder cells. The purified Treg fraction contained more than 90% pure CD4$^+$CD25$^+$ T cells.

Mixed Leukocyte Reaction (MLR) and suppressor activity assays

Responder CD4$^+$CD25$^-$ T cells of recipient and donor were labeled with 2 μM carboxyfluorescein diacetate succinimidyl ester (CFSE) (Molecular Probes, Eugene, OR) and 1.0×10^5 cells per well were stimulated with irradiated (5 Gy) donor LPMC (5×10^4 cells) and recipient PBMC (5×10^4 cells) in a 96-well round bottom plate. Cells were cultured in RPMI-1640 with L-glutamin (Cambrex Bioscience, Verviers, Belgium) supplemented with 10% pooled heat-inactivated human serum, 100 IU/ml penicillin and 100 μg/ml streptamycin in a total volume of 200μl. To determine the suppressive activity of CD4$^+$CD25$^+$ T cells isolated from LPMC and PBMC increasing numbers of CD4$^+$CD25$^+$ T cells (1.0×10^4 and 3.0×10^4) were added to the cultures. Cultures were performed in duplicates or triplicates. At day 4 of culture, 100μl of culture medium was replaced and the concentration of IFN-γ was measured by ELISA (U-CyTech, Utrecht, The Netherlands). After 5 days, T-cell divisions were analyzed by flowcytometry by staining the cells with CD3 and CD4 antibodies. CFSE-flowcytometry data were analyzed by ModFit™ software version 3.0 (Verity Software House, Topsham, USA). The Proliferation Index (PI), which is the sum of the cells in all generations, divided by the computed number of original parent cells, indicates the extent of T-cell expansion. If the PI is equal to one than no T-cell division took place during the course of the culture.

Statistical analysis

For the MLR and IFN-γ production, statistical analysis was performed by analysis of the logarithmic transformation of the dependent variable with random intercept and random slope using PROC Mixed in SAS version 9.1. (SAS Institute Inc, Cary, NC). Significance of differences between LPMC and PBMC flowcytometric results was determined with the Mann-Whitney test using SPSS Inc. software version 11.0 (Chicago, Ill, USA).

RESULTS

LPMC are of liver origin

Perfusates were collected during the pre-Tx albumin perfusion of donor livers on the bench. With a mean ischemia time of 7±2 hours, the perfusates contained on average 91×10^6 mononuclear cells (range $10\text{-}500 \times 10^6$). Viability as determined by trypan blue exclusion showed that 98 ± 2% of the LPMC were vital. Liver lymphocytes are known to express higher levels of LFA-1 than lymphocytes in peripheral blood [25, 26]. Flowcytometric characterization of LPMC and PBMC showed that within the perfusates a significantly greater proportion of lymphocytes had high LFA-1 expression (Figure 1). The CD4:CD8 ratio in perfusates was 1.0:2.4 (n=22) and was significantly distinct from the ratio in blood (2.0:1.0, n=14, p<0.001). These findings indicate that leukocytes present in perfusates are predominantly liver derived and not derived from residual blood.
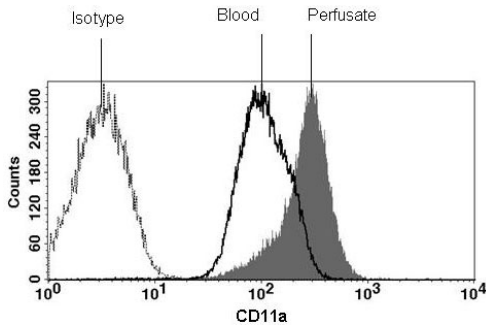
Figure 1A. High LFA-1 expression on CD4^{+} T cells from perfusates. Assessment of LFA-1 expression on CD4^{+} T cells in LPMC and PBMC. Representative histogram showing higher LFA-1 (CD11a) expression on CD4^{+} T cells in LPMC compared to CD4^{+} T cells in PBMC.
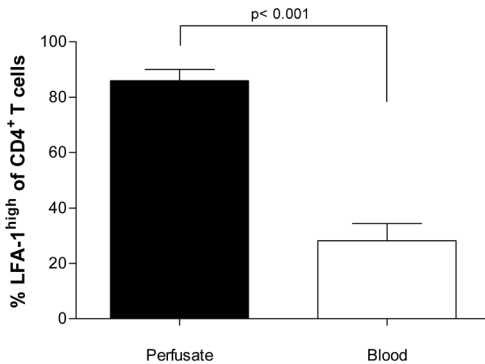
Figure 1B. The increased proportion of LFA-1^{high}CD4^{+} T cells in perfusates suggests that these cells are of liver origin and not derived from residual donor blood. In perfusates (n=9) on average 81% of CD4^{+} T cells are LFA-1^{high} compared to 28% in PBMC of healthy controls (n=10). Shown are mean percentages + SEM.

LPMC are enriched for CD4^{+}CD25^{+}CTLA-4^{+}and CD4^{+}CD25^{+}Foxp3^{+} T cells

In order to determine the presence of T cells with regulatory phenotype, CD25, CTLA-4 and Foxp3 expression within CD3^{+}CD4^{+} T cells was assessed by flowcytometry. Figure 2A shows a representative dot plot of LPMC. As shown in Figure 2B, LPMC contained a median of 4.9% CD4^{+}CD25^{+}CTLA-4^{+} T cells (range 1.8-12.1%), significantly higher compared to 2.2% in PBMC of healthy controls (range 1.1-3.1%).

Figure 2A. LPMC CD4$^+$ T cells contain a higher CD25$^+$CTLA-4$^+$ cell fraction compared to PBMC. FACS profile of CD3$^+$CD4$^+$CD25$^+$CTLA-4$^+$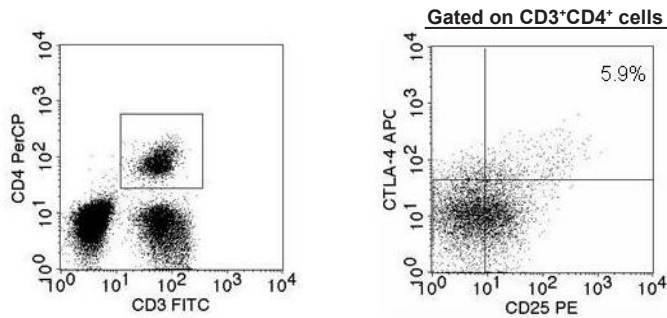 cells. Within the CD3$^+$CD4$^+$ T cell population the proportion of cells expressing membrane CD25 and intracellular CTLA-4 was determined. Shown are representative dot plots from a liver perfusate. Gates were based on isotype matched control stainings.
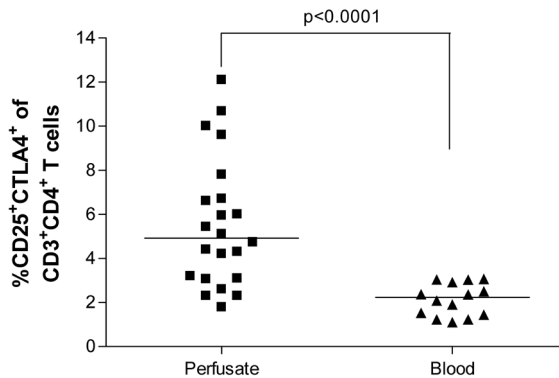
Figure 2B. Increased proportions of CD4$^+$CD25$^+$CTLA-4$^+$ T cells in perfusates compared to peripheral blood. Median percentage of CD25$^+$CTLA-4$^+$ cells within CD3$^+$CD4$^+$ T cells is 4.9% (range 1.8-12.1) in LPMC compared to 2.2% (range 1.1-3.1) in PBMC.

A subset of perfusates was analyzed for Foxp3 expression. In Figure 3A a representative staining for Foxp3 expression in LPMC and PBMC is shown. Comparison of perfusate and peripheral blood revealed increased proportions of CD4$^+$CD25$^+$Foxp3$^+$ T cells in perfusates (Figure 3B). RT-PCR confirmed the presence of Foxp3$^+$ T cells by analysis of Foxp3 transcript levels in perfusates (data not shown). Analysis of CD127, the IL-7 receptor, showed that the majority of perfusate Foxp3$^+$ cells were negative for CD127 identical to Foxp3$^+$ cells in blood (Figure 3C and 3D). In sum, these data indicate that a considerable proportion of helper T cells that detach from the liver during perfusion have a regulatory phenotype.

Figure 3. Increased percentage of CD4+CD25+Foxp3+ cells in LPMC compared to PBMC. Assessment of CD4+CD25+Foxp3+ T cells in perfusate and PBMC. Within the CD3+CD4+ T cell population the p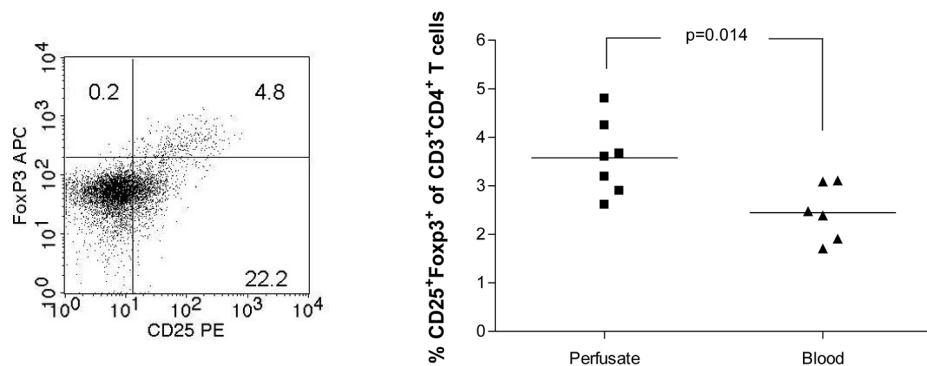roportion of cells expressing CD25 and Foxp3 was determined. **(A)** Representative dot plot of perfusate is shown. **(B)** Increased CD25+Foxp3+ cells in total CD4+ T cells in perfusates compared to PBMC. Almost all CD4+Foxp3+ were positive for CD25.
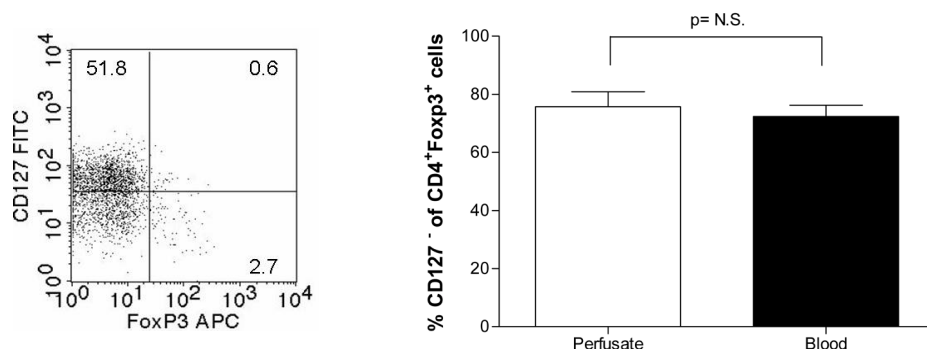
Figure 3C. Representative dot plot showing CD127 expression on perfusate CD4+ cells. **(D)** The majority of perfusate CD4+Foxp3+ cells (n=4) are CD127 negative comparable to the expression in blood CD4+Foxp3+ cells (n=6).

Perfusate CD4+CD25+ T cells inhibit proliferation and IFN-γ production of donor and recipient responder T cells

To test the suppressive activity of CD4+CD25+ T cells in LPMC, we performed a MLR using CFSE fluorescent-labeled responder T cells. First, we tested the suppressive activity of CD4+CD25+ cells in the autologous setting. Donor CD4+CD25- responder cells (1 x 10^5) from LPMC were mixed with two different concentrations of donor CD4+CD25+ T cells leading to a dose-dependent inhibition of proliferation as shown in Figure 4. Also the autologous suppressive activity of recipient CD4+CD25+ T cells from PMBC was demonstrated. Notably, the proliferation of perfusate CD4+CD25- T cells was less extensive compared to recipient blood responder T cells. However, the suppressive activity of perfusate CD4+CD25+ T cells was intact.

Next we tested the suppressive activity of perfusate CD4+CD25+ T cells in an allogeneic setting. As antigen-presenting cells, both irradiated PBMC (5 x 10^4) and LPMC (5 x 10^4) were used together in order to simultaneously activate recipient (responder) and donor (suppressor) T cells. Both perfusate and recipient CD4+CD25+ cells showed no proliferation in response to allogeneic stimulation,
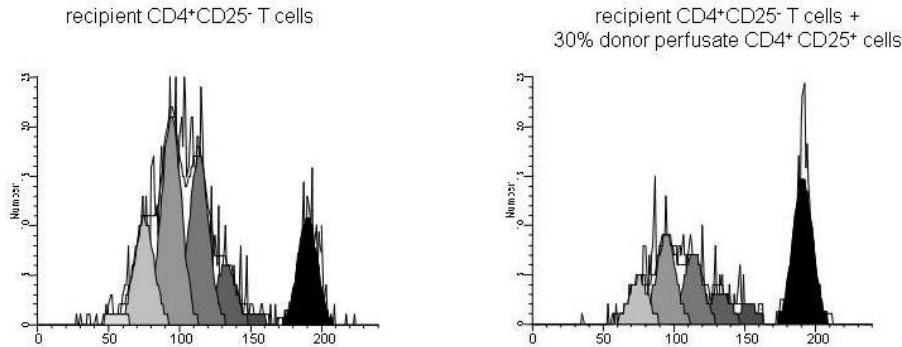
Figure 4A. Perfusate CD4$^+$CD25$^+$ T cells inhibit proliferation of both recipient and donor responder T cells. Representative MLR showing CFSE staining of control recipient CD4$^+$CD25$^-$ T cells (1 x 10^5) in the absence (left) and presence of perfusate CD4$^+$CD25$^+$ T cells (3 x 10^4) (right) upon stimulation with irradiated donor and recipient mononuclear cells. The number of cells in the daughter generations are significantly lowered in the presence of perfusate CD4$^+$CD25$^+$ T cells.
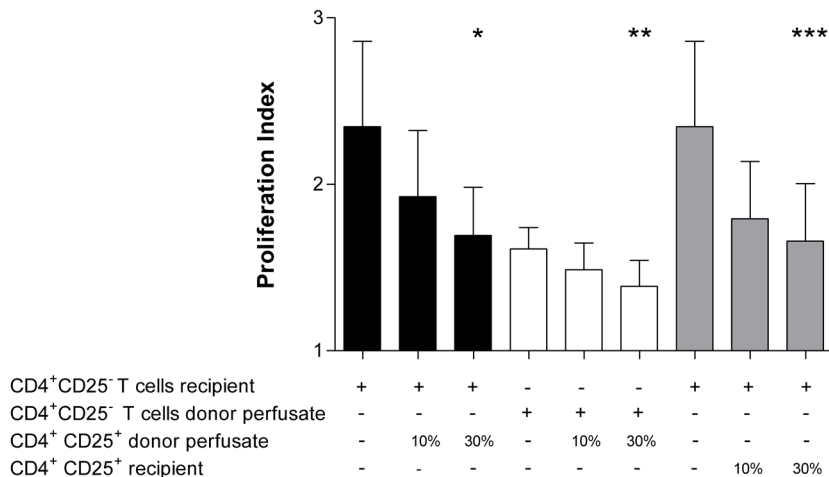

CD4$^+$CD25$^-$ T cells recipient		+	+	+	-	-	-	+	+	+
CD4$^+$CD25$^-$ T cells donor perfusate		-	-	-	+	+	+	-	-	-
CD4$^+$ CD25$^+$ donor perfusate		-	10%	30%	-	10%	30%	-	-	-
CD4$^+$ CD25$^+$ recipient		-	-	-	-	-	-	-	10%	30%

Figure 4B. Effect of perfusate and recipient CD4$^+$CD25$^+$ T cells on proliferation of recipient and donor CD4$^+$CD25$^-$ T cells. Proliferation of recipient (1 x 10^5) and perfusate responder cells (1 x 10^5) was significantly (* p=0.034 and ** p=0.032, respectively) inhibited by perfusate CD4$^+$CD25$^+$ T cells (black and white bars, respectively). Responder cells were stimulated with irradiated donor (5 x 10^4) and recipient mononuclear cells (5 x 10^4). As a positive control the suppressive activity of recipient CD4$^+$CD25$^+$ T cells was also determined (gray bars, *** p=0.003). Significance was tested by analysis of the logarithmic transformation of the dependent variable with random intercept and random slope. Shown is the mean ± SEM of 5 experiments.

indicating a state of anergy (data not shown). When CD4$^+$CD25$^+$ T cells from LPMC were added to recipient CD4$^+$CD25$^-$ responder cells, we observed significant suppression of proliferation comparable to the inhibition observed in the autologous setting (Figure 4). Consistently, when looking at the IFN-γ production in these MLR similar results were found showing dose-dependent inhibition by donor CD4$^+$CD25$^+$ T cells of donor and recipient IFN-γ production (Figure 5). The suppression of both proliferation and IFN-γ production suggests that CD4$^+$CD25$^+$ T cells from the liver are able to suppress the direct-pathway allo-response in vitro.

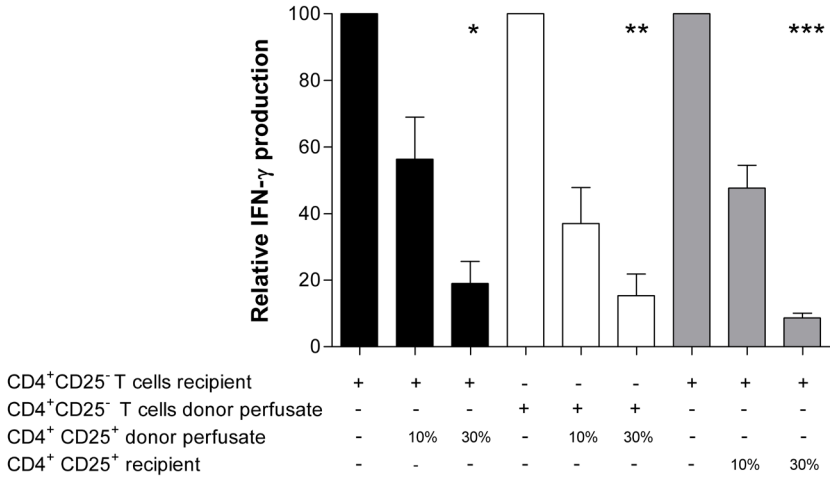| CD4+CD25- T cells recipient	+	+	+	-	-	-	+	+	+
CD4+CD25- T cells donor perfusate	-	-	-	+	+	+	-	-	-
CD4+ CD25+ donor perfusate		10%	30%	-	10%	30%	-	-	-
CD4+ CD25+ recipient	-	-	-	-	-	-	-	10%	30%

Figure 5. Inhibition of IFN-γ production by perfusate CD4+CD25+ T cells.
CD4+CD25+ T cells inhibit IFN-γ production by effector CD4+CD25- T cells. Shown are the mean relative inhibition of IFN-γ production (±SEM) for three experiments, as determined by ELISA. Perfusate CD4+CD25+ T cells inhibit IFN-γ production of recipient responder CD4+CD25- T cells (black bars, p=0.03) and of perfusate responder CD4+CD25- T cells (white bars, p=0.004). Recipient CD4+CD25+ T cells inhibit IFN-γ production of recipient responder CD4+CD25- T cells (grey bars, p=0.07).

CD4+CD25+CTLA-4+ T cells detach from the liver graft after Tx and circulate in recipients

In order to determine whether donor T cells with a regulatory phenotype can be detected in recipients after liver Tx, we performed flowcytometric analysis of PBMC from HLA-A2 negative recipients who received an HLA-A2 positive liver graft. Determination of HLA class 1-A2 allele has previously been shown to be useful to distinguish between donor and recipients cells (27, 28). A representative staining is shown in Figure 6A. In the first week after Tx 3.1± 1.0% SEM of the total CD4+ T cells and 2.5 ± 1.0% SEM of the CD4+CD25+CTLA-4+ cells were found to be of donor origin (Figure 6B). These percentages of donor cells increased initially and gradually declined at one and six months after Tx. Similar results were found when analyzing the percentage of HLA-A2+ cells within the CD4+CD25+Foxp3+ fraction (n=4, data not shown). The proportion of CD25+CTLA-4+ cells within CD4+ cells was significantly higher in donor-derived than in recipient cells (Figure 6C). Also at 6 months there was a difference although the overall percentage of CD4+CD25+CTLA-4+ cells was declined at this time point. These data indicate that donor T cells with a regulatory phenotype migrate from the liver graft into the circulation of recipients.
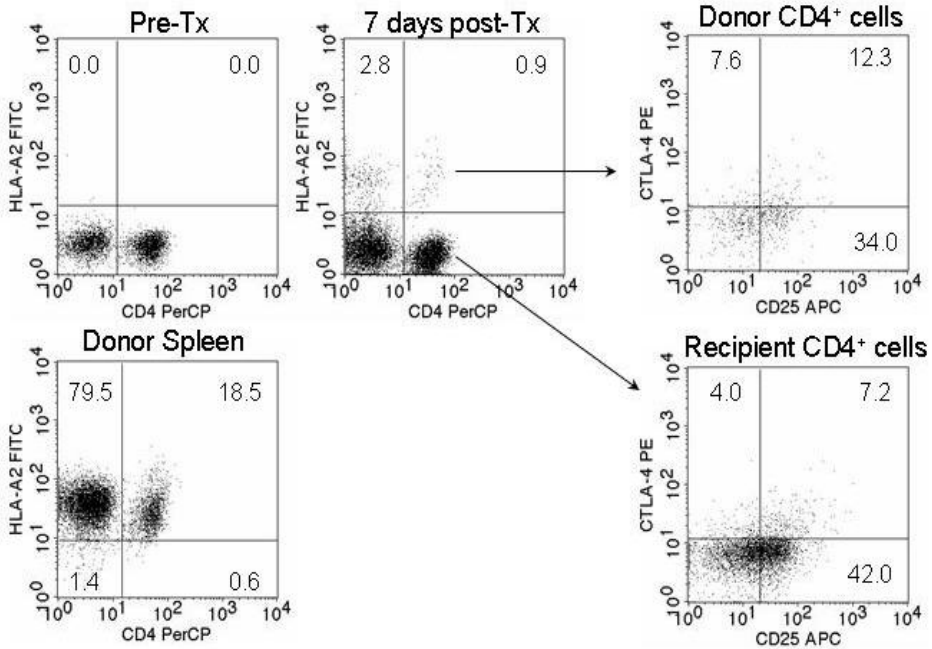
Figure 6A. Donor CD4⁺CD25⁺CTLA-4⁺ T cells circulate in recipients after liver Tx. Representative dot plots showing donor HLA-A2⁺ cells in recipient blood one week after Tx. Pre-Tx recipient PBMC and donor spleen cells serve as negative and positive control, respectively. The expression of CD25 and CTLA-4 of HLA-A2 positive and negative CD4⁺ cells is shown.
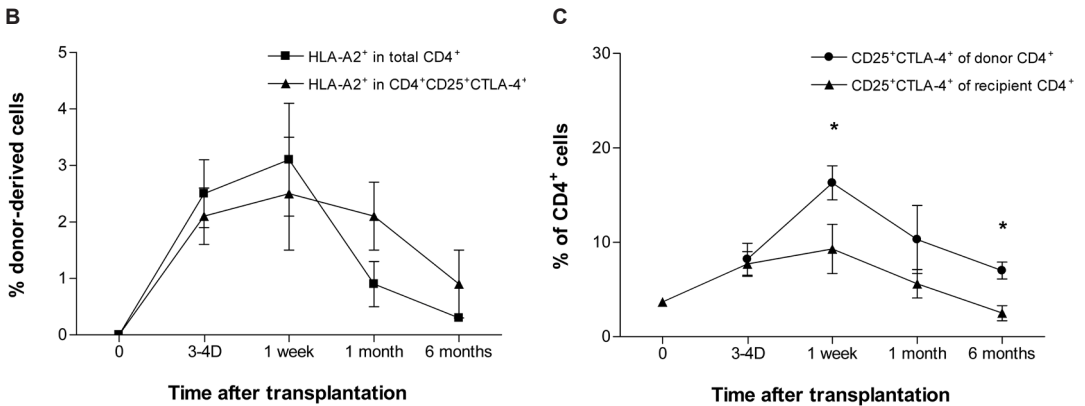
Figure 6B. Proportions of HLA-A2⁺ cells within the CD4⁺ cells and within CD4⁺CD25⁺CTLA-4⁺ cells are highest at one week (n=6) and gradually decrease at one (n=4) and six months after Tx (n=4). **(C)** One week and six months after Tx the proportion of donor CD4⁺CD25⁺CTLA-4⁺ cells within the donor CD4⁺ cells was increased compared to the proportions of CD4⁺CD25⁺CTLA-4⁺ cells within recipient CD4⁺ cells (* $p<0.05$). Both recipient and donor CD4⁺CD25⁺CTLA-4⁺ cells decrease at one and six months after Tx, although not reaching statistical significance due to small sample size.

DISCUSSION

The unique immunological properties of the liver have partly been attributed to the resident leukocyte population. Following liver Tx, donor leukocytes present in the graft are transferred and persist in recipients, a condition referred to as chimerism. In this study we aimed to investigate the presence of Treg in liver graft perfusion solution and in recipient blood after Tx. Within perfusate CD3+CD4+ T cells an increased proportion of CD25-CTLA-4+ and CD25+Foxp3+ T cells were found as compared to peripheral blood of healthy controls (Figures 2 and 3). Foxp3 mRNA expression in LPMC confirmed the presence of Treg (data not shown). Leukocytes in recipient circulation were shown to contain substantial numbers of donor CD4+CD25+CTLA-4+ T cells indicative of a regulatory signature (Figure 6). The functionality of donor Treg was studied in MLR, showing inhibition of proliferation of self- and recipient-responder CD4+CD25- T cells upon stimulation with donor and recipient mononuclear cells (Figure 4). Furthermore, the cytokine production was significantly inhibited, confirming the suppressive activity of CD4+CD25+ T cells within the LPMC (Figure 5).

The number of mononuclear cells obtained from perfusates showed a wide range, but did not correlate with ischemia time or perfusate volume (not shown). Immunophenotypic characterization of LPMC shows clearly differences compared to PBMC, yet comparable with leukocyte subsets obtained from liver tissue (23, 24). Our results show significantly lower proportions of CD4+ T cells and higher proportions of CD8+ within total LPMC T cell population compared to peripheral blood. Previously, it was shown that liver infiltrating T cells have high expression levels of the β2-integrin, LFA-1 (22, 25). Therefore we determined the expression of LFA-1 α-chain (CD11a), which is expressed exclusively on leukocytes, and is involved in migration into tissues. The CD4+ T cells in perfusates have a strong LFA-1 expression, which is significantly higher compared to CD4+ T cells in PBMC (Figure 1). The strong adhesion molecule expression and the reversed CD4:CD8 ratio indicates that these cells originate from the liver tissue and have a pre-activated phenotype (22, 29).

Within the CD4+ T cell population obtained from perfusates, a considerable proportion of T cells with a regulatory phenotype was observed. Treg characterized by CD4, CD25, CTLA-4 and Foxp3 expression were significantly increased compared to peripheral blood levels in healthy controls (Figures 2 and 3). Recently, the absence of the IL-7 receptor expression, CD127, has been suggested as a discriminating factor of Foxp3+ Treg (30, 31). Our analysis showed that the majority of liver-derived Foxp3+ cells were CD127 negative, which was comparable to control blood (Figure 3D). To our knowledge this is the first study to show the presence of CD4+CD25+Foxp3+ T cells as passenger leukocytes in liver grafts. Following liver Tx, a substantial proportion of circulating leukocytes are of donor origin (28) and even higher in case of graft-versus-host disease (32). In the first week after Tx, we have shown that up to 5% of total CD4+CD25+CTLA-4+ T cell population were of donor origin (Figure 6B), equivalent to approximately 5 to 15 x 10^6 cells. The proportion of CD4+ cells which expressed CD25 and CTLA-4 was higher in donor cells than in recipient cells at one and six months after Tx (Figure 6C). Both donor and recipient CD4+CD25+CTLA-4+ cells

decreased in time after Tx. This general drop of CD4$^+$CD25$^+$CTLA-4$^+$ Treg was consistent with our previous studies (33, 34).

Katz et al have shown that hepatic CD4$^+$ T cells are functionally suppressed by environmental factors (35), which may explain the low proliferative capacity of CD4$^+$CD25$^-$ responder cells from LPMC in our study. As we have shown that a relatively increased proportion of CD4$^+$ T cells present in the liver have a regulatory phenotype, one might postulate that hepatic Treg are involved in mediating suppression of responses to antigens presented in the liver. In the context of Tx, these hepatic Treg may suppress not only allo-antigen-specific recipient T cells but also DC and thereby inhibit the immune response against the graft. In our previous study we have shown that hepatic DC have an immature phenotype and produce high amounts of IL-10 (24). Experimental evidence suggests that immature DC can mediate tolerance, presumably by the induction of Treg (36, 37). Furthermore, in vitro, immature DC can induce allo-antigen-reactive Treg (38). Liver-derived DC are able to downregulate immune responses and stimulate T cells to produce IL-10 and IL-4 (39-41). On the other hand, Treg limit the ability of DC to stimulate T cells and enhance the ability of DC to induce anergy concomitant with an increase in CTLA-4 expression (42-44). This way a bidirectional interaction may occur between liver T cells and DC, which may explain the unique hepatic microenvironment that is known to promote tolerance.

In this study we have shown that donor CD4$^+$CD25$^+$ T cells originating from the liver graft are able to suppress responder T cells from both recipient and donor. This observation suggests that Treg can suppress across a MHC barrier. This is consistent with previous findings showing that Treg suppress antigen non-specific once activated through their TCR (45). Furthermore, recently it was shown that allogeneic Treg can inhibit MHC-disparate responder T cells (46). These data indicate that chimerism of donor Treg may contribute to suppression of the direct pathway alloresponse that is the dominant antigen presentation pathway driving rejection early after Tx. The immunological relevance of donor leukocytes for allograft acceptance is particularly evident during the early phase after Tx (3). As we have shown that CD4$^+$CD25$^+$ T cells are functional in suppressing responder cells, one might postulate that these cells participate in the silencing of donor reactive T cells. These findings fit with the possibility that chimerism plays a key role in acquired Tx tolerance.

Donor leukocyte migration and chimerism are associated with transplant tolerance both in the clinical and experimental setting (1, 2, 47). Depletion of donor leukocytes or failure to develop chimerism were shown to result in prompt rejection, but chimerism by itself is not sufficient to prevent rejection. In clinical Tx, the beneficial effect of donor leukocytes was clearly shown by pre-transplant blood transfusions leading to enhanced graft survival (48, 49). It is evident that donor leukocytes are important during the first weeks post-Tx in which maximal donor migration and interaction with host leukocytes occur. The exact mechanism in achieving tolerance through chimerism, however, has not been elucidated. Several mechanisms have been proposed in this

process including clonal exhaustion or deletion, T-cell anergy and active suppression (8, 10). Although the cell interactions are indisputably complex, tolerance to skin transplants following bone marrow Tx was CD4^{+}CD25^{+} Treg dependent mostly so early after Tx (19). Conversely, in bone marrow Tx an important role for CD4^{+} Treg has recently been identified in the induction of chimerism-associated tolerance, which was CD25 independent but CTLA-4 dependent (20). Whether graft-derived donor Treg influence the balance between the immunogenicity and tolerogenicity of organ allografts remains to be determined.

In conclusion, substantial numbers of donor Treg detach from the liver graft during perfusion and continue to migrate into the recipient after Tx. These donor Treg are functional in suppressing the direct pathway allo-responses in vitro and may therefore contribute to chimerism-associated tolerance early after liver Tx.

ACKNOWLEDGMENTS

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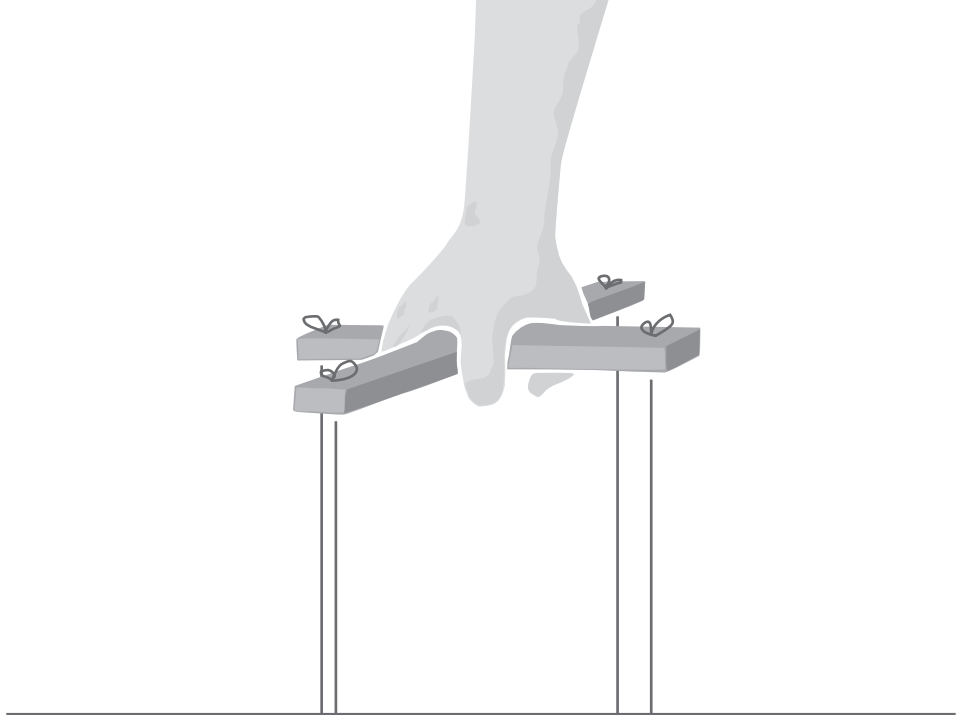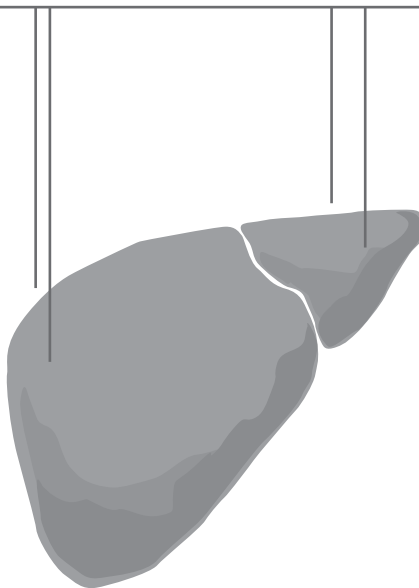
CHAPTER 7

Low IL-10 gene expression levels in liver and blood during acute rejection after liver transplantation

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ABSTRACT

The presence of CD4$^+$Foxp3$^+$ regulatory T cells (Treg) in the transplanted graft, causing local suppression, has been linked to the outcome of transplantation (Tx) in several animal models. The aim of this study was to investigate cytokine expression levels and the early presence of Foxp3$^+$ Treg in the graft after clinical liver Tx using a minimally invasive biopsy technique.

Blood and liver fine-needle-aspiration biopsy (FNAB) samples were taken at 7 to 21 days and 6 to 12 months post-Tx. Quantitative RT-PCR analysis was performed for Foxp3, IFN-γ, IL10, TGF-β, TCR-Cα and 18S mRNA. Flowcytometry was used to determine CD4$^+$Foxp3$^+$ cells.

Early after Tx IFN-γ expression was increased in the liver compared to blood, while at 6 months IL-10 and TGF-β expression was higher in the liver. Recipients developing acute rejection (n=4) showed significantly lower IL-10 expression within the liver (p=0.014) and in blood (p=0.002) during acute rejection, while later no differences were seen. Even though no difference was seen with relative Foxp3 mRNA levels, higher numbers of Foxp3$^+$ cells within the CD4$^+$ T-cell population was observed in the liver compared to blood with flowcytometric analysis of non-rejectors (n=7, p<0.05). This difference was less apparent during acute rejection.

This study shows that aspiration biopsy is a powerful approach to study intragraft gene expression patterns of cytokines and Treg after Tx, which provides relevant information regarding allo-reactive and allo-regulatory responses.

INTRODUCTION

In the past decade CD4^{+} regulatory T cells (Treg) have emerged as critical players in mediating tolerance to self-and non-self antigens. Characterized by Foxp3, a key regulator for Treg function, these cells are able to prevent auto-immune diseases and to induce and maintain transplant tolerance, eliminating the need for high-dose immune suppressive therapy (1-5). In the past decade many studies have been realised to reveal the functional and homeostatic characteristics of these cells. Data from animal studies suggest that at least part of the suppressive activity of Treg occurs in the transplanted organ itself (6, 7), including the liver graft (8). The presence of Foxp3^{+} Treg in the transplanted organ was much higher when the graft was tolerated compared to rejected grafts. This implies that the measurement of Treg presence in the transplanted organ may serve as a marker to determine graft acceptance. Previously, we and others showed that indeed Treg can be detected in the transplanted graft (9, 10). Within the liver graft Foxp3 expression increased in time, while in heart transplants highest Foxp3 expression was observed during acute rejection. Immune activating and immune suppressive cytokines play a central role in the process of rejection and tolerance. A study by Bishop et al. showed that IL-2 and IFN-γ expression are increased during acute rejection and that chronic rejection was associated with low IL-10 expression in the liver graft (11).

Differences between the immunological status in the graft as compared to peripheral blood emphasizes the need to further characterize the intragraft immune events. The major limitation to investigate intragraft parameters in the clinical setting is the risk of complications associated with obtaining tissue biopsies. To obtain tissue from the liver for diagnostic purposes the core-needle biopsy is generally used, which is performed under local anesthetics and can cause serious complications due to damage of intercostal space, intrahepatic blood vessels or biliary tracts. As a less traumatic alternative, the fine-needle aspiration biopsy (FNAB) has been successfully introduced for the detection of allograft rejection and was shown to be as effective as the core-needle biopsy (12-14). The diameter of this needle is significantly smaller and therefore does not require anesthetics and has minimal risk of serious complications. In FNAB from the liver, inflammatory leukocytes (including T lymphoblasts and B plasmablasts), plasma cells, lymphocytes and monocytes are present (15). This technique has been shown to be useful for cytological, immunocytochemical (16) and flowcytometric analysis (17, 18), and gene expression analysis (9).

The aim of the current study is to use FNAB of the liver to determine the presence of Foxp3^{+} Treg and analyze intrahepatic gene expression levels of Foxp3 and cytokines associated with immune activation and regulation. Here, we present data that within the first month after liver Tx IFN-γ expression is increased in liver compared to blood. Furthermore, expression of IL-10 was high in non-rejection and lower when rejection was developed. Cellular analysis showed that early after Tx the helper T-cell population in the liver contains higher levels of Foxp3^{+} Treg compared to blood.

PATIENTS AND METHODS

Patients
Patients who had undergone orthotopic LTx at the Erasmus MC-University Medical Center were consecutively enrolled. Standard immunosuppressive therapy consisted of anti-CD25 mAb (Basiliximab) induction therapy (i.v. 20 mg at day 0 and day 4 post-Tx), calcineurin inhibitions (Cyclosporin A or Tacrolimus) and steroids. Steroid treatment with Prednisone started with 20 mg daily from day 0 and was gradually weaned within 3 months. Acute rejections were clinically and histologically diagnosed in liver biopsies according to the Banff classification criteria. The Medical Ethical Committee of the Erasmus MC approved this study and an appropriate informed consent was obtained from all patients.

Liver aspiration biopsy and peripheral blood mononuclear cells (PBMC)
Fine-needle aspiration biopsies (FNAB) from the liver were performed as previously described (9). Briefly, the liver aspiration was carried out by ultrasound guidance, followed by a macroscopically and microscopically evaluation. Only representative FNAB specimens, i.e. containing at least seven hepatocytes per 100 leukocytes, were analyzed (25). Heparinized peripheral blood was collected at the time of the FNAB. PBMC were obtained by density gradient centrifugation over Ficoll-Paque plus (Amersham Biosciences, Buckinghamshire, UK). The FNAB specimens were partially used for flowcytometric analysis and partially for gene expression analysis.

RNA extraction and real time reverse transcription polymerase chain reaction (RT-PCR)
Isolation of RNA was performed as previously described (9). In summary, erythrocytes were lysed from PBMC (1×10^6 cells) and FNAB with a hypotonic buffer, which also caused lysation of the hepatocytes. The remaining leukocyte fraction was suspended in 0.5 ml TRIzol reagent (Life Technologies, GmbH, Karlsruhe, Germany). Total RNA was extracted and precipitated with ice-cold 80% ethanol (350 µl). RNA was isolated from the precipitated solution using a RNA-isolation kit (Qiagen, Venlo, The Netherlands) according to the manufacturer's instructions. Total RNA was denatured for 10 min at 80°C and then chilled to 4°C.
The isolated RNA was incubated with hexanucleotides (Promega Corporation, Madison, WI) and transcribed with reverse transcriptase (Life Technologies, Gaithersburg, MD) at 42°C for 90 min. The reaction mixture (20 µl) consisted of 2 µl (1000 U) of reverse transcriptase, 8 µl of 5 x reverse transcriptase buffer (250 mM Tris-HCl, pH 8.3, 15 mM MgCl2, 375 mM KCl), 2 µl of dNTP (10 mM), 0.4 µl (800 U) of RNAsin (Promega), 0.4 µl of Random Primers, 4 µl (0.1 M) of dithiothreitol and 3.2 µl of DEPC-H2O.
Quantification of Foxp3, IFN-γ, IL-10 and TGF-β mRNA was done with RT-PCR. As reference genes the constant region of the T cell receptor α chain (TCR-Cα) and the ribosomal 18S RNA were used. For relative quantification of the cytokines IL-10 and TGF-β, 18S RNA was used, as these genes are not exclusively expressed in T cells. Amplifications were performed

using the ABI 7700 sequence detector system (Applied Biosystems). Negative controls (no template), and positive reference samples were included repetitively for each determination, thereby verifying run-to-run variations. Samples negative for the TCR-α or 18S gene were excluded from further analysis. The relative gene expression was calculated based on the comparative ΔΔCt method with the TCR-α or the 18S expression in peripheral blood of blood-bank donors (18). The following formula was used: ΔΔCT (Ct Target – Ct TCR-α) $_{sample}$ - (Ct Target – Ct TCR-α) $_{control}$. The fold change was calculated using $2^{-\Delta\Delta Ct}$. Primers and probes for Foxp3, IFN-γ, IL-10, TGF-β and 18S were obtained from Assays-on-Demand Gene Expression Product and pre-developed Taqman PDAR assays (Applied Biosystems). The choice of primer and probe for the measurement of TCR-α transcripts was defined by using the primer express software (Applied Biosystems, Foster City, CA). The used sequences were for TCR-α were as previously described (10).

Flow Cytometric Analysis

PBMC were obtained by density gradient centrifugation over Ficoll-Paque plus (Amersham Biosciences, Buckinghamshire, UK). After washing twice with phosphate-buffered saline (PBS) containing 0.3% bovine serum albumin (BSA) PBMC and FNAB specimens were stained with monoclonal antibodies in PBS/0.3%BSA (30 minutes at 4ºC). The following fluorochrome-conjugated monoclonal antibodies were used: CD25-PE, CD4-PerCP-Cy5.5 from Becton Dickinson (San Jose, USA), CD8-FITC and IgG2a-PE from Immunotech (Marseille, France), Foxp3-APC and isotype IgG2a-APC from eBiosciences (San Diego, USA). Intracellular Foxp3 was stained after fixation and permeabilization using proper agents supplied by eBiosciences. Flowcytometric analysis was performed using FACS Calibur and CELLQuest Pro software (Becton Dickinson, San Jose, CA).

Statistics

Statistical analysis was performed using software package SPSS version 11.5 (SPSS, Chicago, IL). Significance was tested using the Wilcoxon paired test or the Mann-Whitney U unpaired test. P-values less than 0.05 were considered significant.

RESULTS

Cytokine expression kinetics in liver versus blood

From eleven liver transplant recipients FNAB were taken in the first month after liver Tx. None of the recipients developed complications due to the FNAB procedure. The quality of FNAB specimens was confirmed by microscopic analysis of the hepatocyte/leukocyte ratio and biopsies with blood contamination were excluded from further analysis. In four patients FNAB were taken during acute rejection confirmed with core needle biopsy. IFN-γ, IL-10 and TGF-β expression at early time points were compared to expression levels at six to twelve months after Tx in another cohort of patients (n= 20).

The expression of IFN-γ, IL-10 and TGF-β cytokines associated with immune activation and regulation, were determined by real-time RT-PCR. Relative expression was calculated based on the TCR-Cα mRNA levels, thereby allowing a comparison of T cell-related gene expression within liver and blood compartments. The universally expressed ribosomal 18S RNA was used as a reference gene for relative quantification of the cytokines IL-10 and TGF-β, which are not predominantly expressed in T cells. As shown in Figure 1, compared to blood the liver had a significantly higher expression of IFN-γ early after Tx (p=0.002), while at 6 months and later the expression was not different (Figure 1A). On the contrary, the expression of IL-10 and TGF-β was comparable early after Tx, but was significantly higher expressed in the liver at six to twelve months after Tx. These data suggest that early after Tx immune activating cytokines are predominantly expressed within the liver followed by dominating suppressive cytokine expression, suggesting a regulatory environment.
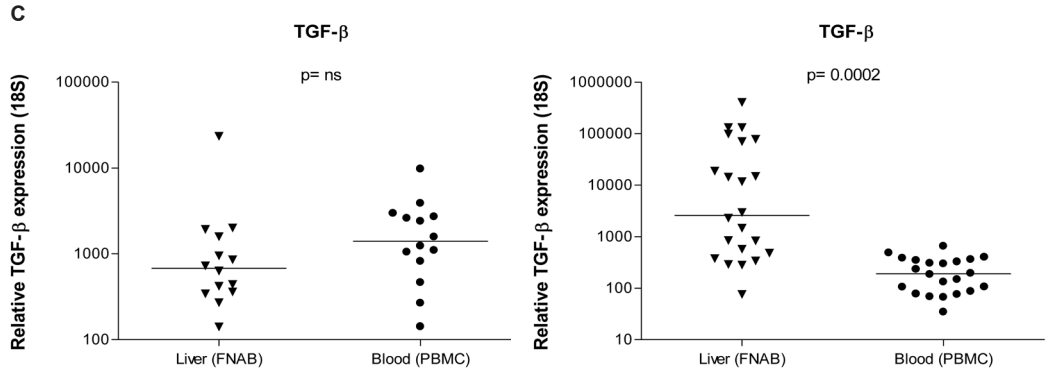
A

B

C
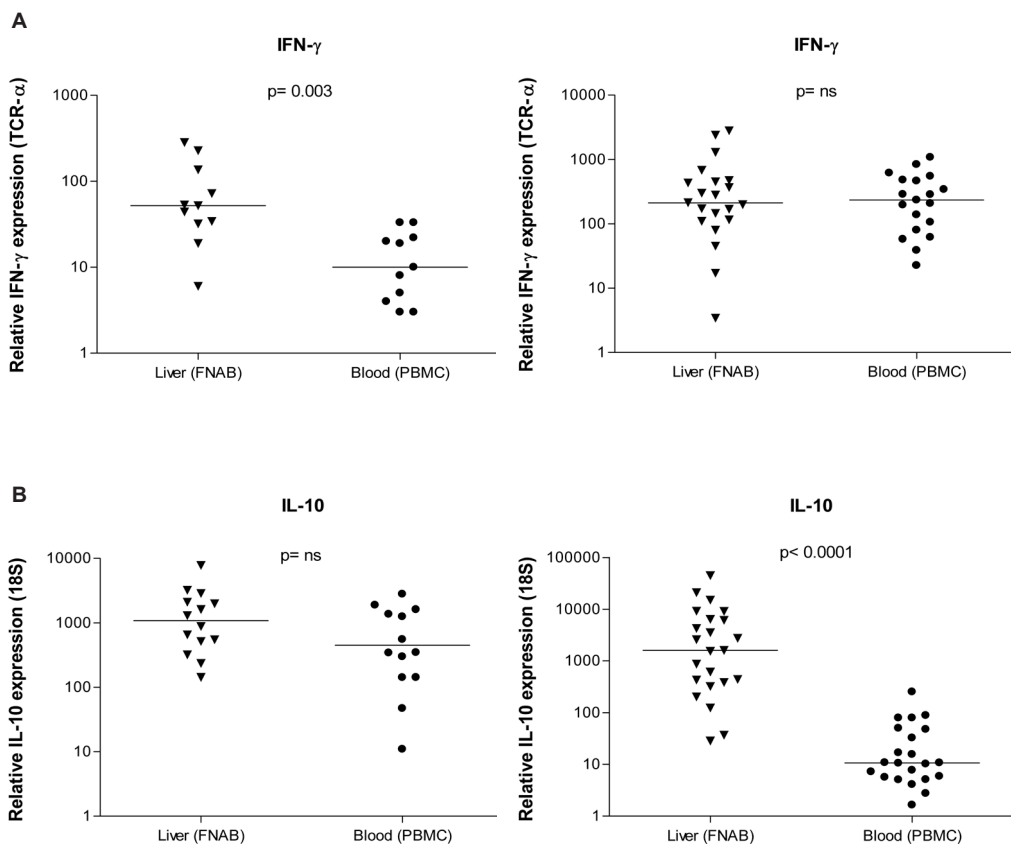
Figure 1. Differential IL-10, TGF-β and IFN-γ gene expression in liver and blood early (first 21 days) and late (6-12 months) after Tx. Gene expression was analyzed by quantitative RT-PCR for fourteen FNAB and blood samples at one month (left graphs) and twenty three at six to twelve months (right graphs) after Tx. IFN-γ expression was significantly higher expressed in the liver (A). At six to twelve months after Tx IL-10 and TGF-β expression was significantly higher in liver, but not IFN-γ (B and C). Comparisons were done using the Wilcoxon signed ranked test. Bar indicates median.

Cytokine expression in liver and blood during acute rejection

The gene expression in relation to the occurrence of acute rejection within one month after Tx was determined in liver and blood. Four of eleven recipients experienced an episode of biopsy-proven (RAI ≥5) acute rejection. As shown in Figure 2A, relative IL-10 levels in the liver of rejectors were significantly lower compared to non-rejectors (median 414 versus 1792, p=0.014). Also IL-10 gene expression in peripheral blood was significantly different between rejectors and non-rejectors (Fig 2B). TGF-β levels in liver and blood showed no difference between these groups (data not shown). Relative expression of IFN-γ was not determined.
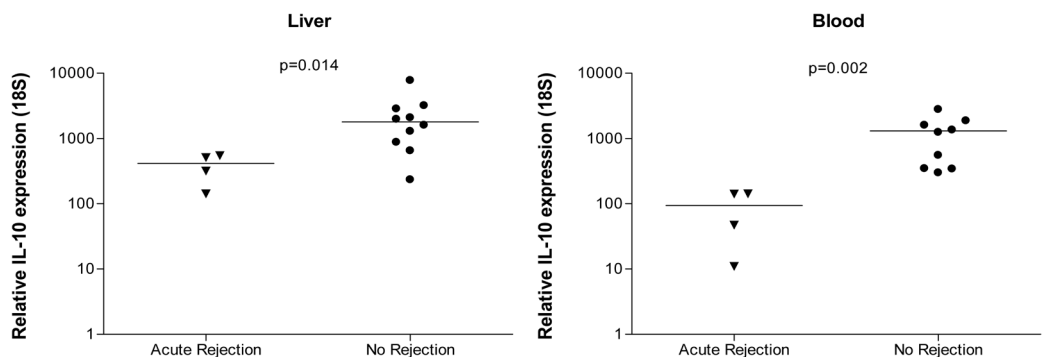

Figure 2. Acute rejection is associated with low IL-10 expression in liver and blood. In the first month after Tx, liver transplant recipients who were free of rejection had significantly higher IL-10 expression compared to recipients who developed acute rejection. Comparisons were done using the Mann Whitney U test. Bar indicates median.

Foxp3+ Treg levels in liver and blood early after Tx

To determine the presence of T cells with a regulatory phenotype, CD25 and Foxp3 expression within CD4+ T cells was assessed by flowcytometry in a subset of patients. Figure 3A shows representative dot plots of Foxp3+CD4+ T cells in liver and blood samples. As shown in Figure 3B, in recipients who did not develop rejection CD4+ T cells contained significantly higher numbers of Foxp3+ cells in liver compared to blood (median 8.4 vs 5.8%, p=0.031). On the contrary, intrahepatic Foxp3 mRNA expression levels were not different compared to blood (n=14). Comparison of CD25 expression within the CD4+ T cells revealed a higher proportion in the liver compared to blood (median 37.3 vs 28.7%, Figure 3C). The number of patients developing rejection was not sufficient for statistical analysis. This suggests an increased T cell activation state and elevated Treg levels in the liver graft early after Tx as compared to the blood compartment.
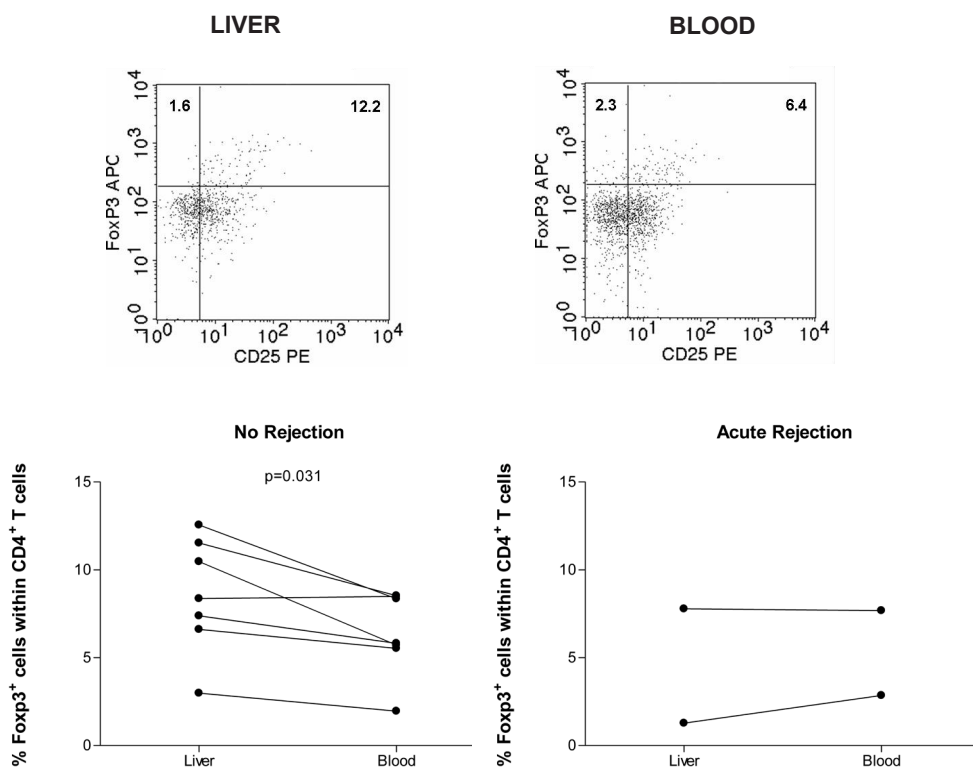
Figure 3. Increased proportion of Foxp3+CD4+ Treg in the liver compared to blood. (A) Representative dot plots showing flowcytometric analysis of CD4+Foxp3+ Treg in liver and blood. Within the intrahepatic CD4+ T-cell population of non-rejectors, an increased proportion of T cells expressing Foxp3 was observed compared to blood (B). Analysis of CD25+ cells within total CD4+ T cells showed higher percentages within the liver early after Tx. No statistical analysis could be performed for recipients who developed acute rejection due to small numbers.

No Rejection

Acute Rejection
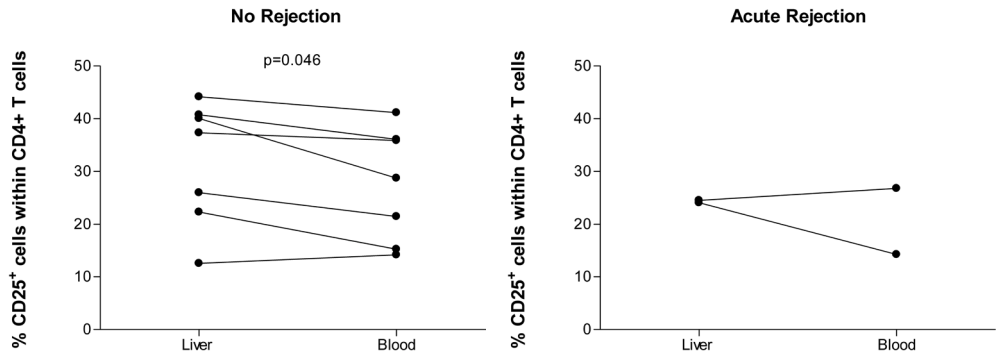
DISCUSSION

The ability to accurately quantify anti-donor immunity is an important issue in clinical Tx. Better understanding of immune reactivity and immune regulation may facilitate safe adjustments in immunosuppressive treatment or even complete withdrawal in case of apparent transplant tolerance. Major research efforts are done to develop methods for immune monitoring and detection of transplant tolerance. Our analysis of gene expression with RT-PCR revealed differential expression of the immune activating cytokine IFN-γ in liver compared to blood (Figure 1), suggesting an increased activation status in the liver. No differences were observed for the other investigated genes in the first month. The expression of IFN-γ in relation to acute rejection requires further analysis. Interestingly, levels of IL-10 and TGF-β mRNA remained highly expressed in the liver at 6 to 12 months after Tx and dropped in blood, which indicates an immune controlled environment within the liver. During acute rejection the relative expression of IL-10 was significantly lower in liver and blood, while Foxp3 expression did not differ. Also, in the non-rejectors no difference was observed in Foxp3 expression between liver and blood. In contrast, analysis of Fopx3+ cells within the CD4+ T-cell population in the liver, revealed an increased level of Foxp3+ cells compared to levels in blood. The discrepancy between the gene expression data and flowcytometric protein analysis may be due to the method of analysis. With flowcytometry the Foxp3+ cells are determined within the CD4+ T-cell population, as for the PCR analysis the expression of Foxp3 was determined relative to the TCR-Cα mRNA, which is also expressed in the extensive population of CD8+ cells in the liver, which practically do not express Foxp3 in the liver (Claassen, unpublished data).

IL-10 has been implicated in controlling cellular immune responses (20, 21), indicating that IL-10 may be crucial in the unique immune tolerogenic environment of the liver. In the setting of liver Tx, the addition of neutralizing IL-10 antibody showed remarkable increase of the anti-donor immune response as measured in a trans-vivo delayed type hypersensitivity response (22). Our results support these data showing lower expression of IL-10 during acute rejection,

suggesting an important role for IL-10 in the development or course of rejection. A recent study by Gras and collegues showed that liver transplant recipients who accepted their graft showed markedly increased IL-10 levels in blood early after Tx, whereas recipients with graft rejection did not (23). Furthermore, T cells from tolerant liver transplant recipients showed increased IL-10 production, associated with increased CD4+CD25high Treg (24). In kidney Tx, the ratio between IFN-γ and IL-10 gene expression has been shown to be relevant for immune monitoring (25). The importance of IL-10 in transplant outcome was shown by Yang and colleagues suggesting a close relationship of IL-10 and induction of long-term liver allograft survival (26). IL-10 recombinant protein was shown to augment the expression of Foxp3 and downregulate the expression of IL-2 and IFN-γ. Importantly, this was accompanied by the generation of CD4+CD25+Foxp3+ and CD8+CD25+Foxp3+ cells, which was blocked by the administration of IL-10 antibody. In addition, Foxp3+ Treg are shown to give rise to peripheral IL-10+ Treg, underlining the close relationship between Foxp+ Treg and IL-10 (27). In the liver, an enrichment of Foxp3+IL-10- Treg was observed. Our data showed a lower expression level of IL-10 during acute rejection. Whether this results in lower Foxp3+ Treg numbers needs to be determined. Several studies have linked IL-10 expression to hyporesponsiveness against the graft. Bishop et al. noticed that in chronic rejected liver transplants IL-10 expression was significantly lower compared to acute rejection and normal liver (11). In heart Tx high IL-10 expression was associated with donor-specific cytotoxic hyporesponsiveness. Altogether these findings suggest an important role for the immunomodulatory cytokine IL-10 in the course of rejection/tolerance.

Acute allograft rejection episodes are characterized by an influx of donor-reactive T cells in the graft. Accordingly, this is accompanied by a prominent increase in mRNA of pro-inflammatory cytokines, such as IL-2 and IFN-γ (11, 28, 29). A recent study by Baan et al showed that during a rejection episode of cardiac allografts relative expression of Foxp3 increased (10). This finding could indicate an influx or local induction of Foxp3+ T cells in the transplanted organ at the time of rejection. Our results for liver Tx showed a significant higher proportion of Foxp3+ cells within the helper T cells in the liver. The level of Foxp3+ Treg in relation to acute rejection requires further analysis. Interestingly no differences were seen in Foxp3 mRNA expression early after liver Tx, while relative expression was increased at 6 months after Tx (9). In kidney Tx levels of Foxp3 mRNA present in urine have been suggested as a biomarker of acute-rejection outcome, with lower levels associated with irreversible acute rejection and even graft failure (31). Altogether, better understanding of the relationship between Foxp3, immune activating/regulating cytokines and acute rejection may help to distinguish transplant outcome.

Increasing evidence shows that measurement of intragraft immunological parameters is as important or even more important compared to parameters in blood. Here, we show that the FNAB may be a favourable alternative for intragraft immune monitoring by intragraft analysis of cell composition and gene expression. In liver transplant recipients the proportion of CD4+ T

cells with a regulatory phenotype was increased compared to blood within the first month after Tx. This finding could indicate that Foxp3^{+} cells of the recipient traffic into the graft or may be locally induced after liver Tx. However, whether this Treg accumulation represents the beginning phase of tolerance to the liver graft remains speculative. The relation of Treg proportion and the development of acute rejection requires further analysis.

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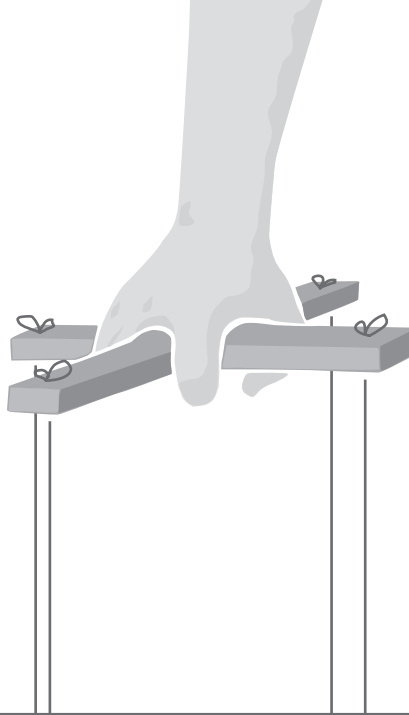
CHAPTER 8

Intrahepatic detection of Foxp3 gene expression after liver transplantation using minimally invasive aspiration biopsy

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Hugo W. Tilanus and Luc J.W. van der Laan*
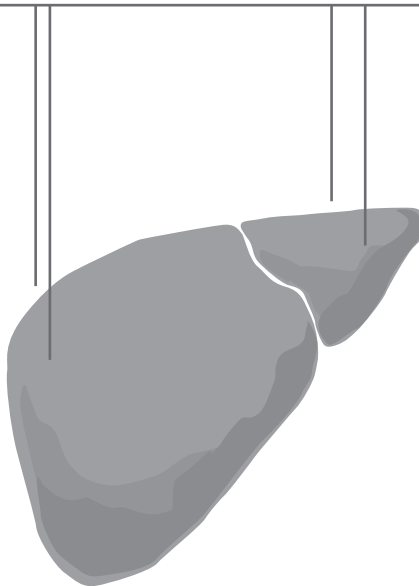
ABSTRACT

Intragraft accumulation of Foxp3$^+$ regulatory T cells (Treg) is associated with local suppression of allo-responses in transplantation models. In the current study the utility of the minimally invasive fine needle aspiration biopsy for the intragraft detection of Foxp3 and IFN-γ mRNA expression was investigated in clinical liver transplantation (LTx). Intragraft Foxp3 increased within the first year after LTx, but not in blood. Elevated Foxp3, but not IFN-γ expression, in the liver was observed after Hepatitis C virus (HCV) re-infection and after a previous episode of acute rejection. These data show the feasibility of aspiration biopsy for intragraft monitoring of gene expression. Intrahepatic Foxp3 levels are associated with HCV re-infection, a history of acute rejection and increased within the first year after LTx. Differences in gene expression between the graft and blood underline the importance of local immune monitoring.

INTRODUCTION

Immune regulation is vital for maintenance of tolerance to self antigens (1-3) and non-self antigens (4-6). Forkhead box P3 (Foxp3)-positive regulatory T cells (Treg) have been identified as important regulators in the immune homeostasis. Genetic defects, most notably Foxp3 deficiency, or impaired activity of Treg have been associated with ineffective suppression of auto-reactive cells leading to the development of autoimmunity (7-10). Further evidence suggests that pathogens can exploit Treg to create a favorable immunological environment in order to escape an adequate immune response. For instance, in chronic viral infections increased frequencies of circulating CD4$^+$CD25$^+$ T-cells have been reported (11-13). In chronic HCV infection, activation of virus-specific CD8$^+$ T-cells is suppressed by CD4$^+$CD25$^+$ Treg and this may contribute to the inadequate immune response against the virus (14).

In experimental organ transplantation, CD4$^+$CD25$^+$ Treg can suppress anti-donor immune responses, thereby preventing the development of rejection (15-17). Recent data suggest that at least part of the suppressive activity of Treg occurs locally in the transplanted allograft (18). The suppression of T cells mediating graft rejection is an active process that involves the persistent presence of Treg at the site of the tolerated graft. Consistently, Foxp3 expression in tolerated skin, cardiac and liver allografts is significantly enriched compared to rejected grafts (19-21). In the clinical setting, Li et al have shown that Foxp3 expression in tolerant liver grafts predominates over markers of immune activation (22). In heart allografts, Foxp3 expression is increased during acute rejection (23).

Here, we used the safe and minimally invasive biopsy technique, the Fine Needle Aspiration Biopsy (FNAB), to obtain liver tissue for the analysis of Foxp3 expression after human liver transplantation (LTx). Previously the FNAB has been successfully introduced for the detection of allograft rejection and liver inflammation (24-29), but no report has been made for the detection of intrahepatic gene expression in LTx.

PATIENTS AND METHODS

Patients who underwent orthotopic LTx between June 2004 and July 2005 were consecutively enrolled. Recipient characteristics are summarized in Table 1. Immunosuppressive therapy consisted of anti-CD25 mAb (Basiliximab) induction therapy (i.v. 20 mg at day 0 and day 4 post-Tx), a calcineurin inhibitior (Cyclosporin A or Tacrolimus) and steroids. Steroid treatment with prednisone started with 20 mg daily from day 0 and was gradually weaned. Acute rejections all occurred within three months after LTx and were histologically diagnosed in liver biopsies according to the Banff classification criteria (1997). This study was approved by the Medical Ethical Committee of the Erasmus MC and an appropriate informed consent was obtained from all patients. Ultrasound guided FNAB specimens were obtained from the liver allograft of twenty liver transplant recipients at six and/or twelve months after LTx, as

previously described (30). From four patients paired (six and twelve month) samples were obtained. When the aspirate contained predominantly blood at macroscopic evaluation, an additional FNAB was taken.

Only representative FNAB, i.e. containing at least seven hepatocytes per 100 leukocytes in cytospins, were included (25). Blood was collected at the time of the FNAB and peripheral blood mononuclear cells (PBMC) were obtained by density gradient centrifugation. Erythrocytes were lysed from PBMC (1 x 10^6 cells) and FNAB with a hypotonic buffer. Under these conditions the majority of hepatocytes (70-90%) in the FNAB were lysed. The remaining leukocyte fraction was suspended in 0.5 ml TRIzol reagent (Life Technologies, GmbH, Karlsruhe, Germany) and 20 μg poly A (Boehringer, Mannheim, Germany) was added. The TRIzol ly-

Patient	Sex	Age	Disease[a]	Acute Rejection	CNI[b]	Steroids[c]	FNAB 6 M	FNAB 12 M	ALT (U/L) 6 M	ALT (U/L) 12 M
1	M	59	CLC	-	TAC	-		X		33
2	V	33	PSC	-	TAC	+		X		12
3	M	47	CLC	-	CsA	+		X		29
4	M	44	HCV	-	TAC	+	X	X	126	194
5	M	41	CLC	-	TAC	+	X	X	30	29
6	M	57	HBV	-	TAC	-		X		44
7	M	50	HCV	-	TAC	-		X		250
8	M	25	ALF	+	TAC	+	X		11	
9	M	56	PSC	-	CsA	+	X		18	
10	M	55	ALC	-	CsA	+	X		36	
11	M	58	PBC	-	TAC	-		X		31
12	M	48	HCV	-	TAC	-	X	X	102	171
13	V	38	PSC	-	TAC	+	X	X	20	15
14	M	29	PSC	+	TAC	-	X		150	
15	M	47	HCV	+	TAC	-		X		107
16	M	49	HCV	-	TAC	-		X		51
17	M	60	ALC	-	TAC	+		X		21
18	M	58	HBV	+	TAC	+	X		56	
19	V	50	PBC	-	TAC	+	X		19	
20	V	22	ALF	-	TAC	-	X		25	

Tabel 1. Patient characteristics. [a] CLC Cryptogenic Liver Cirrhosis, PSC Primary Sclerosing Cholangitis, HCV Hepatitis C Virus Infection, HBV Hepatitis B Virus Infection, ALF Acute Liver Failure, ALC Alcholic Liver Cirrhosis, PBC Primary Biliary Cirrhosis, [b] CNI Calcineurin Inhibitor, TAC Tacrolimus, CsA Cyclosporin A, [c] + prednisone (5-10 mg/day) use at time of FNAB, - no steroid use at time of FNAB. Hepatitis B virus (HBV) infected recipients (n=2) were additionally treated with anti-HBV immunoglobulines and lamivudine to prevent re-infection of the liver graft. HCV-positive recipients (n=5) did not receive any anti-viral therapy.

sates were directly stored at -80ºC. Total RNA was extracted with 160 μl of cold chloroform-isoamylalcohol and subsequently precipitated with ice-cold 80% ethanol (350 μl). The precipitated solution was then loaded on a RNA-isolation column from the Qiagen RNeasy isolation kit (Qiagen, Venlo, The Netherlands) and RNA was isolated according to the manufacturer's instructions. Total RNA was denatured for 10 min at 80°C and then chilled to 4°C. First-strand cDNA synthesis was performed as previously described (31). Real-time RT-PCR was used to quantify Foxp3 , IFN-γ mRNA and as reference genes the constant region of the T cell receptor α chain (TCR-Cα) and CD3ε. Primers and probes for Foxp3, IFN-γ and CD3ε were obtained from Applied Biosystems (Foster City, CA, USA). The used sequences for TCR-Cα transcripts were as previously described (23). Amplifications were performed using the ABI 7700 sequence detector system (Applied Biosystems).

RESULTS AND DISCUSSION

None of the twenty liver transplant recipients experienced complications due to the FNAB. When comparing FNAB taken at six or twelve months after LTx, Foxp3 expression significantly increased in the liver, while relative expression in peripheral blood did not significantly change (Figure 1). The relative increase of Foxp3 was confirmed in four individual patients with paired FNAB samples taken at six and twelve months (median Foxp3 level of 47 at six- versus 128 at twelve-months). When CD3ε mRNA was used as a reference (n=15), the increase of Foxp3 levels in the graft at 12 months (p=0.045) was confirmed. No significant differences in liver or blood were seen for expression of IFN-γ related to TCR-Cα or CD3ε (not shown). The observed increase of Foxp3 expression could indicate that Foxp3+ cells of the recipient traffic
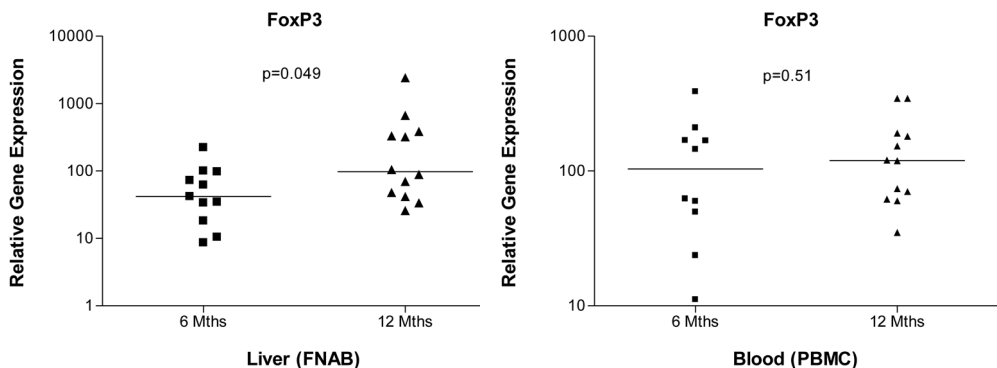
Figure 1. Intrahepatic enrichment of Foxp3 expression in time after transplantation. Analyzes of mRNA was performed by RT-PCR of liver FNAB and PBMC at six- (n=11 and 10, respectively) and 12-months (n=12) post-LTx. mRNA was quantified by measuring threshold cycle (Ct). (**A**) Relative to TCR-Cα Foxp3 mRNA levels in the liver were increased at 12 months after LTx compared to 6 months. (**B**) Levels in blood showed no significant difference for Foxp3 mRNA at these time points. The increase of Foxp3 in the liver was confirmed in a subset of samples with CD3ε as T-cell specific reference gene (n=15, p=0.045, not shown). The bar indicates median. Statistical analysis was performed with the Wilcoxon paired test (SPSS version 11.5, Chicago, IL).

Figure 2. Increased intrahepatic Foxp3 levels after HCV re-infection and acute rejection. Comparison of mRNA expression in HCV-positive (n=7), resolved acute rejection (n=3) and non-HCV/non-rejection FNAB (n=13). (A) Relative F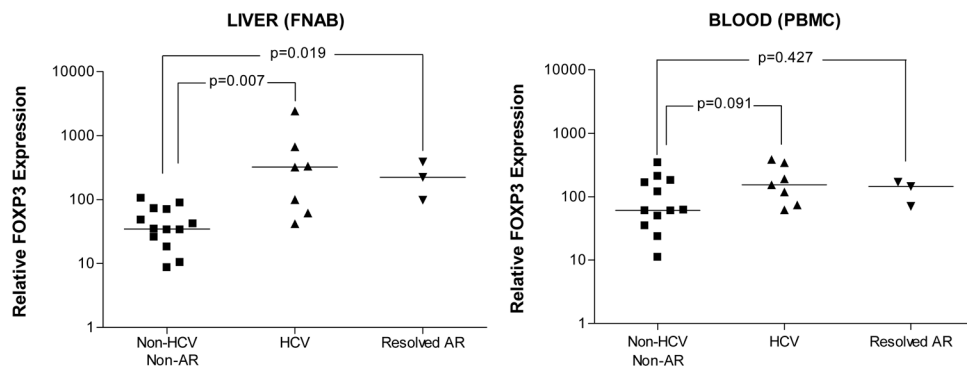oxp3 expression in the liver at was significantly higher in HCV recipients and in patients with a history of acute rejection as compared to non-HCV and non-rejectors. The increased Foxp3 in the liver of HCV recipients was confirmed in a subset of samples where CD3 could be determined as an additional T-cell reference gene (p<0.025). (B) In peripheral blood Foxp3 expression was not different between the groups.

into the graft and accumulate within time after LTx. However, whether the increase in Foxp3 expression represents a tolerant state of the liver graft, remains speculative.

To investigate the effect of HCV recurrence on Foxp3 gene expression, we compared HCV-positive and -negative recipients. All five HCV positive recipients developed recurrence of disease as indicated by biopsy histology and high viral load (range 1.15-2.68x10^7 geq/ml). Serum AST levels were elevated in most of these patients, indicating active liver disease (Table 1). As shown in Figure 2A, in FNAB of HCV-positive recipients Foxp3 expression was significantly increased compared to non-HCV recipients. In blood no significant differences were detected (Figure 2B). The increased Foxp3 in the liver was confirmed in a subset of samples where CD3ε could be determined as an additional T-cell reference gene (p<0.025) and no such differences were seen in blood (not shown). The relative expression of IFN-γ in liver and blood was not different between HCV and non-HCV recipients (Figure 2C and D).

It is known that HCV employs different strategies to evade an adequate immune response. One of these immunological escape mechanisms that has been reported is the induction of virus-specific Treg. Increased levels of these cells were shown to suppress HCV-specific CD8^{+} T cells (32, 33). Our data, showing an increased expression of Foxp3 in liver grafts from HCV positive recipients, suggest an intrahepatic enrichment of Treg (Figure 2). The presence of Foxp3^{+} Treg in liver FNABs was recently confirmed by flowcytometric analysis in chronically HCV patients in our laboratory (Claassen et al. unpublished data).

The impact of an acute rejection episode on Treg-associated gene expression at 6 and 12 months post-LTx was determined in liver and blood. Three of fifteen non-HCV recipients patients experienced an episode of biopsy-proven (RAI≥5) acute rejection, which resolved spon-

taneously. As shown in Figure 2A, relative Foxp3 levels in the liver of rejectors were significantly higher compared to non-rejectors, while levels in blood were comparable. IFN-γ mRNA expression was not different in liver or blood (Figure 2C and D).

Acute allograft rejection episodes are characterized by an influx of donor-reactive T cells within the graft. This is accompanied by a prominent increase in mRNA of pro-inflammatory cytokines, such as IL-2 and IFN-γ (34, 35). Baan et al showed that during a rejection episode of cardiac allografts relative expression of Foxp3 increased (23). This could indicate an influx or local induction of Foxp3+ T cells in the transplanted organ during rejection. Our results for LTx, indicate an increased Foxp3 mRNA long after the occurrence of a spontaneously resolved rejection episode (Figure 2). At this time-point the IFN-γ mRNA was not elevated. Whether this high Foxp3 expression is associated with a more immune tolerogenic status or associated with immune activation remains to be determined. Interestingly, a recent study in kidney transplant recipients suggests that levels of Foxp3 mRNA may serve as a mechanistically informative biomarker of acute-rejection outcome, with lower levels associated with irreversible acute rejection and even graft failure (36). Though statistically significant differences were observed in our study, the overlap of Foxp3 expression between the controls, HCV positive recipients and resolved acute rejectors currently limits the use of intragraft Foxp3 as a clinically relevant biomarker in LTx.

In summary this study demonstrates that FNAB can be used to monitor gene expression profiles in the liver allograft. Using aspiration biopsy it is possible to safely and frequently sample the graft. The diameter of this needle is significantly smaller than the classical needle and therefore does not require anesthetics and has minimal risk of serious complications. The differences between peripheral blood and the liver highlight the importance of local immune monitoring, which may be important for the detection of transplant tolerance.

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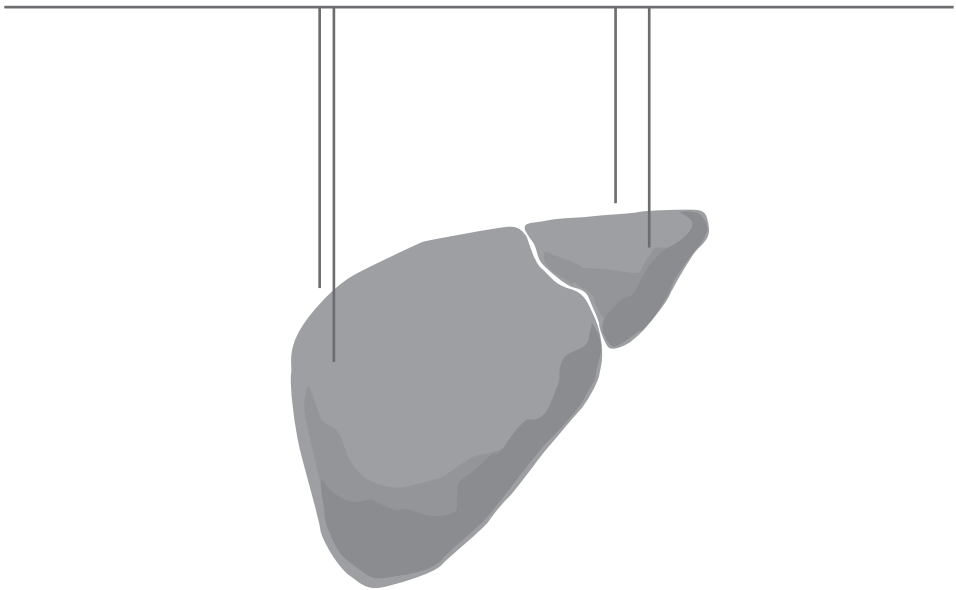
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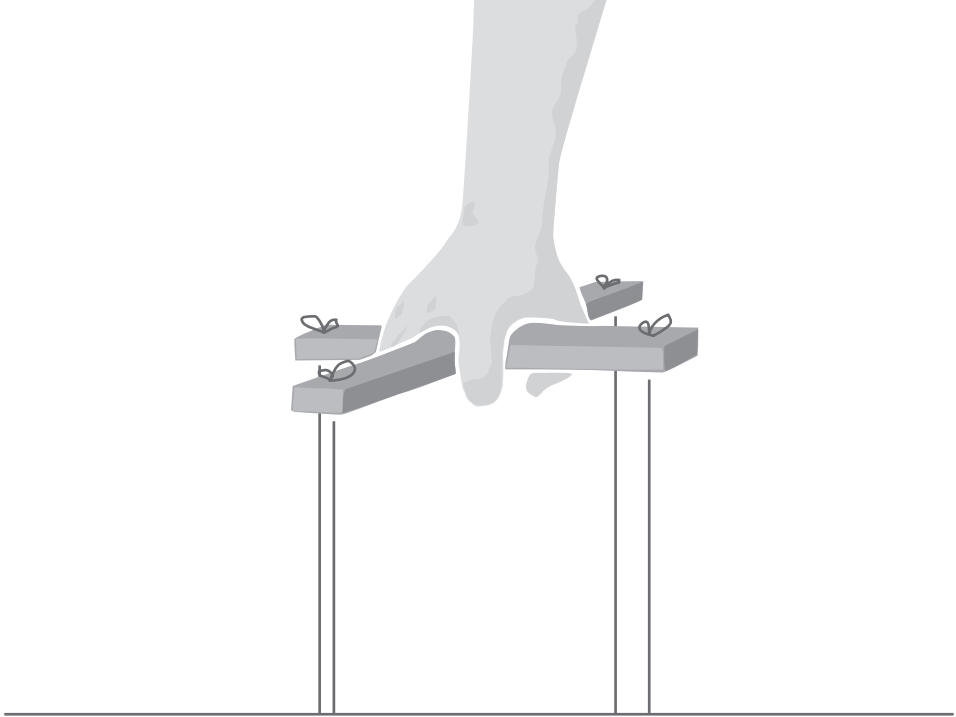
CHAPTER 9

Summary and Discussion

Ahmet Demirkiran

The success of solid-organ transplantation depends on the continuous administration of immunosuppressive drugs to prevent rejection. Most of the currently used immunosuppressive drugs, however, are toxic and target the immune response in a nonspecific fashion. Life long use of immunosuppressive drugs is accompanied by many debilitating side effects influencing long-term graft and patient survival and quality of life. Therefore, reducing the necessity of immunosuppressive drugs by inducing transplant tolerance is the ultimate challenge in the organ transplantation field. One of the possibilities to achieve a state of tolerance is to exploit regulatory T cells (Treg). In various experimental transplantation models Treg have been shown to play a vital role in tolerance (1-3). Although several subsets of Treg have been described to play a role in transplant tolerance, the CD4$^+$CD25$^+$Foxp3$^+$ Treg appear to play a prominent role in controlling the allo-response after transplantation. This subset is capable to induce and maintain immunological non-responsiveness, also referred to as operational tolerance. However, this does not exclude a possible role for other significant regulatory populations, like IL-10 producing Tr1 cells (4), CD8$^+$ suppressor T cells (5) and NKT cells (6), in clinical transplant tolerance. As a matter of fact, these distinct regulatory cell populations may all be involved in the immunological outcomes following transplantation, individually or synergistically. In this thesis we focused on the CD4$^+$CD25$^+$Foxp3$^+$ Treg subset.

The induction of donor-specific Tregs and the potential use of these cells in transplantation tolerance is one of the main topics of research in transplantation immunology. The nonspecific inhibition of the immune system by immunosuppressive drugs may impact the development and function of these Treg and therefore the effect of immunosuppressive drugs on Treg should be made clear to optimize tolerance induction strategies. In **chapter two** we reviewed recent knowledge on the effect of immunosuppressive medication on the homeostasis and function of Treg in vitro and in clinical transplantation.

Circulating regulatory T cells

In part II of this thesis we investigated Treg levels in relation to immunosuppressive therapy, to the occurrence of acute rejection and to time after liver transplantation. Our data suggest that immunosuppressive therapy, in particular the combinational use of steroids and calcineurin inhibitors (CNI), reduce the numbers of CD4$^+$CD25$^+$ Treg in peripheral blood. This finding highlights the possible contrasting effects of immunosuppression: preventing acute rejection by blocking the activation of effector T cells, but also possibly inhibiting the development of donor-specific tolerance by suppressing Treg. In **chapter three** and **chapter four** we showed that after human liver transplantation the percentage of Treg expressing CD4, CD25, CD45RO and CTLA-4, changes dramatically. Levels of these Treg in peripheral blood decreased after transplantation. Importantly, we found significant differences in Treg levels between patients who experienced acute rejection versus non-rejectors. Despite these quantitative changes, the suppressive activity of isolated CD4$^+$CD25$^+$ T cells from liver transplant recipients, both rejectors and non-rejectors, was comparable to that of healthy controls. Furthermore, we showed an association between steroid treatment for acute rejection and sustained low levels of

Treg, which illustrates the effect of immunosuppression on Treg. Taken together, these data imply that immunosuppressive therapy effects Treg levels in blood and that upon allogeneic stimulation the suppressive capacity of total Treg may affect the course of immune activation causing rejection or non-responsiveness.

The suggested negative effect of CNI on Treg was confirmed in **chapter five** showing a significant increase of Foxp3^{+} cells within the CD3^{+}CD4^{+} T cells after discontinuation of CNI and a decrease after re-introduction of CNI. We conducted a conversion study in long term liver transplant recipients with renal impairment. To study the effect of CNI conversion on Treg and to improve renal function we changed the immunosuppressive therapy from CNI to mycophenolate mofetil (MMF). We observed that conversion to MMF caused the percentage of Foxp3^{+} cells to increase after six months as well as an increase of CD25 expression on Foxp3^{+} cells suggesting that Treg may become more susceptible to IL-2 signals. These findings are in line with previous studies showing a decrease of Treg after CNI treatment and higher levels of Treg in patients treated with a non-CNI based immunosuppressive regimen (7-10). The possible negative effect of CNI on tolerance is accompanied with non-immunological side effects, in particular nephrotoxicity, causing significant morbidity. This study showed that conversion from a CNI based immunosuppressive regimen to MMF monotherapy improves renal function with a low risk of acute rejection, supporting previous conversion studies in liver transplant recipients. Conversion from CNI to MMF in liver transplant recipients increases the proportions of circulating CD4^{+}CD25^{bright} T cells, CD4^{+}CD25^{+}CTLA-4^{+} T cells and CD4^{+}Foxp3^{+} Treg, which may have important implications on graft immunity and tolerance. Clinically, conversion results in an improvement of renal function and a decrease of blood pressure.

Intrahepatic regulatory T cells
In part III we aimed to study the presence of Treg within the donor liver before and after transplantation. The unique immunological properties of the liver have partly been attributed to the resident leukocyte population. Following liver transplantation, donor leukocytes present in the graft are transferred and persist in recipients, a condition referred to as chimerism. In **chapter six** we investigated the presence of Treg in the liver graft by analysis of the perfusion solution obtained prior to transplantation. We also studied the presence of donor Treg in recipient blood after transplantation. Within perfusate helper T cells, an increased proportion of CD25^{+}CTLA-4^{+} and CD25^{+}Foxp3^{+} T cells were found as compared to peripheral blood of healthy controls. We confirmed the presence of Treg by analysis of Foxp3 mRNA expression in liver perfusate mononuclear cells. Furthermore, we demonstrated that donor leukocytes detaching from the liver were present in recipient circulation and contained substantial numbers of donor CD4^{+}CD25^{+}CTLA-4^{+} T cells. In a mixed leukocyte reaction we were able to show that donor Treg could suppress the direct pathway alloresponse as shown by inhibition of proliferation and cytokine production of recipient-responder T cells upon stimulation with donor cells. This observation suggests that Treg can suppress across a MHC barrier. These

data indicate that chimerism of donor Treg may contribute to suppression of the direct pathway alloresponse that is the dominant antigen presentation pathway driving rejection early after transplantation. Donor leukocyte migration and chimerism are associated with transplant tolerance both in the clinical and experimental setting. Depletion of donor leukocytes or failure to develop chimerism was shown to result in prompt rejection (11-13), but chimerism by itself is not sufficient to prevent rejection. In clinical transplantation, the beneficial effect of donor leukocytes was clearly shown by pre-transplant donor blood transfusions leading to enhanced graft survival (14, 15). It is evident that donor leukocytes are important during the first weeks after transplantation, in which maximal donor migration and interaction with host leukocytes occur (16). The exact mechanism in achieving tolerance through chimerism, however, has not been elucidated. Whether graft-derived donor Treg influence the balance between the immunogenicity and tolerogenicity of organ allografts remains to be determined. We have shown that donor Treg are functional in suppressing the direct pathway allo-responses in vitro and may therefore contribute to chimerism-associated tolerance early after liver transplantation.

In **chapter seven** and **chapter eight** we studied the expression profiles of immune activating and immune suppressive cytokine genes as well as Treg associated genes. We also performed a flowcytometric analysis of leukocytes present in the liver. To obtain liver tissue we chose the fine-needle aspiration biopsy technique, which has been shown to be a minimally invasive procedure enabling us to study intrahepatic parameters. In chapter seven we aimed to study the changes of Treg associated gene expression and several cytokine genes early after transplantation. We presented preliminary data on the early presence of Treg within the graft and cytokine expression profiles in relation to the occurrence of acute rejection. This study showed that acute rejection is associated with reduced RNA expression levels of the immunosuppressive cytokine IL-10 in the liver and in blood. In the early phase after transplantation there was a higher IFN-γ expression, while at later time points IL-10 expression remained high in the liver and IFN-γ expression levels were not different from blood. This suggests that in the first weeks after transplantation immune activating genes predominate, followed by immune suppressive gene expression at 6 and 12 months after transplantation. Differences in IL-10 expression and possibly Foxp3$^+$ and Foxp3$^-$ T cell ratio may have a predictive value for the development of acute rejection. Moreover, cytokine expression/production patterns may effect the immunological course leading to graft acceptance or rejection. The observed differences in the limited numbers of patients highlight the importance of early immune profiling. Further study and expansion of the study group is required to understand the clinical relevance of these findings. Important issues remaining to be answered are the origin of the different expressed cytokines and the relationship of Treg with other cells present in the liver.

In **chapter eight** we demonstrated that one year after transplantation Foxp3 expression significantly increased in the liver compared to six months, while relative expression in peripheral blood did not significantly change. This observed increase of Foxp3 expression could reflect

an intrahepatic accumulation of Foxp3+ cells within time after liver transplantation. Whether the increase in Foxp3 expression represents a tolerant state of the liver graft, remains speculative. Interestingly, we observed differential expression of Foxp3 between Hepatitis C Virus (HCV)-positive and -negative transplant recipients. In HCV-positive recipients Foxp3 expression in the liver, but not in blood, was significantly increased compared to non-HCV recipients. This finding suggests an intrahepatic enrichment or activity of Treg. HCV is thought to persist and escape an adequate immune response by manipulating the host immune system. One of the immunological escape mechanisms of HCV to avoid an adequate immune response that has been reported is the induction of virus-specific Treg. Increased levels of Treg were shown to suppress a specific anti-HCV T cell response. The increased levels of Foxp3 expression within the liver may reflect a more tolerogenic environment towards the virus. Further characterization of intrahepatic Treg may advance our understanding of HCV recurrence and persistence, which could be used for the enhancement of anti-HCV immune response/therapy.

An episode of biopsy-proven (RAI≥5) acute rejection was associated with increased Foxp3 levels in the liver compared to non-rejection, while levels in blood were comparable. Acute allograft rejection episodes are characterized by an influx of donor-reactive T cells within the graft. This is accompanied by a prominent increase in mRNA of pro-inflammatory cytokines, such as IL-2 and IFN-γ. Our results indicate an increased Foxp3 mRNA long after the occurrence of a spontaneously resolved rejection episode. At this time-point the IFN-γ mRNA was not elevated. Whether this high Foxp3 expression is associated with a more immune tolerogenic status or associated with immune activation remains to be determined. The aspiration biopsy, as an alternative to classical needle-biopsy, has been shown to be safe and to be utile to monitor gene expression profiles in the liver allograft. The differences between peripheral blood and the liver underline the importance of local immune monitoring, which may have important implications of our understanding of rejection and transplant tolerance.

The ability to accurately quantify anti-donor immunity is an important issue in clinical transplantation. The ultimate aim is to enable identification of transplant recipients who can be weaned from immunosuppressive therapy based on immuno-assays in order to raise the quality of life and improve transplantation outcome. Several mechanisms of tolerance and tolerance induction have been suggested including active immune regulation, chimerism, T-cell and B-cell deletion, anergy and clonal exhaustion. All of these mechanisms may represent one of the "ways to Rome", however, up to date evidence to link these mechanisms are lacking and require further exploration to fully understand their role in clinical transplantation. Most of these mechanisms have been studied in an experimental setting and need further analysis in the clinic. We aimed to identify and study changes of Treg in clinical liver transplantation. In this thesis we have shown that functionally active Treg are present in the liver and in the circulation of liver transplant recipients. The choice of immunosuppressive therapy has been shown to influence the fate of these cells, thereby interfering with the natural homeostatic regulatory status and possibly tolerance induction. This reflects the two edges of

the immunosuppressive sword; preventing rejection on one hand, but also inhibiting tolerance mechanisms on the other hand.

Future Perspectives

Longstanding evidence shows that a subset of liver transplant recipients is tolerant for the graft and theoretically does not require any immunosuppression. In addition, a substantial number of patients is thought to be over-immunosuppressed. Lerut et al. elegantly summarized the several studies showing liver transplant tolerance (17). Safe adjustments and weaning of immunosuppression without rejection requires prognostic tests that identify patients who are hypo-responsive or even tolerant for their graft. Currently, no such tests exist to differentiate between tolerant and non-tolerant patients. Hence, to improve long-term transplant outcome, several major challenges remain, including detection of transplant tolerance based on diagnostic tests and minimizing the level of immunosuppression to prevent side effects.

The development of tolerance assays and the implementation of clinical tolerance trials are closely dependent upon each other. Introduction of tolerance induction protocols in organ transplantation will require the identification of simple, robust, ideally non-invasive biological markers reflecting the immune allo-reactivity of the recipient towards his/her donor graft. In the past decades much progress has been made in understanding how the immune system is regulated, with a great deal of recent interest in CD4$^+$ Treg that actively engage in the maintenance of immunological self-tolerance and immune homeostasis (1, 18). Therefore it is tempting to use Treg as a possible marker. In order to do so, complete dissection of the molecular framework and cellular processes concerning the development and function of Treg is a must. Once accomplished, exciting possibilities may emerge for the manipulation of regulatory T-cell pathways in controlling anti-donor immune responses. The identification of the transcription factor forkhead box p3 (Foxp3) as being the master transcription factor, regulating Treg development and function, has made it possible to further characterize these cells (19, 20). A key question that has emerged from these findings is how Foxp3 orchestrates the cellular and molecular programs involved in Treg function. Recent studies have shown that Foxp3 binds to other transcription factors such as NFAT (nuclear factor of activated T cells), thereby interfering with the transcription of the gene encoding for IL-2 (21, 22). Furthermore, Foxp3 up-regulates the expression of CD25 and other Treg cell–associated molecules. As shown in this thesis, current immunosuppressive agents have an effect on these pathways and may therefore disturb the natural pathway of tolerance induction. Further confirmation and specification of these findings in experimental models will provide major insight on the effects of immune suppressive therapy on tolerance. Changes in immunological status after single immune suppressive therapy and in combination, as used in the clinic, need further investigation to determine the optimal strategy without interfering with the induction of tolerance.

Understanding of the distinct effects of immunosuppression on Treg and tolerance is required to be able to adjust or chose the optimal immunosuppressive strategy. Further analysis in *in vivo* models is necessary to fully understand the impact of different immunosuppressive strategies on tolerance. One possibility is to set up an experimental liver transplantation model and investigate the effect of immunosuppression on Treg in relation to naturally developing tolerance. This setup will also allow studying the effect of Treg on other cell subsets *in vivo* in relation to tolerance. Up to date most studies performed on Treg are based on *in vitro* models, which may not adequately reflect *in vivo* Treg activity. We do know now that Foxp3+ Treg can both directly and indirectly suppress the activation and proliferation of many cell types including T cells, B cells, Dendritic Cells, Natural Killer cells and Natural Killer T cells (23, 24). All of these cells are known to be present in the liver. These important findings suggest that Foxp3+ Treg cells do not suppress immune responses by a single mechanism, but may exploit various pathways in a context-dependent manner. In addition, another key challenge remaining in the understanding of Treg mediated tolerance is the elucidation of the exact mechanism of suppression by Treg. So far, it is established that cell-cell contact is mandatory, but the position of immunosuppressive cytokines, like IL-10 and TGF-β, remains controversial (25-27). Several membrane-bound components have been identified as possible candidates involved in the cell-to-cell interaction between Treg and effector cells. CTLA-4, a co-inhibitory molecule, was shown to be highly expressed in Treg and is functional in Treg-mediated suppression (23, 28). Other candidates, such as membrane-bound TGF-β (29) and GITR (30), have also been suggested, but conclusive evidence is still lacking (31, 32). In sum, clarification on the interactions between Treg and other cell types involved in the allogenic immune response and the effect of immunosuppression on these interactions is necessitated.

An important issue raised in the past years is the actual site in which Treg are functional. Analysis of circulating Treg has been very informative on the proportions and functionality of these cells, however, whether the circulating pool of Treg reflects the actual pool of Treg involved in the anti-donor immune response remains unknown. The major limitation of most studies is the analysis of Treg present in peripheral blood, which might not mirror the condition inside the transplanted organ and the lymphoid tissue. In the clinical setting it is nearly impossible to obtain lympoid tissue, which is a major hurdle in Treg research. In animal models the presence of these cells in the graft was linked to the development of acute rejection, suggesting local suppression within the graft. Using fine-needle-aspiration biopsy it is now possible to easily obtain liver tissue and study intragraft cells and gene expression. The possibility to analyze gene expression patterns with gene array chips holds great promise for discovering non-invasive biomarkers for monitoring of intragraft events. Therefore, micro-array analysis should be the next step in the intragraft analysis following transplantation. Differences of immune parameters in the transplanted organ and in blood, as shown in this thesis, highlight the importance of analyzing multiple aspects and behaviours of the immune system. The combination of careful immune monitoring and intragraft assessment of immunological events may hold the key to

detect tolerant patients. Recent advances in our understanding of the molecular mechanisms that control the development of Treg cells have provided new avenues of investigation, but key questions concerning the antigen specificity of Treg cells, their homeostasis, and mechanism of action remain.

The liver is known for its unique immune privilege and in transplantation setting is more often tolerated compared to other organs, reflected by lower rejection rates and dosage of immuno-suppressive drugs. The tolerogenic environment of the liver is well illustrated by several infec-tious diseases, such as Malaria and hepatitis inducing viruses, which often result in chronic infections. The resident immune cells in the liver are thought to play a crucial role in the liver's immune privilege. For this, the structural organization of the liver is thought to be essential. Lar-ge populations of resident leukocytes are present in the liver, which differ from those in blood. These cells are distributed through the sinusoidal spaces and are also organized in lymphoid aggregates in the portal tracts, allowing close cell-cell interactions. Antigen presenting cells are abundantly present in the liver and include Kupffer cells, liver sinusoidal endothelial cells (LSEC) and dendritic cells, which phenotypically and functionally differ from those in blood. The dendritic cells are predominantly immature and are prone to capture and process antigens. In-terestingly, immature dendritic cells have been shown to have tolerogenic properties, including the ability to induce Treg (33, 34). High IL-10 and TGF-β expression by Kupffer cells and LSEC are thought to create a unique cytokine micro-environment that may render resident dendritic cells tolerogenic (35-37). Therefore, it is essential to identify tissue-specific factors that influ-ence the balance between Treg and effector T cells in distinct tissue sites and to understand the bi-directional interactions of the different cells types present in the liver in order to envision the exact processes involved in tolerance. Selective depletion of the different cell types in a liver transplantation model may provide us the specific roles of the different cell populations in tolerance induction. This knowledge can then be implemented in a strategy to manipulate the immune system causing transplant tolerance.

From a clinical perspective, the assessment of allo-reactivity using immunological monitoring in transplant recipient may be directed at the following aims: (1) early, non-invasive detection of allograft rejection, (2) determination of the level of immunosuppression required (choice of immunosuppression, dosage adjustment) and (3) analysis of the immunological phenotype related to operational tolerance and safe immunosuppression withdrawal in a subgroup of re-cipients. Essential for this is an adequate approach in a multidisciplinary fashion requiring ap-propriate interactions between clinicians and immunologists, which should ideally be organized in a prospective fashion from the time of transplantation. Eventually, with innovations in the detection of both reactive- and regulatory-cells we will get a better insight into the immune status of transplant recipients. Accurate visualisation of the host immune reactivity will allow us to stratify patients towards more tailor-made immunosuppressive regimens, thereby improving long term survival and quality of life .

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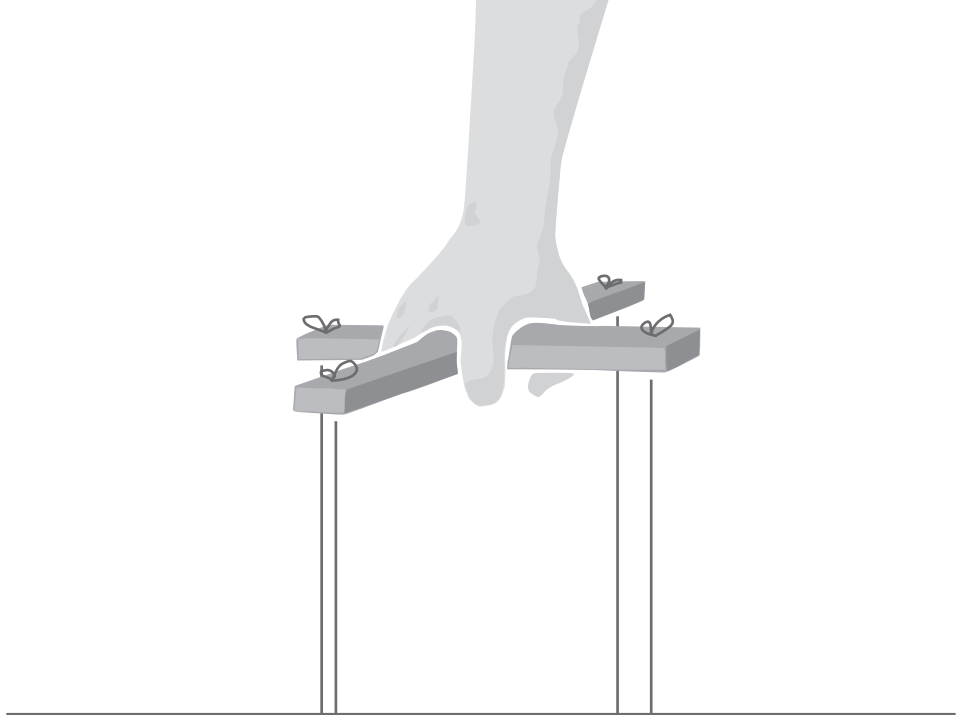
CHAPTER 10

Dutch Summary (Samenvatting)
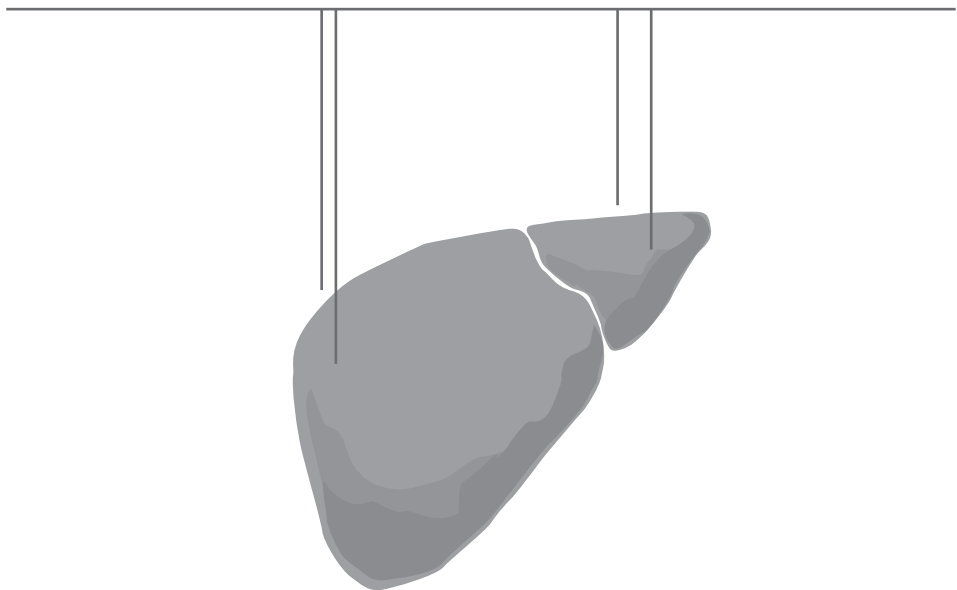
Levertransplantatie is de behandeling bij uitstek wanneer het eindstadium van leverziekte is bereikt. Na transplantatie wordt het donor orgaan door het immuun systeem van de ontvanger als niet-eigen herkend en ontstaat er een afstotingsreactie. Ter preventie van afstoting krijgen alle patiënten die een transplantatie ondergaan levenslang afweeronderdrukkende medicatie (immunosuppressiva) voorgeschreven. Echter, immunosuppressiva onderdrukken de algehele afweer op een niet specifieke manier, waardoor de ontvangers extra gevoelig zijn voor bacteriële, virale en schimmelinfecties en voor het ontwikkelen van kanker. Nierfalen, botontkalking en hart- en vaatziekten behoren tot de meest voorkomende bijwerkingen. In de huidige Rotterdamse patiëntenpopulatie kampt ongeveer tweederde van de patiënten na transplantatie met de bijwerkingen van de toegepaste immunosuppressiva.

Na onttrekking van immunosuppressiva aan transplantatie patiënten die al jaren een stabiele transplantaatfunctie hebben, blijkt zich in een deel van de betrokkenen geen afstoting te ontwikkelen. Het is onbekend waarom bij sommige patiënten blijkbaar tolerantie voor het transplantaat optreedt, terwijl bij anderen immunosuppressiva niet onttrokken kunnen worden zonder dat dit tot afstoting leidt. Wel is bekend dat tolerantie vaker optreedt na levertransplantatie in vergelijking met andere organen. Het verminderen of zelfs stoppen van de immunosuppressieve medicatie zou leiden tot een belangrijke reductie van de morbiditeit en daardoor ook mortaliteit na transplantatie. Momenteel is het echter niet mogelijk om te bepalen welke patiënt wel of geen immunosuppressiva hoeft te gebruiken.

Onderzoek in diermodellen heeft aangetoond dat regulatie een cruciaal element is voor het bereiken van tolerantie. Aangetoond is dat een speciale subset van witte bloedcellen, genaamd regulatoire T-cellen, een belangrijke rol spelen bij transplantaat tolerantie. Deze regulatoire cellen zijn in staat om de functies van reactieve T-cellen, die de afstotingsreactie in gang zetten, te remmen.

Het eerste gedeelte van dit proefschrift (Part I) omvat een korte introductie, het doel en de opzet van dit proefschrift (**hoofdstuk 1**). **In hoofdstuk 2** is er een literatuuroverzicht beschreven over de effecten van afweeronderdrukkende medicatie (immunosuppressiva) op regulatoire T-cellen in klinische orgaan transplantatie. De rol van deze regulatoire T-cellen in het induceren en in stand houden van transplantaat tolerantie in experimentele diermodellen heeft vele onderzoekers gestimuleerd om onderzoek te doen op dit gebied. Een van de belangrijkste werkingsmechanismen van de huidige immunosuppressiva is het remmen van reactieve T-cellen, die onder andere betrokken zijn bij de afstotingsreactie, te remmen. Als onderdeel van de helper T-cel populatie is het dus mogelijk dat immunosuppressiva ook een remmend effect hebben op regulatoire T-cellen, en dit zou kunnen resulteren in een ongewenste remming van transplantaat tolerantie. In verschillende diermodellen is reeds aangetoond dat onder andere het toedienen van calcineurine remmers (Cyclosporine A en Tacrolimus), een van de meest gebruikte immunosuppressiva, juist een negatief effect hebben op tolerantie en dat het transplantaat werd afgestoten na het staken van de immunosuppressiva. Een van de hypothetische verklaringen voor dit fenomeen is het remmende effect van calcineurine rem-

mers op regulatoire T-cellen. De recente literatuur toont verschillende effecten op de functie en overleving van regulatoire T-cellen bij de verschillende immunosuppressiva die gebruikt worden. Met name blijken de calcineurine remmers de verhouding tussen regulerende en reactieve T-cellen te veranderen. Dit is een belangrijke bevinding en kan van invloed zijn op de keuze en mate van immunosuppressieve medicatie in de toekomst. Hiervoor dienen de specifieke effecten van immunosuppressiva nader onderzocht te worden, met name het effect op regulatoire cellen. Uiteindelijk kan dit leiden tot geïndividualiseerde immunosuppressieve behandeling en in een deel tot compleet stoppen, waardoor de kans op complicaties van de behandeling aanzienlijk zal worden gereduceerd.

In het tweede gedeelte van dit proefschrift (Part II) worden regulatoire T-cellen in de bloedcir- culatie gekarakteriseerd en de effecten van verschillende immunosuppressieve behandeling op de cellen beschreven. In **hoofdstuk 3 en 4** hebben wij laten zien dat patiënten met een eindstadium leverziekte verhoudingsgewijs meer regulatoire T-cellen in het bloed hebben en dat na een levertransplantatie het aantal significant daalt. Er is daarbij een associatie gecon- stateerd tussen immunosuppressiva gebruik en de daling van het aantal regulatoire T-cellen. Alle patiënten in deze studie kregen immuunsuppressie op basis van calcineurine-remmers, maar dat is nog geen bewijs dat deze medicatie verantwoordelijk was voor de daling. Ech- ter, duidelijk bleken patiënten die een acute afstotingsreactie hadden ontwikkeld en daarvoor werden behandeld met steroïden, significant minder regulatoire T-cellen te hebben. Deze bevindingen hebben wij nader willen onderzoeken in een prospectief onderzoek (hoofdstuk 5) waarbij patiënten die een calcineurine remmer gebruikten werden geconverteerd naar een ander type immunosuppressivum, mycophenolaat mofetil. Gedurende zes tot negen maanden werd van deze patiënten bloed afgenomen om het effect van de conversie te onderzoeken. Na het stoppen van de calcineurine remmer steeg het percentage regulatoire T-cellen van het totaal aantal T-cellen, bevestigd middels een verhoogd Foxp3 genexpressie. Daarbij waren er tevens fenotypische veranderingen van de regulatoire T-cellen, onder andere een verhoogd IL-2 receptor expressie. Een belangrijke klinische observatie in deze studie was dat er een significante verbetering van de nierfunctie was opgetreden. Twee patiënten ontwikkelden een afstotingsreactie die adequaat behandeld konden worden met steroïden. Geen van deze ver- anderingen werden gezien in de controle patiënten, die de calcineurine remmer behandeling hebben gecontinueerd. Concluderend blijken er duidelijke verschillende effecten te bestaan van de verschillende afweeronderdrukkende medicijnen op het immuunsysteem. Wij hebben in levertransplantatie patiënten functionele regulatoire T-cellen aangetoond die onder invloed van calcineurine remmers dalen in het bloed. In diermodellen zijn er nu sterke aanwijzingen dat de keuze van behandeling tolerantie negatief kan beïnvloeden. Het specifieke effect hier- van op de anti-donor reactiviteit in de mens dient verder geanalyseerd te worden.

In het derde gedeelte (Part III) wordt getracht meer inzicht te geven in de immunologische ka- rakteristieken van de getransplanteerde lever. Hiervoor hebben we op verschillende tijdstippen

gekeken naar onder meer de aanwezigheid van regulatoire T-cellen in het levertransplantaat. Er zijn aanwijzingen dat de aanwezigheid van regulatoire T-cellen in het getransplanteerde orgaan een belangrijke rol spelen bij het reguleren van de immuun respons en tolerantie kunnen induceren. Dit suggereert dat de onderdrukking van de anti-donor reactiviteit door regulatoire T-cellen (deels) lokaal plaats vindt, dus in het getransplanteerde orgaan. Een ander bekend fenomeen wat een duidelijke link heeft met tolerantie is het optreden van chimerisme, gedefinieerd als het persisteren van donor afkomstige immuun cellen in de ontvanger. In **hoofdstuk 6** hebben we perfusievloeistof van de donor lever opgevangen en geanalyseerd. Onder meer is er gekeken naar de samenstelling van de aanwezige immuun cellen, met name de aanwezigheid van donor regulatoire T-cellen. Wij hebben aangetoond dat donor regulatoire T-cellen aanwezig zijn in perfusievloeistof en dat deze cellen na transplantatie vrij komen en circuleren in de ontvanger. Bovendien waren deze regulatoire cellen in staat om de anti-donor respons door de ontvanger cellen te remmen. Dit pleit voor de veronderstelling dat er een belangrijke rol is voor regulatoire T-cellen in chimerisme-geassocieerde tolerantie. De directe link tussen de aanwezigheid van donor regulatoire T-cellen in de lever en de uitkomst na transplantatie dient nader onderzocht te worden.

Om op een veilige en patiënt vriendelijke wijze leverweefsel na transplantatie te verkrijgen hebben wij gekozen voor een alternatieve methode: de dunne-naald aspiratie biopsie. Om post-operatief de regulatoire T-cellen in de lever te kunnen monitoren hebben wij middels dunne-naald-aspiratie leverweefsel verkregen en geanalyseerd. Deze techniek blijkt een goed alternatief voor de klassieke dikke naald biopsie, die kan leiden tot ernstige complicaties. Doelstelling van deze studie was om beter inzicht te krijgen in de immunologische karakteristieken van de getransplanteerde lever. Hiervoor is er op een veilige en patiënt vriendelijke wijze leverweefsel middels dunne naald biopsie afgenomen van lever transplantatie patiënten in de eerste maand (**hoofdstuk 7**), op 6 maanden en 12 maanden (**hoofdstuk 8**) na transplantatie. In de eerste weken na transplantatie blijkt er in vergelijking met het bloed een verhoogd lever expressie te zijn van IFN-γ, een T-cel activerend cytokine. Op 6 maanden na transplantatie is IFN-γ niet meer verschillend tussen lever en bloed (**hoofdstuk 7**). De verhoogde IFN-γ expressie ging gepaard met relatief meer regulatoire T-cellen in de lever vergeleken met bloed. Deze bevindingen impliceren een verhoogde mate van activiteit en regulatie vroeg na transplantatie. Een interessante observatie was dat de expressie van het immuunsuppressieve cytokine IL-10 aanvankelijk niet verschillend is tussen lever en bloed in de eerste weken na transplantatie, maar op den duur wel degelijk verschillen toont op 6 en 12 maanden na transplantatie. Dit suggereert een immuunsuppressief milieu in de lever die ontstaat na verloop van tijd. Vroeg na transplantatie blijkt er tijdens een acute afstotingsreactie een lager expressie te zijn van IL-10 in de lever en in bloed vergeleken met patiënten die geen afstotingsreactie ontwikkelden. Collega Dr. M.C. Warlé heeft in 2002 aangetoond dat polymorfismes van cytokine genen, waaronder het IL-10 gen, een genetische risicofactor kan zijn voor het ontwikkelen van acute levertransplantaat afstoting. Onze studie bevestigt een belangrijke rol voor IL-10

in de eerste fase na levertransplantatie. Nader onderzoek dient te worden gedaan of er een causale verband bestaat tussen IL-10 expressie/productie en het ontwikkelen van afstoting. Langer na transplantatie blijkt er naast een toegenomen IL-10 expressie ook een toename te zijn van Foxp3 expressie (**hoofdstuk 8**), wat suggestief is voor een accumulatie van regulatoire T-cellen. Dit toont het onderlinge verband tussen IL-10 en Foxp3, zoals eerder reeds is beschreven. Patiënten die vroeg na transplantatie afstoting ontwikkelden blijken een hoger Foxp3 expressie in de lever te hebben dan patiënten die geen afstoting ontwikkelden. Ook hebben patiënten met een recidief hepatitis C infectie een hogere Foxp3 expressie. Deze verschillen duiden op een toegenomen aantal, of een verhoogde activiteit van regulatoire T-cellen. Bij patiënten met hepatitis C infectie zou dit een ontsnappingsmechanisme kunnen zijn van het virus, waardoor deze niet adequaat geëlimineerd kan worden omdat virus-speciefieke T-cellen geremd worden door de regulatoire cellen.

Deze beide studies tonen dat middels lever dunne naald biopsie het verloop van de immunologische processen zoals tolerantie op een veilige manier gevolgd kunnen worden. De gevonden verschillen tussen patiënten suggereert een niet generaliseerbaar immuun status van de verschillende patiënten en pleit voor geïndividualiseerde immunosuppressieve behandeling.

Dit proefschrift heeft regulatoire T-cellen beschreven in de lever en bloed van levertransplantatie patiënten en vormt een basis voor verder onderzoek gericht op functionele capaciteiten van regulatoire T-cellen in relatie tot de uitkomst van een transplantatie. Een juiste balans tussen reactieve T-cellen en regulatoire T-cellen is cruciaal om tolerantie te verkrijgen. De diverse immunosuppressiva blijken verschillende effecten te hebben op de functie en overleving van regulatoire T-cellen, wat pleit voor de ontwikkeling van nieuwe immunosuppressieve strategieën die er op gericht zijn donor transplantaat tolerantie te bewerkstelligen. Wij stellen dat door het bepalen van de immuun status op basis van tolerogene kenmerken, waaronder regulatoire T-cel activiteit en cytokine profiel in relatie tot de anti-donor reactie, bij een subset van de patiënten de immuunsuppressiva kunnen worden afgebouwd. Dit zou dan leiden tot een aanzienlijke verbetering van de kwaliteit van leven na een transplantatie.

Appendix

Acknowledgements (Dankwoord)
Curriculum Vitae Auctoris
List of publications

Acknowledgements (Dankwoord)

Hooggeleerde heer Tilanus, beste Huug, ik ben u uiterst dankbaar voor de kansen om onderzoek te doen in zowel binnen- als buitenland. Onvergetelijke, bijzonder leerzame ervaringen. Inmiddels ken ik u al langer dan een decennium. Als middelbare scholier kreeg ik een rondleiding op de operatiekamer, waar u toen een lever transplanteerde. Jaren later heeft u mij aangenomen als promovendus en heeft u mij begeleid in mijn wetenschappelijke en chirurgische ontwikkeling. Als chirurg en mentor bent u een bron van inspiratie en ik bewonder uw visie en kunde in de chirurgie.

Weledelzeergeleerde van der Laan, beste Luc, in 2002 zijn wij na maandenlang tevergeefs pogen een assay op te zetten voor de indirecte antigeenpresentatie, een ander pad opgeslagen, een goed pad, wat heeft geleid tot dit proefschrift. Als jouw eerste promovendus ben ik je bijzonder dankbaar voor de vele leermomenten en begeleiding van dit onderzoek. Jouw standvastigheid, focus en creatieve oplossingen maakt je een volwaardig chirurgisch wetenschapper. Veel succes met de vele projecten die lopen en zullen gaan lopen.

Hooggeleerde heer Metselaar, beste Herold, ik weet nog als de dag van gisteren onze eerste ontmoeting tijdens de 'lever' lunch. Nederland had verloren en dan stond er ook nog eens een Turk voor je die heel enthousiast tolerantie zou gaan detecteren. Ik respecteer je wetenschappelijke en klinische visie en dank je voor jouw inbreng en morele steun. Jij weet het probleem altijd direct te benaderen en te presenteren, waardoor je voor een internist verdacht wordt van chirurgische praktijken.

Hooggeleerde heer Ijzermans, beste Jan, ik bewonder uw klinische en wetenschappelijke vaardigheden. Het cysteuze cadeau tijdens een levertransplantatie zal ik nooit vergeten. Ik ben u dank verschuldigd om meerdere redenen en gebruik deze mogelijkheid om u te danken voor het beoordelen van dit proefschrift, voor uw bijdrage aan een van de artikelen en voor uw vele adviezen het afgelopen jaar. Ik kijk uit naar mijn retour in het Erasmus MC en hoop veel van u te kunnen leren.

Hooggeleerde heer Weimar, wie had ooit gedacht dat de vader van een middelbare school klasgenoot 10 jaar later in mijn promotiecommissie zou zitten. Dank voor het beoordelen van dit proefschrift en de stimulerende gedachtewisselingen op het jaarlijkse bootcongres.

Hooggeleerde heer Laman, ook u wil ik danken voor het beoordelen van dit proefschrift en uw nuttige suggesties. Als expert in de immunologie bent u onmisbaar in dit gezelschap.

Hooggeleerde heer Lerut, dank voor uw bereidheid om zitting te nemen in de commissie. In de afgelopen jaren heb ik meerdere artikelen van u met veel belangstelling gelezen en ik vind

het een eer dat u deel uitmaakt van de commissie.

Weledelzeergeleerde Kazemier, beste Geert, jouw veelzijdigheid en chirurgisch vermogen verdienen terecht mijn ontzag voor jou. Samen op weg naar Leiden, een onvergetelijke rit, wat uiteindelijk heeft geleid tot een veelbelovend project. De vele mooie, leerzame momenten, de zuivere koffie en de 'Less is More' principe zullen mij altijd bij blijven.

Weledelzeergeleerde Baan, beste Carla, jij hebt een belangrijke bijdrage geleverd aan meerdere artikelen en dus ook aan het totstandkomen van dit proefschrift. Ik heb veel van je geleerd en mijn dank daarvoor is groot.

Weledelzeergeleerde Hilbrands, beste Luuk, veel dank voor de stimulerende discussies tijdens het jaarlijkse bootcongres en de bereidheidheid om plaats te nemen in de commissie.

Weledelzeergeleerde Kwekkeboom, beste Jaap, dank voor de vele keren dat je kritisch naar mijn werk hebt gekeken en de waardevolle adviezen, die je hebt gegeven.

Weledelzeergeleerde Alwayn en weledelgeleerde Tran, beste Ian en Khe, zonder jullie bijdrage had ik één van de kopstukken van dit proefschrift niet kunnen schrijven. Dank voor het verzamelen van perfusaten en de goede tijd op de afdeling. Ian, nog veel dank voor het beoordelen van mijn aanvraag en je adviezen. Khe, veel dank voor de leerzame tijd op 8 noord.

Weledelgeleerde Kok, lieve Alice, waar was ik zonder jou? Jouw ongekende toewijding en altijd vrolijke humeur is tot op de dag van vandaag een gemis op het lab. Ik respecteer jouw bijdrage aan het onderzoek en wens je veel succes met je nieuwe loopbaan.

Weledelgeleerde Tha-In, lieve Thanya, samen met Alice vormen jullie mijn 'Angels'. As the Americans would say: 'we go back, all the way to 1998'. Van de collegebanken naar het transplantatielab en vandaar naar de wonderlijke wereld van de chirurgie. Ik koester de vele mooie momenten en wetenschappelijke discussies, die ik met jou heb gevoerd en nog steeds voer. Ik wens je veel succes met het afronden van je onderzoek en hoop ooit samen met jou aan tafel te kunnen staan. Veel dank voor je onmisbare hulp de afgelopen tijd.

Weledelzeergeleerde de Jonge, beste Jeroen, ben zeer verheugd en toch ook gerustgesteld dat ik jou aan mijn zijde heb. Milaan was een waanzinnig goede tijd en ik hoop op nog meer mooie momenten in de toekomst.

Weledelzeergeleerde Terkivatan, lieve Türkan, in de korte tijd op 7 noord/8 noord heb ik veel van je geleerd en wil je graag daarvoor danken. Ik ben ervan overtuigd dat je een uitstekende chirurg bent en wens je veel succes bij het vinden van je plekje.

Weledelgeleerde Claassen, beste Mark(us), dank voor de goede tijd die we samen als Treg-lotgenoten en als kamergenoten hebben gedeeld. Het was een genot om met jou over de Treg filosofie te discussiëren. 'Treg is a way of life', right?. Veel succes met de verschillende veelbelovende projecten.

Weledelgeleerde Bosma, beste Brenda, veel dank voor je bijdrage aan één van de artikelen en de goede tijden op de verschillende congressen. Veel succes met de afronding van je proefschrift en je nieuwe baan.

Weledelgeleerde Hendrikx, beste Thijs, dank voor je inbreng aan het manuscript die we samen hebben geschreven en veel succes met de laatste loodjes.

Het MDL-lab, onder andere Martine, Angela, Jan, Anthonie, Marjolein, Antoine, Anneke, Andre en Linda wil ik danken voor de goede werksfeer en mooie avonden.

Collega-onderzoekers, arts-assistenten en stafleden van de Heelkunde Erasmus MC, IKAZIA en MCRZ, Amici, ik dank jullie voor de stimulerende en leerzame omgeving. De chirurgencup, ski-weekenden en overige sociale events maakt ons een bijzonder genootschap.

Lieve Lara, Anneloes, Sylvia, Elly, Miranda en Fatma, zonder jullie was dit allemaal niet mogelijk geweest, heel veel dank voor jullie inzet. In het bijzonder wil ik Elly danken die een cruciale rol heeft gespeeld bij de inclusie van patiënten voor meerdere studies. Tevens worden bij deze alle patiënten bedankt voor participatie aan de verschillende studies.

Mijn twee beste vrienden, Elwin Bes en Ayhan 'Baykus' Beyazit, ik had geen mooier cover en lay-out kunnen verzinnen en complimenteer jullie met dit sublieme resultaat. Elwin, jouw creatieve geest kan niet worden evenaard. Inmiddels hebben we al een aardige historie samen en het verhaal van G1 en G2 wordt hoe langer, hoe mooier. Ayhan, ik ken je al mijn hele leven en altijd als ik je nodig heb sta je voor me klaar. De vele zware, doch memorabele nachten, die we samen in dit werk hebben zitten zal ik nooit vergeten. Woorden kunnen niet beschrijven hoe groot mijn dank is voor jullie vriendschap.

Vrienden, lieve Rosa, Maria, Edson, Karsten, Stefan, Ivana, Ali en Alime geweldig dat jullie er altijd zijn voor de mooie en minder mooie momenten des levens. Met een ieder van jullie heb ik een bijzondere band en ik ben blij dat ik jullie tot mijn vriendenkring mag rekenen.

Lieve ouders, mijn grote broertje en lieve zusje, tot de dag van vandaag hebben jullie me altijd liefdevol geholpen en gesteund. Dit proefschrift en al het andere had ik zonder jullie niet kunnen bereiken. Aytur, ik ben ervan overtuigd dat jij een uitstekende bouwkundige zal worden en wens je veel succes met je opleiding. Betül, New York wacht op ons, dus....

Curriculum Vitae Auctoris

Ahmet Demirkiran was born on the 5th of December 1979 in Rotterdam. After finishing secondary school at the Erasmiaans Gymnasium in 1998, he started medical school at the Erasmus University Rotterdam. In the second year of his study his interest in immunology began after an elective immunology course under supervision of prof.dr. H.A. Drexhage. In the following year he visited the University of Pretoria in South Africa where he worked at the emergence department (Dr. Marx). He finished the fourth year with a research project at the Mayo Clinics, Rochester MN. Under supervision of prof. dr. M.D. Stegall, dr. D. Ninova and prof. dr. H.W. Tilanus he studied anti-blood group antibodies in kidney transplantation. He was also trained in micro surgery and performed kidney transplantations in rats with dr. M. Covarrubias. Before starting his internship he was offered a position as PhD-student at the Department of Surgery of the Erasmus Medical Center Rotterdam. Under supervision of prof.dr. H.W. Tilanus, dr. L.J.W. van der Laan and dr. H.J. Metselaar his PhD training focused on regulatory T cells in liver transplant recipients, combining his both interests in immunology and surgery. Several studies were performed in collaboration with the Department of Internal Medicine (prof.dr. W. Weimar and dr. C.C. Baan). In 2006 he became team leader of the operating theatre and laboratory support team in liver transplantation. In his final year of training he studied the outcomes of non-heart-beating liver transplantation and biliary complications after liver transplantation (dr. G. Kazemier and dr. J. de Jonge). He obtained his medical degree in 2006. In July 2007 he started his residency in General Surgery at the Medical Center Rijnmond Zuid (dr. E. van der Harst). He will finish his residency at the Erasmus Medical Center (prof.dr. J.N.M Ijzermans).

List of publications

Ahmet Demirkiran, Alice Kok, Jaap Kwekkeboom, Herold J. Metselaar, Hugo W. Tilanus and Luc J.W. van der Laan
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Submitted