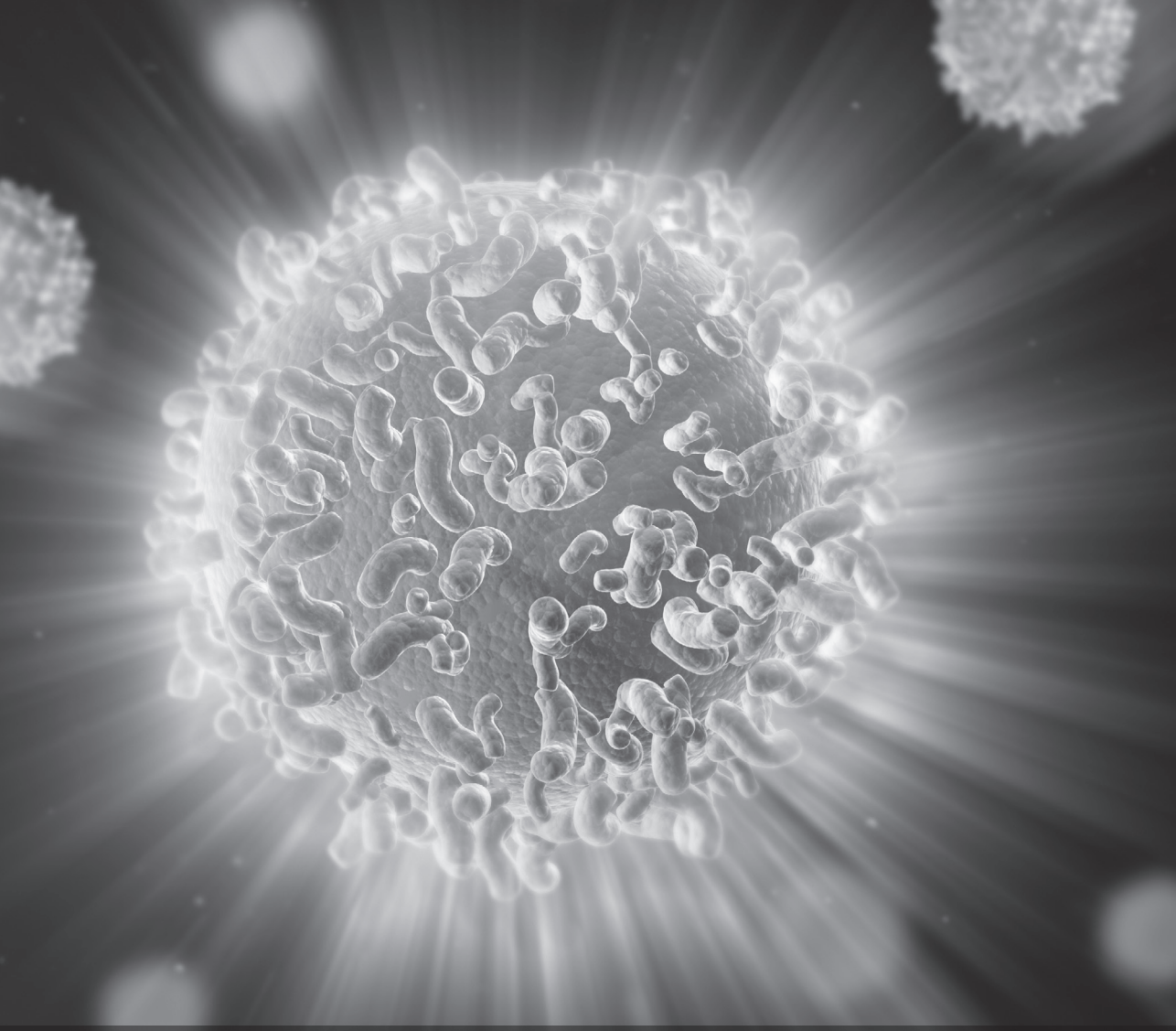

APPROACHES TO OPTIMIZE IMMUNOSUPPRESSION AFTER LIVER TRANSPLANTATION

ÖZLEM TAPIRDAMAZ

Approaches to optimize immunosuppression after liver transplantation

Özlem Tapirdamaz

The studies described in this thesis were performed at the Department of Gastroenterology and Hepatology, Erasmus MC University Medical Center, Rotterdam, The Netherlands

The research included in this thesis was supported by a Mosaic Grant of The Netherlands Oranization for Scientific Research (NWO 017.003.031)

ISBN: 978-94-6169-598-7

Cover design: Optima Grafische Communicatie, Rotterdam, The Netherlands
Layout and printed by: Optima Grafische Communicatie, Rotterdam, The Netherlands

Approaches to optimize immunosuppression after liver transplantation

Methoden van aanpak ter optimalizatie van immunosuppressie na levertransplantatie

Proefschrift

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

Prof. dr. H.A.P. Pols

en volgens besluit van het College voor Promoties.
De openbare verdediging zal plaatsvinden op

17 december 2014 om 13.30 uur

door

Özlem Tapirdamaz

geboren te Eindhoven

Promotiecommissie:

Promotor(en): Prof.dr. H.J. Metselaar

Overige leden: Prof.dr. C.C. Baan
Prof.dr. F.H.J. Claas
Prof.dr. T. van Gelder

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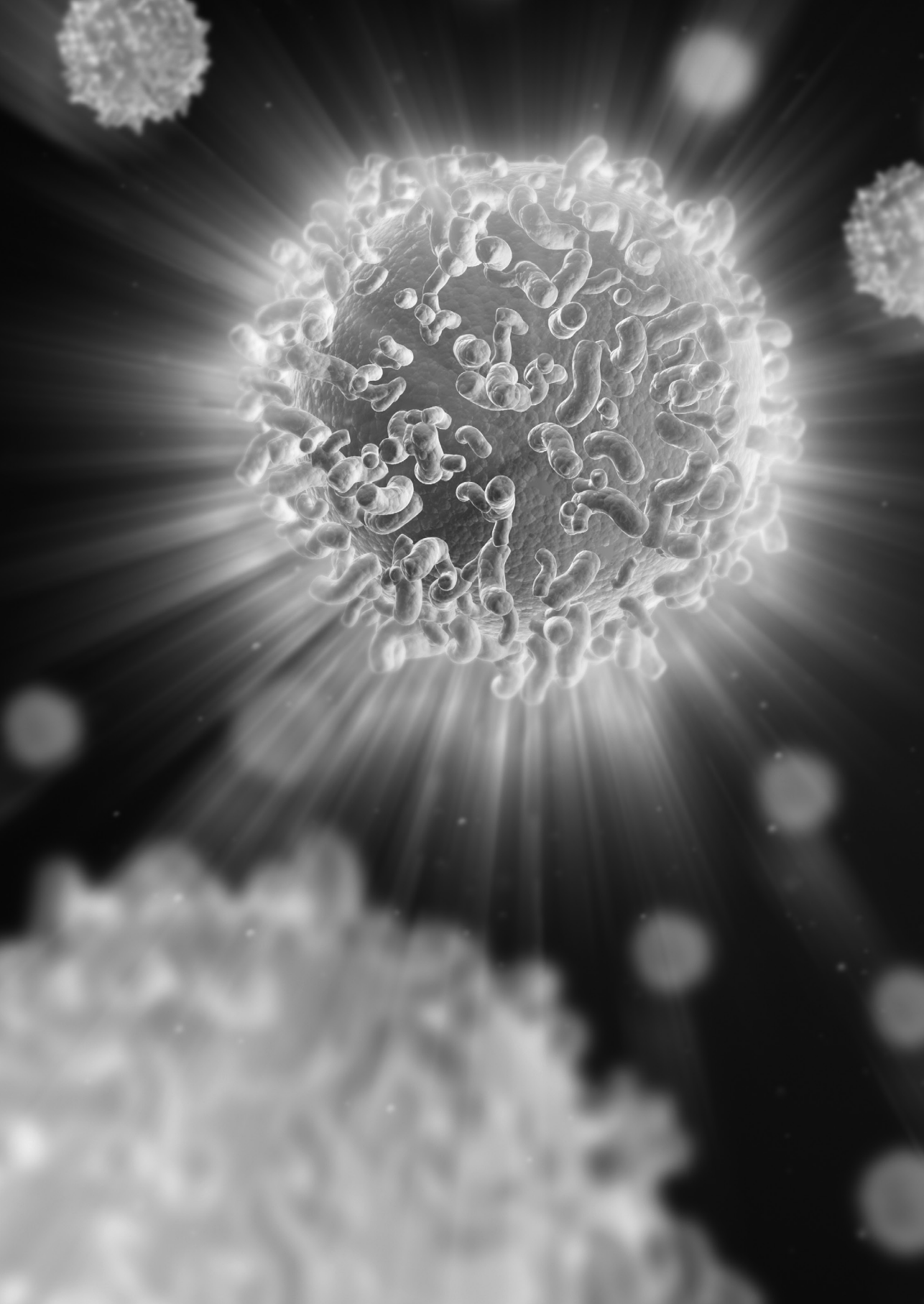
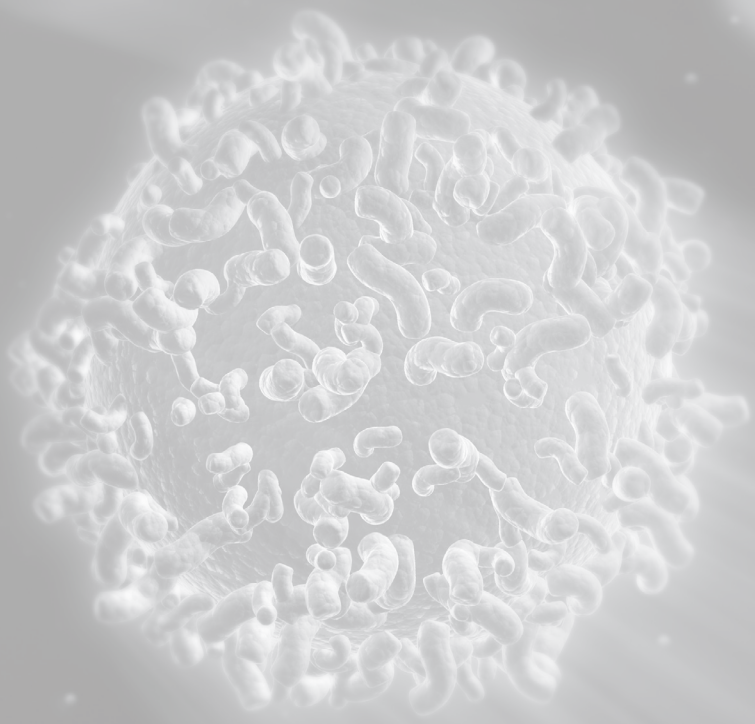
Chapter Outline



Chapter 1

General introduction and outline of the thesis



General introduction

Since its advent 51 years ago [1], liver transplantation (LT) has progressed from an experimental treatment to an accepted therapeutic modality that has reversed the gloomy prognosis of end stage liver disease. The great success of LT is for the major part due to calcineurin inhibitors (CNI) like Cyclopsorin A and Tacrolimus. These are powerful immunosuppressants (IS), which prevent graft rejection and are the cornerstone of post-transplant patient management. The introduction of Cyclosporine in March 1980 marked great clinical advance for graft and patient survival [2]. Following the introduction of cyclosporine in LT, one-year patient survival more than doubled (from approximately 33 to 68%).[2,3], with an increment to above 80% nowadays. *(www.eltr.org) [4,5]*.

Although immunosuppressive regimens are indispensible therapeutics for LT recipients, the downside is their major adverse effects on the long-term. The obligatory lifelong use of these drugs increases patients' morbidity by increasing their susceptibility for infections [6,7], cancer[8], cardiovascular diseases [9-11], kidney failure [12,13] and de-novo diabetes [14], which impair long-term survival and quality of life after transplantation. These adverse effects are largely caused by the non-specificity of the immunosuppressive medications. In addition CNI also exert undesirable side effects on vasoactive compounds resulting in vasculopathy [15-17], and on LDL receptor bile acid synthesis from cholesterol [18] and lipoprotein lipase activity [19], resulting in dyslipidemia.

Because of genetic and immunologic differences between patients, the effectiveness of immunosuppressive drug treatment as well as the adverse effects of these drugs show large inter-individual variations [20-23]. Not every patient responds similarly to the same dose of the same medication. Moreover, clinical experience shows that minimization or even complete withdrawal of immunosuppressive medication is possible in some liver transplant patients without graft rejection or impairment of graft and patient survival [22,24,25]. Minimization of IS improves long-term metabolic parameters, cardiovascular risk factors and renal function [26]. Unfortunately, no tools are currently available that accurately predict the risk of liver graft rejection, or which patients will develop immunosuppression-related complications and which patients not. Therefore, clinicians are compelled to maintain long-term treatment with CNI and other IS at recommended doses. Prognostic markers that predict the risk of graft rejection and/or the risk of adverse effects of IS after transplantation will enable to individualize IS therapy, which will be beneficial for patient morbidity and mortality.

For this purpose, it is important to have a clear understanding of the immunological responses of patients to foreign liver grafts after transplantation, since these are main determinants of graft-survival. In addition, it is important to acquire a good knowledge of the genetic variations that influence the immunological responses to allogeneic

grafts, as well as genetic differences that affect processes regulating the metabolism of IS. Better understanding of these subjects may lead to new therapeutical and diagnostical developments which may subsequently contribute to individualisation of patient management after LT.

The immune system and its major components

By detecting and eliminating invading pathogens and foreign cells, the immune system is the protection mechanism of the human body against diseases . It detects a wide variety of agents, including bacteria, parasites, viruses and altered dangerous self-cells and can discriminate these from healthy self-cells in order to function properly.

The cells of the immune system originate from the bone marrow, and differentiate in the central lymphoid organs, which are bone marrow and thymus. They circulate via the blood and lymphatic vessels through the body and migrate via those vessels into the peripheral or secondary lymphoid organs, like the spleen and lymph nodes. The peripheral lymphoid organs provide the environment for cooperative interactions between antigen presenting cells (APC), T cells and B cells resulting in lymphocyte activation [27-29]. Lymphocytes recirculate between those organs and blood until they encounter an antigen.

The two major types of lymphocytes are B cells, which mature in the bone marrow; and T cells which migrate to the thymus and mature in this organ. B cells are mainly involved in the humoral immune response; after differentiation to plasma cells they produce specific antibodies that provide defence against pathogens [30,31]. Additionally, they have the capacity to produce cytokines, including interferon-gamma (IFN-γ), interleukin-6 (IL-6) and lymphotoxin α [31,32], express MHC class I and II molecules as well as costimulatory molecules and therefore can behave as APC, priming and reactivating antigen-specific T cells [31,33-36].

T cells can be divided into CD4+ or T-helper cells and CD8+ or cytotoxic T cells. CD4+ T cells have the ability to "help" via production of stimulatory cytokines, or via cell-cell interactions, macrophages and CD8+ T cells, which are the key components of the effector arm of the immune system, to become fully functional.

CD4+ T cells comprise of helper 1 (Th1), Th2, Th17, and regulatory T cells (Tregs), all with different cytokine production profiles [37]. Th1 cells produce mainly interleukin 2 (IL-2), interferon-γ, and tumor necrosis factor-α, and play an important role in helping CD8+ T cells, macrophages, and other immune cells to acquire effector capacity and execute cell-mediated immune responses [37]. Th2 cells produce mainly IL-4, IL-5, and IL-13 and play an important role in humoral responses [37]. Th17 cells produce IL-17A and IL-17F and mediate inflammatory responses against bacteria and fungi. Tregs are identified by their phenotype CD4+CD25+Foxp3+, produce IL-10 and transforming growth factor β (TGF-β), and provide negative feedback to activated T cell responses,

which is required to limit potential collateral damage [37]. In transplantation, Th1 and Th17 have been shown to be detrimental, whereas Th2 may sometimes be beneficial for graft survival. Treg cells are critical in preventing graft rejection and contribute to induction and maintenance of immunological tolerance to allogeneic grafts [38].

Activated CD8+ T cells are capable of eliminating pathogens, infected, allogeneic, and altered self cells, either by releasing cytotoxins, including perforin, granzymes [39] and granulysin [40], or by cell-surface interactions that activate apoptotic pathways in the target cells [41,42], resulting in cell death . The cytotoxin pathway is dominant in CD8 cytotoxic T lymphocytes. It depends on the pore-forming molecule perforin, which enables delivery of granzymes A and B to target cells, where they trigger apoptosis through both caspase-dependent and caspase-independent pathways [39]. Cytotoxic cell surface interactions are mediated by Fas ligand (FasL) which is expressed on the CD8+ T cell surface upon activation. Engagement of FasL and Fas receptor on target cells can trigger their death [41-43].

Naïve T cells are a subset of T cells which have never encountered an antigen before. They are activated when they recognize a specific antigenic peptide presented by an HLA molecule and subsequently can differentiate into effector T cells or memory T cells. NaiveT cells express high levels of chemokine receptor CCR7 and CD62L which enables them to migrate via high endothelial venules into the secondary lymphoid organs, where they encounter antigens ([44,45]. Naïve T cells are further characterized by the cell surface expression of the CD45RA isoform. The relative contribution of naïve T cells to allo-responses against transplanted organ in humans requires further study.

Memory T cells are a subset of T cells that have encountered their cognate antigens, persist in the human body, and are able to quickly expand to large numbers of effector T cells upon re-encountering their cognate antigen [46], thus providing the immune system with "memory" against past antigens. Memory T cells themselves again comprise of two subtypes: central memory T cells (T_{CM} cells) and effector memory T cells (T_{EM} cells), which differ in their homing properties [47], cytotoxic and proliferative capacities [48]. T_{CM} cells express high levels of chemokine receptor CCR7 and adhesion molecule CD62L [47,49,50] that enables them to migrate from the blood circulation via high-endothelial venules into secondary lymphoid organs, whereas T_{EM} cells express CD62L at variable levels but lack CCR7 [47,50] and are therefore prohibited from migration into secondary lymphoid organs. However T_{EM} cells have transendothelial migration capacity allowing them to directly enter peripheral tissues from the circulation and react rapidly against invading pathogens [45]. Memory T cells can be either $CD4^+$ or $CD8^{+,}$ but typically express the cell surface protein CD45RO, an isoform of CD45. Memory T cells have been demonstrated to be the main players in the vigorous allo-response against organ grafts in experimental animal studies [51,52], but the relative contributions of naïve and memory T cells to graft rejection in humans has not been fully elucidated.

Optimal T-cell activation requires two signals, an antigen specific signal via the interaction between the T-cell receptor (TCR) and peptide-major histocompatibility complex (MHC), and an antigen independent signal, called costimulation, via the interaction between the costimulatory molecule CD28 on the T-cell surface membrane and the molecules B7-1 (CD80) or B7-2 (CD86) on the APC [53,54]. CTLA-4, which is only presented on the surface membrane of activated T-cells, is a homologue of CD28. By interacting with the B7 ligands it delivers inhibitory signals to T cells, resulting in downregulation of T cell responses [53-55]. Genetic polymorphisms in the CTLA-4 gene can result in an altered function of the CTLA-4 protein, which can influence the strength of T cell responses [21,56,57].

The mechanism of allo-response

Organ grafts are recognized by the recipient's immune system as foreign tissue, because the graft is derived from an allogeneic (=genetically different) individual. The recipient's immune system recognizes an organ graft as allogeneic because of the expression of "transplantation antigens", which are proteins encoded by genes of the Major Histocompatibility Complex (MHC). These proteins, called Human Leukocyte Antigens (HLA), are highly polymorphic, i.e. show large differences between individuals. The physiological function of HLA-molecules is to present antigenic peptides to T-cells. The MHC consists of two classes of genes: class I genes encode HLA-A, -B and –C molecules, which present antigenic peptides to CD8+ cytotoxic T-cells; class II genes encode HLA-DP, -DQ and –DR molecules, which present peptides to CD4+ T-helper cells.

There are two major pathways of allo-recognition [58]. In the direct pathway recipient T cells recognize intact allogeneic MHC molecules on non-self cells. In the indirect pathway recipient T cells recognize donor MHC-derived peptides presented by self-MHC molecules expressed by recipient cells, which is basically the mechanism of the conventional way of antigenic recognition by T cells. Both types of allo-antigen presentation can occur during pregnancy, upon blood transfusion, or after allogeneic transplantation of cells, tissue or organs. The semi-direct pathway has recently been introduced as a third pathway of allo-recognition, in which intact donor MHC molecules are transferred to host APC activate recipient T-cells[59]. Figure 1 displays the three pathways of allorecognition

The role of direct and indirect donor HLA-recognition in allo-response

Since every individual has large numbers of T-cells with direct allo-specificity, allogeneic transplantation without IS therapy will inevitably be followed by an extraordinary allogeneic immune response to the allogeneic graft. However, the direct pathway is probably not the cause of rejections late after transplantation. There is growing evidence that

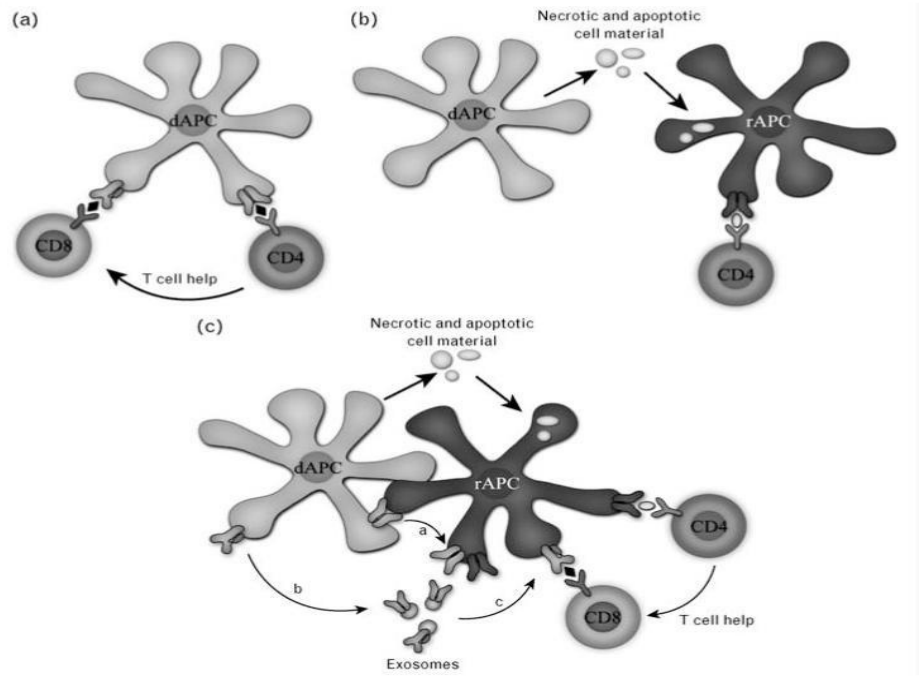


Figure 1: Direct, indirect and semi-direct pathways of allorecognition (a) Direct pathway. Recognition of intact foreign MHC on donor APC primes $CD4^+$ and $CD8^+$ recipient T cells. $CD4^+$ cells then provide T cell help for the effector function of $CD8^+$ cells. (b) Indirect pathway. Recipient APCs traffic through transplanted organs, phagocytose allogeneic MHC shed from foreign cells through cell necrosis and apoptosis and present the processed peptides in the context of self-MHC class II to MHC class II-restricted $CD4^+$ T cells. (c) Semi-direct pathway. Cell-to-cell contact between donor and recipient APC may transfer intact membrane components including intact allo-MHC (a). Likewise, donor APC can release small vesicles, known as 'exosomes' containing intact MHC (b), which fuse with the membrane of recipient APCs (c). Recipient APCs, now chimeric for MHC, stimulate direct pathway CD4 and CD8 responses through intact foreign MHC and indirect responses through processing and presentation of peptides of foreign MHC acquired from necrotic and apoptotic cell material. Given that the same APC stimulates both CD4 and CD8 cells, linked help can occur. APC, antigen-presenting cells; dAPC, donor APC; MHC, major histocompatibility complex; rAPC, recipient APC.
Afzadi et al, Curr Opin Organ Transplant. Aug 2008; 13(4)

the numbers of T cells directly recognizing donor HLA-molecules decrease in time after transplantation [60-63].

Therefore, it is generally assumed indirect allo-response plays a role long after transplantation. There is strong evidence from animal experiments that the strength of the indirect T-cell response is sufficient to reject allografts [64,65]. However, current tests for T cells with indirect allo-specificity are insensitive, and do not detect these T-cells in 50% – 70% of organ transplant recipients [60,61,66-69].Therefore the role of the indirect pathway in rejection of allogeneic grafts in humans remains unclear.

The role of viral infections in transplantations

Viral infections, including CMV infection, prevent tolerance induction to allogeneic grafts in experimental animal models [51,70,71]. One of the mechanisms contributing to this phenomenon is heterologous immunity resulting from anti-viral T cells that recognize allo-HLA antigens via the direct pathway and contribute to allograft rejection [72]. Indeed, cross-reactive viral-specific memory T cells are common in humans, and many CMV-specific T-cells are cross-reactive to at least one allogeneic HLA molecule [72,73]. However, whether and how CMV infection influences allogeneic T-cell responses and the risk of graft rejection in humans is unknown.

Immunosuppressive management

A cornerstone of successful post-transplantation patient management is the prevention of graft rejection with IS therapy. IS regimens to prevent both acute and chronic rejection have been designed based on the understanding of the mechanism of graft rejection and how immune-mediated tissue injury can be prevented, while at the same time minimizing the risks associated with immunosuppression. Because it is believed that T cells are the main role-players in graft rejection, the development of immunosuppressive regimens has been based on the understanding of T-cell biology and has focused almost exclusively on suppression of T-cell functions, including T-cell survival, trafficking capacity, antigen-driven activation, proliferation, and effector functions. Although current IS target mainly T cells, they have also effects on other immune cells and non immune cells[15-18]. In addition, suppression of immunity by IS is non-specific, meaning that IS also inhibit immune responses to pathogens and altered self cells. Therefore, , all immunosuppressive regimens have undesired side effects, the spectra of which vary and in some cases overlap [6,8,9]. Compromised immunity caused by these regimens is also responsible for reactivation of latent viral infections in recipients, of which cytomegalovirus (CMV) is the leading cause of viral infections after organ transplantation [74,75]. Transplantation of an allograft from a CMV seropositive donor can cause primary infection in previously uninfected patients, while previously infected patients can develop reactivation or reinfection, which subsequently may lead to an impaired post transplantation outcome [7].

Generally, most liver transplant centers use mono- bi- or triple therapy to prevent allograft rejection. This includes a CNI, either cyclosporine or tacrolimus, combined with mycophenolate mofetil or, less commonly, azathioprine, and a glucocorticoid such as prednisone. According to the liver function tests, when normal and stable, immunosuppression is tapered down to monotherapy, which is usually a CNI.

Calcineurin inhibitors

Cyclosporin is the first calcineurin inhibitor used in the clinic, and it was introduced in LT on 9th of March 1980. The treated patient was a 28-years old female. In 1983 the use of cyclosporine after LT was approved [3]. Since then, other CNI have been developed, like tacrolimus. CNI bind to the cytosolic protein cyclophilin and this formed complex inhibits calcineurin, which is responsible for the dephosphorylation of NFAT, a transcription factor that activates cytokine production in T cells. Subsequently, the production of cytokines like interleukin-2, -4, -7, -9 and -15, and interferon-y are inhibited, leading to reduced proliferation of T cells and thereby immunosuppression [76,77].

Adverse of CNI include high susceptibility for infections [6], malignancies [8], cardiovascular diseases [9-11] and diabetes [14]. Another obstacle to the use of CNIs is nephrotoxicity [12,13]. The longterm use of CNIs has been associated with irreversible renal failure in organ transplant recipients caused by afferent arteriole vasoconstriction of the renal arteries. This leads to deterioration of the glomerular filtration rate. Also vasoactive compounds and pathways which are stimulated by CNI, like the renin-angiotensin system, prostaglandins, endothelin and nitric oxide, contribute to chronic vasculopathy [15,16] which may result in renal deterioration. Additionally it has been shown that CNI induce the production of TGF-beta, an immunoregulatory and pro-fibrotic cytokine, which stimulates cells to produce extracellular matrix and decreases the production of extracellular matrix-degrading proteases, leading to interstitial fibrosis [17]. TGF-beta interacts also with various growth factors, cytokines and other peptides, like endothelin and angiotensin-II. The latter is known for its role in the development of chronic renal disease.

The clinical use of the CNI is complicated by their highly variable pharmacokinetics between individuals [78,79]. Interindividual differences in exposure to these drugs are related to genetic polymorphisms in the drug-metabolizing enzyme cytochrome P450 (CYP) CYP3A5, and the drug transporter ABCB1 (previously known as P-glycoprotein), which are responsible for the clearance of these drugs [80,81]. These genetic polymorphism are associated with an altered metabolism of CNI, resulting in the need of higher or lower doses of CNI in affected patients.

Aims of this thesis

The protocol of post transplantation liver transplant patient management provides general recommendations, and all patients are treated with standard IS regimens and doses, although we know that the responses of individual patients vary. Some of the patients suffer more from severe side effects of IS than others. Despite powerful IS therapy, some patients experience one or more acute rejection episodes. In addition, we know that part of patients with stable liver graft functions will not reject without taking any immunosuppressive drugs, and these are therefore over-immunosuppressed. Together,

these considerations suggest that clinical benefit can be achieved by individualisation of IS therapy. However, since there are currently no tools available to predict whether an individual patient has a high or low risk of graft rejection, or which patient will be at risk of suffering from IS-related complications, LT patients are treated according to general IS protocols. Therefore, identification of accurate predictive markers for the risk of undesirable effects, like rejection and nephrotoxicity of IS., would be helpfull to individualise the dose of immunosuppression To achieve this, a good understanding of the mechanism of liver graft rejection and IS metabolism is required.

The aims of this thesis were to identify genetic factors that determine inter-individual differences in the risk of graft rejection or IS-related complications (chapters 2 & 3), to develop accurate and sensitive assays for quantification of allo-reactive T cells in transplant recipients (chapter 4 & 5), and to study the effects of viral infection on allogeneic T cell responses and acute rejection (chapter 6). Such knowledge may be useful for development of predictive diagnostic parameters and tools that identify LT patients at risk for these complications, in order to facilitate tailoring of IS for each individual patient to prevent on the one hand under-immunosuppression which may lead to graft rejection and on the other hand decrease IS-related morbidity, thereby optimizing patient survival. In Chapter 2 we studied whether genetic polymorphisms in the CTLA-4 gene are associated with the incidence of acute rejection after LT. We determined two single nucleotide polymorphisms (SNPs) in the CTLA4-gene in 485 LT-patients from three centers, and analysed retrospectively their association with the risk of acute rejection. Chapter 3 discusses whether genetic polymorphisms in the ABCB1 and CYP3A5 genes are associated with the incidence of chronic kidney disease after LT. It has been described that polymorphisms in these genes are associated with CNI-induced nephrotoxicity after renal transplantation. However, renal dysfunction after kidney transplantation has many causes other than the use of CNI, and even kidney biopsy (still considered the gold standard) may not be able to differentiate between CNI-induced kidney damage and other causes of renal dysfunction. Therefore, the genetic basis of chronic kidney disease (CKD) may be better studied in LT recipients. In this chapter we investigated whether SNPs in these genes in LT donors or recipients are related to Tacrolimus-induced post-transplantation chronic kidney dysfunction in a single center cohort of 125 Caucasian LT patients.

Chapter 4 starts with the immunological part of the thesis. We studied the longitudinal course of circulating CD4+ and CD8+ T cells with direct allospecificity, utilizing an optimized assay, in 18 LT recipients. This study showed new insights into the kinetics of the direct donor-specific T cell response during the first year after LT.

In chapter 5, we determined the influence of CMV infection on T cell differentiation and alloreactivity, as well as on the risk of graft rejection after LT. Circulating T cell subsets (T_{CM}, T_{EM}, T_N and T_{EMRA}) and CD4+ and CD8+ with direct donor-specificity were quantified

in LT patients with different CMV serostatus. Additionally, the association between CMV serostatus and graft rejection was analysed.

Chapter 6 is a methodological description of the construction of a novel technique for quantification of human T cells with indirect allospecificity, which has several theoretical advantages compared to existing techniques.

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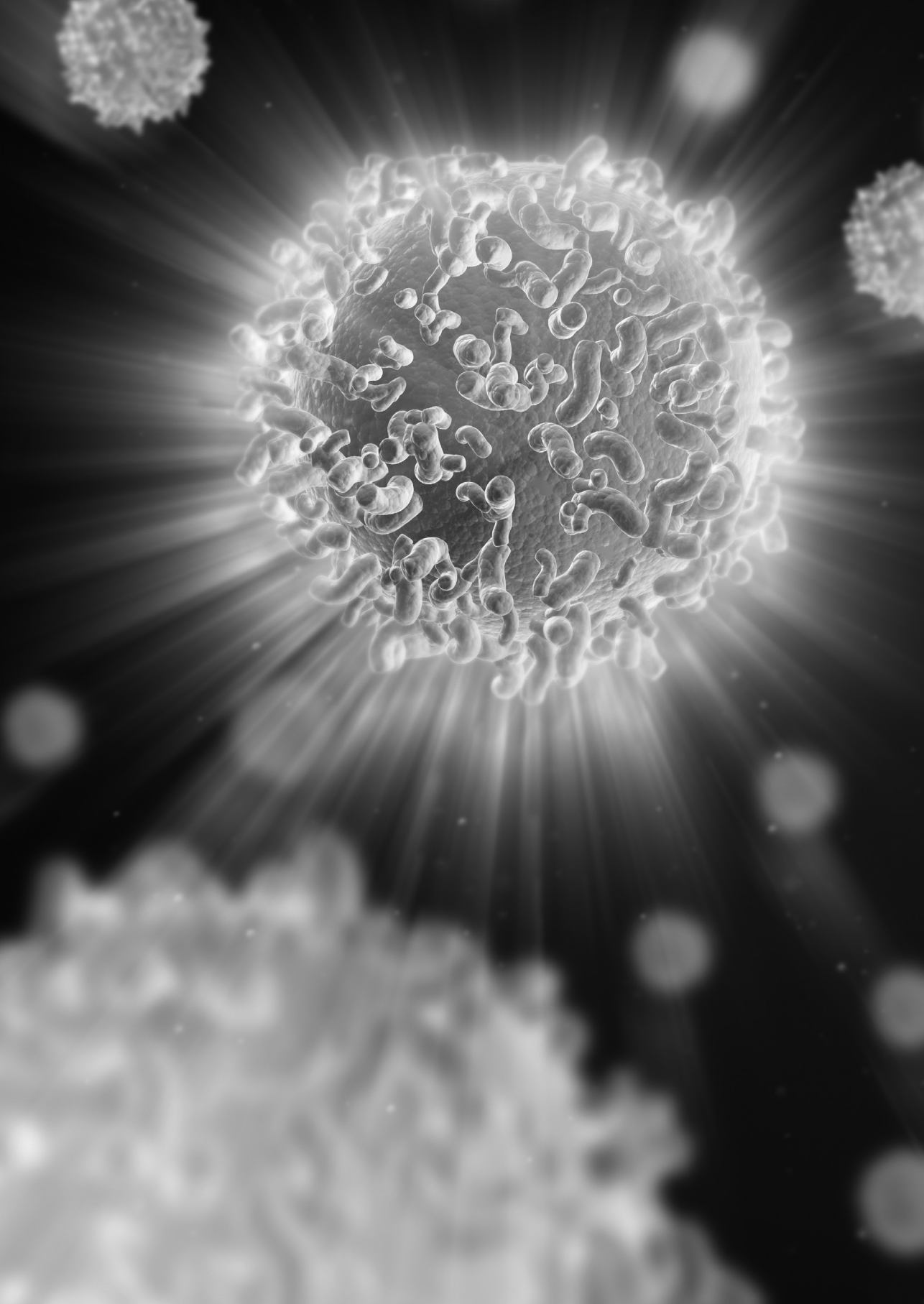

Chapter 2

Polymorphisms in the T cell regulatory gene cytotoxic T lymphocyte antigen 4 influence the rate of acute rejection after liver transplantation

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Tapirdamaz et al; 2006 Gut. 55(6):863-8

Abstract

Background: The cytotoxic T lymphocyte antigen 4 (CTLA-4) gene encodes for a membrane bound (mCTLA-4) and a soluble (sCTLA-4) isoform, which are both involved in regulation of T cell function. The CTLA-4 +49A/G single nucleotide polymorphism (SNP) influences expression of mCTLA-4; +6230G/A SNP affects the production of sCTLA-4.
Aim:To examine whether these functional SNPs influence the rate of rejection after liver transplantation.
Patients and methods: Liver graft recipients (n = 483) were genotyped for both SNPs, and haplotypes were reconstructed. Association with rejection was tested by the log rank test using the Kaplan-Meier method with time to the first acute rejection episode as outcome. Multiple analysis of SNPs together with demographic factors was performed by Cox regression.
Results: Three haplotypes were observed in the cohort: +49A/+6230A, +49A/+6230G, and +49G/+6230G. The +49A/+6230G haplotype was significantly and dose dependently associated with acute rejection (p = 0.01). Of the demographic factors tested, only underlying liver disease was significantly associated with rejection. Adjusted for underlying liver disease, each additional +49A/+6230G haplotype allele resulted in a significantly higher risk of acute rejection (risk ratio 1.34 (95% confidence interval 1.04–1.72); p = 0.02). Patients who lacked this haplotype had the lowest, carriers an intermediate, and homozygotes the highest risk of acute rejection.
Conclusion: The CTLA-4 +49A/+6230G haplotype, which encodes for normal mCTLA-4 expression but reduced sCTLA-4 production, is a co-dominant risk allele for acute rejection after clinical liver transplantation. This implies that even under immunosuppression, CTLA-4 is critically involved in the regulation of the human immune response to allogeneic grafts.

Introduction

Cytotoxic T lymphocyte antigen 4 (CTLA-4; CD152) is a homologue of CD28, which is expressed on the cell surface of activated memory T cells and on $CD4^+CD25^+$ regulatory T cells, and is critically involved in downregulation of T cell responses. Several mechanisms may account for its inhibitory effects. Firstly, CTLA-4 has a higher affinity for the B7 molecules CD80 and CD86 compared with CD28, and thereby serves as a competitive antagonist of CD28 for B7 binding [1]. Secondly, on binding to B7 molecules, CTLA-4 actively suppresses interleukin (IL)-2 production and cell cycle progression of T cells [2]. Thirdly, CTLA-4 is one of the inhibitory molecules by which $CD4^+CD25^+$ regulatory T cells exert their suppressive function on effector T cell activation [3]. Finally, an alternative splice form of CTLA-4, which is secreted by resting T cells [4], can suppress allogeneic T cell activation [5]. This soluble CTLA-4 (sCTLA-4) isoform is present in human serum [4] and its levels are enhanced in the serum of patients with autoimmune thyroid disease. [6]

Several autoimmune diseases have been found to be associated with allelic variations in the CTLA-4 gene. The strongest associations have been observed with the single nucleotide polymorphisms (SNPs) CTLA-4 +49A/G and +6230G/A. The +49 A/G SNP results in substitution of threonine by alanine in the leader peptide of the newly formed CTLA-4 molecule. It was found to be associated with, for example, Graves' disease [7], diabetes mellitus type 1 [8], primary biliary cirrhosis [9], and autoimmune hepatitis [10]. The +6230G/A SNP is situated in the 3′ untranslated region of the CTLA-4 gene, and was recently found to be more strongly associated with Graves' disease compared with the +49A/G SNP [11]. In addition, associations between the +6230G/A SNP and type 1 diabetes [12], and clearance of hepatitis B virus (HBV) infection [13] were found.

In view of the important role of CTLA-4 in regulating rejection activity against allogeneic organ grafts in experimental animals,[14,15] we examined whether genetic variations in the CTLA-4 gene influenced the rate of acute rejection after liver transplantation. In a previous single centre study aiming to explore whether SNPs in costimulatory molecules influenced the risk of acute rejection after liver transplantation, we found evidence for an association of the +49A/G SNP with rejection [16]. Here we present data from a multicentre study with a larger cohort of patients which aimed to determine to what extent the functional CTLA-4 +49A/G and +6230G/A SNPs influence the probability of rejection after liver transplantation.

Patients and methods

Patients

In this retrospective study, 483 liver transplant recipients, derived from three centres (126 from Rotterdam, 204 from Birmingham, and 153 from Newcastle), who received an orthotopic liver transplant between 1987 and 2001, were included. Patients with a follow up period of less than 90 days and patients treated with anti-IL-2 receptor monoclonal antibodies as part of their immunosuppressive induction treatment were excluded from the study. All included patients received standard immunosuppressive therapy consisting of ciclosporin or tacrolimus and prednisone, with or without azathioprine. Mean follow up was five years. Written consent was obtained from all participating patients, and the Medical Ethics Committee of the Erasmus MC in Rotterdam approved the study. Patient characteristics and data on acute rejection were derived from computerised databases or extracted from patient files. To correct for a possible influence of underlying liver disease on the risk of rejection, patients were classified according to their diagnosis for liver transplantation into the following categories: viral liver infection (acute or chronic HBV or hepatitis C virus (HCV) infection), autoimmune related liver diseases (primary biliary cirrhosis, primary sclerosing cholangitis, or autoimmune hepatitis), alcoholic liver cirrhosis, fulminant hepatic failure, or other (which contained all other diagnoses). Acute rejection was defined as an episode with increasing liver enzymes with no other identifiable cause, together with moderate or severe rejection activity in a liver biopsy, which was responsive to high dose corticosteroid or antilymphocyte immunoglobulin treatment.

Determination of SNPs

DNA was isolated from blood by the classic salting out method, involving proteinase K digestion and ethanol precipitation. Samples from Rotterdam and Birmingham were analysed in Manchester by allele specific polymerase chain reaction (PCR) by one of the investigators (ÖT) who was blinded to the clinical data. Allele specific forward primers and generic reverse primers were designed using the sequence of the CTLA-4 gene published in the Nucleotide Data bank (http://www.ncbi.nlm.nih.gov). The sequences of the primers are depicted in table 1. Each of the allele specific primers was mixed with the associated generic primer in a 1:1 ratio and separate reactions were performed for determination of each allele. An internal control amplification of the human growth hormone (HGH) gene was used as a positive control for the PCR reaction. DNA was amplified in Mastermix (Abgene, Epsom, UK) supplemented with 5 µM CTLA-4 and HGH primers. PCR was run in a PTC-100 PCR machine (MJ Research, Massachusetts, USA) according to the following programme: 95°C 60 seconds, 10 cycles of 95°C 15 seconds, T1°C 50 seconds, 72°C 40 seconds, and thereafter 20 cycles of 95°C 20 seconds, T2°C 50 seconds, and 72°C

50 seconds. The primer specific annealing temperatures T1 and T2 are depicted in table 1. PCR products were monitored after electrophoresis on a 2% agarose gel containing ethidium bromide.

Samples from Newcastle were genotyped by a PCR-restriction fragment length polymorphism method [10]. The sequences of the primers used to amplify the SNP flanking regions are shown in table 1. *Bbv*I was used to digest the G allele of +49 SNP and *Hpy*CH4IV to digest the G allele of the +6230 SNP. Alleles were identified after electrophoresis in an agarose gel.

Table 1 Polymerase chain reaction (PCR) primers used in the study

SNP	PCR primer sequences	Annealing temp (°C)
	Primers used for allele specific PCR	
CTLA-4 +49A/G	5'CAAGCCAGATTGGAGTTA 3'	T1 = 64
	5'CTCAGCTGAACCTGGCTA/G 3'	T2 = 58
CTLA-4 +6230G/A	5'AGGAAGGCAGATCAAAATGC 3'	T1 = 65
	5'CACCACTATTTGGGATATAACA/G 3'	T2 = 59
HGH	5'GCCTTCCCAACCATTCCCTTA 3'	
	5`TCACGGATTTCTGTTGTGTTTC 3'	
	Primers used for PCR-RFLP	
CTLA-4 +49A/G	5'CCACGGCTTCCTTTCTCGTA 3'	56
	5'AGTCTCACTCACCTTTGCAG 3'	
CTLA-4 +6230G/A	5'CATCCTTGCATTTGAATATTGTG 3'	59
	5'AAGAATCACAGAGGGCCCAGTG 3'	

CTLA-4, cytotoxic T lymphocyte antigen 4; SNP, single nucleotide polymorphism; HGH, human growth hormone; RFLP, restriction fragment length polymorphism

Haplotype- genotype estimation

The linkage disequilibrium coefficient (D') between the two SNPs was calculated using Phase software version 2 [17] (http://archimedes.well.ox.ac.uk/pise/PHASE.html). This program was also used to estimate the most possible haplotype pair for each individual.

Statistical analysis

The Kaplan-Meier method was used to determine the cumulative probability of the first episode of acute rejection in patient groups with different genotypes or haplotypes, and the log rank test was applied to compare rejection curves. Cox regression analysis was used to assess the influence of patient characteristics (age, sex, ethnicity, year of transplantation, and underlying disease) and the numbers of haplotypes on the risk of acute rejection, using the time to the first episode of acute rejection as outcome. To adjust the contributions of genetic CTLA-4 haplotypes for those of patient characteristics

to the risk of acute rejection, multiple Cox regression analysis was performed. In all Cox regression analyses patients were stratified on centre to correct for the influence of the different centres on outcome. A p value of <0.05 was considered statistically significant.

Results

Patient characteristics and acute rejection

The incidence of acute rejection in the investigated cohort was 39% (189 out of 483 patients). Table 2 shows the influence of demographic patient characteristics on the rate of acute rejection. Risk ratios of acute rejection were calculated by Cox regression using the time to the first acute rejection episode as outcome and stratifying patients on centre. Female recipients had a higher incidence of rejection compared with male recipients. Patients with a viral liver infection (either HCV or HBV) as the indication for liver transplantation had about a twofold decreased risk of acute rejection compared with patients with other underlying liver diseases, except alcoholic cirrhosis. Recipient age and year of transplantation were not significantly related to risk of acute rejection. The latter indicates that changes in immunosuppressive treatment over the years did not significantly influence the rate of acute rejection.

Table 2 Association between patient characteristics and time to first acute rejection, as analysed by Cox regression and stratified on centre.

Patient Characteristics	HR (95% CI)	p Value
Sex		0.04
Male (N=259)	1	
Female (n=224)	1.35 (1.01-1.79)	
Age (per year)	0.99 (0.98-1.01)	0.29
Year of transplantation	0.96 (091-1.03)	0.25
Ethinicity		0.10
Caucasian (n=449)	1	
Black African (n=19)	0.43 (0.13-1.36)	
Asian (n=15)	0.41 (0.10-1.65)	
Diagnosis		0.01
Chronic viral hepatitis (n=94)	1	
Autoimmune related (n=172)	2.06 (1.25-3.39)	
Alcoholic cirrhosis (n=80)	1.37 (0.77-2.44)	
FHF (n=44)	2.37 (1.29-4.34)	
Other (n=93)	1.79 (1.04-3.09)	

Groups with a hazard ratio (HR) of 1 were chosen as the reference categories. 95% CI, 95% confidence interval. P values were derived from the log likelihood test. FHF, fulminant hepatic failure

Single nucleotide polymorphisms and acute rejection

Figure 1 shows the cumulative probabilities of acute rejection in patient groups with different genotypes of the +49A/G and +6230G/A SNPs. Overall, neither SNP was significantly associated with acute rejection but pairwise comparisons of the genotypes showed a significantly lower risk of rejection in patients with the +6230AA genotype compared with those with the AG genotype.

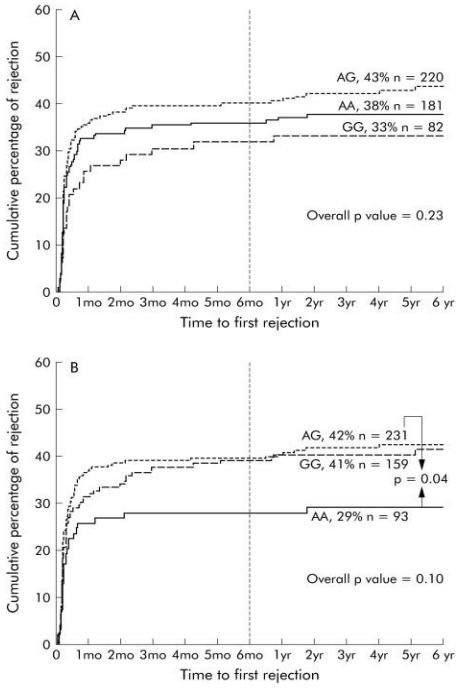

Figure 1: Cumulative percentages of acute rejection (Kaplan-Meier curves) in liver transplant recipients classified according to their cytotoxic T lymphocyte antigen 4 (CTLA-4) +49A/G (A) or CTLA-4 +6230G/A (B) genotype. The outcome used was the time to the first episode of acute rejection. Percentages indicate cumulative incidences of acute rejection in each group, eight years after transplantation. For better visualisation of the kinetics of rejection, the x axis of the first half year was spread out and curves were cut off at six years. No acute rejections occurred after that time. p values were derived from the log rank test.

Haplotypes and acute rejection

The +49A/G and +6230G/A SNPs were in complete linkage disequilibrium (D′ = 1). Haplotype reconstruction from the SNP by Phase software showed that only three of the four possible haplotype combinations were present in the cohort—namely, +49A/+6230A, +49A/+6230G, and +49G/+6230G. To establish the associations between these haplotypes and acute rejection, we grouped patients by haplotype copy numbers

(non-carriers, heterozygous carriers, and homozygous carriers) for each haplotype allele. Kaplan-Meier curves of the cumulative incidences of acute rejection in the different groups are depicted in fig 2. As the +6230A allele was completely linked to the +49A allele, the curves of the +49A/+6230A haplotype (fig 2A) were exactly the same compared with the curves of the +6230G/A SNP (fig 1B), and the rate of rejection was significantly lower in patients homozygous for the +49A/+6230A haplotype compared with heterozygous carriers. However, the opposite haplotype +49G/+6230G

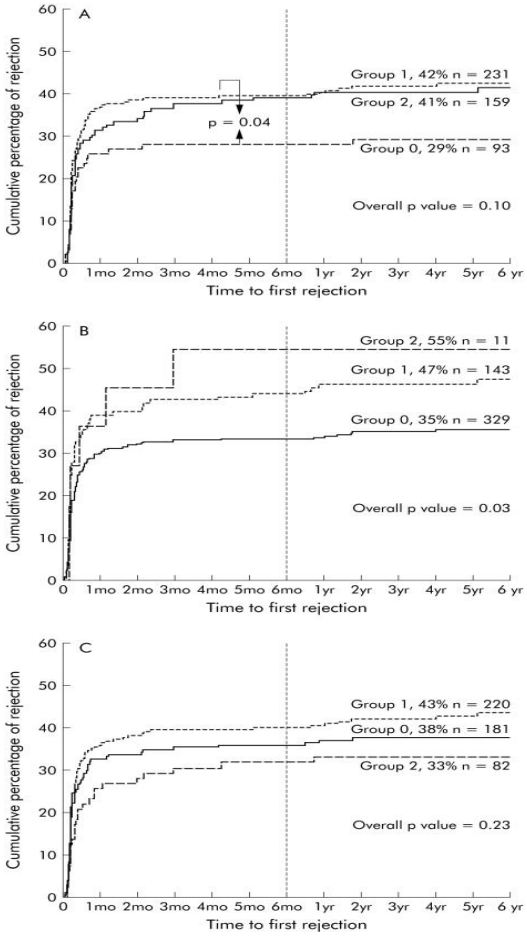

Figure 2: Cumulative percentages of acute rejection in liver transplant recipients classified according to the numbers of CTLA-4 +49/+6230 haplotype copies they carried. This is shown for haplotypes +49A/+6230A (A), +49A/+6230G (B), and +49G/+6230G (C). Patients who lacked the haplotype are termed group 0, heterozygous carriers group 1, and homozygous carriers group 2. The outcome used was the time to first episode of acute rejection. Percentages indicate cumulative incidences of acute rejection six years after transplantation. p values were derived from the log rank test

was not a risk allele for acute rejection (fig 2C). Log rank analysis showed that overall only the +49A/+6230G haplotype (fig 2B) was significantly associated with the rate of acute rejection. To investigate whether the influence of this haplotype on the risk of rejection was dose dependent, a univariate Cox regression model was used in which the +49A/+6230G haplotype was introduced as a continuous variable. In this model, the +49A/+6230G haplotype demonstrated a significant dose-allele effect with a hazard rate of 1.39 (95% confidence interval 1.08–1.79; p = 0.01). Collectively, these data indicate that the CTLA-4 +49A/+6230G haplotype is a co-dominant risk allele for acute rejection after liver transplantation. When patients were classified according to their CTLA-4 +49/+6230 haplotype genotypes, Kaplan-Meier plots confirmed that homozygous and heterozygous carriers of the +49A/+6230G haplotype allele had the highest rates of acute rejection (fig 3), and homozygous carriers of both of the other haplotypes (49A/+6230A and +49G/+6230G) the lowest.

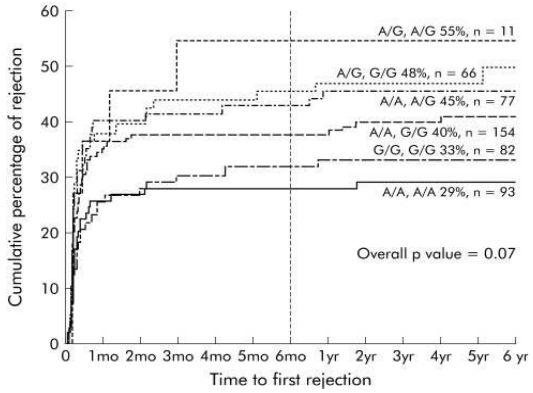

Figure 3: Cumulative percentages of acute rejection in liver transplant recipients classified according to their haplotype genotypes. Each curve represents a group of patients with one genotype, which is depicted above or underneath the curve with the +49/+6230 genotypes, separated by a comma. The outcome tested was the time to first episode of acute rejection. Percentages indicate cumulative incidences of acute rejection six years after transplantation. The p value is derived from the log rank test

Multivariate analysis of the association of CTLA- 4 haplotypes and patient characteristics with the rate of acute rejection

To determine whether the CTLA-4 haplotypes contributed to the risk of acute rejection independently of patients characteristics, a Cox regression model which included all haplotypes and patients characteristics was used. Non-significant parameters were removed stepwise. Table 3 shows that only the +49A/+6230G haplotype and underlying liver disease were significantly associated with rejection. Although in the univariate analysis a significant association between recipient sex and acute rejection was found,

this was not the case in multivariate analysis. Adjusted for the effect of underlying diseases, the +49A/+6230G haplotype was dose dependently associated with acute rejection, with a risk ratio of 1.34 for each additional +49A/+6230G haplotype allele. This means that heterozygous carriers had a 1.34 higher relative risk of acute rejection compared with patients who lacked this haplotype, and that homozygous carriers had a 1.34 higher relative risk compared with heterozygous carriers.

Table 3 Multiple Cox regression analysis of the influence of cytotoxic T lymphocyte antigen 4 (CTLA-4) +49/+6230 haplotypes and patient characteristics stratified on centre on the rate of acute rejection

	Risk ratio	95% CI	p Value
Haplotype +49A/+6230G (per allele number)	1.34	1.04-1.72	0.02
Diagnosis			
Chronic viral hepatitis	1		0.01
Automimmune related	2.05	1.24-3.35	<0.01
Alcoholic cirrhosis	1.34	0.75-2.39	0.33
FHF	2.25	1.23-4.14	0.01
Others	1.76	1.02-3.04	0.04

In the Cox regression model each of the three different CTLA-4 +49/+6230 haplotypes were included as a continues variable, together with underlying liver disease, year of transplantation, and recipient ethnicity, seks and age. The diagnosis group with a hazard ratio of 1 was chosen as a reference category. Only those parameters which were significantly associated with acute rejection are shown. The overall p value was 0.003 (log likelihood test). FHF, fulminant hepatic failure, 95% CI, 95% confidence interval.

Discussion

In the present study, we analysed the influence of two functional SNPs in the CTLA-4 gene on the rate of acute rejection after liver transplantation. The +49A/G SNP is located in the signal peptide of the molecule and influences expression of the full length isoform on the T cell membrane. Full length CTLA-4 molecules encoded by the G allele are incompletely glycosylated, leading to retrograde transport of a portion of the molecules to the cytoplasm for degradation. This results in reduced expression on the T cell surface [18] and impaired inhibitory function of CTLA-4 on T cell activation in individuals homozygous for +49G [7]. The +6230G/A SNP is located in the 3′ untranslated region of the CTLA-4 gene and was reported to influence the production rate of the soluble alternative splice form of CTLA-4. sCTLA-4 mRNA encoded by the +6230G-allele is produced at a reduced rate compared with mRNA encoded by the +6230 A allele [11]. As the soluble isoform is a suppressor of allogeneic T cell activation [5], it is conceivable that carriers of the +6230G allele may be more susceptible to rejection after organ transplantation. This was indeed what we found on analysis of the association between the +6230 SNP and

rejection: homozygous CTLA-4 +6230A liver transplant recipients which lacked the G allele showed a reduced rate of rejection compared with carriers of the G allele.

However, haplotype analysis showed that the single +6230G allele was not the risk allele for acute rejection. The +6230G allele was not associated with an increased risk of rejection when it was combined in a haplotype with the +49G allele, but only when it was combined with the +49A allele. Thus the risk allele for acute rejection after liver transplantation (+49A/+6230G) combines the genetic predisposition for reduced capability to produce sCTLA-4 (+6230G) with a normal capacity to express the full length CTLA-4 isoform on the T cell membrane (+49A). The effect of this haplotype on rejection was dose dependent, meaning that each additional allele results in a higher risk of rejection. The conclusion that this specific combination of single alleles confers risk to acute rejection is further substantiated by the observation that carriers of one +49A/+6230A allele and one +49G/+6230G allele had a rate of rejection intermediate between carriers of the +49A/+6230G risk haplotype and homozygous carriers of the protecting +49A/+6230A and +49G/+6230G haplotypes (fig 3). Conceivably, the single risk alleles +49A and +6230G can also exert their combined effect on rejection when they are located on different chromosomes.

The finding that only the +49A/+6230G haplotype is the risk allele for acute rejection is remarkable. The +49A/+6230G and +49G/+6230G haplotypes were observed to be associated to a similar extent with Graves' disease [11]. For type I diabetes, the +49G/+6230G is a risk allele but the association of the +49A/+6230G haplotype with disease is either positive or negative, depending on the allele of the −319C/T SNP which is cotransmitted [12]. It is difficult to imagine how increased expression of the inhibitory mCTLA-4, as encoded for by the +49A allele, contributes to an enhanced rate of rejection. A major difference between acute rejection of clinical organ grafts and autoimmune diseases is that rejection develops under the cover of continuous immunosuppression. Calcineurin inhibitors such as ciclosporin and tacrolimus inhibit the upregulation of mCTLA-4 expression on the cell surface after T cell activation [19]. As sCLTA-4 is produced by resting, but not by activated T cells, production of this isoform is probably not suppressed by calcineurin inhibitors. Therefore, the ratio between levels of mCTLA-4 and sCLTA-4 expression in organ transplant recipients may be quite different from that in patients with developing autoimmune diseases. As a consequence, the effects of high or low mCTLA-4 expression on acute rejection may differ from those on development of autoimmune diseases. Furthermore, an additional unknown single allele which influences CTLA-4 expression of function may be linked to the +49A+6230G haplotype, but not the +49G+6230G allele. The effect of this allele may only penetrate into a clinical effect under conditions of immunosuppression.

In a previous explorative single centre study, we found that liver transplant recipients who were homozygous for the CTLA-4 +49 G allele had a lower incidence of acute rejec-

tion after liver transplantation compared with homozygous or heterozygous carriers of the A allele [16]. A trend towards a similar association was found in the present study, but this was not statistically significant. Our previous finding seemed to contradict observations that the CTLA-4 +49G allele is a disease predisposing allele for several autoimmune diseases.[7,8,9,10] In addition, *Slavcheva* and colleagues [20] found no association between the +49 A/G polymorphism and acute rejection in a combined cohort of liver and kidney transplant recipients. The results of the present study may explain why differences in associations between the +49A/G polymorphism and acute rejection can be obtained in different study cohorts. Individuals with genotype +49G/G lack the risk haplotype allele +49A/+6230G, which makes them less susceptible to rejection, but carriers of +49A alleles can either carry the risk haplotype +49A/+6230G or the protective haplotype +49A/+6230A in conjunction with the +49A allele. Therefore, the association of the +49A/G genotype with rejection is dependent on the proportion of +49A alleles that are linked to the +6230G allele in the study cohort. In the present study, more than half (93/181) of the patients with the +49A/A genotype were homozygous carriers of the +49A/+6230A haplotype allele, and therefore had a low rate of acute rejection (fig 3). Consequently, in the cohort investigated in the present study, no significant association between the +49A/G polymorphism and rejection was found. The same may have been the case in the cohort studied by Slavcheva and colleagues [20].

The results of the present study do not contradict the association between liver graft survival and recipient CTLA-4 +49 genotype observed by Marder and colleagues [21]. These authors reported that liver transplant recipients with the +49G/G genotype have a decreased graft survival in comparison with patients with +49A/A or A/G. The shortened graft survival was, however, not due to a higher incidence of acute rejection in this group but mainly related to recurrence of viral hepatitis. Therefore, the decreased graft survival in the +49G/G group was probably due to an increased immune response of the recipient to reinfection of the liver graft with hepatitis virus. Indeed, the CTLA-4 +49G allele has been associated with an enhanced immune response against HBV [13].

The only patient characteristic that was significantly associated with acute rejection on multiple analysis was underlying liver disease. Liver transplant recipients with viral hepatitis or alcoholic cirrhosis as indication for liver transplantation had a twofold reduced risk of acute rejection. The first group included patients with HBV and HCV infection, transplanted for either acute liver failure or cirrhosis due to chronic infection. The reduced incidence of rejection is this patient category was probably due to prophylactic treatment of HBV positive patients with high dose anti-hepatitis B immunoglobulins to prevent reinfection of the graft. Recently, we confirmed the original observation of Farges and colleagues [22] that treatment with intravenous anti-hepatitis B immunoglobulins protects against rejection, and we demonstrated that these immunoglobulins

inhibit both T cell and dendritic cell function.[23] The relatively low incidence of rejection in patients with alcoholic cirrhosis has also been observed in other studies.[24]

In conclusion, the CTLA-4 +49A/+6230G haplotype is a co-dominant risk allele for acute rejection after clinical liver transplantation. This implies that, even under immunosuppression, CTLA-4, in common with experimental animals, is critically involved in the regulation of the human immune response against allogeneic grafts.

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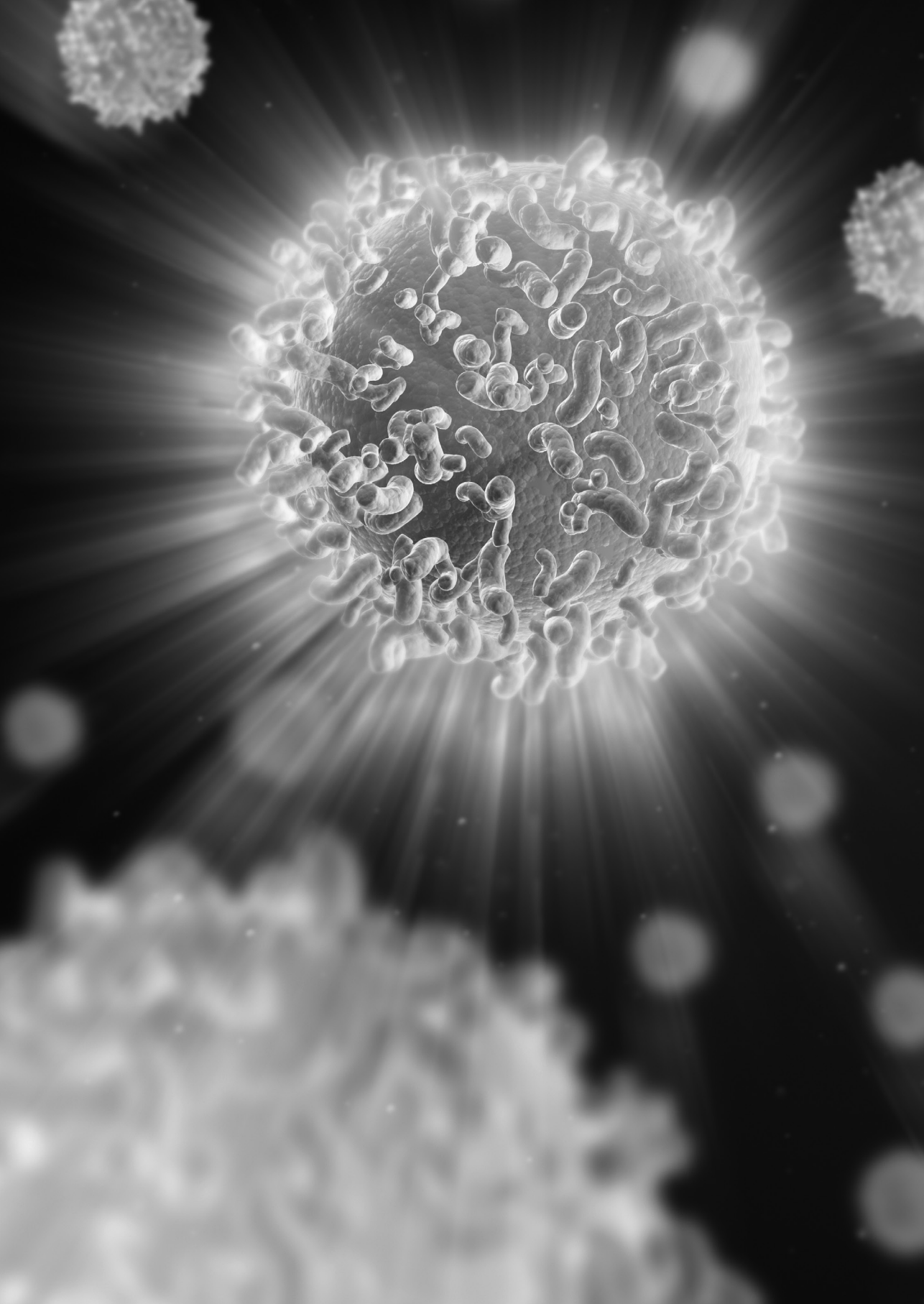

Chapter 3

Genetic variance in ABCB1 and CYP3A5 does not contribute to the development of chronic kidney disease after liver transplantation

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Tapirdamaz et al; 2014 Pharmacogenet Genomics 24(9):427-35.

Abstract

Introduction: Chronic kidney disease (CKD) after liver transplantation (LT) is a major clinical problem that appears to be associated with non-genetic, as well as genetic determinants. Calcineurin inhibitor (CNI) use is considered to play a major role in the development of CKD after LT. We studied the influence of single-nucleotide polymorphisms (SNPs) in the genes of the donor and recipient CNI-metabolizing enzyme *CYP3A5* and the CNI-transporting *ABCB1*, on the development of chronic kidney disease (CKD) after liver transplantation (LT).

Methods: Tacrolimus (Tac) predose concentrations at different time-points after transplantation and the *CYP3A5* 6986A>G and *ABCB1* 3435C>T SNPs were determined in 125 LT recipients and their respective donors to study the influence of Tac predose levels and genetics on CKD development.

Results: After a median follow up of 5.7 ± 2.9 years, CKD developed in 47 patients (36%). The Tac predose levels were not correlated with the development of CKD. Neither did we find a correlation between the investigated SNPs in either donor or recipient *ABCB1* and *CYP3A5* genes (or combinations thereof) and the development of CKD. These genetic variatiants did not relate to Tac predose blood concentrations in our study.

Conclusion: An individual's risk to develop CKD after LT is not associated with genetic variation in either recipient or donor *CYP3A5* or *ABCB1* genotype status.

Introduction

Tacrolimus (Tac) is currently the most commonly used calcineurin inhibitor (CNI) after liver transplantation (LT) to prevent allograft rejection. The clinical use of Tac is challenging because of variable pharmacokinetics between individuals [1-3]. In most transplant centers, the dosage of Tac is based on predose (whole-) blood concentrations to find a balance between under- and over-immunosuppression and its associated risks of rejection, nephrotoxicity, or infection. Nonetheless, undesired effects of Tac treatment cannot be totally prevented by performing therapeutic drug monitoring [4].

One of the mechanisms of inter-individual differences in Tac exposure is the activity of the polymorphically-expressed CNI-metabolizing enzyme cytochrome P450 (CYP) CYP3A5, and the drug transporter ABCB1 (previously known as P-glycoprotein).[5,6,7] CYP3A5 and ABCB1 are both expressed in the intestine, the liver and the kidney.[5,6,8-11] ABCB1 is responsible for decreasing the systemic exposure to Tac, by increasing its efflux into the intestinal, canalicular and tubular lumina. CYP3A5 is responsible for the clearance of Tac and is co-expressed with ABCB1 in liver and kidney. Through the action of ABCB1 local Tac concentrations are kept low, ensuring efficient CYP3A5-mediated Tac metabolism.

Several single-nucleotide polymorphisms (SNPs) have been identified in the *CYP3A5* and *ABCB1* genes. The best-studied SNP in *CYP3A5* is that in intron 3, genomic 6986A>G.[12,13] The variant allele, referred to as *CYP3A5*3* (corresponding with the G allele), leads to the absence of functional CYP3A5 protein and occurs homozygously in 80% of the Caucasian and in 30% of the black population. Several investigators have demonstrated that patients carrying the *CYP3A5*1* (wild-type) allele and who therefore express functional CYP3A5, need a Tac dose which is on average 50% higher compared to patients with the *CYP3A5*3/*3* genotype (non-expressers) [14]. In addition, an individual's *CYP3A5* genotype may determine one's risk of developing Tac-related nephrotoxicity [15].

More than 25 SNPs have been identified in *ABCB1*. The best-studied SNP is 3435C>T in exon 26, which may lead to the formation of an ABCB1 protein with an altered conformation and activity, and possibly intracellular accumulation of Tac.[15,16] There is ample evidence that intra-renally expressed ABCB1 is implicated in the pathogenesis of CNI-induced renal dysfunction [17]. Additionally, several studies have provided evidence that genetic variation in *ABCB1* may predispose patients to the nephrotoxic effects of CNIs after kidney transplantation.[18,19]' .

Most studies on the role of genetic variation in *CYP3A5* and *ABCB1* in CNI-related nephrotoxicity were performed in renal transplant recipients.[15,19,21-23] However, renal dysfunction after kidney transplantation has many causes other than the use of CNIs, and even kidney biopsy (still considered the gold standard) may not be able to differentiate between CNI-induced kidney damage and other causes of renal dysfunction.

Therefore, the genetic basis of chronic kidney disease (CKD) may be better studied in LT recipients. So far, three studies have investigated the role of *ABCB1* and *CYP3A5* in the development of CKD after LT [24-26]. However, these studies were performed in Asian (rather than Caucasian) or pediatric populations and included relatively small numbers of patients. In the present study, we investigated if genetic variation in *CYP3A5* and *ABCB1* is associated with CKD in a Caucasian, adult LT population.

Materials & Methods

Patients

This retrospective study included clinical data of patients who underwent a liver transplantation between September 1997 and November 2009 and were followed at the outpatient clinic of the Erasmus MC, University Medical Center Rotterdam, the Netherlands. Three months post-LT was considered as time-point 0, meaning the starting point of kidney function measurement. We chose this time point to minimize kidney dysfunction associated with liver failure and surgical related complications.

Starting in 1996 there has been a gradual transition from cyclosporine to the use of Tac, with Tac becoming the main immunosuppressant after the year 2000. The main immunosuppressant at 3-months post-LT was considered as the immunosuppressive regimen that the patient received. Patients received a CNI from day one post-LT, unless there was a medical reason to postpone the first gift. The doses of Tac were adjusted to achieve and maintain whole-blood predose concentrations of 10 to 15 ng/mL in the first month post-LT, and between 5 to 10 ng/mL from 1 month onwards. Doses were adapted for presumed CNI-related toxicity or allograft rejection.

For this study we used the following inclusion criteria: 1) Use of Tac at 3 months after LT, 2) Caucasian ethnicity, 3) availability of DNA of both donor and recipient 4) a follow-up time of at least one year after LT. Exclusion criteria were 1) a combined kidney-liver transplantation, 2) a previous kidney transplantation, 3) recipient age <18 years, 4) death within the first year after LT, 5) a follow-up period of less than one year after LT (for reasons other than death), 6) a re-LT within one year after the first LT, 7) receiving immunosuppression other than Tac in the first three months and 8) an ethnicity other than Caucasian. Patients in need of a re-transplantation were censored at the time of their second transplantation.

The institutional medical-ethical review board of our hospital approved the current study and informed consent was obtained from all patients.

Renal function

The estimated glomerular filtration rate (eGFR) was calculated by use of the Modification of Diet in Renal Disease study equation (MDRD) [25]. Serum creatinine values (µmol/L) were collected at 3, 6 and 12 months after LT and yearly thereafter. The same creatinine assay was used during the whole study period. Patients were categorized according to the Kidney Disease Outcomes Quality Initiative (KDOQI) groups: patients were considered to have mild renal insufficiency when their eGFR was between 30 and 60 mL/min per1.73 m^2 (CKD stage 3). Moderate renal failure was categorized as CKD stage 4 (eGFR between 15 and 30 mL/min per 1.73 m^2), and end-stage kidney disease or CKD stage 5 (eGFR < 15 mL/min per 1.73 m^2 or the need for renal replacement therapy).

Tacrolimus assay

Tacrolimus predose concentrations were determined throughout the study period in whole blood by use of immunoassays [the Emit 2000 assay (Syva Company, Dade Behring Inc., Cupertino Calif.) and the ACMIA-Flex assay (Siemens HealthCare Diagnostics, Inc., Newark, DE)] on several analyzers [the IMX (Abbott), the Cobas Mira Plus analyzer (Roche Diagnostic Systems, Basel, Switzerland), the V-twin and Dimension XPand (both Siemens HealthCare Diagnostics, Inc, Newark, DE)]. The upper limit of detection was 30 ng/mL and the lower limit of detection 1.5 ng/mL. Details on the sensitivity, reproducibility, and sensitivity of the Emit assay in our laboratory were published previously [26]. Proficiency samples were obtained from the United Kingdom Quality Assessment Scheme (Dr Holt, St George's Hospital Medical School, London, United Kingdom).

DNA isolation and genotype determination

Deoxyribonucleic acid (DNA) was extracted from venous blood from recipients and splenocytes of deceased donors, by using a DNA Purification kit (Promega, Madison USA) according to the manufacturer's guide. The subjects were genotyped for the *CYP3A5* (6989A>G) and *ABCB1* (3435C>T) SNPs as previously described [27]. In brief, 50 ng of DNA was used in a total PCR volume of 50 µL containing 1x buffer (10-mmol/L Tris-hydrochloric acid, pH 8.3; 1.5 mmol/L magnesium chloride; 50-mmol/L potassium chloride; and 0.001% [wt/vol] gelatin [PerkinElmer, Inc, Wellesley, Mass]), 0.2-mmol/L of each deoxyribonucleoside triphosphate (Roche), 1.25 U of Ampli*Taq* Gold (Perkin-Elmer) and 40 pmol of each forward and reverse primer. For *ABCB1*, the forward primer 5'-CATGCTCCCAGGCTGTTTAT-3' and the reverse primer 5'-GTAACTTGGCAGTTTCAGTG-3' were used. PCR conditions were as follows: 7 minutes at 94°C; 35 cycles of 1 minute at 94°C; 1 minute at 55°C, 1 minute at 72°C and finally 7 minutes at 72°C. The PCR products were digested with *SspI* (*CYP3A5*) or *DpnII* (*ABCB1*) in a total volume of 15 µL for 2 hours at 37 °C and subsequently analyzed on agarose/Tris-borate-ethyl-enediaminetetraacetic acid gel with ethidium bromide staining.

Statistical analysis

SPSS 17.1 was used for data analysis. Differences in parametric en nonparametric values between groups were tested with the unpaired t-test and Pearson's Chi Square, respectively, for categorized variables and one-way ANOVA for continuous variables. Linear regression was used for differences between Tac predose concentrations between groups. Survival curves according to Kaplan Meier log rank comparison were calculated for the univariate analysis of multiple covariates (tested separately) in correlation with CKD. Deviations from the Hardy-Weinberg equilibrium were studied using appropriate Chi Square testing, using a calculator on http://www.oege.org/software/hwe-mr-calc.shtml. P values < 0.05 were considered significant.

Results

Baseline characteristics

In the study period, a total of 328 patients underwent a liver transplantation of which 52 subjects used cyclosporin as main immunosuppressive therapy. Within the first year after LT, 15 patients died and 43 underwent a re-LT. Of the remaining 218 patients, 2 were excluded because of kidney transplantation prior to LT and 2 patients were excluded because of combined kidney-liver transplantation. Of 52 subjects there was no DNA available and 25 were not Caucasian. Of 5 patients we did not have sufficient (clinical) data to calculate the eGFR. Seven patients were lost to follow-up, leaving a total of 125 patients for the final analysis (Figure 1A).

The baseline characteristics of the 125 studied patients are depicted in Table 1, categorized in patients with and without renal dysfunction. The overall mean follow up time was 5.7 ± 2.9 years with a total follow up of 11.2 years. Patients developing kidney dysfunction were more often of female gender, older and had pre-transplant diabetes mellitus. Besides, there was a difference in primary indication for liver transplantation between the two groups. There were no differences between the pre-existent hepatitis C infection (HCV) and genetic distribution of the SNPs between the two groups.

In this study population, renal function remained normal in 62% (n = 78), 36% of patients (n = 45) developed mild renal dysfunction, and only 2% (n = 2) developed moderate renal failure (CKD 4). None of the patients developed end-stage renal disease [eGFR < 15 mL/min/1.73 m^2] (Figure 1B). Figure 1C displays the dynamics of the mean renal function between patients with a normal eGFR and patients with renal impairment. Patients with an impaired renal function started with a significantly lower eGFR at 3 months after liver transplantation.

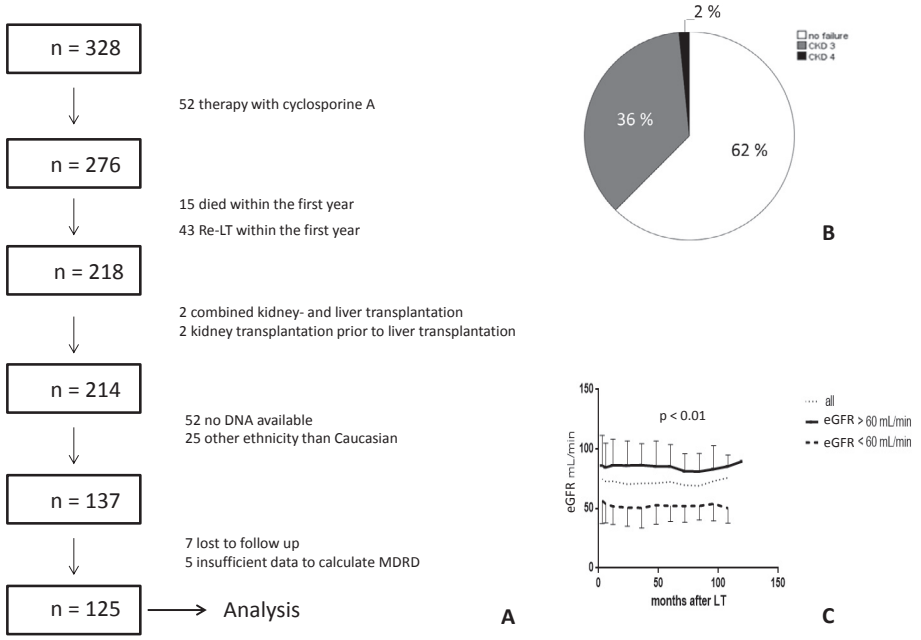

Figure 1: A) Flow chart of study cohort, between September 1997 and December 2009, displaying how many subjects were excluded, leaving 125 LT recipients for final analysis B) Pie chart of distribution of chronic kidney failure classification; majority does not develop kidney failure (62%), 36% develops mild kidney failure (CKD 3) and only 2 % develops a eGFR between 15- and 30 mL/min (CKD 4); C) Mean eGFR levels through time between patients with a eGFR > 60 mL/min en eGFR < 60 mL/min. Patients developing CKD start with lower eGFR than patients with a normal kidney function, which is significant ($p < 0.01$)

Renal clearance and Tac predose levels

Tac predose blood concentrations were collected at several time-points for each patient, starting at month 1 after LT. The mean exposure to Tac in the early phase after transplantation was 10.4 ± 4.8 mg/L (with a range of <1.5 mg/L and 26.4 mg/L) with a decrease thereafter and with a mean at month 48 of 5.8 ± 3.2 mg/L (range between <1,5 and 14.4 mg/L). When analysis was performed of exposure of patients with an eGFR >60 mL/min and those with a eGFR <60 mL/min, there was no significant difference (Figure 2A) and both groups had Tac predose concentrations within the target range. Analysis of exposure to Tac predose levels in patient groups with different genotypes was performed and shown in figure 2B to 2D, and no significant differences were observed. The Tac predose levels of the two patients with CKD 4 were on average lower than those of patients with normal renal function or those with CKD stage 3. After month 6, the predose levels of this group was below the target range of 5 mg/L (data not shown).

Table 1: Baseline characteristics of recipients of liver transplantation with and without chronic kidney failure (CKD)

		EGFR>60 mL/min Total N=78(%of N)	EGFR<60 mL/min Total N=47(%of N)	p-value
Gender	Male	59 (76)	21 (45)	0,01
	female	19 (24)	26 (55)	
BMI (kg/m^2)	mean ± SE	25,7 ± 4,3	24,7 ± 2,9	0,15
Age	mean ± SE	44,3 ± 12,6	50,6 ± 8,7	0,03
Primary disease	acute hepatic failure	4 (5)	9 (19)	0,01
	cholestatic disease	23 (30)	5 (11)	
	alcoholic disease	9 (12)	11 (23,4)	
	viral hepatitis	9 (12)	8 (16)	
	HCC	7 (10)	3 (6)	
	metabolic disease	3 (4)	1 (2)	
	polycystic disease	1 (1,3)	5 (11)	
	other	22 (28)	5 (11)	
Diabetes mellitus	No DM	63 (81)	28 (60)	0,34
	Post LT	6 (8)	7 (15)	
	Pre-existent	9 (11)	12 (25)	
Hepatitis C	negative	71 (91)	41 (87)	0,53
	positive	7 (9)	6 (13)	
R_ABCB1 C>T	CC	23 (30)	13 (28)	0,32
	CT	31 (41)	22 (47)	
	TT	22 (29)	12 (25)	
R_CYP3A5 A>G	AA	0	0	0,42
	AG	10 (13)	4 (8)	
	GG	65 (87)	43 (92)	
D_ABCB1 C>T	CC	17 (22)	9 (20)	0,91
	CT	40 (60)	20 (56)	
	TT	20 (26)	11 (23)	
D_CYP3A5 A>G	AA	2 (3)	0 (0)	0,28
	AG	6 (7)	7 (16)	
	GG	70 (90)	38 (84)	

Baseline characteristics of LT recipients of Caucasian race, who received Tac as main immunosuppressant, genotyped for the CYP3A5 6986A>G and ABCB1 3435C>T SNPs (BMI = Body mass index). Gender, age en primary indication for transplantation and pre-existent diabetes mellitus are significantly different in between these groups.

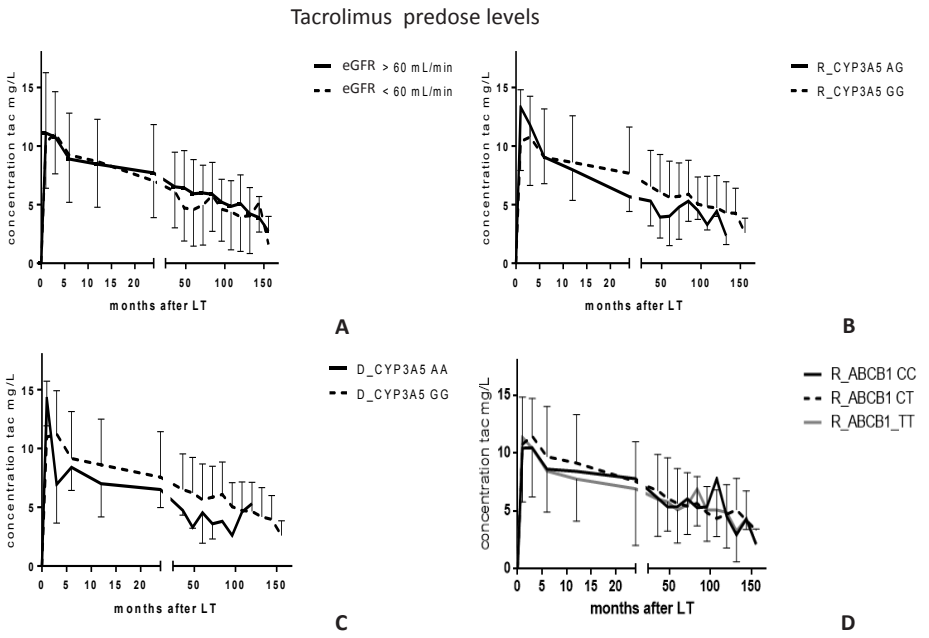

Figure 2: Tac predose levels A) No difference of Tac predose levels between patients with a normal renal function (> 60 mL/min) and an impaired renal function post-LT (< 60 mL/min) B) CYP3A5 non-expressers in the kidney (R_CYP3A5 GG) do not have higher Tac predose level than CYP3A5 expressers (R_CYP3A5 AG) C) Patients who received a donor liver which is not expressing the CYP3A5 protein have equal Tac predose levels compared to patients who received an organ expressing CYP3A5 (D_CYP3A5 GG) D) Tac predose levels of LT recipients with expression of different forms of intra-renal ABCB1 protein, which do not significantly differ in between.

Influence of SNPs in CYP3A5 and ABCB1 on CKD development

All liver donors were Caucasians. Table 2 shows the allele frequencies and the genotype distributions of the SNPs in recipients and donors of our cohort, which were all in the Hardy-Weinberg equilibrium. The incidence of CKD was not associated with *ABCB1* or

Table 2: Allele distribution in Caucasian

SNP	Allele frequency (of variant allele)	wt/wt	wt/m	m/m
R_CYP3A5 6986 A>G	0,94	0/122	14/122	108/122
D_CYP3A5 6986 A>G	0,93	2/123	13/123	108/123
R_ABCB1 3435 C>T	0,49	36/123	53/123	34/123
D_ABCB1 3435 C>T	0,52	26/123	66/123	31/123

Allele frequencies of CYP3A5 6986A>G and ABCB1 3435C>T SNPs do not significantly vary between donor and recipients (both Caucasian). Wt= wild type allele (allele A for CYP3A5 6986 and allele C for ABCB1 3435), m = mutant allele. Allele frequencies are displayed for the mutant allele.

CYP3A5 genotype, either when considering donor or recipient genotype, as depicted in Figure 3. With univariate analysis, CYP3A5 expressers (*CYP3A5*1* carriers) and non-expressers (*CYP3A5*3/*3* genotype) were compared with regard to the incidence of CKD. The same was done for *ABCB1* genotype (*ABCB1* 3435 C allele or wildtype carriers *vs*. *ABCB1* TT homozygotes*)*. No relation between *CYP3A5* and/or *ABCB1* genotype and the incidence of CKD was observed.

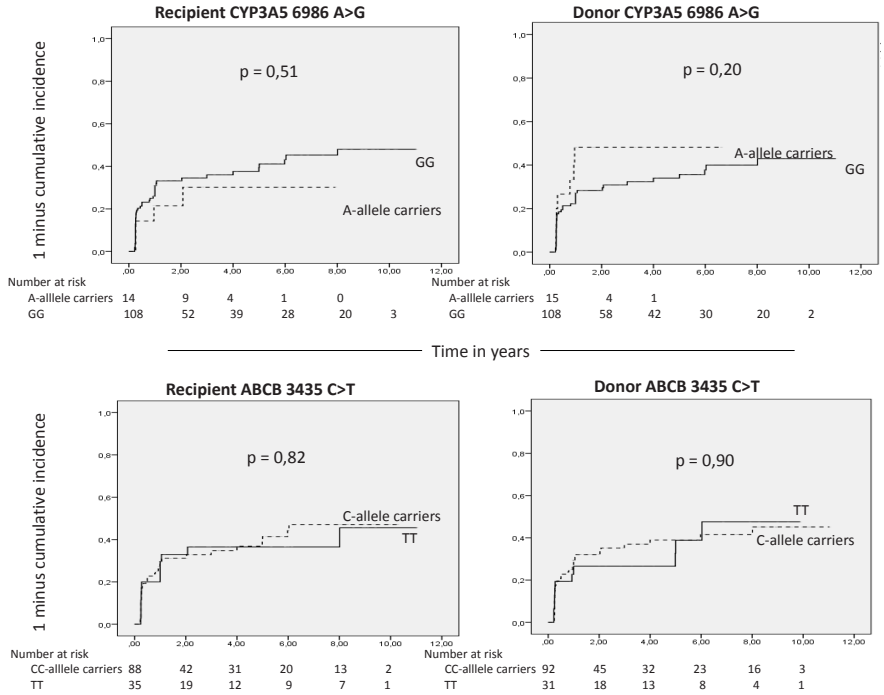

Figure 3: Association of CYP3A5 6986A>G and ABCB1 3435C>T SNPs with the development of CKD
No differences in the cumulative incidence of CKD were observed between CYP3A5 6986 A allele carriers (CYP3A5 expressers) and non-expressers (A and B). Likewise, there were no significant differences in the risk of developing CKD between ABCB1 3435 TT homozygotes and variant C allele carriers (C and D).

Subsequently, we analyzed if the combined *ABCB1* and *CYP3A5* genotype was associated with the incidence of CKD. Patients with *ABCB1TT* who received an allograft from a *CYP3A5AA* donor did not have significant incidence of CKD (figure 4). However, in our study population only 4 of such combinations were present. The possibility of performing a multivariate analysis to establish risk factors for CKD was considered. However, in the univariate analysis, the p-value of the genetic factors was higher than 0.2, indicating that these risk factors would not be significant in a multivariate analysis.

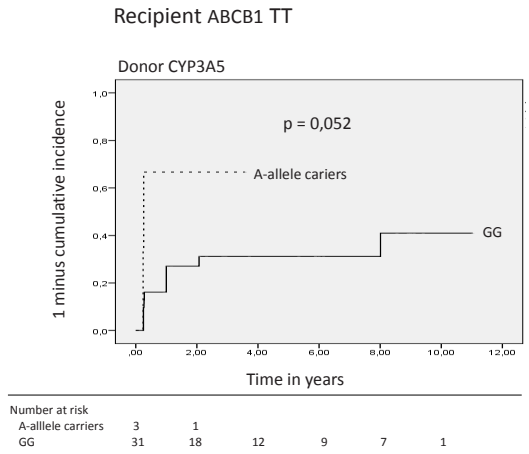

Figure 4: Sub-analysis of native ABCB1 mutants combined with graft CYP3A5 expressers
A trend of higher incidence of CKD development is observed in patients with native ABCB1 expression who received a graft with CYP3A5 expression, which is not significant.

Discussion

In this study, we analyzed the incidence of CKD after LT in a single-centre cohort consisting of 125 adult, Caucasian patients and in relation to *ABCB1* and *CYP3A5* genotype of both donor and recipient. CKD developed in 38% of the LT patients, which is in line with the CKD incidence reported in other studies (between 20 and 70%).[30,31] The allele frequencies of the investigated SNPs in our population were also comparable to those of other studies.[19,32,33]

Our data show that neither the *CYP3A5* 6989A>G nor the *ABCB1* 3435C>T genotype of either donor or recipient were associated with CKD risk after LT. Even our sub-analysis with combining recipient *ABCB1* TT genotype with the donor *CYP3A5* genotype did not show a significant difference in the incidence of CKD. However, we cannot rule that the low numbers could have masked an effect.

Other studies, which previously did identify an association between *CYP3A5* and/or *ABCB1* genotype and renal dysfunction differed from ours in terms of the study population, ethnicity, type of CNI prescribed, and primary endpoint.[24-26,34] For example, in one study, nephrotoxicity was defined as a 30% reduction in eGFR [25]. Other studies investigated the effect of genotype on Tac C/D ratios rather than renal endpoints.[25,26,34] Finally, the activity of CYP3A depends on a subject's age (older patients require a lower CNI dose than younger ones) and therefore, the outcome of genetic association studies in young patients may not be extrapolated to the adult and elderly [35].

Most investigations into the association of post-transplant renal failure and *CYP3A5* and/or *ABCB1* genotype performed up until now, were conducted in cohorts of kidney transplant recipients.[4,15,19,21,23,36,37,47] Although several authors have identified genetic risk factors for renal insufficiency after kidney transplantation, the findings of these studies are also conflicting.[13,18] In addition, in the kidney transplant setting, many factors other than the use of CNIs may influence renal function and these include recurrence of original kidney disease, ischemia-reperfusion injury, allograft rejection and quality of the kidney transplant. It should be taken into account that a kidney biopsy as the "gold" standard to diagnose CNI-induced renal insufficiency was not performed in several studies. Therefore, some of the reported findings may have been spurious associations or misdiagnoses. Nonetheless, even a renal biopsy may not always reliably establish a diagnosis of CNI-induced nephrotoxicity [39].

For the present analysis we chose eGFR as a measure of renal function. eGFR measurements are routinely used in everyday clinical practice and we do not take renal biopsies in all patients with a decreased renal function. This is a limitation of the study but we feel that the proportion of patients that was misdiagnosed as having CKD resulting from prolonged Tac use, will be limited. First, certain causes of renal insufficiency such as rejection or recurrence of primary kidney disease do not apply to the LT setting. Second, although CKD after LT may occur for reasons other than the use of CNIs[30,31,40], patients underwent extensive clinical investigations which often included a renal biopsy, whenever a glomerulonephritis or tubulo-interstitial nephritis was suspected.

Although CYP3A and ABCB1 play an important role in the pharmacokinetics of Tac, there is at present no proof that intra-renal concentrations of Tac (or its metabolites) are related to the development of Tac-induced nephrotoxicity. Until recently, because of analytical difficulties, it was not possible to measure intra-renal Tac concentrations [41]. However, even if tissue Tac concentrations turn out to be an important determinant of its toxic effects, it remains to be demonstrated that intra-renal Tac concentrations are altered to a significant degree by the studied genetic variants. A recent study by Zheng *et al*. provided some evidence that *CYP3A5* genotype may be a determinant for intra-renal Tac concentrations. However, the influence of ABCB1 *3435C>T* on the pharmacokinetics of Tac is questionable and probably limited.[46,48]

In the present study there was no association between Tac predose concentrations and the development of CKD, which is supported by previous studies [40]. Although this may seem counter-intuitive at first view, this finding may be explained by the fact that LT recipients suffering from renal insufficiency underwent Tac dose reductions at some point during their posttransplant course in an attempt to limit the nephrotoxic effects of this agent.

In this cohort, patients developing CKD had a lower "baseline" eGFR at month 3. This could indicate that renal injury occurring before transplantation or in the early postop-

erative phase, is critical for development of CKD. In our cohort, renal function remained stable after month 3 in most patients during the average follow up of 5.7 years. This relatively short follow up can`t exclude that tacrolimus is not a nephrotoxic drug. A longer observation may show deterioration of renal function in some patients treated with tacrolimus. *Matas et al.* questioned the chronic nephrotoxicity of CNI.[43,44] However, a recent published prospective, randomized study in *de novo* liver transplant recipients comparing everolimus and reduced dose tacrolimus, standard dose tacrolimus and everolimus without tacrolimus from day 30 after transplantation clearly shows that early introduction of everolimus with reduced-exposure to tacrolimus provided a significantly better renal function at 2 years posttransplant [49]. This study confirms the potential nephrotoxic effect of tacrolimus in liver transplant recipients.

In summary, genetic variations in the *CYP3A5* and *ABCB1* genes were not significant risk factor for the development of CKD after LT. Tac whole-blood exposure was also not associated with the CKD risk. Genotyping LT recipients or their donors for SNPs in these genes is therefore unlikely to aid the clinician in preventing CKD.

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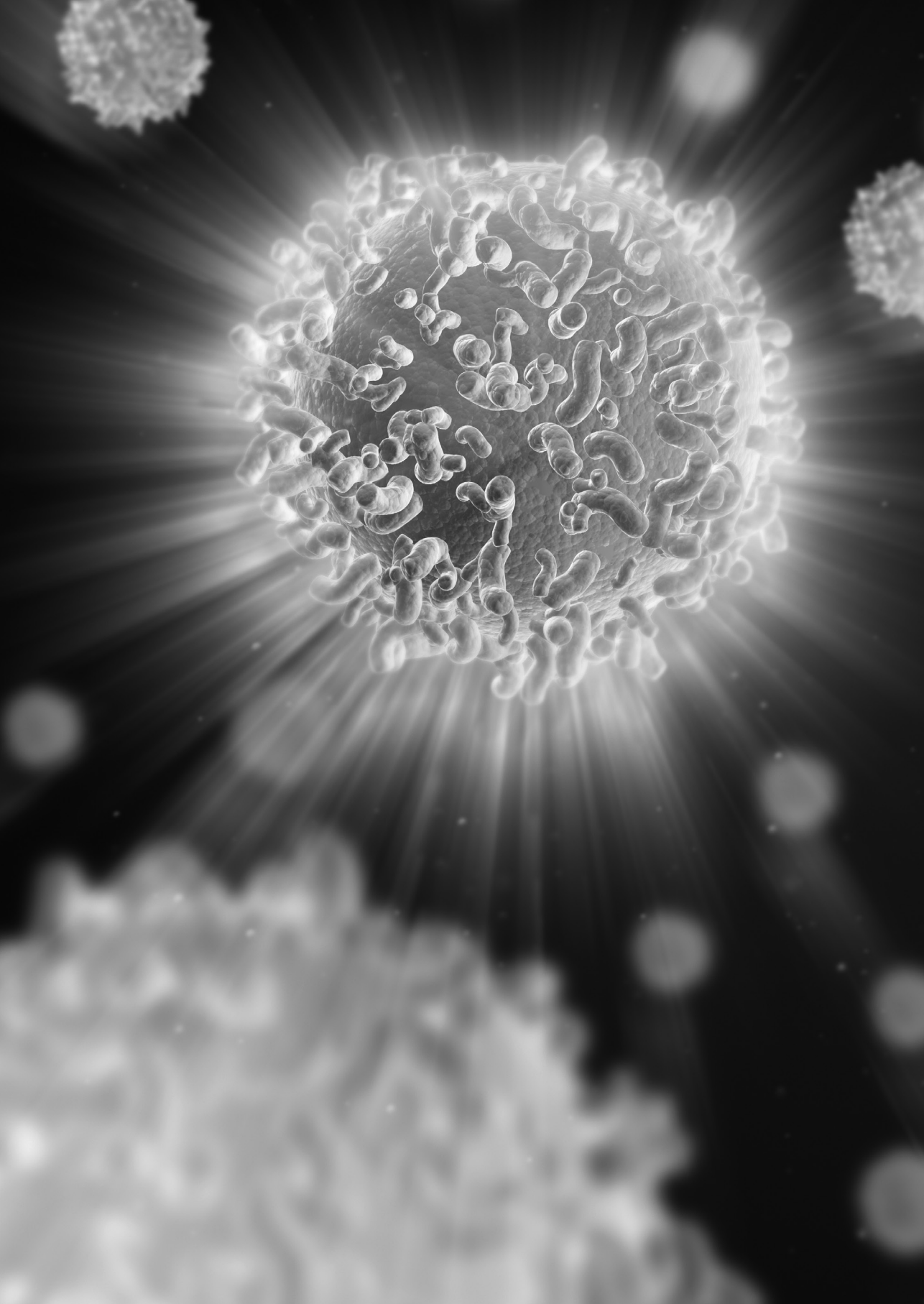

Chapter 4

Detailed kinetics of the direct allo-response in human liver transplant recipients: new insights from an optimized assay

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Tapirdamaz et al; 2010 PLoS One. 29;5(12):e14452

Abstract

Conventional assays for quantification of allo-reactive T-cell precursor frequencies (PF) are relatively insensitive. We present a robust assay for quantification of PF of T-cells with direct donor-specificity, and establish the kinetics of circulating donor-specific T cells after liver transplantation (LT).

B cells from donor splenocytes were differentiated into professional antigen-presenting cells by CD40-engagement (CD40-B cells). CFSE-labelled PBMC from 13 recipients obtained before and at several time points after LT, were stimulated with CD40-B cells. PF of donor-specific T cells were calculated.

Compared to splenocytes, stimulations with CD40-B cells resulted in 3 to 5-fold higher responding T-cell PF. Memory and naïve T-cell subsets responded equally to allogeneic CD40-B cell stimulation. Donor-specific $CD4^+$ and $CD8^+$ T-cell PF ranged from 0.5 to 19% (median: 5.2%). One week after LT, PF of circulating donor-specific $CD4^+$ and $CD8^+$ T cells increased significantly, while only a minor increase in numbers of T cells reacting to 3rd party allo-antigens was observed. One year after LT numbers of $CD4^+$ and $CD8^+$ T cells reacting to donor antigens, as well as those reacting to 3rd party allo-antigens, were slightly lower compared to pre-transplant values.

In conclusion, our alternative approach enables detection of allo-reactive human T cells at high frequencies, and after application we conclude that donor-specific T-cell PF increase immediately after LT. However, no evidence for donor-specific hypo-responsiveness of the direct pathway was obtained, underscoring the relative insensitiveness of previous assays.

Introduction

After allogeneic transplantation, recognition of major allo-antigens by recipient T-cells occurs via two different pathways: 1) the direct pathway whereby intact donor MHC is presented by donor-derived Antigen-Presenting Cells (APC) to recipient T cells; 2) The indirect pathway, whereby processed donor MHC is presented to recipient T cells as peptides on self-MHC molecules expressed on self-APC [1-2].

Direct pathway T cells are activated by donor-derived APC that migrate from the graft into recipient secondary lymphoid tissues [3-6]. Animal studies have shown that donor APC migration is a transient phenomenon after transplantation [3, 5]. Recently, we observed that a similar transient migration of donor-derived APC into recipients occurs after liver transplantation (LT) in humans [7]. Therefore, the general presumption is that direct pathway responses dominate during the early post-transplant period, but subside thereafter [2]. However, due to their cross-sectional approach [8-13] not any study has described the kinetics of recipient T-cell alloreactivity after organ transplantation in humans in detail.

A common assumption is that frequencies of allogeneic T cells stimulated by the direct pathway are 100- to 1000-fold higher than responses to pathogens [2, 14]. Animal studies showed that frequencies of T cells with direct allo-specificity range between 0.01 and 21% [15-21]. However, the reported alloreactive T cell frequencies in humans are generally lower, ranging from 0.001 to 0.1% [8, 12-13, 22-23]. This raises the question if this difference in reported frequencies is due to an underestimation of alloreactive T cells in humans caused by suboptimal sensitivity of conventional assays.

Traditionally, allo-reactive T cells have been quantified by limiting dilution analysis (LDA). It is now known that LDA detect frequencies of pathogen-specific T cells that are one to two logs lower than those detected by MHC tetramer staining [24-25]. Hence, a more robust assay for quantification of alloreactive human T cells is required. Because the restricted availability of MHC-tetramers (especially of MHC class II tetramers) is insufficient to cover the enormous heterogeneity of HLA, and the knowledge of peptides involved in direct pathway allo-recognition is limited[26], we chose an alternative approach.

The first aim of the present study is to set up a sensitive assay for quantification of frequencies of direct pathway alloreactive T cells. For this purpose, we used CD40-activated donor-derived B cells instead of donor leukocytes from spleen or blood, as stimulator cells, and calculated frequencies of responding recipient T-cells from division patterns measured by flow cytometric analysis of carboxyfluorescein succinimidyl ester (CFSE) fluorescent dye dilution. CFSE-dilution has a sensitivity similar to MHC-tetramer staining [27] [28-30], and CD40-B cells are a uniform source of professional APC [31-34]. The

second aim was to determine the kinetics of the direct pathway allo-response after liver transplantation by applying this assay.

Materials and Methods

Ethics Statement:

"The Ethics committee of the Erasmus Medical Center has approved the collection of blood in patients after liver transplantation for immunologic monitoring. A written consent from patients was obtained prior to the study."

Patients

This study included 13 patients who underwent an orthotopic liver transplantation between 1997 and 2000 at Erasmus University Medical Centre. All patients received a graft from a deceased donor. Indication for LT was: post-alcoholic liver cirrhosis (n=4), primary sclerosing cholangitis (n=4), chronic hepatitis C (n=1), chronic hepatitis B (n=1), auto-immune hepatitis (n=1), Morbus Wilson (n=1), and heamangio-endothelioma (n=1). Initial immunosuppressive therapy consisted of cyclosporine A (CsA) or tacrolimus together with prednisone, and with or without azathioprine. Patient follow up was at least one year.

Patient and donor cells

Splenic tissue from donors was obtained during multi-organ donation procedure, and single cell suspensions were made. Venous blood from patients was collected before transplantation and at several time points after transplantation. For optimization of the assay conditions, blood from healthy individuals was obtained from the blood bank. Peripheral Blood Mononuclear Cells (PBMC) and splenocytes were isolated by Ficoll-Hypaque density gradient centrifugation and stored frozen in 10% DMSO solution.

CD40-B cell generation

B cells were expanded from donor splenocytes or recipient PBMC using a mouse fibroblast cell line stably transfected with human CD40L (L-CD40L) that was kindly provided by prof. Cees van Kooten (LUMC, Leiden, The Netherlands)[35]. L-CD40L cells were irradiated (52 Gy) and plated on 6-well plates (Costar, Cambridge, USA) at a concentration of $0.2x10^6$ cells per well in medium containing RPMI 1640 (Lonza, Basel, Switzerland), 10% heat inactivated FCS (Sigma-Aldrich, St Louis, US) and 1% penicillin/streptomycin (Gibco, California, USA). After overnight culture, L-CD40L cells were rinsed with RPMI. PBMC were seeded at $1x10^6$ cells/ml IMDM (Lonza) with 10% heat inactivated human male AB serum (Lonza), 1% penicillin/streptomycin and 1% insulin-transferrin-selenium

solution (Gibco) (B-cell medium) on L-CD40L cells. Recombinant human interleukin-4 (rhIL-4) (40 IU/ml) (Strathmann Bioscience, Germany) and CsA (1 ug/ml) (Novartis, Basel, Switzerland) to prevent T-cell expansion were added. Every 3-4 days the cultured cells were transferred to freshly irradiated L-CD40L cells in a ratio of 1 CD40L cell: 4 $CD19^+$ cells. Proportions of B- and T cells were checked at regular intervals. When the percentage $CD19^+$ cells was > 85%, CsA was discontinued for at least 3 days before their use in T-cell stimulators. When the purity of $CD19^+$ cells was > 95%, CD40-B cells were used in assays.

CFSE-MLR

Before labeling with CFSE (Invitrogen, Paisley, UK), PBMC were thawed and recovered during overnight culture at 37°C with 5% CO_2. Labeling was performed with 0.5 µM CFSE. CFSE-labeled PBMC ($1x10^5$) were stimulated with $2x10^5$ irradiated (30 Gy) donor CD40-B cells, third party CD40-B cells, or autologous PBMC-derived CD40-B cells in 96-wells U-bottom plates in a final volume of 200 µl B-cell medium. Each assay was performed in triplicate. PHA-stimulated recipient PBMC were included as positive controls to test viability of thawed PBMC. Results were only included if PBMC showed responsiveness to PHA. Unless otherwise stated in the results section, flow cytometric analysis was performed after 6 days of culture at 37˚C and 5% CO_2.

Data Analysis with ModFit® Software

Precursor frequencies were calculated using ModFit LT® software (Verity Software House, USA). The basic principles of the calculation model of the program are that the cell number duplicates at each division and that CFSE is equally distributed over the daughter cells, resulting in a two-fold reduction of the CFSE intensity after each division cycle [36] as explained in Figure 1. The software calculates backwards the *precursor frequency (PF),* which is the proportion of the total cells calculated to have been present at the start of the experiment that responded to the allo-stimulus by dividing. Because the width of the intensity histogram of T cells in the parent generation spreads slowly during culture, it is difficult to differentiate cells that have divided once from non-divided cells. Supported by other studies [28, 37], we considered cells that had undergone at least two divisions as responders in our data analysis. Only ModFit® plots with a reduced chi-square (which is a measurement for the association of the calculated model and the real CFSE-dilution histogram: or in other words, a measurement for the fit of the model into the dilution histogram) smaller than 5 were included in the analysis. Mean PF were calculated from triplicate cultures. To assess the accuracy of the technique, we calculated the coefficient of variation of triplicates in 6 patients using the following formula: $SD_{triplicate}/mean_{triplicate}$.

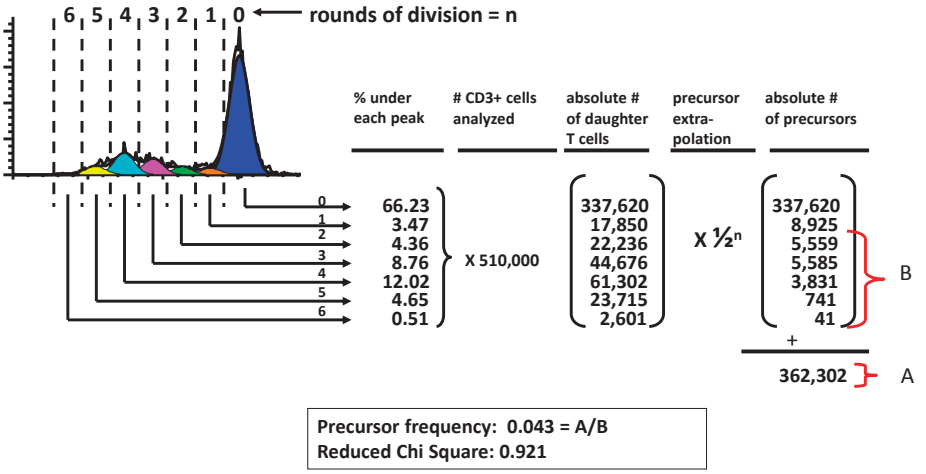

Figure 1: Analysis of CFSE-dilution patterns by ModFit® software
ModFit-derived CFSE-pattern of $CD3^+$ T cells after 6 days of stimulation with allogeneic CD40-B cells. The software draws peaks within the CFSE-histogram centered on halving intensity values from the parental peak CFSE-intensity. Non-divided T cells are $CFSE^{high}$ and are depicted blue, while the peaks left from it correspond to divided cells. Based on the enumerated proportions of T cells detected in each generation (=% under each peak) and the total number of $CD3^+$ T cells analyzed by the flowcytometer, the program calculates the absolute numbers of daughter T cells in each generation. The numbers of precursors which gave rise to the daughter cells are extrapolated by dividing the absolute numbers of T cells in each generation by 2^n, in which n stands for the division cycle (=absolute # of precursors). The PF is calculated by dividing the numbers of precursors from generation 2 onwards (=B) by the total number of precursors in all generations including the parent peak and generation 1 (=A). In this example the calculated PF= (362,302 – (337,620 + 8925) / 362,302) x 100 = 4.3%. The fit of the derived Gaussian peaks in the CFSE-dilution pattern is indicated by the reduced chi-square value. Values below 5 were considered as a good fit according to the manufacturer's instructions, and were included in the analysis.

FACS data acquisition

Cultured cells were stained for 15 minutes at room temperature in 96-wells U-bottom plates with appropriate concentrations of CD3-PE (Biolegend, San Diego, USA), CD4-APC (eBioscience, San Diego, US) and CD8-Pacific Blue (BD Biosciences, San Jose, CA, USA) antibodies in a final volume of 50 µl PBS. In individual experiments indicated in the Results section, cultured cells were stained with CD8-Pacific Blue mAb, after which cells were fixed and permeabilized with Fix & Perm® reagent (ADG Bio Research GmbH, Vienna, Austria) and incubated with PE-labeled anti-granzyme B (Sanquin, Amsterdam, The Netherlands) and anti-perforin (BD Pharmingen) mAb to analyze intracellular expression of cytotoxic molecules. 7AAD (BD Biosciences, San Jose, CA, USA) was added just before measurement started to exclude dead cells. Data acquisition was performed with a Canto II Flowcytometer from BD Biosciences (San Jose, CA, US).

FACS sorting

PBMC from healthy subjects ($200x10^6$) were labeled in PBS with sterile CD14-PE (BD Biosciences, San Jose, US), anti-BDCA1-PE (Miltenyi, Bergisch Gladbach, Germany), CD19-PE, CD123-PE, CD56-PE , CD45RA-FITC and CD45RO-APC (all from Beckman Coulter, Miami, US) in a final volume of 500 µl for 30 minutes at 4˚C, followed by a double wash step with 3 ml PBS (Lonza) . The cell pellet was resuspended to a concentration of $50x10^6$ PBMC/ml PBS. Naïve and memory T-cell populations were sorted with a FACS Aria (BD Biosciences, San Jose, CA, USA) by exclusion of PE-positive cells and gating on the CD45-RA^{high} and CD45-RO^{high} cells. $CD3^+$ purity was checked after sorting with CD3-PerCP-Cy5 (Biolegend, San Diego, US).

Statistics

Differences between groups were analyzed using the Wilcoxon test and were considered statistically significant if the p-value was ≤ 0.05.

Results

CD40-B cells expand and differentiate from splenocytes and PBMC into professional antigen presenting cells

Within 21 days, co-culture of human splenocytes or PBMC with L-CD40L cells in the presence of IL-4 and CsA resulted in a 10^4-fold increase of total B-cell numbers, followed by a decrease after 27 days (Figure 2A). The decrease was due to loss of CD40 expression on CD40-B cells (data not shown). B-cell purity > 95% was reached between day 9 and 12. CD40-B cells did not only expand, but also differentiated to professional APC, expressing CD38 (activation marker), HLA-DR and co-stimulatory molecules (Figure 2B). Contrary, human splenocytes contained 47 ± 5% HLA-DR+ APC, of which only a small fraction expressed co-stimulatory molecules (CD80+: 0.9±0.9%, CD86+: 7.1±5.2%; n=6) (Figure 2C), indicating that CD40-B cells are a more uniform preparation of APC. CD40-B cells did not differentiate to plasma cells, as all cells lacked CD138.

Optimization and validation of CFSE-MLR

To determine the optimal culture period for quantification of T cells reacting to allogeneic stimulation with CD40-B cells, CFSE-dilution patterns of $CD3^+$, $CD4^+$ and $CD8^+$ cells were analyzed daily till day 7 of culture using ModFit® software. Proliferation of T cells started at day 4, and new T cells were recruited to proliferate until day 5, thereafter PF reached a plateau value (Figure 3A). After day 6 PF increased again, which is probably due to non-specific bystander activation [27]. Therefore, we decided to determine PF at day 6 of culture. $CD8^+$ T cells acquired cytolytic molecules from day 4 onward, and at

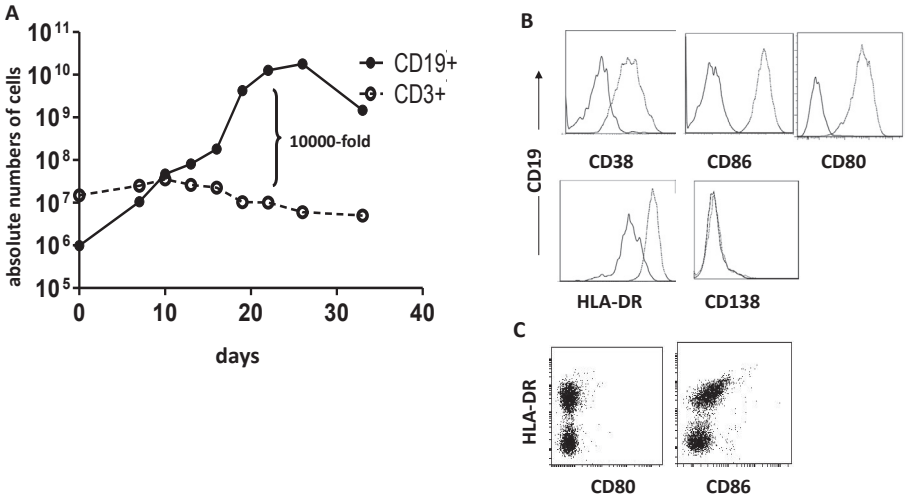

Figure 2: Expansion and differentiation of B cells using L-CD40L cells

A. Human splenocytes or PBMC were co-cultured with L-CD40L cells, IL-4 and CsA. Within 3 weeks, a 10^4 fold increase of the numbers of B cells was obtained with a purity of > 95%. T cells did not expand due to the presence of CsA. The current graph represents expansion from PBMC, splenocytes gave similar results.

B. Cell surface marker expression on CD40-B cells harvested at day 12 of culture. CD40L-expanded B cells were activated ($CD38^+$) and expressed the co-stimulatory molecules CD80 and CD86, and MHC class-II, indicating that they became professional APC. However, they did not become plasma cells, since they lacked CD138. Solid lines represent the expression of surface markers at day 0; dotted lines represent the expression at day 12 after culture.

C. Donor splenocytes contained about 50% of $HLA\text{-}DR^+$ APC, however these lacked CD80 and showed low expression of CD86.

day 7 about 50% of $CD8^+$ T-cells that proliferated in response to allogeneic CD40-B cells (=$CFSE^{low}$ $CD8^+$ T cells) expressed granzyme B and/or perforin, indicating that they had acquired cytolytic effector function (Figure 3B).

In calculating PF, ModFit® assesses the total number of T cells at the start of the allogeneic stimulation using backward calculation from the cells analyzed at the end of the culture. In contrast, LDA and ELISPOT calculate PF based on the numbers of T cells that are put into culture. To assess whether this difference would influence the outcomes of the different approaches of calculating PF, we counted daily the numbers of vital PBMC and calculated the proportions of $CD3^+$, $CD4^+$ and $CD8^+$ T cells in CD40-B cell-stimulated CFSE-MLR. Figure 3C shows that numbers of all T-cell subsets decreased during the first 2 days of cultures by 36% for the $CD3^+$, 34% for the $CD4^+$ and 38% for the $CD8^+$ T cells, a phenomenon called preparation-induced cell death [38]. Thereafter, numbers of T cells increased. Most likely, T cells alive at day 2 are the real starting population from which allo-reactive T cells are recruited to proliferate, suggesting that LDA and ELISPOT underestimate PF, while CFSE-based assays may yield more accurate PF.

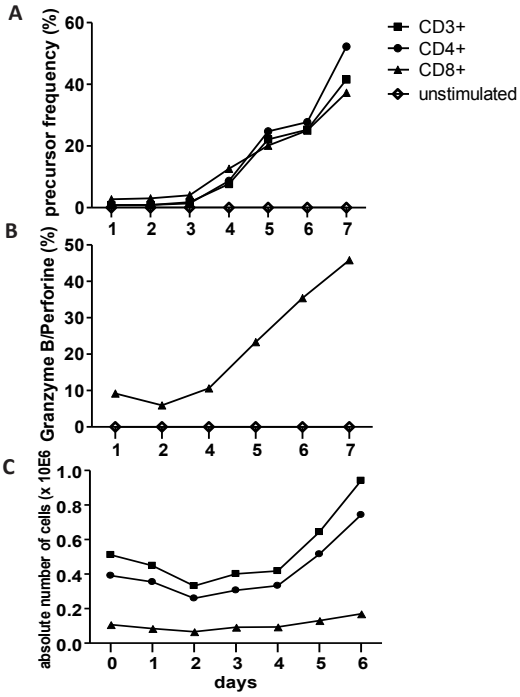

Figure 3: Kinetics of allogeneic T-cell responses to CD40-B cell stimulation
CFSE-labeled PBMC from a healthy individual were cultured for 7 days with allogeneic splenocyte-derived CD40-B cells. A. CFSE-dilution patterns of $CD3^+$, $CD4^+$, $CD8^+$ T cells were analyzed daily, and PF were calculated with ModFit® software. B. Intracellular granzyme B and perforin expression were analyzed daily in $CD8^+$ T cells. Depicted are the percentages of $CD8^+CFSE^{low}$ T cells that expressed granzyme B and/or perforin.
C. Absolute numbers $CD3^+$, $CD4^+$ and $CD8^+$ T cells during co-culture of CFSE-labelled PBMC with allogeneic CD40-B cells. Vital PBMC were counted daily using trypan blue, and proportions of $CD3^+$, $CD4^+$ and $CD8^+$ T cells were analyzed by flowcytometry. Absolute numbers of T-cell subsets were calculated by multiplying the number of vital PBMC with the proportions of T cells obtained from flowcytometric analysis.

Comparison of CD40-B cells with conventional splenocytes as stimulators in CFSE-MLR

To compare the efficacy of CD40-B-cells with conventional splenocyte stimulation, CFSE labeled PBMC from patients were cocultured with donor splenocyte-derived CD40-B cells or with non-manipulated splenocytes from the same donor. Figure 4A displays that at day 6 T cells responded abundantly to stimulation with CD40-B cells, while splenocyte stimulation resulted in a much lower response. The results of paired comparisons between stimulations of LT recipient PBMC with CD40-B cells or splenocytes are summarized in Figure 4B and C. Although stimulation with autologous CD40-B cells also resulted in T-cell proliferation, responses to allogeneic CD40-B cells were significantly higher (4B). The PF of T cells specifically responding to donor-derived CD40-B calculated in this ways were 6.8 fold, 5.9-fold and 8.6-fold higher PF for CD3+, CD4+ and CD8+ T cells, respectively, compared to specific responses to donor-derived splenocytes. Specific allogeneic responses to donor-derived CD40-B cells were calculated by subtraction of PF reacting to autologous CD40-B cells, and specific allogeneic responses to donor splenocytes by subtraction of PF of unstimulated T cells (4C). These data also reveal that comparable proportions of alloreactive precursors are detected within $CD4^+$ and $CD8^+$ subsets upon CD40-B cell stimulation (median PF: 6.4% ± 4.0 for the CD4+ and 8.45% ± 5.4 for the CD8+ subpopulation). Finally, CD40-B cell stimulation resulted in less variation between triplicate PF measurements than splenocyte stimulation; the coefficient of variation of measured triplicates was 3-fold lower (Figure 4D).

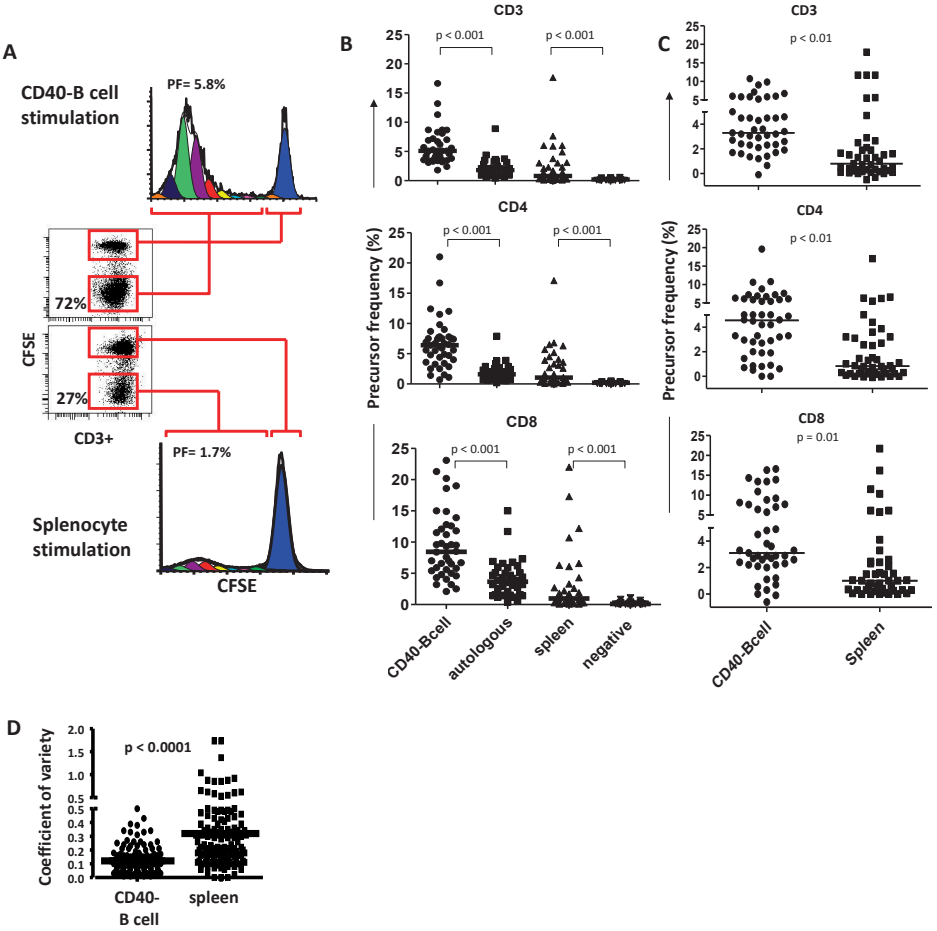

Figure 4: Comparison of CD40-B cell and splenocyte stimulation

A. CFSE-labeled PBMC from an LT-recipient were stimulated for 6 days with allogeneic splenocyte-derived CD40-B cells or with splenocytes from the same donor. Dot-plots and CFSE-dilution graphs of $CD3^+$ T cells show increased T-cell proliferation upon stimulation with CD40-B cells compared to splenocytes. Percentages in dot plots depict % dividing ($CFSE^{low}$) T cells. Percentages in histograms are calculated PF.

B: Paired comparisons of PF of LT-recipient $CD3^+$, $CD4^+$ and $CD8^+$ T cells upon stimulation with donor-derived CD40-B cells, autologous patient CD40-B cells, splenocytes or without stimulus. Sixty CFSE-labelled PBMC-samples of 6 LT-recipients, obtained either before, or at different time points after transplantation, were stimulated with donor-derived or autologous CD40-B cells or with splenocytes from the same donor for 6 days. Bars represent median values.

C: Donor-specific T-cell responses obtained by subtraction of PF reacting to autologous CD40-B cells from PF responding to donor-derived CD40-B cells, or subtraction of PF in the absence of allo-antigen (negative controls) from PF responding to donor splenocytes. Bars represent median values.

D: Paired comparisons of the coefficients of variation of triplicate measurements of LT-recipient $CD3^+$ PF in 108 PBMC-samples of 6 LT-recipients upon stimulation with donor-derived CD40-B cells or donor-derived splenocytes. Bars represent median values.

Relative contributions of naïve and memory T cells to the alloresponse measured by CFSE-dilution

To dissect the contributions of naïve ($CD45RA^+$) or memory ($CD45RO^+$) T cells to the allogeneic responses measured by CSFE-MLR upon stimulation with CD40-B cells, we sorted $CD3^+CD45RA^+$ and $CD3^+CD45RO^+$ cells (Figure 5A) and stimulated them separately with CD40-B cells. Figure 5B shows that after 6 days of culture memory and naïve subsets contributed equally to the alloresponse, with median $CD3^+$ T-cell PF of 6.7% and 7.7%, respectively.

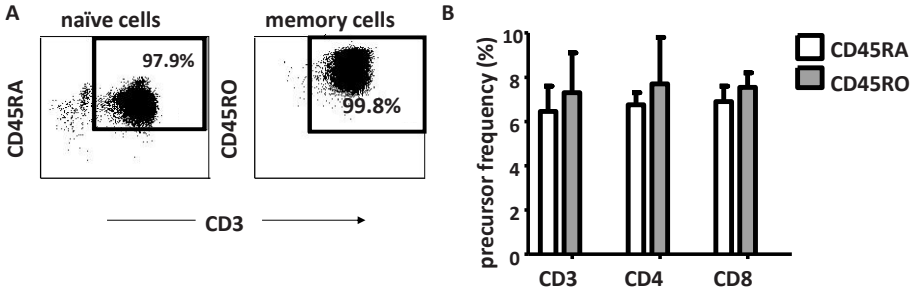

Figure 5: Relative contributions of naïve and memory T cells to the allo-response in CFSE-MLR upon stimulation with CD40-B cells

CFSE-labelled naïve $CD3^+CD45RA^+$and $CD3^+CD45RO^+$ memory T cells were isolated from PBMC of healthy individuals by flowcytometric sorting, and stimulated with allogeneic CD40-B cells for 6 days.

A. Purity of $CD3^+CD45RO^+$ and $CD3^+CD45RA^+$ T cells after sorting.

B. PF of $CD3^+CD45RO^+$ and $CD3^+CD45RA^+$ T cells proliferating upon stimulation with allogeneic CD40-B cells. Depicted are means ± SD of data from two independent experiments, each with 3 replicates.

Kinetics of the direct pathway donor-specific T-cell response after LT

To determine the kinetics of donor-specific T cell reactivity after LT, we quantified PF of donor-specific T cells in PBMC of 13 LT-recipients using donor-spleen derived CD40-B cells as stimulators. PBMC were collected before transplantation, and 1 week, 1 month, 3 months and 1 year after transplantation. To evaluate the specificity of variations in responses to donor allo-antigens, we stimulated CFSE-labeled patient PBMC also with 3rd party spleen-derived CD40-B cells or with CD40-B cells derived from autologous PBMC. Recipients and donors differed on the average in 1.5 HLA-AB alleles and in 1.7 HLA-DR alleles. Third-party stimulators were mismatched with recipients in 1.7 HLA-AB alleles and 1.8 HLA-DR alleles. Donor-derived and 3rd party-derived stimulator cells differed on the average at 1.8 HLA-AB loci and 1.5 HLA-DR loci. In all samples measured, PF of T cells reacting to autologous CD40-B cells were low compared to PF of T cells reacting to donor or 3rd party CD40-B cells. In addition, autologous responses did not change over time after transplantation. On the contrary, the numbers of circulating

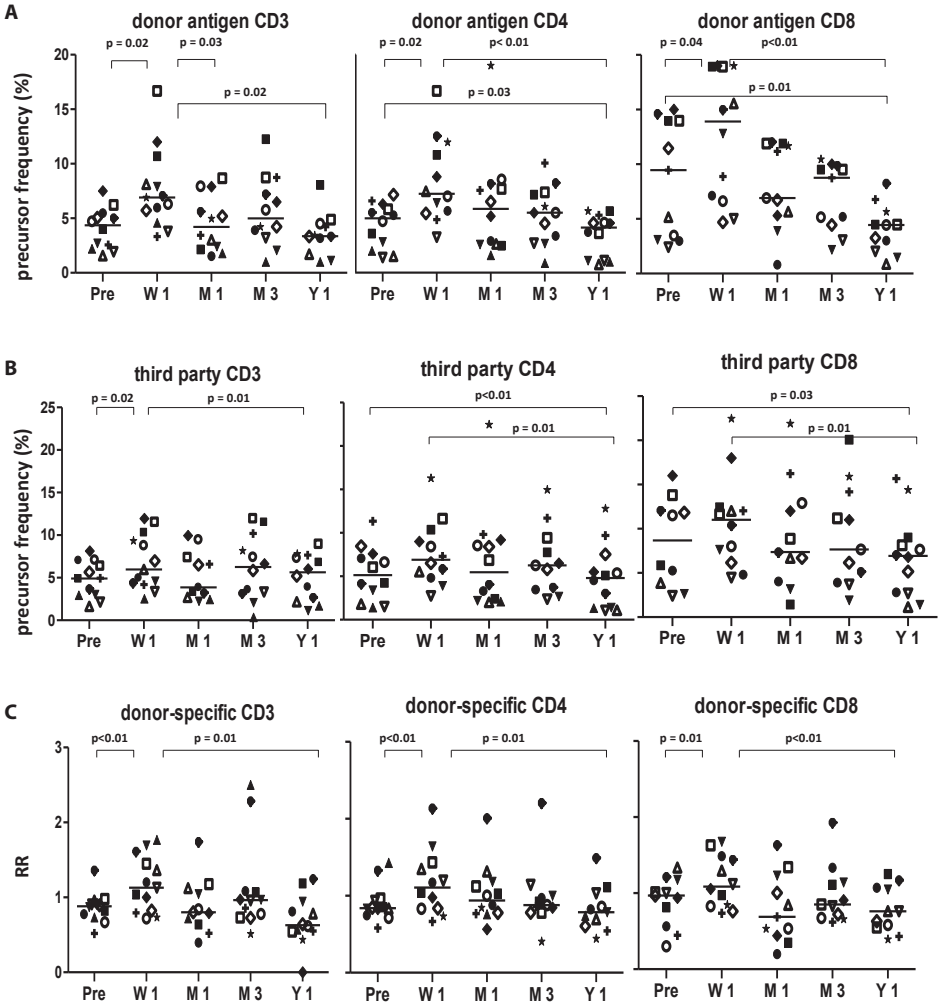

Figure 6: Longitudinal course of circulating donor-specific T-cell precursor frequencies in LT-recipients
CFSE-labeled PBMC of 13 LT-recipients obtained before transplantation (Pre), or at 1 week (W1), 1 month (M1), 3 months (M3) or 1 year (Y1) after transplantation, were co-cultured for 6 days with donor-derived splenic CD40-B cells, 3rd party splenic CD40-B cells or autologous PBMC-derived CD40-B cells. PF of $CD3^+$, $CD4^+$ and $CD8^+$ T cells responding to these stimulators were determined.
A. Numbers of T-cell precursors responding to donor-derived CD40-B cells increased significantly 1 week after transplantation in all T-cell subsets, followed by a decrease to values below pre-transplant levels.
B. Non-specific variations in allo-responses were determined by stimulating patient PBMC with third-party spleen-derived CD40-B cells. Changes in PF of $CD4^+$ and $CD8^+$ T cells responding to 3rd party CD40-B cells between subsequent time points were generally smaller compared to those in donor-specific PF.
C. Donor-specific responses were calculated by dividing PF responding to donor-alloantigen by third-party PF to obtain the relative responses (RR). RR increased significantly 1 week after transplantation in all T-cell subsets, followed by a significant decrease at 1 year after LT.

T cells reacting to donor-derived CD40-B cells increased significantly at 1 week after LT in all T-cell subsets, followed by a decrease to values below pre-transplant levels 1 year after LT (Fig 6A). Variations in T-cell PF reacting to 3rd party allo-antigens showed the same trend, although the differences in PF between subsequent time points were generally smaller, and the increases in the first week after LT were not significant in the separate CD4$^+$ and CD8$^+$ T-cell subsets (Figure 6B). To correct variations in the response to donor allo-antigens for nonspecific changes in numbers of circulating allo-reactive T cells, we calculated the donor-specific responses (relative response; RR) by dividing PF to donor antigens by PF to third parties. Figure 6C shows that the RR significantly increased in all T-cell subsets during the first week after LT compared to the pre-transplant levels (CD3$^+$: 1.4-fold, CD4$^+$: 1.3-fold and CD8$^+$: 1.1-fold), followed by a gradual decrease after week 1. However, after correction for responses to 3rd party allo-antigens, none of the donor-specific responses declined below pre-transplant values. Together these data show a donor-specific increase in direct pathway T cell PF immediately after LT, followed by a non-specific decline of allo-reactive T cells numbers to values slightly below pre-transplant levels within the first year after LT.

Discussion

Combining CFSE- dilution as a read out with CD40-B cells as stimulator cells, we developed a robust assay that detects human T cells with direct allospecificity at frequencies comparable to those predicted by animal experiments and 1- to 3-logs higher than PF detected with conventional LDA or ELISPOT [19-21, 39-40]. This CFSE-MLR assay is able to discriminate between CD4 and CD8 PF and detects both naïve and memory T-cell responses to allo-antigens in the same culture. By application of this assay we found that, even with immunosuppressive therapy, numbers of circulating CD4$^+$ and CD8$^+$ T cells with direct donor-specificity increase immediately after LT, indicating that both subsets are primed by the graft, followed by a gradual decrease within the first year. Importantly, after correction of changes in responses to 3rd party allo-antigens, no decrease below pre-transplant values was observed, showing that CD4$^+$ and CD8$^+$ T cells with direct donor-specificity persist in the circulation up to at least 1 year after LT. However, a non-specific decrease in frequencies of circulating allo-reactive CD4+ and CD8+ T cells to values slightly below pre-transplant values was observed 1 year after LT.

The first improvement contributing to the robustness of the described assay is utilizing CD40-B cells as stimulators, resulting in 3- to 5-fold higher PF compared to stimulations with splenocytes. CD40-engagement induces B-cell differentiation into professional APC with uniform expression of co-stimulatory molecules, that are able to prime both memory and naïve T-cell reponses [31, 34, 41-43] [44]. Contrary, splenocytes contain only about 50% APC, of which a minority express co-stimulatory molecules. CD40-B cells can be expanded from thawed splenocytes or PBMC, even when these have a low

viability, and their expansion enables repeated measurements of donor-specific T-cell reactivity in cases of limited supply of donor cells. Importantly, the absence of T cells within CD40-B cell preparations precludes that donor-derived T cells are included in CFSE-profiles of responder T cells, which would lead to wrong PF calculations.

The second improvement is the use of CFSE-dilution in combination with software that calculates PF as a read-out technique. Detection of responding cells by this technique is not dependent on the number of progeny cells in each individual culture, which in LDA may lead to underestimation of PF when small clones of progeny cells remain undetected [17, 24-25, 45]. Moreover, estimated PF are not negatively influenced by "preparation-induced cell death" of responder T cells at the beginning of the cultures, because PF are calculated from the sum of precursors present at the end of the cultures instead from cell input at the start of the culture. We showed that allogeneic stimulations are prone to significant loss of responder T cells during the first 2 days of culture. Since dying cells do not respond, techniques calculating PF from numbers of cells present at the start of the culture, like LDA and ELISPOT, underestimate actual PF [38]. Conversely, the CFSE-MLR may overestimate PF if more non-proliferating cells compared to proliferating cells die during culture. It is difficult to analyze whether non-proliferating cells die, but the appearance of a plateau phase of PF between day 5 and 6 of culture indicates that at least during this last phase of the cultures no preferential death of non-proliferating T cells occurs.

ELISPOT may also underestimate PF by ignoring allo-reactive T cells that do not secrete the particular cytokine detected. In contrast to short-term ELISPOT assays which detect only the rapidly reacting allo-reactive memory T cells [23], the CFSE-MLR detects allo-reactive precursors in memory and naïve T cells simultaneously. Because the allo-reactive repertoire contains both naive and memory T cells [10, 46-47], we judge that simultaneous quantification of both subsets is an advantage of the current technique compared to ELISPOT. Together, these differences may explain why published PF of donor-specific T cells in human organ transplant recipients detected by LDA [8-10, 13, 48-49] or ELISPOT [22-23, 50-52] are significantly lower compared to those observed in this study with the CFSE-MLR.

Importantly, use of CD40-B cells as stimulators to detect allogeneic T-cell responses [53], and CFSE-dilution as a technique to measure the proliferative response of T cells to allo-antigens [45][46] have both been described, but to our best knowledge these techniques have never been combined. A limitation of the CFSE-MLR in its current form is that it detects T-cell proliferation only and not effector functions. However, we observed that about 50% of $CD8^+$ T cells that proliferated in response to allogeneic CD40-B cells also acquired cytolytic molecules. We have not investigated cytokine production, but Zand et al [53] have shown that CD40-B cells are much more efficient in stimulating cytokine production by allogeneic T cells compared to PBMC. Therefore, we believe that

the majority of T cells that proliferate in response to allogeneic CD40-B cells also acquire effector function. In a future application, simultaneous measurement of T-cell proliferation and effector functions may allow discrimination between proliferating T cells that acquire effector functions and those that do not.

It is generally assumed that recipient T-cell responses against directly presented donor allo-antigen peak shortly after transplantation [2], but this has never been formally proven in humans. So far, the only other study that quantified donor-specific T-cell responses longitudinally after human organ transplantation [50] actually found a nadir in frequencies of donor-specific T cells at 1 week after kidney transplantation using IFN-γ ELISPOT. We observed, after correction for changes in responses to 3rd party allo-antigens, a significant, but modest, increase of circulating donor-specific CD4^{+} and CD8^{+} T-cell numbers immediately after LT, followed by a decrease to levels equal to numbers of T cells responding to 3rd party allo-antigens within the first year. This increase was not due to variations in immunosuppression, because it was corrected for variations in 3rd party responses. The observed increase in T-cell PF reacting to 3rd party allo-antigens, which was smaller compared to the increase in donor-specific T-cell PF, may be explained by partial overlap in HLA between donor and 3rd party stimulator cells spleen CD40-B cells. Due to limited availability of banked splenocytes, complete HLA mismatching between donor and 3rd party stimulators was not always possible. Our results are congruent with recent observations in mice showing a significant increase in direct pathway donor-specific T-cells shortly after transplantation [54].

With LDA, it has repeatedly been shown that after organ transplantation in humans, numbers of circulating donor-specific T cells decrease below pre-transplant values and below numbers of T cells responding to 3rd party allo-antigens [8-11, 13, 22, 55]. Donor-specific hypo-responsiveness was already observed at 1 month after LT [13]. This phenomenon was attributed to induction of anergy in donor-specific T cells [56] or to suppression exerted by CD4^{+}Foxp3+ regulatory T cells [11]. Of notice, donor-specific hypo-responsiveness in cytotoxic T-cell PF detected by LDA after lung transplantation, could not be confirmed using flow-cytometric detection of CD8^{+} T-cell activation and IFN-γ production [57]. Therefore, the lack of donor-specific T-cell hypo-responsiveness observed in the present study is probably due the higher sensitivity of the CFSE-MLR compared to LDA. Our data are consistent with those of Kusaka et al [58], who showed high levels of donor HLA-specific T-cell clonotype mRNAs in PBMC late after renal transplantation. Both studies imply that T cells which recognize donor allo-antigens via the direct pathway remain present in the recipient circulation for at least one year after transplantation. If donor-specific hypo-responsiveness might develop later after transplantation will be subject of a future study.

In conclusion, by using a novel technical approach, we showed for the first time an increase of donor-specific T-cell frequencies shortly after LT in humans. In addition, we

observed that T cells reacting to donor allo-antigens presented via the direct pathway remain present in the recipient circulation for at least 1 year after transplantation. To determine the role of the direct pathway in liver graft damage late after transplantation, quantification of donor-specific T-cell PF during acute or chronic rejection late after LT should be performed.

Acknowledgements:
We would like to acknowledge Prof. R. De Boer from the department of Theoretical Biology of the University of Utrecht for his expert advice on PF calculations, Dr. N.M. Van Besouw and drs. M. Crop from the department of Internal Medicine, Erasmus University Medical Centre, for their help in interpreting HLA matching and ModFit® software analysis, and Dr. E. Kondo from the department of Haematology of the University of Cologne for his advices on CD40-B cell expansion.

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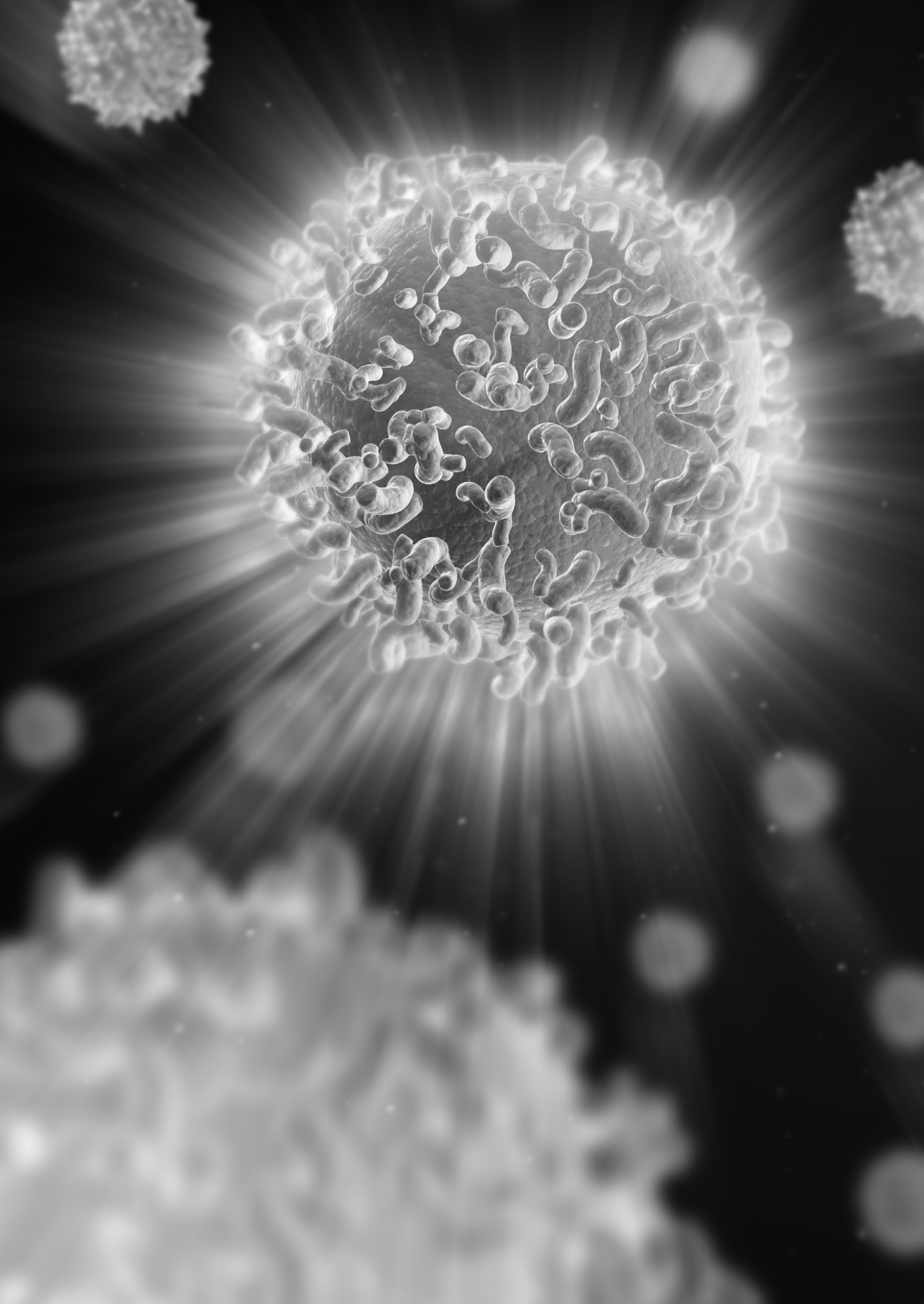

Chapter 5

CMV primary infection is associated with donor-specific T-cell hyporesponsiveness and fewer late acute rejections after liver transplantation

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Submitted for publication

Abstract

Background & Aims: Cytomegalovirus (CMV) is a leading viral infection after liver transplantation (LT) with immune-modulatory effects. Viral infections, including CMV, abrogate transplantation tolerance in animal models. Whether this occurs in humans as well remains elusive. We investigated how CMV infection affects T cells and rejection episodes after LT.

Methods: Phenotype and alloreactivity of peripheral and liver allograft-infiltrating T cells of LT patients with different CMV status were analyzed by flow cytometry. The association of CMV status with early and late acute rejection (EAR and LAR) was retrospectively analyzed in a cohort of 639 LT patients.

Results: CMV infection was associated with rapid expansion of circulating $CD4^+$ and $CD8^+$ effector memory (T_{EM}) and $CD8^+$ terminally differentiated effector memory T cells (T_{EMRA}) after LT. CMV primary infection (CMV-PI) patients showed donor-specific hyporesponsiveness in $CD8^+$ T cells and $CD4^+$ T_{EM}. Moreover, while T_{EMRA} comprised the majority of circulating donor-specific $CD8^+$ T cells in CMV-PI patients, they were hardly present in liver allografts. In the retrospective analysis, CMV-PI was significantly associated with lower incidence of LAR. Multivariate Cox regression analysis showed that CMV-PI was an independent protective factor for LAR (hazard ratio=0.17, 95%CI=0.04-0.83, *P*=0.01). In addition, CMV-PI patients showed the highest Vδ1/Vδ2 T-cell ratio, which is associated with operational tolerance after LT.

Conclusions: CMV-PI patients develop donor-specific T-cell hyporesponsiveness after LT, and they are protected from LAR and show signs of operational tolerance. CMV infection may promote immunological tolerance to liver allografts, and CMV status should be taken into account when tapering immunosuppression.

Introduction

Cytomegalovirus (CMV) is a prevalent β-herpesvirus causing lifelong latency in humans, and a leading viral infection after solid organ transplantation [1]. Immune responses to viruses, including CMV, have been proposed as one of the main barriers to the achievement of transplantation tolerance [2], as they prevent tolerance induction in experimental animal models [3-5]. However, associations between CMV infection and graft rejection in humans vary between different types of organ transplants and show conflicting results [6]. Therefore, no consensus has been made on the effect of CMV infection on graft rejection or tolerance after LT.

Acute rejection (AR) is primarily initiated by recipient T lymphocytes (T cells) that recognize non-self antigens derived from donor [7]. T cells can be classified into different subsets based on their differentiation status, reflecting distinct migration patterns and effector functions upon antigenic stimulation. While naive (T_{Naive}) and central memory (T_{CM}) T cells proliferate robustly in response to antigen, effector memory (T_{EM}) and terminally differentiated effector memory (T_{EMRA}) T cells produce high amounts of pro-inflammatory cytokines and cytolytic mediators[8, 9]. In addition, T_{Naive} and T_{CM} recirculate between secondary lymphoid organs, while T_{EM} home to inflamed peripheral tissues [10]. On one hand, accumulation of $CD8^+$ T_{EMRA} is a typical characteristic of CMV-driven immune senescence [11], which is associated with increased susceptibility of the elderly to infections, and poor responses to vaccinations[12, 13]. On the other hand, CMV-specific memory T cells are hypothesized to be detrimental to allografts as they can be cross-reactive to allogeneic HLA[14, 15]. However, no literature is available on how CMV infection alters T-cell alloreactivity after LT.

To address these issues, using *ex vivo* isolated cells from peripheral blood and from liver allografts explanted during re-LT, we investigated the effect of CMV infection on T-cell differentiation and alloreactivity, as well as on γδ T-cell subset distribution, which has been shown to be associated with operational tolerance after LT[16, 17]. In addition, we performed a retrospective study in a cohort of 639 LT patients, to analyze the associations of CMV infection with both early (< 6 months) and late (> 6 months) acute rejection (EAR and LAR).

Patients and methods

Study subjects

Peripheral blood samples were collected from 75 patients that underwent primary orthotopic LT at Erasmus MC, the Netherlands (summarized in Supplementary Table 1).

Liver allograft biopsies were obtained from explants from 8 patients that underwent re-LT (summarized in Supplementary Table 2).

In the retrospective analysis to study the impact of CMV on graft rejection, 639 patients that underwent LT at Erasmus MC from 1992-2010 were included. Demographic

Table 1: Demographic and baseline clinical characteristics of patients included in the retrospective analysis

Variable	Total: 639 patients
Recipient age (median, range), years	50 (16-71)
Recipient, female	264 (41.3%)
Recipient BMI (mean±SD), kg/m^2	25±4
Primary liver disease	
AHF	119 (18.6%)
HCC	64 (10.0%)
PBC/PSC/AIH	149 (23.3%)
HBV/HCV	84 (13.1%)
Alcoholic cirrhosis	71 (11.1%)
Cryptogenic cirrhosis	41 (6.4%)
Others	111 (17.4%)
Donor age (median, range), years	46 (8-78)
Donor, female	333 (52.1%)
DCD donor	49 (7.7%)
Cold ischemia time (mean±SD), minutes	493±166
Warm ischemia time (mean±SD), minutes	45±24
Re-LT	75 (11.7%)
Basiliximab as induction immunosuppression	375 (58.7%)
Calcineurin inhibitor	
Cyclosporin A	249 (39.0%)
Tacrolimus	367 (57.4%)
CMV serostatus	
R^-/D^-	127 (19.9%)
R^-/D^+	122 (19.1%)
R^+	390 (61%)
CMV viremia	152 (23.8%)
Timing of CMV viremia detection after LT (median, range), days	35 (2-2502)
Timing of first CMV viremia after LT < 180 days	140 (92.1%)
Early acute rejection	144 (22.5%)
Timing of early acute rejection (median, range), days	9 (2-166)
Late acute rejection	41 (6.4%)
Timing of late acute rejection (median, range), days	487 (186-6368)

details of donors and recipients are summarized in Table 1. Patients were followed up until graft loss, death, or the end of the study period on 31 December 2011.

Written informed consent was obtained from all patients before collection of samples. The medical ethics committee of the Erasmus MC approved this study.

Cell isolation and flow cytometry

Protocols for cell isolation, list of antibodies, and flow cytometry details are in Supplementary Materials and Methods.

Quantification of alloreactive T-cell frequencies

Alloreactive T cells were analyzed by determination of the activation-induced CD137 expression on $CD4^+$ and $CD8^+$ T cells after allogeneic stimulation for 24 hours by either donor or third-party (mismatched at HLA-A, B and DR loci with both donor and recipient) splenocytes. This assay has been used in the identification and isolation of viral, tumor, and importantly allospecific T cells regardless of their differentiation stage or cytokine production profile[18, 19]. Details are described in Supplementary Materials and Methods.

Immunosuppression

The standard immunosuppressive therapy in our center consisted of prednisone, cyclosporine or tacrolimus, with or without azathioprine or mycophenolate mofetil (MMF). Since 1998, basiliximab was introduced as induction immunosuppression and was used in 58.7% of all patients in the retrospective cohort (Table 1), and meanwhile the use of cyclosporine was gradually replaced by tacrolimus. Immunosuppressive regimens were similar in patients from whom blood samples were collected, consisting of corticosteroids, MMF, tacrolimus, and induction with basiliximab in 85% of patients (Supplementary Table 1).

Viral diagnostic, treatment, and CMV status definition

CMV serostatus of patients was defined as: CMV negative (neg: R^-/D^-), primary infection (PI: R^-/D^+), and non-primary infection (NPI: R^+). Viral diagnostic and treatment details are in Supplementary Materials and Methods.

Definition of early and late acute rejection

AR was defined as: graft dysfunction accompanied by moderate or severe rejection activity (RAI≥5) detected in the liver biopsy according to Banff criteria, and responsiveness to additional immunosuppressive treatment. While early acute rejection (EAR) was defined as rejection occurring within 180 days after LT, late acute rejection (LAR) was defined as those occurring after 180 days after LT. Associations of CMV with EAR or LAR

were analyzed separately, as EAR is most common during the first few weeks after LT, generally preceding CMV infection.

Statistical analysis

For data obtained from *ex vivo* analysis, differences between groups were analyzed using the nonparametric Mann-Whitney U test for unpaired data or the Wilcoxon matched pairs test for paired data. *P*-values < 0.05 were considered significant.

In the retrospective analysis, data were summarized using mean with standard deviation or median with range for continuous variables and percentage for discrete variables. The cumulative incidences of EAR and LAR were estimated using the Kaplan-Meier method and differences between curves were analyzed using a log-rank test. Analysis of risk factors for EAR and LAR was performed using the Cox proportional-hazards regression model with a likelihood ratio test. For multivariate analysis, we first performed univariate analysis for each potential independent variable. Independent variables with *P*-values less than 0.2 were included in the multivariate analysis together with CMV serostatus and viremia. Linearity of continuous variables and clinical relevant interactions were tested. Where

multiple pairwise comparisons were made, a Bonferroni correction on the alpha level was applied. SPSS v. 21 was used for statistical analysis, and *P*-values < 0.05 were considered significant.

Results

CMV infection is associated with the expansion of effector memory T-cell subsets in peripheral blood after liver transplantation

We prospectively collected PBMC samples from 34 patients before, at 1 month and at 6 months after LT. Peripheral T-cell subsets were analyzed by flow cytometry on basis of CCR7 and CD45RO expression [10], as shown in representative plots (Fig.1A). The proportion of $CD8^+$ T_{Naive} decreased continuously within the first 6 months after LT (pre-LT: 48%; 1 month: 42%; 6 months: 22%). In contrast, the percentages of $CD8^+$ T_{EM} and especially T_{EMRA} increased (Fig.1B), and T_{EMRA} became the predominant $CD8^+$ T-cell subset 6 months after LT (pre-LT: 23%; 1 month: 28%; 6 months: 43%). No significant changes were observed in $CD4^+$ T-cell subsets within the first month after LT (Fig.1C). However the fraction of $CD4^+$ T_{Naive} decreased significantly 6 months after LT (pre-LT: 51%; 1 month: 52%; 6 months: 39%), which was mainly compensated by an increased percentage of T_{EM} (pre-LT: 16%; 1 month: 16%; 6 months: 23%).

To determine whether the observed changes in $CD4^+$ and $CD8^+$ T-cell subsets were attributed to CMV, we divided patients into three groups based on CMV status: CMV

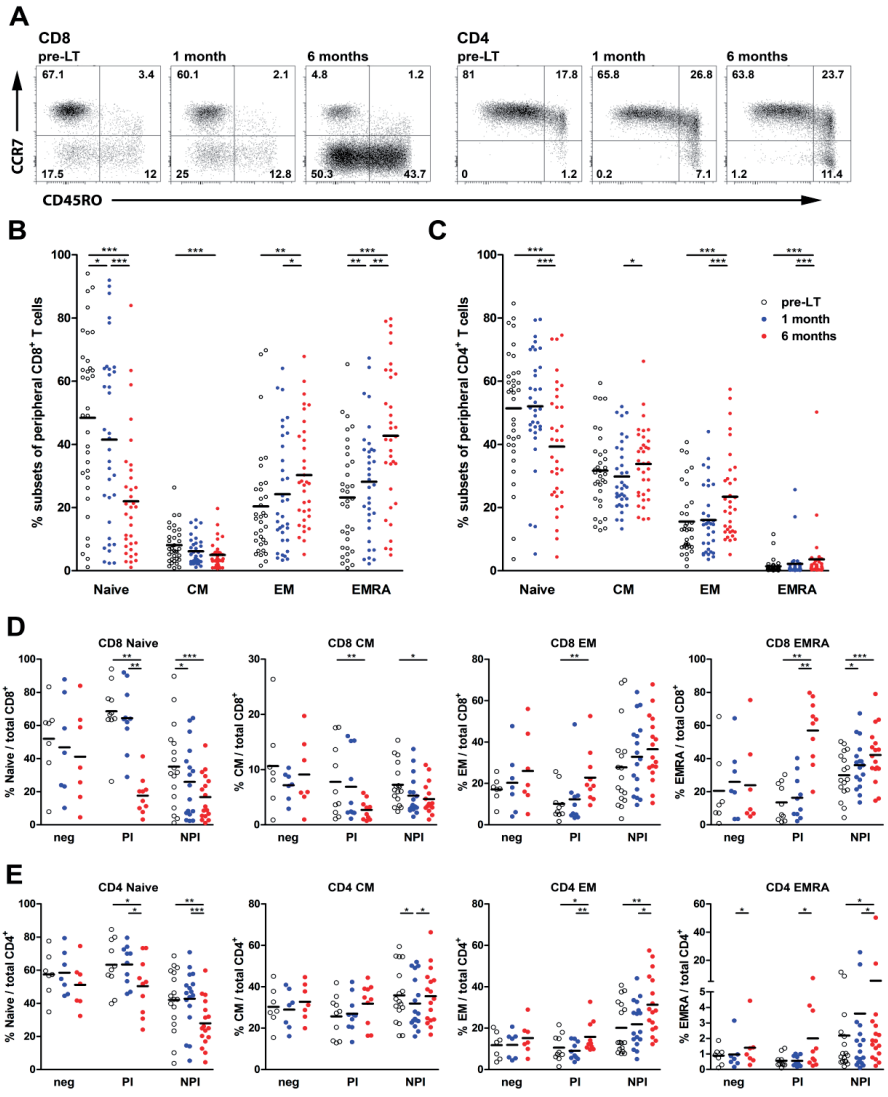

Figure 1: Changes of circulating $CD4^+$ and $CD8^+$ T-cell subsets after LT in relation to patient CMV status. (A) T-cell subsets were defined as T_{Naive} ($CCR7^+CD45RO^-$), T_{CM} ($CCR7^+CD45RO^+$), T_{EM} ($CCR7^-CD45RO^+$), and T_{EMRA} ($CCR7^-CD45RO^-$), shown as representative FACS plots from one patient. Distribution of (B) $CD8^+$ and (C) $CD4^+$ T-cell subsets before (white), at 1 month (blue) and at 6 months (red) after LT were compared (n=34). Then patients were grouped based on CMV status, and changes of each (D) $CD8^+$ and (E) $CD4^+$ T-cell subset were compared between CMV negative (neg, n=7), CMV primary infection (PI, n=10), and CMV non-primary infection (NPI, n=17) patients. Horizontal lines indicate mean values. *P<0.05, **P<0.01, ***P<0.001.

negative (neg: R^-/D^-), primary infection (PI: R^-/D^+), and non-primary infection patients (NPI: R^+). With the exception of a small increase of $CD4^+$ T_{EMRA} in CMV negative patients, all the changes in the subset distribution of $CD8^+$ (Fig.1D) and $CD4^+$ (Fig.1E) T cells after LT were observed only in CMV positive patients (both PI and NPI). Particularly $CD8^+$ T-cell subsets from PI patients underwent the most dramatic changes (T_{Naive}: pre-LT 69%, 6 months 18%; T_{EMRA}: pre-LT 14%, 6 months 57%). Together, these data show that CMV infection is associated with the expansion of effector memory T-cell subsets in peripheral blood after LT.

$CD8^+$ T cells from CMV primary infection patients develop donor-specific hyporesponsiveness

We hypothesized that the expansion of effector memory T-cell subsets driven by CMV might increase the frequencies of alloreactive T cells, as cross-reactive viral-specific memory T cells are common [15]. Thus we quantified the frequencies of donor-specific and third party-reactive T cells in 51 patients at minimum 6 months after LT by measuring the allogeneic activation-induced CD137 expression (Supplementary Fig.1A, B).

Overall $CD8^+$ T cells showed donor-specific hyporesponsiveness (Fig.2A, left panel). We did not observe higher frequencies of alloreactive T cells in CMV positive patients than CMV negative patients. Unexpectedly, CMV-PI patients showed prominent donor-specific hyporesponsiveness in $CD8^+$ T cells (Fig.2B, left panel), which was evident in T_{Naive}, T_{CM} and T_{EM} (Fig.2C). In contrast, no donor-specific hyporesponsiveness was observed in CMV negative patients, while in CMV-NPI patients significant donor-specific hyporesponsiveness was only observed in T_{EM}.

Next, we assessed the subset composition of alloreactive $CD8^+$ T cells ($CD8^+CD137^+$ T cells) (Supplementary Fig.1B). Compared to CMV negative and CMV-NPI patients, donor-specific T cells from CMV-PI patients were predominantly T_{EMRA} (Fig.2E, F), suggesting an important role of T_{EMRA} in the anti-donor $CD8^+$ T-cell response in these patients. Altogether, our data show that CMV-PI is associated with the development of donor-specific hyporesponsiveness in $CD8^+$ T cells after LT.

$CD4^+$ effector memory T cells from CMV positive patients are less alloreactive than those of CMV negative patients

Overall $CD4^+$ T cells did not develop significant donor-specific hyporesponsiveness (Fig.2A, right panel). Alloreactive T-cell frequencies were also similar in patients with distinct CMV status (Fig.2B, right panel). However when we zoomed into each $CD4^+$ T-cell subset, we found that both $CD4^+$ T_{EM} and T_{EMRA} subsets in CMV-PI and NPI patients were less reactive to donor as well as to third-party stimulation than those of CMV negative patients (Fig.2D). In addition, $CD4^+$ T_{EM} of CMV-PI and NPI patients showed donor-specific hyporesponsiveness.

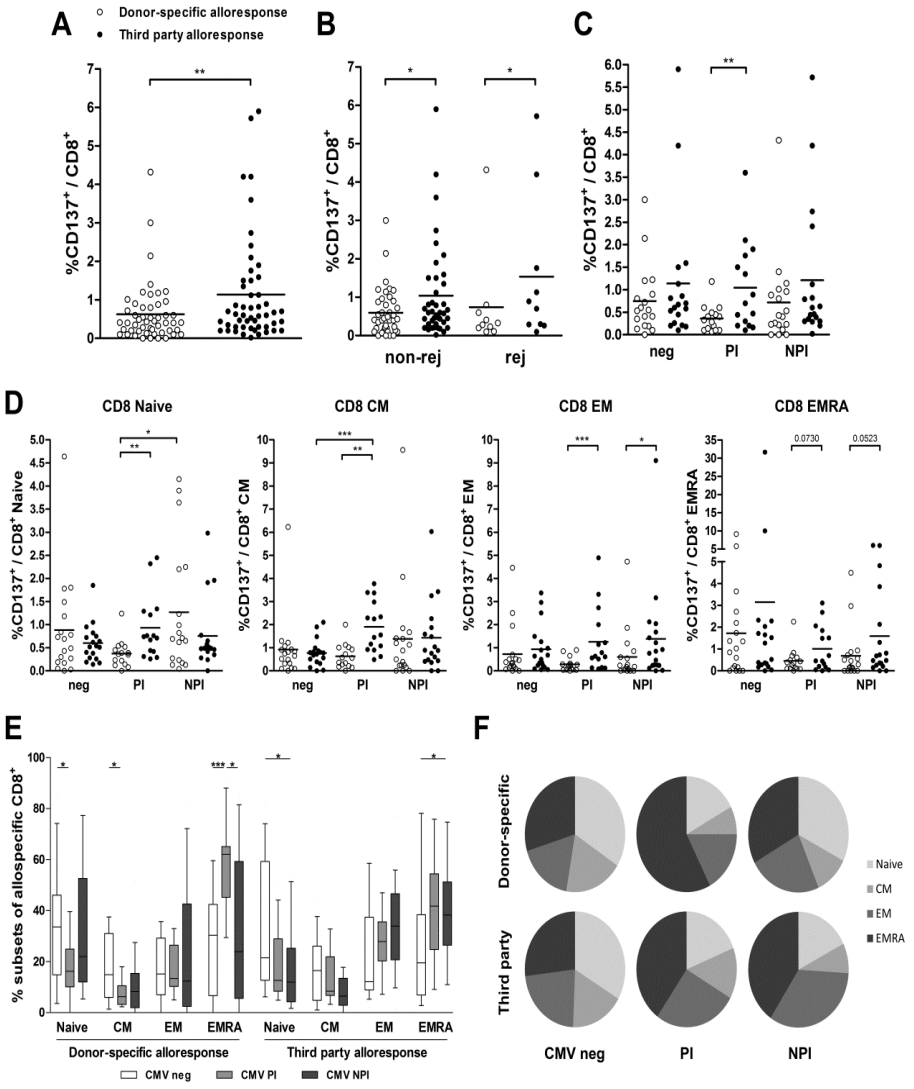

Figure 2: CMV primary infection is associated with the development of donor-specific T-cell hyporesponsiveness after LT. The frequencies of peripheral alloreactive $CD8^+$ and $CD4^+$ T cells were analyzed by flow cytometric determination of CD137 expression on T cells after allogeneic stimulation. (A) Overall frequencies of donor-specific and third party-reactive $CD8^+$ (left panel) and $CD4^+$ (right panel) T cells were compared (n=51). Alloreactive T-cell frequencies of (B) total $CD8^+$ (left panel) and $CD4^+$ (right panel) T cells, and of each (C) $CD8^+$ and (D) $CD4^+$ T-cell subset were compared between patients with different CMV status (CMV neg n=18, PI n=15, NPI n=18). Horizontal lines indicate mean values. Subset composition of alloreactive $CD8^+$ T cells was assessed by measuring CD45RO and CCR7 expression on $CD137^+CD8^+$ T cells, and was compared between patients with different CMV status. (E) Proportions of each T-cell subset within donor-specific and third party-reactive $CD8^+$ T cells are shown as median values and interquartile ranges, (F) also summarized as pie charts presenting mean proportions. *P<0.05, **P<0.01, ***P<0.001.

T_{EM} are the major T-cell population infiltrating liver allograft and are enriched for donor-specific $CD8^+$ T cells

As T_{EMRA} were the major $CD8^+$ T-cell subset present in peripheral blood after LT, and comprised the majority of residual donor-specific $CD8^+$ T cells in CMV-PI patients, we asked whether $CD8^+$ T_{EMRA} can infiltrate liver allograft and thereby contribute to allograft rejection. To investigate which T-cell subsets infiltrate liver allograft, we isolated intrahepatic lymphocytes (IHLs) from liver allografts which were explanted during re-LT (n=8). IHLs contained mainly $CD4^+$ and $CD8^+$ T_{EM} and hardly any T_{Naive} and T_{CM} (Fig.3A, B). $CD8^+$ T_{EMRA} were present in the liver allografts but accounted for a significantly smaller proportion than in paired PBMC samples. The ratio between $CD8^+$ T_{EM} and T_{EMRA} was on average 5-fold higher in liver allografts than in peripheral blood (Fig.3C).

Sufficient amounts of IHLs were isolated from two of the liver explants to measure their reactivity to donor and third-party stimulation. In the absence of allogeneic stimulation *ex vivo*, considerable percentages of $CD4^+$ and $CD8^+$ T cells expressed CD137, indicating that they were activated in the liver allografts *in vivo* (Fig.3D). $CD8^+$ T_{EM} were enriched for donor-specific T cells, as 20% and 33.9% of $CD8^+$ T_{EM} from two IHLs samples respectively were reactive to donor splenocytes, while 8.8% and 7.5% of them responded to third-party stimulation (CD137 expression in conditions without allogeneic stimulation were subtracted). $CD8^+$ T_{EMRA} contained less alloreactive cells than T_{EM}. $CD4^+$ T_{EM}, but not T_{EMRA}, contained high numbers of alloreactive T cells, but the percentages were similar for donor and third-party stimulation. Altogether, these data indicate that T_{EM} preferentially infiltrate liver allografts, and that infiltrating $CD8^+$ T_{EM} are enriched for donor-specific T cells which may contribute to intragraft alloreactivity. In contrast, $CD8^+$ T_{EMRA} largely remain in the circulation, even though they contain the majority of donor-specific $CD8^+$ T cells in the peripheral blood of CMV-PI patients.

CMV primary infection protects patients against the occurrence of late acute rejection

To study whether the donor-specific T-cell hyporesponsiveness that we observed in CMV-PI patients has any clinical relevance, we performed a retrospective study on 639 patients that underwent LT in our center between 1992 and 2010 to investigate the impact of CMV infection on EAR and LAR. One, 3, or 6 months after LT have been variably used in literature as the cut-off to define EAR and LAR [20]. In our center, the first episodes of CMV viremia were detected at median 35 days after LT, 92.1% of which were within the first 6 months (Table 1). Thus we choose 6 months as the cut-off to define LAR in order to focus on the effect of CMV infection on graft rejection and not the other way around. Of the 639 patients, 144 (22.5%) developed EAR (median 9 days after LT; range 2-166 days), and 41 (6.4%) developed LAR (median 487 days after LT; range 186-6368 days) (Table 1).

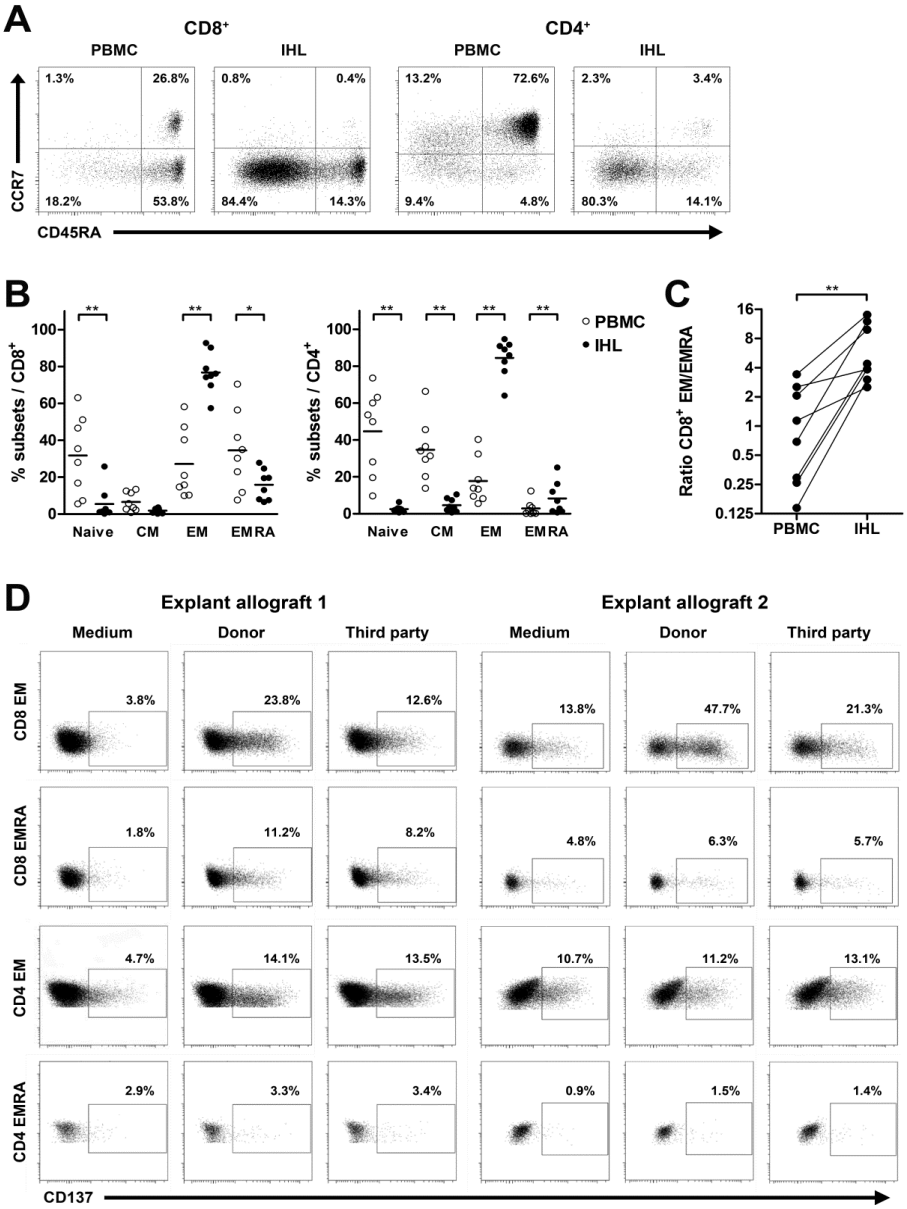

Figure 3: T_{EM} are the major T-cell population infiltrating liver allograft and are enriched for donor-specific $CD8^+$ T cells. Intrahepatic lymphocytes (IHLs) were isolated from explant liver allografts to study the subset composition and alloreactivity of graft infiltrating T cells. $CD8^+$ and $CD4^+$ T-cell subsets between paired PBMCs and IHLs were compared, shown (A) as representative FACS plots from one patient and (B) as summarized (n=8). (C) Ratios $CD8^+$ T_{EM} and T_{EMRA} were also compared between paired PBMCs and IHLs. Large amounts of IHLs were isolated from two explant allografts and were co-cultured with donor and third-party splenocytes. Donor-specific and third party-reactive T cells were identified by CD137 upregulation. (D) Results are shown by FACS plots and percentages of $CD137^+$ T cells for each subset are depicted in the plots for two experiments. *P<0.05, **P<0.01.

EAR and LAR were set as the endpoints for risk factor analysis, and CMV serostatus and viremia were analyzed. CMV serostatus was not associated with the cumulative incidence of EAR (P=0.77) (Fig.4A), while the incidence of LAR in CMV-PI (R^-/D^+) patients was significantly lower than in CMV negative (R^-/D^-) patients (*P*=0.014) and CMV-NPI (R^+) patients (*P*=0.017) (Fig.4B). In univariate Cox regression analysis, recipient age (*P*=0.007), recipient BMI (*P*=0.026), donor gender (*P*=0.034), warm ischemia time (*P*<0.001), basiliximab induction (*P*<0.001), tacrolimus as CNI (*P*<0.001), and CMV viremia (*P*=0.003) were significantly associated with EAR (Supplementary Table 3). Meanwhile, CMV serostatus (*P*=0.015) was the only factor associated with LAR (Table 2).

In the multivariate Cox regression analysis, recipient age (*P*=0.048, hazard ratio [HR]=0.99), donor gender (*P*=0.019, HR=0.66), basiliximab induction (*P*=0.014, HR=0.59), tacrolimus as CNI (*P*<0.001, HR=0.48), and CMV viremia (*P*=0.008, HR=1.70) were considered as independent factors associated with EAR (Supplementary Table 3). In contrast, CMV serostatus was the only independent factor associated with LAR (*P*=0.014; HR for R^-/D^+=0.17; HR for R^+=0.97)(Table 2). Altogether, these data show that CMV-PI after LT protects patients against the occurrence of LAR, corroborating the *ex vivo* donor-specific T-cell hyporesponsiveness that we observed in CMV-PI patients.

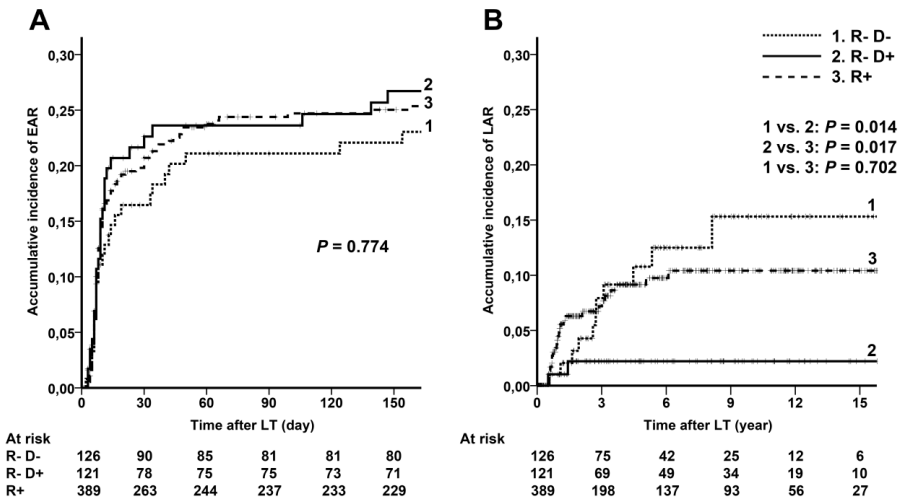

Figure 4: CMV primary infection protects patients against the occurrence of late acute rejection. Patients were grouped based on CMV serostatus before LT as follows: Group 1: R^-/D^-; Group 2: R^-/D^+; Group 3: R^+. The cumulative incidences of (A) early acute rejection (EAR) and (B) late acute rejection (LAR) were estimated using the Kaplan-Meier method and differences between curves were analyzed using a log-rank test. The number of patients at risk are depicted below the graphs.

Table 2. Risk factor analysis for late acute rejection following liver transplantation

	Univariate analysis			**Multivariate analysis**		
Variable	HR	95% CI	*P* Value	HR	95% CI	*P* Value
Recipient age, year	0.99	(0.97-1.02)	0.455			
Recipient, female	1.29	(0.70-2.39)	0.416			
Recipient BMI, kg/m^2	1.04	(0.97-1.12)	0.309			
Primary liver disease			0.189			0.225
HBV/HCV (Ref)	1.00			1.00		
AHF	1.45	(0.49-4.34)		1.72	(0.56-5.33)	
HCC	0.29	(0.03-2.50)		0.35	(0.04-2.98)	
PBC/PSC/AIH	1.54	(0.56-4.29)		1.88	(0.66-5.36)	
Alcoholic cirrhosis	0.49	(0.10-2.54)		0.60	(0.12-3.14)	
Cryptogenic cirrhosis	2.04	(0.59-7.07)		2.19	(0.62-7.77)	
Others	0.81	(0.24-2.81)		1.03	(0.29-3.67)	
Donor age, year	0.99	(0.97-1.01)	0.239			
Donor, female	1.35	(0.72-2.51)	0.342			
DCD donor	1.43	(0.44-4.65)	0.575			
Cold ischemia time, 10 min	0.99	(0.97-1.01)	0.344			
Warm ischemia time, 10 min	1.01	(0.89-1.15)	0.877			
Re-LT	0.94	(0.33-2.63)	0.900			
Basiliximab induction	0.97	(0.51-1.85)	0.920			
Calcineurin inhibitor, Tacrolimus	0.63	(0.34-1.19)	0.154	0.70	(0.37-1.32)	0.268
Early acute rejection	0.90	(0.44-1.86)	0.781			
CMV serostatus			0.015			0.014
R^-/D^- (Ref)	1.00			1.00		
R^-/D^+	0.18	(0.04-0.81)		0.17	(0.04-0.83)	
R^+	0.87	(0.43-1.76)		0.97	(0.47-2.02)	
CMV viremia	0.93	(0.45-1.89)	0.832	1.18	(0.55-2.54)	0.670

CMV primary infection patients shows the highest Vδ1/Vδ2 γδ T-cell ratio after LT

To further investigate whether CMV-PI patients show signs of tolerance, we measured peripheral Vδ1 and Vδ2 γδ T-cell subsets, and calculated the Vδ1/Vδ2 γδ T-cell ratio, which is associated with operational tolerance after LT[16, 17]. CMV-PI patients contained the highest percentage of Vδ1 γδ T cells within peripheral $CD3^+$ cells, while CMV negative patients contained the lowest (CMV-neg: 0.65%, PI: 4.69%, NPI: 2.29%; median values) (Fig.5A). Meanwhile the percentages of Vδ2 γδ T cells were similar (CMV-neg: 0.56%, PI: 0.37%, NPI: 0.42%; median values) (Fig.5B). Moreover, CMV-PI patients showed higher Vδ1/Vδ2 γδ T-cell ratio than CMV-neg and NPI patients (CMV-neg: 1.76, PI: 11.1, NPI: 4.62; median values) (Fig.5C).

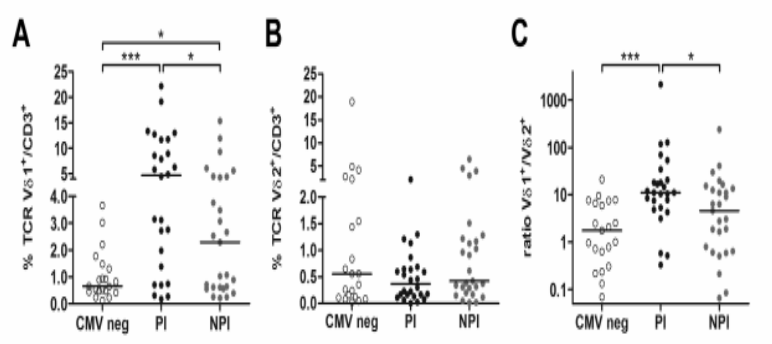

Figure 5: CMV primary infection patients show the highest peripheral Vδ1/ Vδ2 γδ T cell ratio after LT. Peripheral blood Vδ1 and Vδ2 γδ T-cell subsets were analyzed by flow cytometry in LT patients. The percentages of (A) Vδ1 and (B) Vδ2 γδ T cells within total $CD3^+$ T cells, and (C) the subsequent Vδ1/ Vδ2 γδ T-cell ratio were compared between patients with different CMV status (CMV neg n=21, PI n=26, NPI n=27). Horizontal lines indicate median values. *P<0.05, ***P<0.001.

Discussion

Heterologous immunity induced by anti-viral T cells to allo-HLA antigens is proposed to promote allograft rejection and prevent the establishment of tolerance [14]. Cross-reactive viral-specific memory T cells are common in humans. About 45% of viral-specific, including CMV-specific, T-cell clones are cross-reactive to at least one allogeneic HLA molecule [15]. Unexpectedly, and contrary to our initial hypothesis, we did not find higher frequencies of alloreactive T cells in CMV positive LT patients. In contrast, the frequencies of alloreative T cells were generally lower in $CD4^+$ effector memory T-cell subsets of CMV positive patients and, most conspicuously, we found a robust donor-specific $CD8^+$ T-cell hyporesponsiveness in CMV-PI patients. In accordance with the observed donor-specific hyporesponsiveness, CMV-PI patients had a significantly lower risk to develop LAR in our retrospective analysis, and showed the highest ratio of peripheral Vδ1/Vδ2 γδ T cells, which is associated with operational tolerance after LT. To our knowledge this is the first study showing that CMV-PI remarkably reduces donor-specific T-cell reactivity and is a protective factor against the occurrence of LAR after LT, suggesting a role of CMV in achieving transplant tolerance to liver allografts in humans.

We first found that CMV infection, and particularly CMV-PI, is associated with the expansion of $CD4^+$ and $CD8^+$ effector memory T-cell subsets in peripheral blood after LT. Since T_{EM} and T_{EMRA} subsets can mount rapid effector responses upon allostimulation [8], they are hypothesized to be detrimental to allografts [21]. However our data do not support a detrimental role of $CD8^+$ T_{EMRA} after LT, as we found that CMV-PI patients, in which $CD8^+$ T_{EMRA} are the major circulating $CD8^+$ T cell subset and contain the majority of the remaining donor-specific $CD8^+$ T cells, have a reduced incidence of LAR. Similarly,

a recent report shows that increased numbers of circulating $CD8^+$ T_{EMRA} before kidney transplantation are associated with a reduced incidence of acute rejection [22]. These results may be explained by our finding that $CD8^+$ T_{EMRA} largely remain in circulation and scarcely migrate into liver allografts. Similarly, few $CD8^+$ T_{EMRA} were found in rejecting kidney allografts [23]. However we cannot rule out the possibility that T_{EMRA} may change their phenotype into T_{EM} upon infiltration.

An intriguing question is: how does CMV infection induce donor-specific T-cell hyporesponsiveness? CMV infection is known to drive immunosenescence, manifested by inflation of CMV-specific effector memory T cells. It has been postulated that the massively expanded CMV-specific effector memory T-cell pool competes with newly generated T cells for niches and survival factors, and as a consequence T-cell diversity and responses to other pathogens are restricted[24, 25]. Indeed both in humans and mice CMV infection causes impaired T-cell immunity to other pathogens[26, 27], and CMV infection after organ transplantation is associated with a higher incidence of opportunistic infections [28]. The global immunosenescence driven by CMV may explain the lower numbers of alloreactive $CD4^+$ T_{EM} and T_{EMRA} that we observed in CMV positive patients. This is supported by previous findings showing that high numbers of CMV-IE-1-specific memory T cells are associated with lower numbers of alloreactive T cells and improved renal allograft function [29], and that high CMV-specific $CD4^+$ T-cell responses correlate with protection from cardiac allograft rejection [30]. We hypothesize that the donor-specific T-cell hyporesponsiveness observed in CMV-PI patients could be related to the multifaceted immune evasion capacity used by CMV to establish latency, in particular its capacity to modulate antigen presentation [31]. Alexander et al. reported the development of hematopoietic chimerism and donor-specific hyporesponsiveness in a patient with severe CMV disease early after LT [32]. We also reported 3 cases of long-term hematopoietic chimerism within liver allografts [33], interestingly all 3 patients were R^+/D^+ with detection of viremia in two of them (unpublished data). The immune-modifying effects of CMV may have contributed to the engraftment of donor cells, leading to subsequent donor-specific hyporesponsiveness. Moreover, dendritic cells present in liver grafts are the main instigators of T-cell immunity against the graft [34], but CMV infected dendritic cells are impaired in their ability to stimulate allogeneic lymphocytes [35]. The exact mechanisms attributing to this phenomenon remain to be investigated.

In the retrospective analysis, we found that CMV viremia was positively associated with EAR. However, EAR occurred on median 9 days after LT, preceding the detection of CMV viremia in general. This finding is in line with the hypothesis that alloimmune stimulation triggers CMV replication from the latency stage. In contrast, we found that CMV-PI was an independent protective factor against LAR, while CMV negative patients had the highest incidence of LAR. As there is no indication that in CMV negative and NPI patients immunosuppression was prescribed differently causing more LAR, the lower in-

cidence of LAR is probably a reflection of the pro-tolerogeneic status of CMV-PI patients. This finding indicates that CMV during active replication or in a quiescent state may have differential effects on graft rejection, which may also contribute to the discrepancy of EAR and LAR in relation to CMV infection. Careful distinguishing between CMV-PI and CMV-NPI, and between EAR and LAR in our analysis, may be a possible reason why this association has never been reported before.

Immunosuppression can be completely discontinued in more than 40% of adult, stable LT patients[36, 37]. Whether CMV infection plays a role in achieving operational tolerance after LT has yet not been investigated. However, studies sought to identify biomarkers for operational tolerance have found expansion of peripheral Vδ1 γδ T cells, and an increased Vδ1/Vδ2 ratio in tolerant LT patients. Vδ1/Vδ2 ratio has also been used as a surrogate marker to predict operational tolerance[16, 17]. Interestingly, expansion of Vδ1 γδ T cells is also a feature that is shared by CMV infection[38, 39], arguing in favour of a potential association between CMV infection and liver graft tolerance. In addition to previous findings, we found CMV-PI patients showed the highest Vδ1/Vδ2 ratio. This finding corroborates the lower incidence of LAR in our retrospective analysis and suggests that CMV-PI patients may have the highest chance to achieve operational tolerance after immunosuppression discontinuance. However we do not minimize immunosuppression routinely in our center, thus we are not able to demonstrate the direct link between CMV infection and operational tolerance.

To conclude, the primary findings of this study are the development of donor-specific T-cell hyporesponsiveness in CMV-PI patients, and that CMV-PI patients are protected from the occurrence of LAR and show signs of operational tolerance. Further investigations into the role of CMV infection in the development of operational tolerance after LT are necessary. Since CMV status is easily measured without additional effects or costs, this parameter can be taken into account by physicians when tapering immunosuppressive therapy in LT patients. Altogether, for the first time to our knowledge, we show evidences that CMV infection may promote immunological tolerance towards allogeneic liver graft in humans.

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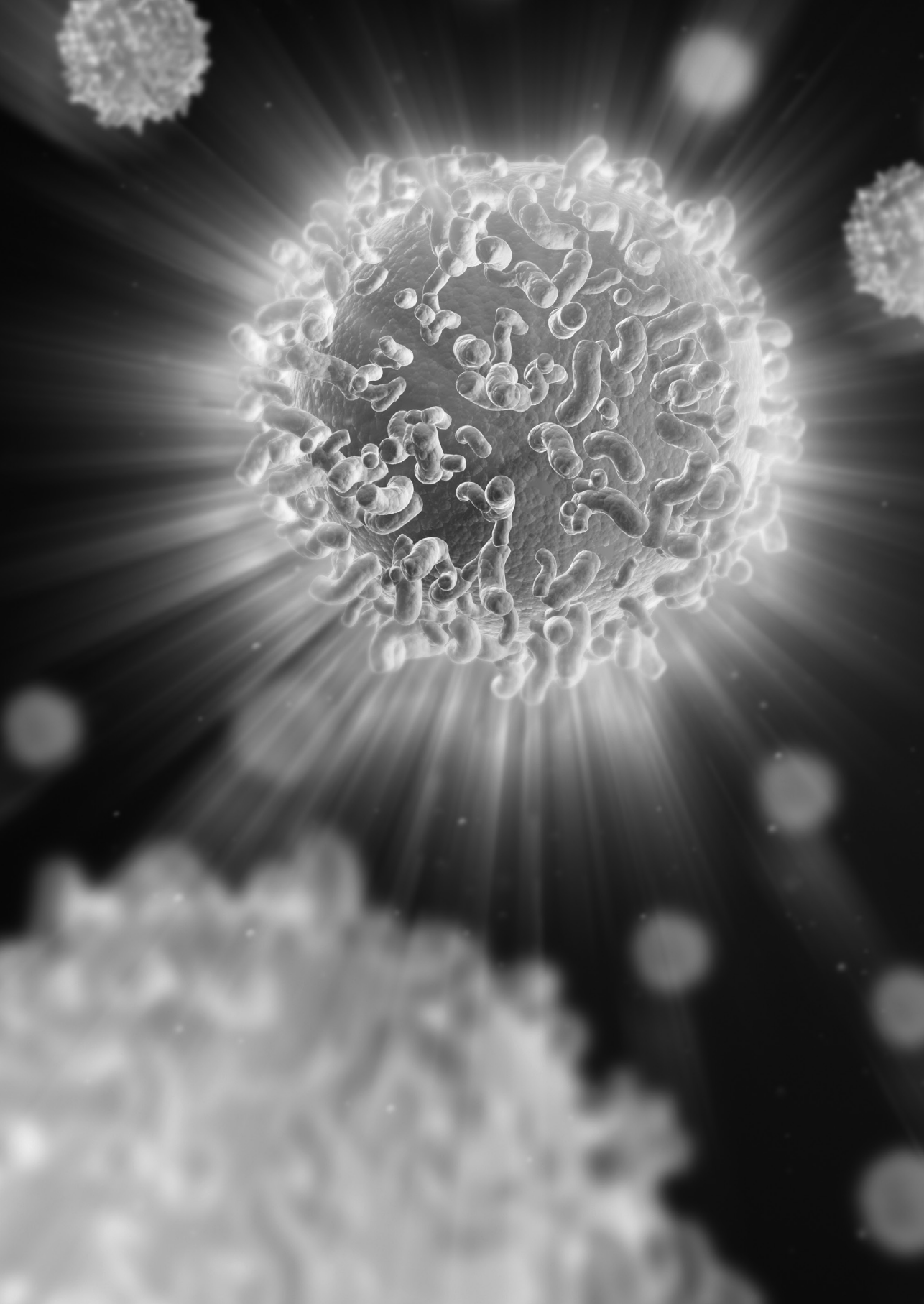

Chapter 6

A new approach for quantification of T-cells with indirect alloreactivity

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Abstract

Studies on indirect allorecognition in transplant patients are hampered by the lack of a reliable assay to detect T cells with indirect allospecificity. The aim of this study is to set up a novel technique based on messenger RNA (mRNA) electroporation to measure indirect T cell responses after organ transplantation. For this purpose, we synthesized mRNA encoding the human leukocyte antigen (HLA) A*0201 by in vitro transcription from a DNA template. To exclude the possibility of direct allorecognition, the transmembrane region of the HLA-A*0201 α-chain was deleted from the DNA construct, while the human DCLamp gene was included to obtain translocation of the translated protein to the MHC class II compartments. By stimulation with CD154-expressing fibroblasts in the presence of IL-4, peripheral blood B cells of HLA-A*0201^{-}/HLA-DR*0101^{+} donors were expanded and differentiated into antigen presenting B cell blasts (CD40-B). These CD40-B cells were electroporated with the HLA-A*0201-encoding mRNA and co-cultured with three different CD4^{+} T cell clones recognizing HLA-A*0201 derived peptides presented in HLA-DR*0101. After electroporation, HLA-A*0201 was not expressed on the surface of the CD40-B cells but was detected intracellularly as soon 2 hours later. The CD4^{+} T cell clones produced high amounts of IFN-y upon co-culture with electroporated CD40-B cells, and IFN-y production was completely abrogated by addition of anti-MHC class II antibody, demonstrating MHC class II restriction.

In conclusion, we set up a novel technique to measure human T cells with indirect allospecificity using mRNA electroporation. This technique may be promising to study indirect allorecognition in patients after organ transplantation.

Introduction

Organ graft rejection is induced by activation of the recipient immune system by allo-antigens which are derived from graft tissue of a genetically disparate donor. MHC-molecules are the major allo-antigens to which the host immune system vigorously reacts, due to their highly polymorphic nature. There are two major pathways of allo-recognition by T cells [1]: 1. The direct pathway in which the recipient T cells recognize intact allogeneic MHC molecules on non-self cells, which can occur during pregnancy, upon blood transfusion, or after allogeneic transplantation of cells, tissue or organs; 2. The indirect pathway in which the recipient T cells recognize donor MHC-derived peptides presented by self-MHC molecules expressed on recipient cells, which is basically the same mechanism as used in recognition of pathogen-derived antigens. The semi-direct pathway has been discovered more recently as a third pathway of allo-recognition in which membrane fragments containing intact donor MHC molecules that are transferred to recipient antigen presenting cells activate recipient T-cells [2]. Many studies have shown the importance of direct pathway donor-specific T cells in immune reactivity to allogeneic organ grafts in humans [3-5]. It is believed that the direct pathway is the major driving force of early acute rejection of allogeneic transplants, since it leads to exceptionally robust inflammatory T-cell responses. The direct pathway response is thought to extinguish after transplantation because of depletion of donor Antigen Presenting Cells (APC). In contrast, when intact donor MHC-molecules have been taken up by host APC and are presented to recipient T cells, the indirect pathway T-cell response becomes active. [6,7] [8] [9]. Previously, it was believed that the evanescence of direct pathway allogeneic T-cells occurred within weeks after transplantation [3-5], however in our recent study in which we used a more robust technique to quantify T-cell responses to directly presented allo-antigens, we showed that direct pathway allogeneic T-cells remain detectable at least after one year after liver transplantation [10].

The indirect T cell alloresponse is thought to be weaker than the direct T cell alloresponse, but long-lived and tending to spread to formerly cryptic determinants on donor and self-tissue specific antigens [11]. This feature of indirect T-cell alloreactivity is presumably associated with the sustained presence of recipient APC that can maintain a chronic inflammatory response similar to that observed in autoimmune diseases [1] [11]. Therefore, it is generally assumed that the indirect pathway contributes to rejection episodes late after transplantation and to chronic rejection. However, current ex vivo assays for measuring T cells that recognize allo-antigens via this pathway are rather insensitive, not enabling detection T cells with indirect allospecificity in 50%-70% of organ transplant recipients [3,12-14]. Because the indirect alloresponse is assumed to play a central role in late rejection episodes it is of clinical importance to develop a sensitive assay for monitoring this pathway.

Different ex vivo techniques for measuring T cells that respond to indirectly presented allo-antigens have been described, but all have important limitations [15]. In one approach, recipient peripheral mononuclear cells (PBMCs) are loaded with donor tissue homogenates made by multiple freeze-thaw cycles to disrupt cell membranes [3,16-19]. Subsequently recipient PBMC internalize donor cell fragments and present donor antigens as peptides on self-MHC molecules. A first technical limitation is that PBMC contain limited numbers of APC, which are moreover in an immature state, together resulting in a suboptimal presentation of antigens, which is crucial for detection of low frequency T-cells with indirect specificity. In addition, a recent study shows that tissue homogenates used as allo-antigen source poorly activate T cells via the indirect pathway, but instead activate direct pathway T-cells [20]. Apparently, intact donor HLA molecules are transferred to autologous APC in the cultures, resulting in activation of recipient T cells via semi-direct presentation.

Another method loads recipient APC with synthetic MHC-peptides corresponding to the donor or mismatched allotype [6,12,13,21].Since it is impossible to add sufficient peptides to cover the complete donor MHC repertoire which is mismatched with the recipient into the ex vivo cultures, a selection of putative peptides representing one or a few dominant donor HLA-alleles is made. Furthermore, because it is not known which peptides are generated during MHC processing in APC, synthetic peptides may present neo-epitopes that are not naturally occurring. Besides, the use of peptides is restricted by the HLA-typing of the recipient, because different HLA allotypes present different peptides, and prediction of HLA class II binding peptides is complex [22]. Recently, a third technique for allo-antigen loading of recipient APC was published which uses pulsing of recipient APC with HLA class-I monomers [20].

In the current study we constructed a technique which has the potential to overcome these problems. The first modification we introduced is the use of a pure source of recipient APC in the *ex vivo* assay to optimize antigen presentation. The second improvement is the development of a technique for transfection of recipient APC with mRNA encoding for mismatched donor MHC in order to achieve efficient presentation of naturally processed donor HLA peptides to recipient T cells. This technique is being used in tumor immunology to achieve presentation of a broad range of natural peptides of tumor antigens to both CD4+ and CD8+ T cells [23-28]. The transfected APC synthesize the antigen(s) encoded by the electroporated mRNA, degrade it by using their natural processing machinery into a natural repertoire of peptides, and present them on self-MHC molecules to T cells, thereby preventing the generation of neo-epitopes. A further advantage of this technique is its independence of the patient's HLA-repertoire. Despite its success in tumor immunology, this technique has not been applied yet in transplantation immunology.

Whereas most studies using mRNA electroporation to achieve antigen presentation to T cells have used monocyte-derived dendritic cells(moDCs) as APC, we decided to use B-cells expanded from PBMC by co-culture with CD40-ligand transfected fibroblasts and IL-4 (CD40-B cells), since B cells expanded by this technique have potent T-cell stimulatory capacity and can be generated in large numbers from a small volume of peripheral blood [10] . MessengerRNA-transfected CD40-B cells have been demonstrated as an alternative source of APC able to induce functional antigen-specific T cell responses *ex vivo* [23,24,29-32] . By electroporation of recipient-derived CD40-B cells with mRNA encoding for one or more genetically disparate MHC alleles, we aimed to simulate *in vivo* indirect allo-antigen presentation to T cells as strict as possible in an *ex vivo* environment.

Materials & Methods

Cell lines

The L-CD40L cell line, a mouse fibroblast cell line stably transfected with human CD40L (kindly provided by prof. C. van Kooten) was cultured in RPMI (Lonza, Breda, The Netherlands) with 10% FCS (Sigma-Aldrich, Zwijndrecht, The Netherlands) and penicillin/streptomycine (Gibco, Bleiswijk, The Netherlands) in T75 culture flasks. For splitting, the cells were detached from the culture plates with 0.5% Trypsin/0.2% EDTA (Sigma-Aldrich, Zwijndrecht, The Netherlands) and washed with serum free RPMI before transferring them at a concentration of 1x10E5 cells/mL into fresh culture medium.

K562 cells, a human immortalised myelogenous leukaemia line which lacks HLA class I surface expression, were cultured in the same medium in T 175 culture flasks.

B cell expansion

HLA-typed buffy coats were obtained for the Sanquin Blood Bank (Rotterdam, The Netherlands) , and PBMC were isolated by Ficoll gradient centrifugation. B cells were expanded from PBMC by co-culture with irradiated mouse fibroblast cells that were stably transfected with human CD40L in the presence of cyclosporin and IL-4 as described previously [10]. When the purity of $CD19^+$ cells was > 95%, CD40-B cells were used in assays.

Construction of vectors for in vitro mRNA generation

A schematic overview of the constructed vectors is depicted in Figure 1, in which the cloning order of the functional parts is shown including the unique restriction sites. Transcription of all vectors is driven by a T7 bacteriophage promotor, which is followed by a signaling peptide which is either exon 1 of the inserted HLA molecule or a synthetic sequence, which is responsible for directing the transportation of the encoded protein

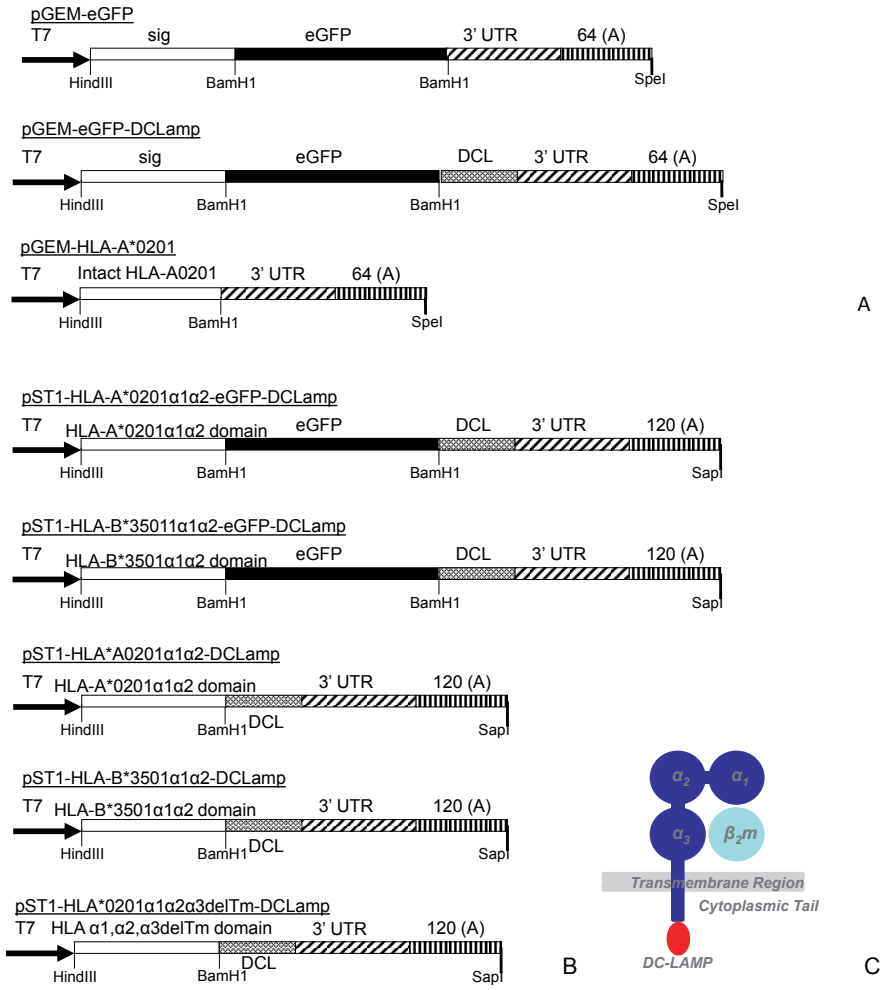

Figure 1: Overview of the DNA constructs used for in vitro transcription.

A] pGEM vectors driven by T7 bacteriophage promotor and encoding for: signaling peptide (sig) which is a synthetic sequence responsible for targeting the encoded protein to the endoplasmatic reticulum, Enhanced Green Fluorescence Protein (eGFP), DCLamp (DCL) which is a targeting signal to the endolysosomes, intact HLA-A*0201 molecule including the sequence for the complete HLA-A*0201 α1α2 and α3 domain, 3`flanked Untranslated Restriction (3`UTR) and 64 bp long poly-A tail both responsible for a slower degradation of the synthesized protein. The positions of the restriction sites, HindII, BamH1 and SpeI are shown. B] pST1 vectors with different encoding parts resembling the pGEM vector, difference with the pGEM vectors are the 120 bp long poly-A tail and the unique restriction site with SapI at the end of the vector. The sequences encoding for the HLA-A*0201α1α2, HLA-B*3501α1α2 and HLA-A*0201α1α2α3 without the transmembrane part (HLA-A*0201α1α2α3delTm) are located directly after the T7 promotor. Different vectors with and without DCLamp (DCL) are shown including the positions of the restriction sites HindIII, BamH1 and SapI.

C] Schematic overview of HLA class II protein on the cell surface. The α1, α2, and α3 domain are shown together with the transmembrane cytoplasmic tail

to the endoplasmatic reticulum. We used the backbone vector plasmid pST1, which was kindly provided by dr. U. Sahin, Mainz, Germany [33], to make constructs that encoded for parts of human HLA molecules. To prevent the expression of intact HLA-molecules resulting in direct pathway allorecognition, we did not include exons 5,6,7 and 8 which encode the transmembrane, cytoplasmic parts and the unspecified part of HLA-molecules, respectively. The first constructs that we made comprised only exons 2 and 3 of HLA-A*0201 or HLA-B*3501, which encode for the for α1 and α2 domains which contain the peptide binding groove of the HLA-molecules. This part is the most polymorphic part, and encodes for the majority of allo-antigens. The gene fragment encoding for α1 and α2 domains of the human MHC alleles were amplified from pcDNA-HLA-A*0201 or from DNA isolated from a HLA-B*3501 transfected Single Antigen expressing Line [34], (both kindly provided by Dr. I.I. Doxiadis, LUMC, Leiden) by PCR using the following primers:

HLA-A*0201	sense:	5`- AAA AAG CTT GCC ACC ATG GCC GTC ATG GCG CCC C-`3
	antisense:	5`-AAG GAT CCG TGC GCT GCA GCG TCT CC-`3
HLA-B*3501	sense	5`- AAA GCT TGC CAC CAT GCG GGT CAC GGC GCC CC-`3
	antisense:	5`- AAA GGA TCC GCG CGC TGC AGC GTC TCC-`3

Primers were designed with a linked restriction site for *HindIII* at the 5' end and for *BamH1* at the 3' end to enable ligation of the amplified sequence into the backbone vector plasmid pST1, which has a *HindIII* restriction site after the T7 promotor region and *BamH1* before the sequence encoding Enhanced Green Fluorescence Protein(eGFP). A Kozak sequence was added for the initiation of the translation, which starts after the restriction site. HLA exon-coding sequences in these constructs are directly followed by sequences encoding eGFP and/or the transmembrane and luminal region of Dendritic Cell Lysosome Associated Membrane Protein (DCLamp), which is a targeting signal for the endo-lysosome, resulting in peptide loading in MHC-II [35]. The 3' UTR (untranslated region) and the 120 poly-A tail are responsible for a slower degradation of the electroporated mRNA, leading to a longer time for translation and peptide-formation. The nonfunctional parts of the plasmid for mRNA synthesis are not shown. These constructs are called pST1-HLA*A0201-α1α2-eGFP-DCLamp and pST1-HLA-B*3501-α1α2-eGFP-DCLamp, respectively. In addition, constructs containing the same HLA-inserts, but lacking the eGFP sequence were generated; called pST1-HLA-A*0201α1α2-DCLamp and pST1-HLA-B*3501α1α2-DCLamp.

The second type of construct that we made in backbone pST1 contains an insert that encodes the complete HLA-A*0201 protein including the α1α2 and α3 domain except the transmembrane part. This insert was synthesized by Geneart (Life Technologies Europe, Gent, Belgium) and is referred as HLA-A2 α1α2α3delTm, when cloned into pST1 vector including eGFP and DClamp it is referred as pST1-HLA-A*0201 α1α2α3delTm-eGFP-DCLamp. For comparison, we used a pGEM construct containing intact HLA-

A*0201 sequence, including the transmembrane and cytoplasmic parts, referred as pGEM-HLA-A*0201. For development of an electroporation protocol suitable for transfections of CD40-B cells, we constructed: The pGEM-eGFP plasmid which encodes for eGFP flanked by the 5′ and 3′ UTRs of Xenopus Laevis β-globin and 64 bp poly A tail of which the backbone pGEM was kindly provided by Dr E Gilboa, Duke University Medical Center, Durham, NC, USA and eGFP was flanked as described by *Meirvenne et al.* and collegues [36][37]; 2. The pGEM-eGFP-DCLamp in which the MHC class II targeting transmembrane/luminal region sequence of DCLamp is inserted at the 3′ end of eGFP.

In vitro transcription of DNA constructs

Prior to *in vitro* transcription, the plasmids in backbone vector pST1 were linearized with *SapI* and plasmids in backbone vector pGEM were linearized with *SpeI*. *In vitro* transcription was performed using the T7 RNA polymerase and ARCA (anti-cap analogue) kit (Ambion m MESSAGE mMachine T7ultra kit; Austin, TX, USA) according to the manufacturers guidelines. The concentration and quality of the *in vitro* transcribed mRNA were assessed by spectophotometry and agarose gel electrophoresis. The obtained mRNA of the constructs were stored at -80°C till utilization.

mRNA electroporation

Before electroporation, B cells were washed twice, first with serum-free IMDM (Lonza, Breda, The Netherlands) and subsequently with Opti-MEM (Invitrogen-Life technologies, Bleiswijk, The Netherlands). Cells were resuspended in Opti-MEM to a final concentration of $20x10^6$ cells/mL. Thereafter, 200 µL of the cell suspension was mixed with 20 µg mRNA in a 0.4-cm gap sterile disposable electroporation cuvette and electroporated with a BioRad Gene pulser Xcell Electroporation system using a program generating square wave pulses of 800 V (or different voltages as indicated in the Figure legends) for 0.5 ms. Immediately after electroporation cells were transferred into fresh cell culture medium, including IMDM (Lonza Breda, The Netherlands) with 10% heat-inactivated human male AB serum (Lonza, Breda, The Netherlands), 1% penicillin/streptomycin and 1% insulin-transferrin-selenium solution (Gibco, Bleiswijk, The Netherlands), and incubated at 37°C in a humidified atmosphere supplemented with 5 % CO_2. Two to 5 hours after electroporation, cells were harvested and washed with PBS (Lonza, Breda, The Netherlands) and resuspended in fresh culture medium for further experiments.

Flowcytometric analysis

For analysis of HLA-A2 expression, mRNA electroporated CD40-B cells were incubated with anti-HLA-A2-Fitc mAb (BD Pharmingen, Breda, The Netherlands). For determination of transfection efficiency, eGFP expression was measured at indicated time points after transfection. Cells were measured using a BD Facs flow cytometer (BD Biosciences,

Breda, The Netherlands), and flow cytometric data were analysed using FacsDiva (BD biosciences, Breda, The Netherlands) or FLowJo software version 8.8.4 (Treestar, San Carlos, CA). To exclude non-viable cells, 7-AAD (BD Biosciences, Breda, The Netherlands) added just before flow cytometric measurement.

CD4+ T-cell clones

HLA-DR*0101 restricted HLA-A*0201 specific CD4+ clones 4.12, 4.43 and 4.44, generated from an HLA-A*0201 negative patient with severe GVHD after administration of HLA-A*0201 positive donor lymphocyte infusion [38] were used as responder cells for testing indirect presentation of HLA-A*0201. Cells were expanded by stimulation with allogeneic PBMC which were irradiated at 35 Gy and supplemented with PHA (2 ug/ml), in T-cell medium containing IMDM (Lonza, Breda, The Netherlands) supplemented with 5% FCS (Hyclone, GE Healthcare Life Sciences, Eindhoven, The Netherlands), 5% human AB-serum (Lonza, Breda, The Netherlands), penicilline/streptomycine (Gibco, Bleiswijk, The Netherlands), L-glutamine (Lonza, Breda, The Netherlands) and IL-2 (2ug/mL) at a T-cell: feeder cells ratio of 1:5. Cells were cultured at 37 °C in a humidified atmosphere supplemented with 5% CO2. Every three days T-cell clones were further expanded by splitting of the cultures and replenishing of fresh culture medium without feeder cells. At day 9, cells were harvested and used for experiments.

Antigen presentation assay

In a 96-wells- U bottom microtiter plate, 1 x 10E4 cells of the T-cell clones were co-cultured in T cell medium without IL-2 together with 1x10E5 HLA-A*0201 – DRB1*0101 + CD40-B cells either or not transfected with mRNA for 24 hours at 37°C in a humidified atmosphere supplemented with 5% CO2. After incubation, cell-free supernatant was collected and the concentrations of IFN-γ were determined by a standard enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions (eBioscience, San Diego, CA). For positive control experiments, synthetic 20-mer peptides corresponding to residues 99-114 or 103-120 of the α1 and α2 domain of the HLA-A*0201 molecule were synthesized by EZbiolab (Carmel, USA). CD-40 B cells were diluted in culture medium including IMDM (Lonza, Breda, The Netherlands) with 10% heat inactivated human male AB serum (Lonza, Breda, The Netherlands), 1% penicillin/streptomycin and 1% insulin-transferrin-selenium solution (Gibco, Bleiswijk, The Netherlands) at a concentration of 1x10E6 cells/mL and the HLA-A*0201 synthetic 20-mer peptides were added at a concentration of 10 ug/ml and incubated between 3 and 12 hours before utilization for stimulation of T cells.

Results

Establishment of optimal conditions for mRNA transfection of CD40-B cells

First we established the optimal conditions for mRNA transfection of CD40-B cells by electroporation at different voltages. Figure 2 shows the transfection efficiency and viability of CD40-Bcells after electroporation of pGEM-eGPF-derived mRNA at different voltages. Transfection efficiencies increased with increasing voltages. The highest transfection efficiency was observed at the highest voltage (800 Volt), while no eGPF expression was observed at 300 Volt (Fig 2A). More than 80% of CD40-B cells expressed eGFP upon electroporation of pGEM-eGPF-derived RNA at 800V, and expression remained stable for 22 hours. After 42 hours still 40% of transfected CD40-B cells expressed eGFP. We observed more cell death at higher voltages (fig 2B). Nevertheless, upon electroporation at 800V 80% of CD40-B cells remained viable up to 22 hours of culture, and after 42 hours still 60% of CD40-B cells were viable. Therefore, in all subsequent experiments we used 800V for electroporation.

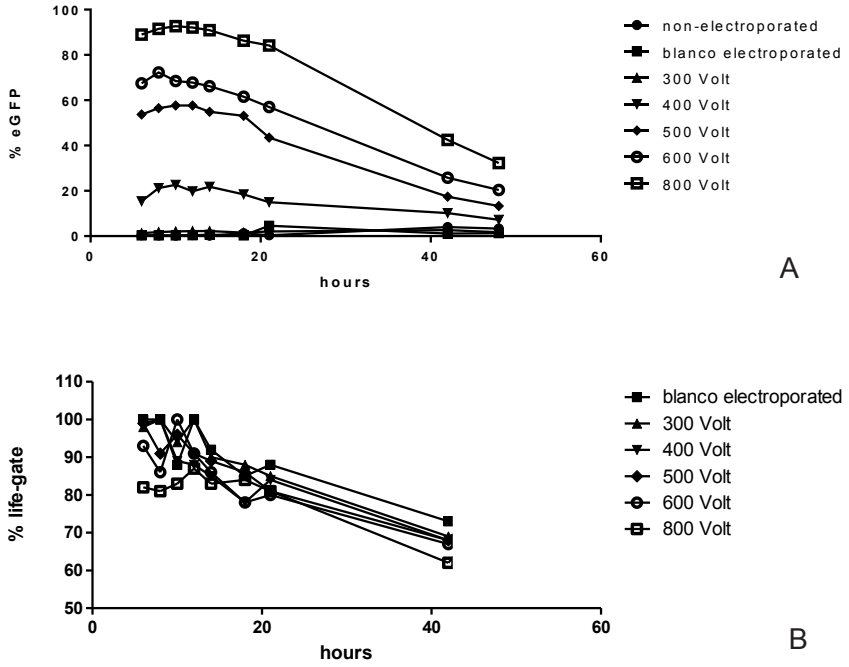

Figure 2: Optimization of the electroporation protocol of CD40-B cells using pGEM-eGPF-derived RNA. A) Transfection efficiency upon electroporation at different voltages. Depicted are % eGFP expressing CD40-B cells after different periods of culture upon electroporation. B) Viability of CD40-B cells as measured by 7-AAD exclusion upon electroporation at different voltages.

To study the effect of DCLamp on protein expression, we compared the transfection efficiency of mRNA derived from pGEM-eGFP to mRNA derived from pGEM-eGFP-DCLamp in CD40-Bcells. The transfection efficiency of pGEM-eGFP-DCLamp RNA was lower than that of RNA without a DCLamp encoding sequence, and also the decline of eGFP expression upon electroporation with RNA containing DCLamp was faster (Fig 3).

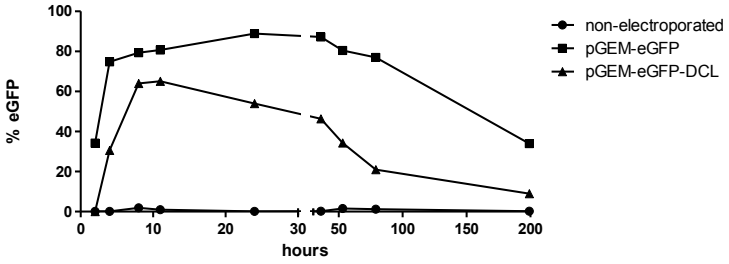

Figure 3: Transfection efficiencies upon electroporation of RNA transcribed from pGEM-eGFP constructs with and without DCLamp in CD40-B cells. DCLamp is responsible for a lower transfection efficiency and faster degradation of eGFP.

No indirect allo-antigen presentation by CD40-B cells electroporated with mRNA encoding for alpha 1 and 2 domains of MHC class I alleles

To study whether electroporation of CD40-B cells with mRNA encoding for a fusion protein of α1 and α2 domains of HLA*A0201 and DCLamp results in presentation of HLA*A0201-derived peptides to CD4+ T cells, HLA-A*0201-negative HLA-DR*0101-positive (HLA* $A0201^{-}DR0101^{+}$) CD40-B cells were electroporated with mRNA derived from pST1-HLA*A0201α1α2-DCLamp, and thereafter co-cultured with HLA-DR*0101-restricted HLA-A*0201 specific CD4+ Tcell clones 4.12 or 4.44. As a positive control, we co-cultured the T-cell clones with HLA*$A0201^{-}DR0101^{+}$ CD40-B cells loaded with the specific HLA*A0201 peptides that are recognized by these CD4+ T cell clones. Figure 4 shows that both T-cell clones reacted in positive control conditions, but did not react to the electroporated HLA*$A0201^{-}DR0101^{+}$ CD40-B cells.

To investigate why electroporation with pST1-HLA*A0201α1α2-DCLamp mRNA did not result in activation of the T cell clones, we analysed whether electroporation with mRNA derived from pST1HLA*A0201-α1α2-eGFP-DCLamp resulted in eGFP expression. As is shown in Figure 5A, electroporation of K562 cells with mRNA derived from this construct cells resulted in eGFP expression in the majority of these cells, but electroporation of CD40-B cells did almost not, suggesting that the failure to achieve antigen presentation to HLA-DR*0101-restricted HLA-A*0201 specific CD4+ clones might be due to lack of translation of mRNA derived from pST1HLA*A0201α1α2-DCLamp into protein in CD40-B cells. However, when HLA-A*0201 negative CD40-B cells were electroporated

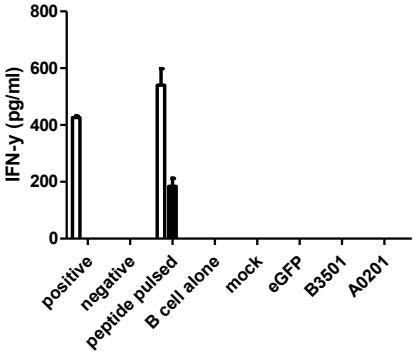

Figure 4: Electroporation of HLA-A*0201$^-$HLA-DR*0101$^+$ CD40-B cells with pST1-HLA-A*0201α1α2-DCLamp mRNA does not result in activation of HLA-DR*0101- restricted HLA-A*0201 specific CD4+ clones. Cytokine production of HLA-DR*0101- restricted HLA-A*0201 specific CD4+ clones upon 24 hours stimulation with the following conditions: negative: T-cell clones in culture medium only, peptide pulsed: T-cell clones stimulated with peptide pulsed HLA-A*0201$^-$HLA-DR*0101 CD40-B cells (positive control), B-cell alone: HLA-A*0201$^-$HLA-DR*0101$^+$ CD40-cells in culture medium, mock: HLA-A*0201$^-$HLA-DR*0101$^+$ CD40-B cells electroporated without mRNA, eGFP: T-cell clones stimulated with HLA-A*0201$^-$HLA-DR*0101$^+$ CD40-B cells electroporated with pGEM-eGFP-DCLamp mRNA, B3501: T-cell clones stimulated with HLA-A*0201$^-$ HLA-DR*0101$^+$ CD40-B cells electroporated with pST1-HLA B*3501α1α2-DCLamp mRNA, A0201: T-cell clones stimulated with HLA-A*0201 HLA-DR*0101$^+$ CD40-B cells electroporated with pST1-HLA -A*0201α1α2-DCLamp mRNA. This figure shows only IFN-y production upon stimulation with peptide-pulsed CD40-B cells.

with mRNA derived from a construct containing intact HLA*A0201 (pGEM-HLA*0201) including the transmembrane and cytoplasmic parts but lacking the DCLamp encoding sequence, we observed HLA-A*0201 expression on the cell surface, as detected with an anti-HLA-A2 specific antibody (Figure 5B). In addition, when HLA*A0201$^-$DR0101$^+$ CD40-B cells were electroprated with mRNA derived from the construct with intact HLA*A0201 lacking DCLamp (pGEM-HLA*A0201), both HLA-A2-specific CD4+ T cells clones were activated (Figure 6). These data demonstrate that, while mRNA derived from pST1-HLA*A0201 α1α2-DCLamp can be translated into protein in K562 cells, it is poorly translated in CD40-B cells and does not result in HLA*A0201 peptide presentation to CD4+ T cells. Conversely, electroporation of CD40-B cells with mRNA derived from a construct containing intact HLA*A0201 without DCLamp (pGEM-HLA*A0201), results translation into protein and presentation of HLA*A0201 peptides to CD4+ T cell clones.

Transfection of CD40-B cells with mRNA encoding for HLA-A2 without transmembrane part results in MHC class II-restricted antigen presentation to CD4+ T cells

Since electroporation of CD40-B cells with mRNA encoding intact HLA*A0201 also resulted in presentation of intact HLA*A0201 on the cell surface, enabling recognition

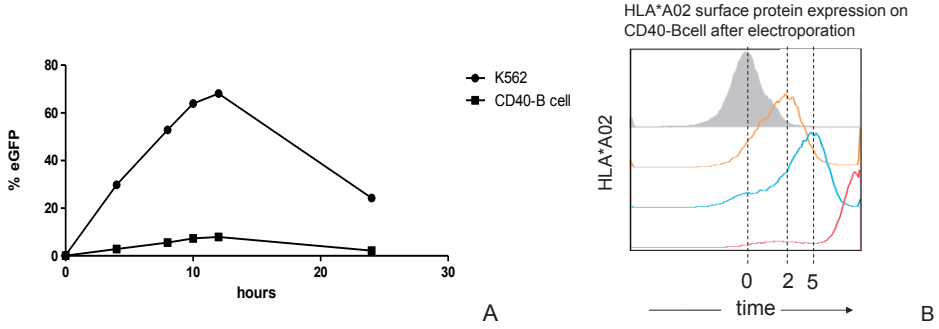

Figure 5: Electroporation of HLA-A*0201$^-$HLA-DR*0101$^+$ CD40-B cells with mRNA encoding intact HLA-A*0201 results in surface expression of HLA-A*0201.
A] eGFP expression of pST1-HLA-A*0201-eGFP-DCLamp mRNA in K562 and CD40-B cells. There is eGFP protein synthesis in K562 but not in CD40-B cells. B] HLA-A2 surface expression on HLA-A2- CD40-B cells after electroporation with pGEM-HLA-A*0201 (containing intact HLA*A0201), which induces HLAA*0201 protein synthesis and presentation on the surface of CD40-B cells. The x-axis shows different timepoints (in hours) after electroporation at which HLA-A*0201 expression was determined.

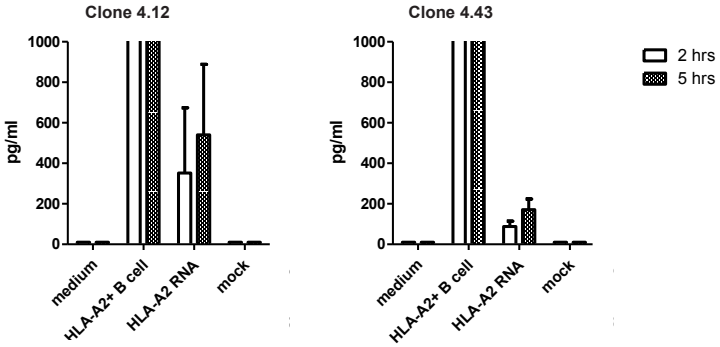

Figure 6: Electroporation of CD40 B cells with mRNA encoding intact HLA-A*0201 results in IFN-y production by HLA-DR*0101 restricted HLA-A*0201 specific CD4+ T-cell clones. IFN-y production by CD4+ T cell clones 4.12 and 4.43 upon 24 hours stimulation in the following conditions:

- Medium: CD4+ clones in T-cell medium
- HLA-A2+: CD4+ clones stimulated with HLA-A*0201$^-$HLA-DR*0101$^+$ + CD40-B cells
- HLA*A2 RNA: CD4+ clones HLA-A*0201$^-$HLA-DR*0101$^+$ CD40-B cells electroporated with pGEM-HLA-A*0201 mRNA
- Mock: CD4+ clones stimulated with HLA-A*0201$^-$HLA-DR*0101$^+$ CD40-B cells electroporated without mRNA

The y-axis shows the concentration of IFN-y in pg/mL and the x-axis the different Stimulation conditions. Open bars show cytokine production of T cells which were added to CD40-B cells 2 hours after electroporation, while hatched bars show cytokine production of T cells added to CD40-B cells 5 hours after electroporation.

by T cells via the direct pathway and electroporation of mRNA encoded by the pST1-HLA*A0201α1α2-DCLamp vector did not result in T-cell activation, a new construct in the pST1 vector was designed in which only the transmembrane region of the HLA-A*0201 α-chain was deleted to exclude the possibility of direct allorecognition, while the hu-

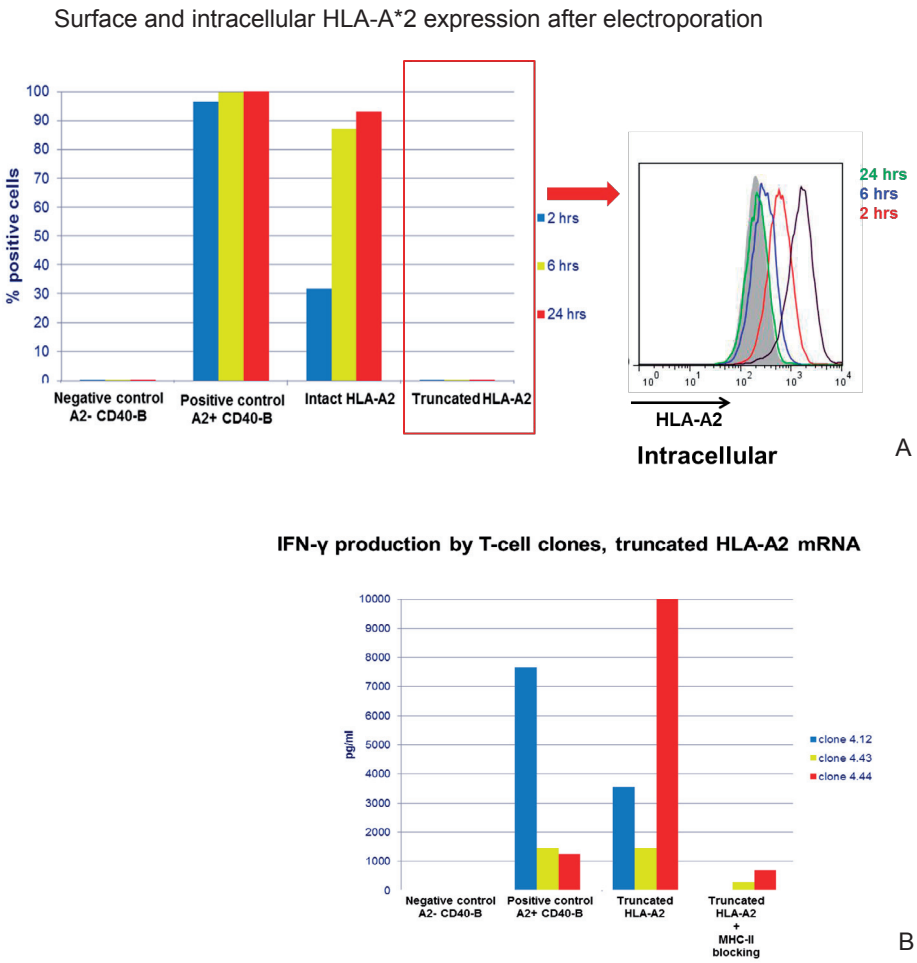

Figure 7: Electroporation of CD40 B cells with mRNA encoding HLA-A*0201 without transmembrane region results in IFN-γ production by HLA-DR*0101-restricted HLA-A*0201-specific CD4+ T-cell clones. A] Surface and intracellular expression of HLA-A2 after electroporation of CD40-B cells with mRNA derived from pGEM-HLA-A*02010-DCLamp (intact HLA-A2) or pST1HLA*A0201α1α2α3delTM- eGFP-DCLamp (truncated HLA-A2). Surface expression of HLA-A*0201 is detected when electroporation with intact HLA-A2 mRNA is performed. Electroporation with truncated HLA-A2 prevents surface expression of HLA-A*0201, however intracellular expression is detected, ensuring that protein translation occurs. B] IFN-γ production of HLA-DR*0101restricted HLA-A*0201-specific CD4+ clones when stimulated with CD40-B cells electroporated with truncated HLA-A2. Since IFN-γ production is abrogated by addition of MHC-II blocking antibody, it is ensured that CD4+ activation is restricted by MHC class II.

man DCLamp gene was included to obtain translocation of the translated protein to the MHC class II compartments (pST-HLA*A0201α1α2α3 delTM -DCLamp). Electroporation of HLA*A0201^{-}DR0101^{+} CD40-B cells with mRNA derived from the latter construct did not result in surface expression of HLA-A2, but intracellular HLA-A2 was detected (Figure 7A). In addition, the HLA-A*0201-specific CD4+ T cell clones were activated by HLA*A0201^{-}DR0101^{+} CD40-B cells electroporated with pST-HLA*A0201 α1α2α3delTM-DCLamp mRNA. Importantly, CD4+ T cell responses could be completely abrogated by addition of an MHC class II blocking antibody, demonstrating that HLA-A*0201 peptides were recognized in an MHC class II-restricted manner (Figure 7B).

Conclusion/ Discussion

In this study we demonstrate that electroporation of CD40-expanded B cells with *in vitro* transcribed mRNA that encodes a genetically disparate HLA-A0201 molecule results in presentation of this molecule as a peptide in cognate MHC class II molecules on CD40-B cells, and that this is suitable technique to activate selectively CD4+ T cells with indirect specificity.

It has been shown that mRNA transfection is as potent in achieving antigen presentation to CD4+ and CD8+ T cells as lentiviral gene transduction [23]. Besides, it is a more time saving method and cleaner system without risk of pseudotransduction phenomenon when compared to lentiviral transduction. Additionally, it is applicable for different cell types, such as PBMCs, monocytes, moDCs and CD40-Bcells [23,24,26,28,30-32,39-45]. When compared to other methods of RNA gene transfer like lipofection and passive pulsation, mRNA electroporation has been shown to be superior in terms of gene transfection efficiency and reduced cell toxicity [26].

Using mRNA encoding for eGFP we optimized the electroporation protocol, showing that CD40-B cells translate the electroporated mRNA into protein, which can be concluded by the fact that eGFP is detectable by flow cytometric analysis (Figure 2). The highest transfection efficiency was obtained with an electroporation voltage of 800 volt. Despite the higher percentage of non-viable cells after electroporation at this voltage, still 80% of the electroporated CD40 B cells remained viable after 18 hours. Comparable results for transfection efficiency and viability of CD40-B cells were described by *van den Bosch et al.,* although they applied a lower electroporation voltage of 300 V [32].

We used square wave pulses to transfect CD40-B cells with mRNA, because this has been described to be more efficient in gene transfer than exponential decay pulses [46] Our goal was to obtain presentation of peptides derived from the transfected mRNA-encoding MHC molecules in MHC-class II. However, since peptides derived from cell-intrinsic proteins are generally presented by MHC-class I and not by MHC class II, we

included as a targeting signal in the mRNA to the endolysosomes a sequence encoding for the luminal and transmembrane parts of DCLamp, which has been shown to enable efficient antigen presentation to $CD4^+$ T cells [24,29,30,32,35,44,45]. After electroporation CD40-B cells with pGEM-eGFP-DCLamp mRNA, expression of eGFP diminished more rapidly than after electroporation with the control construct pGEM-eGFP without DCLamp in CD40-Bcells (Figure3), suggesting indirectly that there is targeting of the protein to the endo-lysosomes followed by degradation.

To prevent expression of intact allogeneic HLA molecules on the cell surface of recipient APC, we designed a plasmid construct containing only the sequences encoding the α1 and α2 regions of HLA-A*0201 molecule, without or with the eGFP gene to enable measurement of transfection efficiency. Similar constructs were generated for HLA-B*3501.For detection of allo-antigen presentation via the indirect pathway, we stimulated CD4+ T-cell clones which specifically recognize HLA-A*0201 peptides restricted by HLA-DRB*0101, with HLA-A*0201-negative HLA-DRB*0101-positive CD40-Bcells electroporated with HLA-A*0201 mRNA derived from the pST1-HLA-A*0201α1α2-DCLamp construct. However, the T-cell clones showed no responses (figure 4). When we verified the protein production after electroporation of pST1-HLA-A*0201α1α2-eGFP-DCLamp mRNA in K562 and CD40-Bcells, we discovered that pST1-HLA-A*0201α1α2-eGFP-DCLamp protein was produced in K562 cells but barely in CD40-Bcells (Figure 5A). Currently we do not know why mRNA derived from the pST1-HLA-A*0201α1α2-eGFP-DCLamp construct is not efficiently translated into protein in CD40-B cells. It could be that synthesis of a fusion protein of the two α-chains of HLA-A*0201 and DC-Lamp is inefficient, or that the resulting protein is unstable and is rapidly degraded. In contrast, electroporation of HLA-$A0201^-$ HLA-$DRB*0101^+$ CD40-B cells with mRNA coding for intact HLA*A0201 (pGEM-HLA*A0201) resulted in rapid membrane expression of HLA-A*0201 (Figure 5B) and in presentation to CD4+ T cells (Figure 6). Since electroporation with mRNA encoding for intact HLA-A*0201 resulted in HLA-A*0201 surface expression, mRNA derived from this construct is not suited for enumeration of patient T cell responses with indirect allospecificity, since allo-recognition in a direct manner cannot be ruled out. Since mRNA of vector pST1-HLA-A*0201α1α2-eGFP-DCLamp did not result in protein synthesis in CD40-B cells, we designed new DNA construct in which only the transmembrane region of the HLA-A*0201 α-chain was deleted to exclude the possibility of direct allorecognition, while the human DCLamp gene was included to obtain translocation of the translated protein to the MHC class II compartment; pST1-HLA-A*0201α1α2α3delTM-DCLamp. Electroporation of HLA-$A*0201^-HLA^-DRB*0101^+$ CD40-Bcells with mRNA derived from the latter construct resulted in intracellular protein expression but not in surface protein expression (Figure 7A). Most importantly, HLA-$A*0201^-$HLA-$DRB*0101^+$ CD40-B cells electroporated with pST1-HLA-A*0201α1α2α3delTM-DCLamp derived mRNA were able

to induce activation of HLA-A*0201-specific HLA-DRB*0101-restricted CD4+ T cells. Antigen presentation was restricted by MHC class II, as was shown by blocking MHC class II.

This new approach of ex vivo antigen loading of CD-40 Bcells to present HLA-peptides via the indirect pathway has several advantages; allorecognition via the direct or semi-direct pathway is excluded, since the sequence encoding the transmembrane region of the HLA-molecule is lacking, which prevents any presentation on the cell surface. Recently *Breman et al* [20] used synthetic HLA class I monomers to achieve indirect presentation to CD4+ T cells. Although they demonstrated that this technique did not lead to semi-direct HLA antigen presentation to a CD8+ T cell clone, the design of their technique does not exclude the possibility of semi-direct antigen presentation to occur when using T cells from patients. Compared to peptides, the technique presented here does not require knowledge of the peptides presented by different MHC molecules and is not restricted by HLA-typing of the patient. CD40-B cells can be charged with the full antigenic spectrum of allo-peptides using mRNA encoding to whole HLA-antigens (only exluding the transmembrane part) without prior identification of HLA-peptides. Additionally, mRNA encoding for different HLA molecule can be co-electroporated together, in case of multiple HLA mismatches between donor and recipient [28]

Although dendritic cells (DC) are considered to be the most professional APC of the immune system, deriving them for ex vivo utilization suffers from serious drawbacks in practice: primary DC constitute only 0·1-0·5% of human peripheral blood mononuclear cells [47]. Ex vivo generation of dendritic cells from patients' monocytes or $CD34^+$ precursor cells is a more practical alternative [48], but requires multiple phlebotomies, leukapheresis or even bone marrow aspiration, especially from immunosuppressed transplant patients in whom circulating immune cells are already repressed due drugs. Instead, our technique uses CD40-expanded B cells as a source of antigen presenting cells, which can be rapidly expanded to large amounts from a limited volume of blood [10,32].

Altogether, we show a proof of principle of this new approach for detection of T cells with indirect allospecificity. However further development of the protocol for detection of CD4+ T cell responses to indirectly presented donor HLA in blood or graft-infiltrating cells is required before this assay can be applied for studying the role of T cell responses to indirectly presented donor HLA after organ transplantation in humans.

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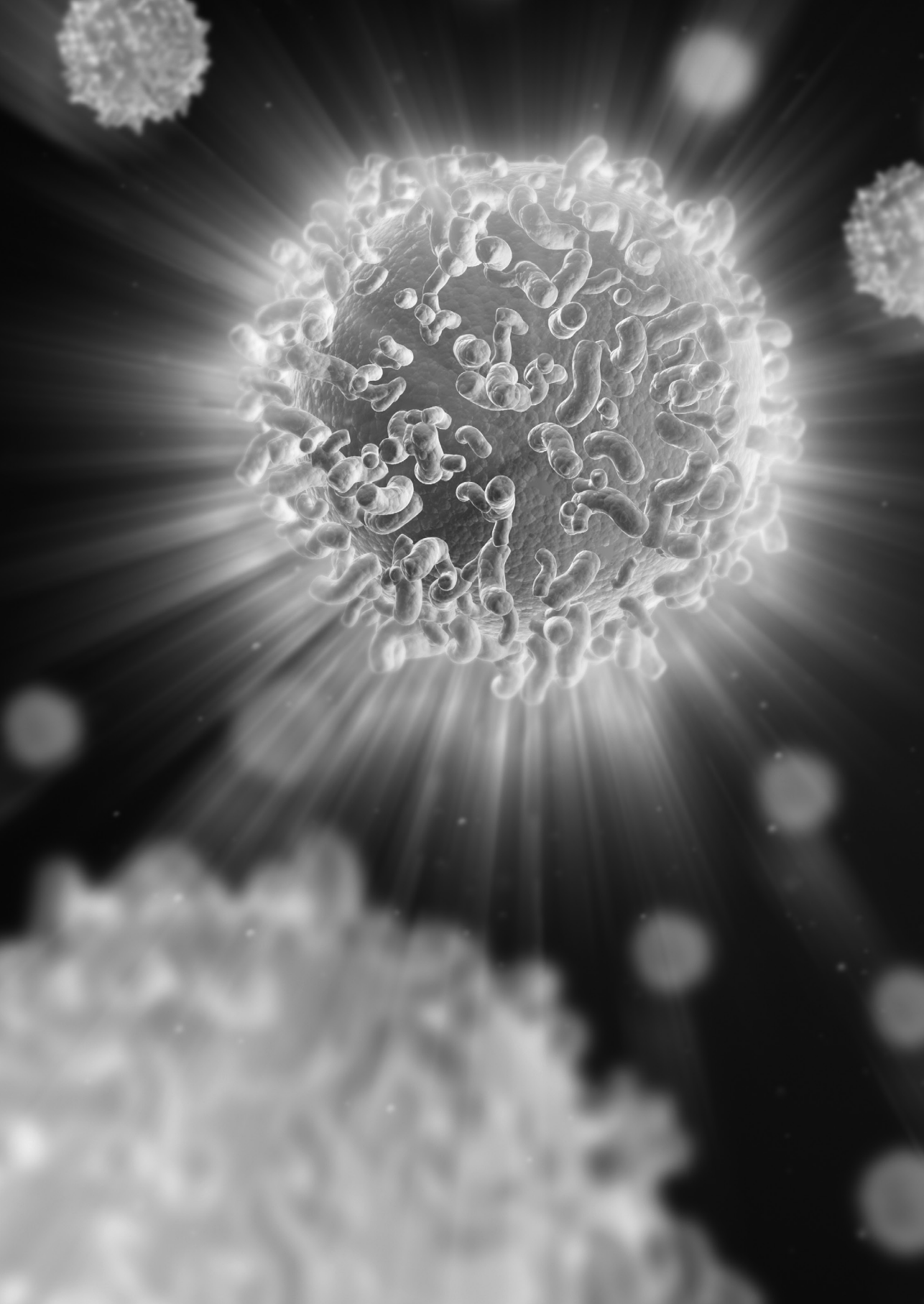
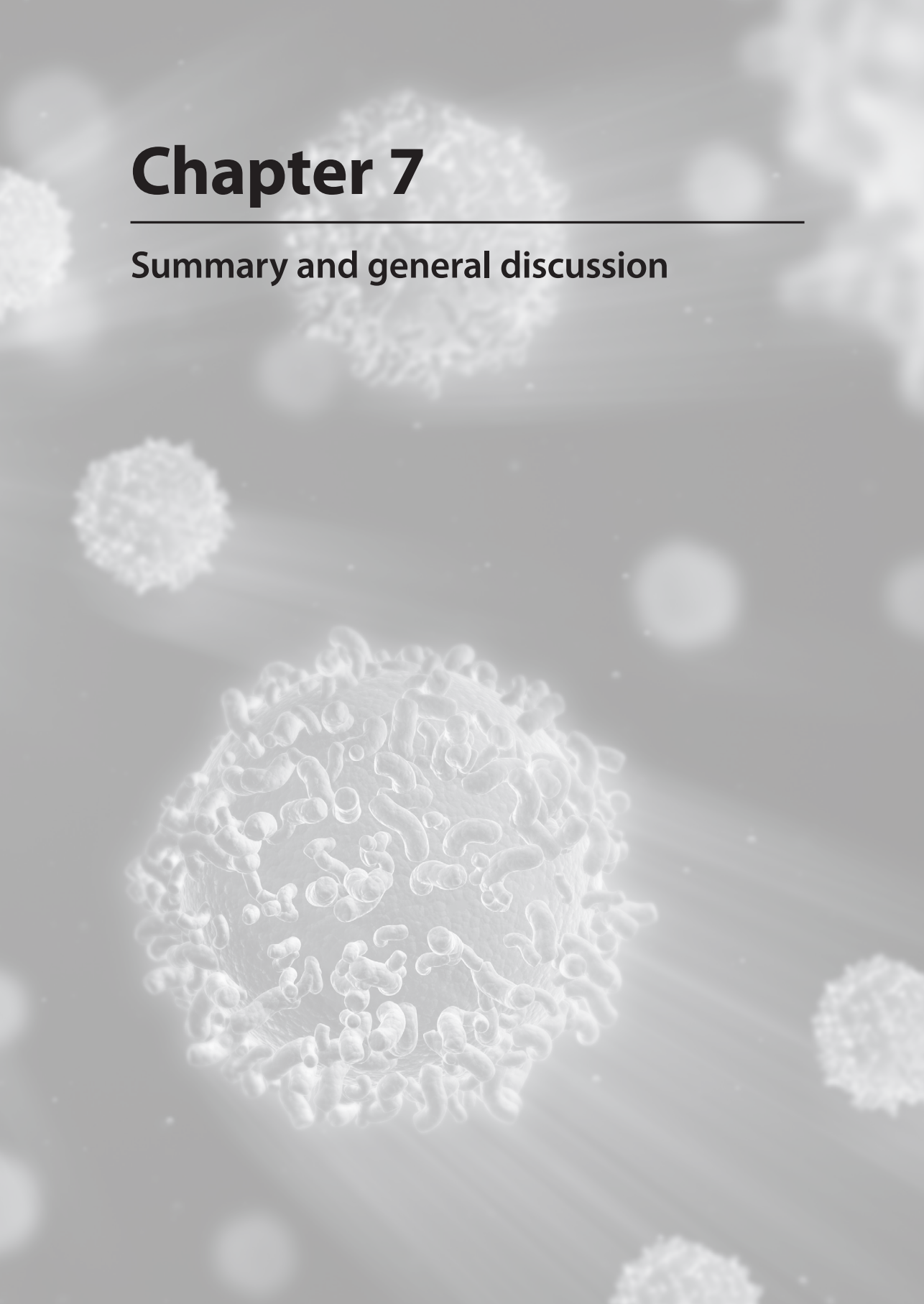


Chapter 7

Summary and general discussion



Liver transplantation is a lifesaving treatment for end stage liver diseases, which has become a routine therapy nowadays. Despite growing experience in surgery and immunosuppression protocols, the long-term mortality after liver transplantation remains much higher than in the general population. Although this is partly due to chronic allograft injury or rejection, the main causes are comorbidities provoked by chronic immunosuppressive drug usage [1-5]. Minimization of these regimens would improve patients' quality of life by decreasing the side effects and subsequently the comorbidities. Therefore research priorities in the transplantation field should be more focussed on minimization or even withdrawal of immunosuppression. Withdrawal of immunosuppression has been described in selected patients after liver transplantation, where approximately 20% of patients could safely discontinue all immunosuppressive drugs [6,7]. However, a recent prospective withdrawal study showed that immunosuppression could be completely discontinued in about 40% of adult LT patients [8]. These patients, maintained stable graft function without signs of rejection and are supposed to be operationally tolerant for their graft. These studies used transcriptional profiles to characterize tolerant (patients in which immunosuppression can be weaned off safely) and non-tolerant (patients in whom immunosuppression cannot be safely weaned off) liver graft recipients. Tolerant recipients exhibited peripheral blood mRNA expression profiles which were significantly enriched in genes preferentially expressed by gamma-delta T cells (γδT cells) and NK cells and in genes encoding for proteins involved in cycle cell arrest. In addition, tolerant patients were observed to differ from non-tolerant patients in the distribution of circulating γδT cells cell subsets (Vδ1:Vδ2 γδT cells) and in the frequency of potentially regulatory Tcells (Tregs) . Finally, tolerant and non-tolerant patients were found to differ in intragraft expression of genes involved in the regulation of iron hemostasis [9-11, 49]. However, there is a difficulty of reproducibility of these results because of the relatively small numbers of patients and variability between patients included in these studies. Therefore, the mechanisms of operational tolerance in LT patients remain obscure, and predicting which patient can be safely withdrawn of immunosuppression is still not possible.

It is therefore important to have a better apprehension of the immunological responses after liver transplantation, including the role of different genetic factors in determining immunological responses to the allogeneic liver graft. Such knowledge may lead to new therapeutic and diagnostic developments, which potentially could lead to individualized immunosuppressive regimens, sufficient for preventing rejection and improving outcome and quality of life in individual patients. Besides, a financial benefit for the society could be achieved, not only because of reduced use of expensive immunosuppressive drugs, but also by reduction of their side effects which cause additional medical and societal costs.

Examples of immunosuppression associated morbidities are renal failure [12-14], opportunistic infections [15], cancer [16], cardiovascular diseases [17,18] and de novo diabetes [19]. Prognostic markers can be investigated on molecular, cellular, immunological, and genetic level. In this thesis we attempted to find a prognostic genetic marker for acute rejection, and investigated if we could predict calcineurin inhibitor-related renal dysfunction after liver transplantation using genetic markers. In addition, we elucidated the longitudinal course of circulating T cells that recognize donor allo-antigens via the direct pathway of allorecognition after liver transplantation, including the contribution of T-cell subsets T_{cm}, T_N, T_{em} and d $T_{emra.}$ Moreover, we studied the influence of CMV infection on donor-specific T cell responses and acute rejection after liver transplantation, and started to develop a novel technique for monitoring T cells with indirect allospecificity, as an aid for tapering down immunosuppressive regimens.

Acute rejection occurs in 27% to 58% of the LT-recipients, depending on several factors such as the type of graft (living or post mortem donor) and the intensity of immunosuppression [20,21]. Prognostic factors for acute rejection could aid in dosing immunosuppressive drugs on an individual base. *Chapter 2* describes the influence of two functional SNPs in the CTLA-4 gene on the rate of acute rejection post-LT. Full length CTLA-4 molecules encoded by the +49G allele are incompletely glycosylated, leading to retrograde transport of a portion of the molecules to the cytoplasm for degradation, resulting in reduced expression on the T cell surface and impaired inhibitory function of CTLA-4 on T cell activation in individuals homozygous for +49G. The +6230G/A SNP was reported to influence the production rate of the soluble alternative splice form of CTLA-4, in which the +6230G allele produces sCTLA-4 at a reduced rate compared with mRNA encoded by the +6230 A allele. As the soluble isoform inhibits allogeneic T cell activation, it is conceivable that carriers of the +6230G allele may be more susceptible to rejection after organ transplantation, as was supported with our analysis of the association between the +6230 SNP and acute rejection: homozygous CTLA-4 +6230A liver transplant recipients which lacked the G allele showed a reduced rate of rejection compared with carriers of the G allele. However, haplotype analysis showed that the single +6230G allele was not the risk allele for acute rejection. The +6230G allele was only associated with an increased risk of rejection when it was combined in a haplotype with the +49A allele. Thus the risk allele for acute rejection after liver transplantation (+49A/+6230G) combines the genetic predisposition for reduced capability to produce sCTLA-4 (+6230G) with a normal capacity to express the full length CTLA-4 isoform on the T cell membrane (+49A). The effect of this haplotype on rejection was dose dependent, meaning that each additional allele results in a higher risk of rejection. The conclusion that this specific combination of single alleles confers risk to acute rejection is further substantiated by the observation that carriers of one +49A/+6230A allele and one +49G/+6230G allele had a rate of rejection intermediate between car-

riers of the +49A/+6230G risk haplotype and homozygous carriers of the protecting +49A/+6230A and +49G/+6230G haplotypes. Unfortunately, even though the risk ratio of the +49A/+6230G was dose dependent and significant, the risk ratio of 1.34 for each additional risk haplotype is too low to be useful for guiding individualized immunosuppressive treatment. Moreover, only 2% of the patients were homozygous for the risk allele. Nevertheless, this study shows that even under conventional immunosuppressive therapy, CTLA-4 is critically involved in the regulation of the anti-donor immune response after liver transplantation. Other SNPs in immune-regulatory genes should be determined too, and a full haplotype analysis with all the functional SNPs should be performed in order to obtain a more robust prognostic marker.

Another common problem in long-term survivors of liver transplantation is chronic kidney disease (CKD) of which the prevalence ranges between 20-70% [12,13] and complicates medical management because of its associated increased morbidity and mortality [14,22-26]. In *chapter 3*, we evaluated the association between SNP ABCB1 3435C>T and CYP3A5 6986A>G and the incidence of CKD after LT in a single-center cohort of 125 adult Caucasian LT-patients with up to 23 years of follow-up. CKD developed in 38% of the LT patients, which is in line with the CKD incidence reported in other studies (between 20 and 70%). The allele frequencies of the investigated SNPs in our population were also comparable to those of other studies. Our data show that neither the *CYP3A5* 6989A>G nor the *ABCB1* 3435C>T genotype of either donor or recipient were associated with CKD risk after LT. Even our sub-analysis in which we combined recipient *ABCB1* TT genotype with the donor *CYP3A5* genotype did not show a significant difference in the incidence of CKD. However, we cannot rule that we did not detect an association because of the low numbers of patients included in the study. Nevertheless, to date our study presents the largest Caucasian adult non-renal organ transplant cohort in which associations between *CYP3A* and/or *ABCB1* SNP in both donors and recipients with CKD have been studied, but did not confirm findings from previous studies in liver transplant recipients which did identify associations between *CYP3A5* 6989A>G and the *ABCB1* 3435C>T SNPs [27-29] Differences in study populations (ethnicity, age) type of CNI prescribed, and primary endpoints studied are possible explanations for our different finding. Moreover, most studies were conducted in renal transplant recipients [30-36] in which confounding factors as recurrence of original kidney disease, ischemia-reperfusion injury, allograft rejection and quality of the kidney allograft could have influenced reported associations.

T cells that recognize donor alloantigens via the direct pathway are activated by donor-derived APC that migrate from the graft into recipient secondary lymphoid tissues [37-40]. This is a transient process, which has been confirmed as well as in animal as in human studies. [37,39,41]. In experimental animals direct pathway T-cell responses dominate during the early post-transplant period and subside thereafter [42], but in the

human transplantation setting the only evidence available is from cross-sectional studies [43-48]. There were no studies describing the kinetics of recipient T-cell alloreactivity after organ transplantation in humans in detail. The kinetics of the direct pathway in LT-recipients was determined *in chapter 4*, by using a novel technical approach. Our data showed for the first time an increase of donor-specific T-cell frequencies shortly after LT and additionally, we observed that T cells reacting to donor allo-antigens presented via the direct pathway remain present at levels similar to pre-transplant levels in the recipient circulation for at least 1 year after transplantation. Previous studies have shown the rapid decrease of the direct pathway within one month to values below pre-LT levels [48]. Our study shows that the direct pathway remains longer active and could be responsible for rejection up till at least 1 year after transplantation. However, to confirm this proposition, blood samples taken during episodes of late acute rejection episodes should be investigated for T cells with direct allospecificity. The different findings between our study and previous studies may be explained by the stronger donor-derived stimulator cells that we have used, resulting in an increased sensitivity compared to conventional assays. use of this assay as a diagnostic tool for for guidance of individualized immunosuppression first requires establishment of the relationship between numbers of circulating T cells that respond to donor allo-antigens presented via the direct pathway and acute rejection long after LT.

The exact mechanism and the cells contributing to immunological tolerance to allogeneic (liver) grafts is still unknown. Whether ignorance, anergy, deletion or regulation of immune cells are involved is still unclear. Studies have demonstrated that tolerant LT patients show expansion of $V\delta1^+$ γδT cells, and related transcriptional profiles, in blood [9,10,49]. Expansion of $V\delta1^+$ γδT cells is also seen after CMV infection [50-52] arguing in favour of a potential association between CMV infection and liver graft tolerance. Moreover, accumulation of $CD8^+$ $T_{EMRA,}$ which also is a typical characteristic of CMV-driven immune senescence [53,54], is associated with increased sensitivity and susceptibility of the elderly to infections, and poor responses to vaccinations [55,56], suggesting compromised immunity. On the other hand, CMV-specific memory T cells are hypothesized to be detrimental to allografts as they can be cross-reactive to allogeneic HLA [57] and anti-viral memory T cells are regarded as one of the main barriers to the achievement of transplantation tolerance. Despite the vast amount of literature on the clinical impact of CMV infection after organ transplantation, it is poorly understood whether and how CMV infection influences T cell allo-reactivity. In *chapter 5* we show that CMV infection after LT is associated with accumulation of differentiated effector memory (T_{EM}) CD4+ and CD8+ T cells and CD8+ terminally differentiated effector memory T cells (T_{EMRA}) in the peripheral blood within the first half year post-transplant. Most importantly, 6 months after transplantation, hyporesponsiveness of circulating CD8+ T cells to directly presented donor allo-antigens was observed in CMV primary infection patients, sug-

gesting that these patients might have developed a certain degree of immunological tolerance to their graft. This conclusion was supported by the observation that LT patients with primary CMV infection showed a higher ratio of circulating Vδ1:Vδ2 γδT cells compared to CMV naïve patients and patients which were already CMV IgG positive before LT. In addition, patient with primary CMV infection demonstrated a reduced incidence of late acute rejection episodes compared to other LT patients. Interestingly, direct pathway donor-specific responsiveness in patients with primary CMV infection was observed in all CD8+ T cell subsets except T_{EMRA}. Apparently $CD8+_{TEMRA}$ are not very detrimental for liver allografts, which might be partially explained by our observation that these cells poorly infiltrate into liver allografts. Thus, our data show an unexpected role of primary CMV infection in the development of hypo-responsiveness of CD8+ T cells to directly presented donor allo-antigens. How primary CMV-infection attributes to this donor-specific hypo-responsiveness in the CD8+ T cell compartment is currently unknown. CMV is known for utilizing multifaceted immune evasion strategies to establish latency, in particular the capacity to modulate antigen presentation [58]. While dendritic cells present in liver grafts are the main instigators of T cell immunity against the graft [40,59], CMV infected dendritic cells are impaired in their ability to stimulate allogeneic lymphocytes[60,61]. Moreover, the myelosuppression effect of ganciclovir treatment cannot be neglected. The exact mechanisms attributing to this phenomenon remains to be investigated.

Chapter 6 is a methodological chapter on the development of a robust assay for determination of T cells with indirect allospecificity. Indirect allorecognition is the recognition by recipient T cells of processed donor antigens presented as peptides in the context of self-MHC. Evidence for this pathway came from the observations that graft rejection still occurred in experimental animal transplant models in the absence of immunogenic donor derived passenger cells in the graft [62,63]. Alloantigens shed from the graft are internalized and processed in the same manner as exogenous antigens, and they are presented by recipient APCs as peptides in self-MHC class II molecules to T-helper cells. Recipient APCs migrate continuously to the donor graft where donor derived peptides are encountered, internalized, processed, and subsequently presented to recipient T cells in the peripheral lymph nodes and spleen. Experimental animal transplantation models have demonstrated that the frequency of T cells with indirect allospecificity is lower than that of T cells with direct allospecificity and the indirect T cell response peaks later [64], but its magnitude is sufficient to induce graft rejection [63]. Current assays for monitoring these T cells in humans are relatively insensitive, and in addition, their accuracy is debated [65]. Because of the complexity of this pathway it is difficult to mimick it *in vitro*. Therefore, little is known about the contribution of T cells that recognize donor antigens via the indirect pathway to graft rejection, or their relevance in development of tolerance to allografts in humans. Different techniques have been described for

detection of T cells with indirect allospecificity. Some studies use donor cell fragments to pulse recipient APC. An advantage is that donor cell fragments theoretically cover the full HLA-repertoire of the donor. However the HLA-specificity of the responding T cells is unknown, and there is evidence that intact donor HLA-molecules present in the fragmented donor cell suspensions are also presented to T cells and thereby direct T cell alloresponses can be induced in this type of *in vitro* stimulations [66]. Other studies use synthetic donor HLA peptides to pulse recipient APC. Disadvantages of this approach are, firstly, that it is impossible to add sufficient peptides to cover all mismatched donor HLA-molecules to the in vitro cultures. Secondly, since we do not know which donor HLA peptides are presented in vivo, synthesized peptides may contain neo-epitopes that do not occur *in vivo*. Therefore, we studied a novel approach for detection of T cells with indirect allospecificity by gene transfection of recipient APC with mRNA encoding for donor HLA. For this purpose, we designed a DNA construct suited for in vitro transcription, that contains the full HLA-A*0201 sequence, except the transmembrane region, thereby preventing the expressionof the intact HLA-A*0201 molecule on the surface after electroporation, which might result in recognition by T cells with direct allospecificity. This DNA-construct was used to synthesize HLA-A*0201-encoding mRNA by in vitro transcription. Electroporation was chosen as the technique for genetic transfection because of high efficiency compared to other transfection techniques. As recipient APC we chose CD40-activated B cells because, in contrast to monocyte-derived dendritic cells which are generally used as APC in this type of assays, they can be expanded from small numbers of PMBC. We showed that the proof of principle for this new approach was successful, when three different CD4+ T cell clones, each recognizing a HLA-A*0201 peptide in the context of a HLA-DR*0101 molecule, responded to HLA-A*0201$^-$ HLA-DR*0101$^+$ CD40-B cells electroporated with HLA-A*0201 mRNA. Addition of a blocking anti-MHC class II antibody completely abrogated stimulation of the CD4+ T cell clones, demonstrating that their activation was MHC class II-restricted. In addition, after transfection of HLA-A*0201$^-$ CD40-B cells with the HLA-A*0201-encoding mRNA, that lacked the transmembrane part of the α3 domain, we observed that HLA-A2 could be detected intracellularly but not on the cell surface, and therefore direct recognition is prevented. An accurate assay for monitoring indirect T cell alloresponses is essential to determine the kinetics and clinical relevance of these T cells in allograft rejection. If these cells critically contribute to allograft rejection, a robust assay for quantification indirect T cell responses may aid in identification of patients in which immune suppressive drugs can be safely tapered. Further studies will reveal whether the assay that we developed is able to accurately quantify CD4+ T cell responses to indirectly presented donor HLA-molecules in transplant recipients.

The main findings from our research were:

1. The CTLA-4 +49A/+6230G haplotype is a co-dominant risk allele for acute rejection after clinical liver transplantation. This implies that, even under immunosuppression, CTLA-4, in common with experimental animals, is critically involved in the regulation of the human immune response against allogeneic grafts
2. Genetic variations in the *CYP3A5* and *ABCB1* genes were not significant risk factors for the development of CKD after LT. Genotyping LT recipients or their donors for SNPs in these genes is therefore unlikely to aid the clinician in preventing CKD
3. By using a novel technical approach, we showed for the first time an increase of donor-specific T-cell frequencies shortly after LT in humans. In addition, we observed that T cells reacting to donor allo-antigens presented via the direct pathway remain present in the recipient circulation for at least 1 year after transplantation.
4. The development of donor-specific T-cell hyporesponsiveness in CMV-PI patients, and that CMV-PI patients are protected from the occurrence of LAR and show signs of operational tolerance meaning that CMV infection may promote immunological tolerance towards allogeneic liver graft in humans.
5. We show a proof of principle of a new approach for detection of T cells with indirect allospecificity, in which CD40-Bcells are electroporated with mRNA of mismatched HLA-protein are able to induce response of T cells with indirect allospecificity

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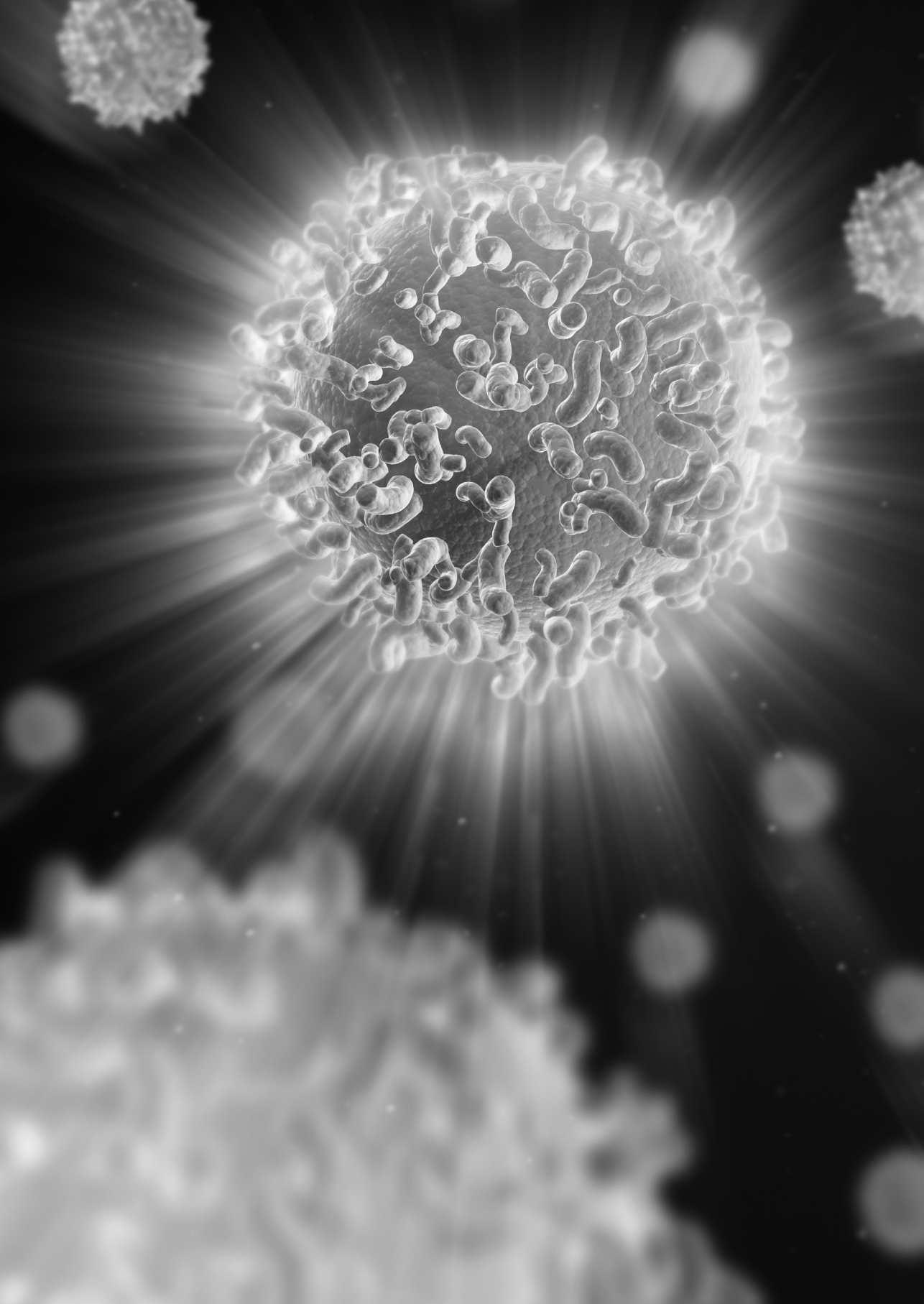
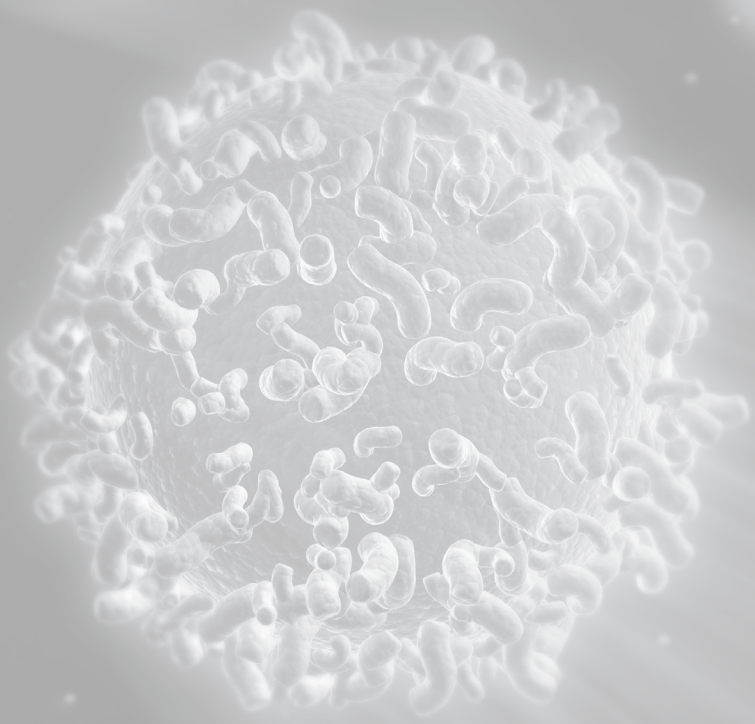


Chapter 8

Dutch summary/ Nederlandse samenvatting



Nederlandse samenvatting

Levertransplantatie is een levensreddende behandeling voor eindstadium leverfalen, welk tegenwoordig als routinematige behandeling wordt toegepast. Ondanks de groeiende ervaring in operatie en immunosuppressie protocollen, blijft de lange termijn morbiditeit hoger bij levertransplantatie patiënten dan bij de algemene populatie. Hoewel dit gedeeltelijk verklaard kan worden door chronische transplantaat beschadiging of afstoting, wordt het merendeel van de co-morbiditeit uitgelokt door het chronisch gebruik van immunosuppressiva [1-5]. Het gebruik van immunosuppressiva na levertransplantatie is noodzakelijk om afstoting van het donororgaan tegen te gaan, echter zijn ernstige bijwerkingen een keerzijde. Minimaliseren van de doses van deze medicatie zou de kwaliteit van leven bij levertransplantatie patiënten verbeteren door vermindering van de bijwerkingen wat tot verlaging van de co-morbiditeit tot gevolg heeft. Onderzoek naar het afbouwen of minimaliseren van immunosuppressiva zou in de transplantatieveld meer prioriteit moeten hebben. In ongeveer 20% van de levertransplantatie patiënten zouden de afweerdonderdrukkende medicatie volledig afgebouwd kunnen worden [6,7]. Echter, een recente prospectieve studie toonde aan dat in 40% van alle volwassen levertransplantatie patiënten, de immunosuppressiva volledig afgebouwd kon worden [8]. Deze patiënten behielden een stabiele orgaanfunctie zonder tekenen van afstoting en zijn operationeel tolerant voor hun donor orgaan. Deze studie maakte gebruik van transcriptie profielen om tolerante patiënten (transplantatie patiënten waarin veilig immunosuppressiva afgebouwd kunnen worden) en niet-tolerante (transplantatie patiënten waarin de immunosuppressiva niet afgebouwd kunnen worden) levertransplantaat ontvangers te karakteriseren. Tolerante ontvangers toonden perifere bloed mRNA expressie profielen welke significant verrijkt waren met genen die bij voorkeur tot expressie worden gebracht door gamma-delta T cellen (γδT cellen) en Natural Killer cellen (NK cellen) en in genen coderende voor eiwitten die betrokken zijn in cel arrest. Tevens verschilden de tolerante patiënten van de niet-tolerante patiënten in de distributie van circulerende γδT cel subset (Vδ1:Vδ2 γδT cells) en in de frequentie van potentiele regulatoire T cellen (Tregs). Als laatste, tolerante en niet-tolerante patiënten verschilden in gen expressie in het donororgaan welk betrokken zijn in de regulatie van ijzer hemostase [9-11, 49]. Echter, zijn de resultaten van deze studies moeilijk te reproduceren omdat het betrekkelijk kleine aantal patiënten betreft en de grote variatie is tussen de patiënten die geïncludeerd zijn in deze studies. Daarom blijft het mechanisme van operationele tolerantie in LT patiënten onduidelijk en is de predictie van patiënten, waarbij de immunosuppressiva veilig afgebouwd kunnen worden, niet mogelijk. Om deze redenen is het belangrijk om een beter inzicht van de immunologische reacties na levertransplantatie te hebben en tevens ook beter inzicht in de rol van verschillende genetische factoren die immunologische responsen bepalen.

Deze kennis zou tot nieuwe therapeutische en diagnostische ontwikkelingen kunnen leiden welke de potentie hebben om de immunosuppressiva te kunnen individualiseren, waarbij de dosis per patiënt voldoende is om afstoting van het donororgaan tegen te gaan maar zo laag dat het weinig tot geen bijwerkingen heeft zodat de kwaliteit van leven per individuele patiënt verbeterd. Daarnaast zou een financiële voordeel voor de samenleving behaald kunnen worden, doordat de kosten van de dure immunosuppressiva dalen door minder gebruik, maar ook door vermindering van ernstige bijwerkingen welke additionele medische kosten met zich meebrengen. Voorbeelden van immunosuppressiva afhankelijke morbiditeit zijn nierfalen [12-14], opportunistische infecties [15], kanker [16], cardiovasculaire aandoeningen [17,18] en *de novo* diabetes [19]. Onderzoek naar prognostische factoren zou verricht kunnen worden op moleculair, cellulair, immunologisch en genetische niveau.

In deze thesis hebben we getracht een prognostische genetische marker te vinden welke gerelateerd is met acute afstoting en we hebben onderzocht of we calcineurin inhibitor (CNI) gerelateerde nierfalen na levertransplantatie konden voorspellen middels een genetische marker. Tevens hebben we meer inzicht gekregen in de longitudinale kinetiek van circulerende T cellen die volgens de directe route allo-antigenen herkennen door gebruik van nieuwe benaderingen. Ook hebben we de invloed van CMV infectie op donor specifieke T-cel responsen en acute afstoting na levertransplantatie onderzocht. Tenslotte beschrijven we een nieuwe benadering voor de ontwikkeling van een diagnostische test om T-cellen met indirecte allospecificiteit te kwantificeren; welke uiteindelijk gebruikt zou kunnen worden als hulpmiddel om immunosuppressiva veilig te indivudualiseren.

Acute afstoting komt in 27% tot 58% van de levertransplantatie patiënten voor, afhankelijk van bepaalde factoren als type donororgaan (levende of post mortem donor) en de sterkte van de immunosuppressie [20,21]. Prognostische factoren voor het ontwikkelen van acute afstoting zouden behulpzaam zijn in het individueel doseren van de immunosuppressiva. Hoofdstuk 2 beschrijft de invloed van twee functionele SNPs in het CTLA-4 gen op de proportie acute afstoting na levertransplantatie. Volledig intacte CTLA-4 moleculen gecodeerd door het +49 allel, worden onvolledig geglycosyleerd dat leidt tot retrograde transport van een deel van de moleculen naar het cytoplasma voor degradatie, resulterende in verminderde expressie van CTLA-4 op het T-cel membraan en verminderde inhibitoire functie van CTLA-4 op T-cel activatie. Dit gebeurt in patiënten die homozygoot zijn voor het +49 G allel. Het +6230G/A SNP heeft invloed op de productie van de oplosbare alternatieve vorm van CTLA-4 (sCTLA-4) waarin het +6230G allel sCTLA-4 in lagere snelheid produceert dan het +6230A allel. Omdat sCTLA-4 allogene T-cel activatie remt is het aannemelijk dat dragers van het +6230G allel een hogere risico lopen voor het krijgen van afstoting na levertransplantatie. In dit hoofdstuk tonen wij een relatie tussen +6230 SNP en acute afstoting waarbij in de groep levertrans-

plantatie patiënten die homozygoot voor CTLA-4 +6230A zijn en waarbij het G-allel ontbreekt, minder acute afstoting voorkomt dan de groep levertransplantatie patiënten die drager zijn van het G allel. Echter, haplotype analyse toonde dat enkel dragerschap van het +6230G allel geen risicofactor is voor het ontwikkelen van acute afstoting. Het +6230G allel is enkel geassocieerd met acute afstoting als het gecombineerd voorkwam als haplotype met het +49A allel. Dus, de genetische predispositie voor het risico allel voor acute afstoting na levertransplantatie (+49A/+6230G) is een combinatie van verminderde capaciteit van sCTLA-4 productie (+6230G) met een normale capaciteit van intact CTLA-4 expressie (+49A) op het T-cel membraan. De invloed van deze haplotype op acute afstoting is dosis afhankelijk, betekenende dat ieder extra gedragen allel resulteert in een hoger risico op acute afstoting. Dit wordt verder onderbouwd door de observatie dan dragers van één +49A/+6230A allel en één +49G/+6230G allel een proportie acute afstoting hebben dat tussen het risico +49A/+6230G haplotype en homozygote dragers van +49A/+6230A en +49G/+6230G haplotypen in ligt. Hoewel het relatieve risico van +49A/+6230G zowel dosis afhankelijk als significant is, is een relatieve risico van 1.34 voor ieder additionele risico haplotype te laag om dit haplotype te gebruiken als hulpmiddel om immunosuppressiva af te bouwen. Een bescheiden 2% van de patiënten waren homozygoot voor het risico allel. Niettemin, toont deze studie aan dat zelfs tijdens het gebruik van immunosuppressiva, het CTLA-4 gen betrokken is bij de regulatie van anti-donor immuun respons na levertransplantatie. Voor het verkrijgen van een robuuste prognostische genetische marker zouden alle functionele SNPs in overige immuun regulatoire genen bepaald moeten worden voor een volledige haplotype analyse.

Een ander veelvoorkomend probleem in levertransplantatie patiënten is chronische nierziekten (CKD) waarvan de prevalentie varieert tussen de 20-70% [12-13], welke is gerelateerd aan verhoogde morbiditeit en mortaliteit [14,22-26]. Polymorfismen in het ABCB1 en CYP3A5 gen die een rol spelen in de metabolisering van CNIs zouden de ontwikkeling van CKD na transplantatie kunnen beïnvloeden. In hoofdstuk 3 hebben we de relatie tussen SNP ABCB1 3435C>T en CYP3A5 6986A>G met de incidentie van CKD na levertransplantatie geëvalueerd. Achtendertig procent van de LT patiënten ontwikkelden CKD, een percentage dat overeenkomt met voorgaande studies (20-70%). De allel frequenties van de onderzochte SNPs waren vergelijkbaar met eerder gepubliceerde studies. Onze data laten zien dat zowel het *CYP3A5* 6989A>G als het *ABCB1* 3435C>T genotype van zowel donor als patiënt geen associatie hebben met het ontstaan van CKD na levertransplantatie. Alhoewel, kunnen we niet uitsluiten dat we geen associatie hebben kunnen tonen door de relatief lage aantallen patiënten die in deze studie zijn geïncludeerd. Niettemin, presenteert onze studie wel het grootste volwassen non-renaal orgaan transplantatie cohort, waarin is gekeken naar de associatie tussen zowel donor als ontvanger CYP3A5 en/of ABCB1 SNP en het risico op CKD na transplantatie. Echter,

de resultaten van voorgaande studies waarin wel associaties tussen *CYP3A5* 6989A>G en *ABCB1* 3435C>T SNPs met CKD werden getoond [27-29], worden in dit hoofdstuk niet bevestigd. Verschillen in studie populatie (etniciteit, leeftijd), type CNI dat gebruikt is en primaire definities van eindpunten waarnaar gekeken is zijn mogelijke oorzaken voor onze resultaat. De meeste studies naar de relatie tussen CNI en ontstaan van CKD na orgaan transplantatie zijn verricht in cohorten met niertransplantatie patiënten [30-36], waarin confounding factoren als recidief van primaire nierziekte, ischemie-perfusie schade tijdens transplantatie, orgaan afstoting en de kwaliteit van het niertransplantaat de associatie met CKD mogelijk hebben beïnvloedt.

T-cellen die allo-antigenen via de directe route herkennen, worden geactiveerd door donor afgeleide APC, die van het donororgaan naar de secundaire lymfoide organen in de ontvanger migreren [37-40]. Dit is een tijdelijk proces, welke zowel in dieren als humane studies is aangetoond [37,39,41]. In dierenexperimenten is aangetoond dat de respons van T-cellen met directe allospecificiteit gedurende de vroege post-transplantatie periode domineert waarna het uitdooft [42]. Maar in humane transplantatie setting zijn er enkel aanwijzingen voor dit effect uit crossectionele studies [43-48]. Er was geen beschrijvende studie naar de kinetiek van de allogene T-cel responsen in mensen. De kinetiek van de directe-route in levertransplantatie ontvangers wordt beschreven in hoofdstuk 4, door gebruik te maken van een nieuwe technische benadering. Onze data laat voor de eerste keer zien dat er een stijging is van donorspecifieke T-cel frequenties kort na levertransplantatie. Aanvullend tonen wij in dit hoofdstuk dat T-cellen met directe allospecificiteit tenminste 1 jaar na transplantatie nog aanwezig zijn in frequenties vergelijkbaar met die van vooraf aan transplantatie (pre-transplantatie niveaus). Eerdere studies beschrijven een snelle daling van de T-cellen met directe allospecificiteit, waarin de frequenties binnen een maand dalen naar onder het pre-transplantatie niveau [48]. Onze studie toont aan dat de directe route langer actief blijft en dat het mogelijk een rol speelt in afstoting episodes die tot een jaar na levertransplantatie ontstaan. Om deze hypothese te bewijzen, zouden bloedmonsters bij levertransplantatie patiënten genomen moeten worden ten tijde van afstotingsreacties, welke vervolgens onderzocht dienen te worden naar de frequentie van T-cellen met directe allospecificiteit. Het verschil tussen onze observatie en voorgaande observaties zou verklaard kunnen worden door de krachtigere donor-afgeleide stimulator cellen die zijn gebruikt, welke hebben geresulteerd in een gevoeligere test vergeleken met eerder beschreven testen. Om deze vorm van technische benadering te gebruiken als diagnostische leidraad ter individualisering van immunosuppressiva, zou eerst de relatie tussen de circulerende T-cellen met directe allospecificiteit en episoden van late acute afstoting onderzocht moeten worden.

De exacte mechanisme en de cellen die een rol spelen in immunologische tolerantie voor allogene (lever) transplaten is nog steeds onduidelijk. Studies hebben laten zien

dat tolerante LT patiënten een expansie van de Vδ1+ γδT cellen vertonen met daaraan gerelateerde transcriptionele profielen in perifeer bloed [9,10,49]. Expansie van Vδ1+ γδT cellen wordt ook gezien na een CMV infectie [50-52], wat zou kunnen betekenen dat er mogelijk een relatie bestaat tussen CMV infectie en levertransplantaat tolerantie. T_{EMRA} accumulatie is een typisch kenmerk van CMV geïnduceerd ouder en zwakker wordende immuunsysteem[53-54].Tevens is accumulatie van CD8+ T_{EMRA} gerelateerd aan verhoogde risico op infecties bij ouderen en een zwakke respons op vaccinaties, wat een zwak immuun systeem suggereert [55-56]. Aan de andere kant CMV specifieke memory T cellen kunnen schadelijk zijn voor het donororgaan indien zij kruisreactiviteit vertonen tegen allogene HLA moleculen [57]. Ondanks de vele studies die zijn verricht is het tot op heden nog steeds onduidelijk hoe CMV infectie de T-cel alloreactiviteit beïnvloed. In hoofdstuk 5 laten we zien dat CMV infectie na LT geassocieerd is met accumulatie van gedifferentieerde effector memory (T_{EM}) CD4+ en CD8+ T-cellen en met CD8+ terminaal gedifferentieerde effector memory cellen (T_{EMRA}) in perifeer bloed in het eerste half jaar na levertransplantatie. Meest belangrijke observatie is dat 6 maanden post-LT, hyporesponsiviteit van circulerende CD8+ T cellen tegen donor allo-antigenen die via de directe route gepresenteerd worden, in patiënten met een primaire CMV infectie. Deze observatie suggereert dat deze patiënten wellicht een soort van immunologische tolerantie voor hun donororgaan hebben ontwikkeld. Dit wordt ondersteund door de observatie dat LT patiënten met primaire CMV infectie een hogere ratio circulerende Vδ1:Vδ2 γδT-cellen hebben vergeleken met patiënten die geen CMV infectie hebben en patiënten die reeds CMV IgG positief vooraf aan de levertransplantatie waren. Ook tonen we in dit hoofdstuk dat patiënten met een primaire CMV infectie minder kans op late episodes van acute afstoting hebben. Directe pathway donorspecifieke respons in patiënten met primaire CMV infectie was bij observatie in alle CD8+ subsets, behalve in te T_{EMRA} hoog. Blijkbaar zijn T_{EMRA} niet erg schadelijk voor lever transplantaten, welk mogelijk verklaard kan worden door de observatie dat zij niet goed het transplantaat kunnen infiltreren. Onze data laat een onverwachte rol zien van primaire CMV infectie op de ontwikkeling van hyporesponsiviteit van CD8+ T-cellen tegen allo-antigenen die via de directe pathway gepresenteerd worden. Welk mechanisme ervoor zorgt dat CMV infectie invloed heeft op de deze donorspecifieke hyporesponsiviteit van de CD8+ T-cellen is niet duidelijk. CMV staat bekend om zijn gebruik van diverse strategieën voor invasie van het immuunsysteem, om aan te zetten tot latentie, in het bijzonder door het moduleren van antigeen presentatie [58]. Terwijl de dendritische cellen in levertransplantaten voornamelijk de aanstichters zijn van T-cel immuniteit tegen het transplantaat [40,59], zijn CMV geïnfecteerde dendritische cellen niet in staat om allogene lymfocyten respons te stimuleren [60,61]. Alhoewel, het myelosuppressieve effect van ganciclovir behandeling mag niet genegeerd worden. Het exacte mechanisme dat resulteert in dit fenomeen zou verder opgehelderd moeten worden door verder onderzoek.

Hoofdstuk 6 is een methodologische beschrijving over de ontwikkeling van een robuuste test om T-cellen met indirecte allospecificiteit te bepalen. Indirecte pathway allopeptide herkenning is respons van ontvanger T-cellen op donor antigenen, die bewerkt zijn en als peptiden in eigen MHC moleculen gepresenteerd worden. Bewijs voor deze pathway van afstoting werd geleverd door de observatie dat transplantaat afstoting nog steeds kon plaatsvinden in experimentele diertransplantatie modellen, waarbij de immunogene donorgeleide cellen in het transplantaat afwezig waren. [62,63]. Allo-antigenen die worden afgeworpen van het transplantaat worden geïnternaliseerd en verwerkt op dezelfde manier als exogene antigenen. Vervolgens worden zij door ontvanger APC als peptiden in eigen MHC klasse II moleculen aan T-helper cellen gepresenteerd. Ontvanger APCs migreren continue naar het donor transplantaat alwaar donor afgeleide peptiden worden aangetroffen, vervolgens geïnternaliseerd, verwerkt en gepresenteerd aan ontvanger T-cellen in perifere lymfeklieren en in de milt. Experimentele diertransplantaat modellen laten zien dat de frequentie van T-cellen met indirecte allospecificiteit lager is dan T-cellen met directe allospecificiteit en dat de T-cellen met indirecte respons later pieken dan T-cellen met directe respons [64]. Hoewel de omvang van de indirecte T-cel respons lager is, is het wel voldoende om afstoting te induceren [63]. Huidige testen om T-cellen met indirecte allospecificiteit te kwantificeren zijn relatief ongevoelig en staat de accuraatheid ter discussie [65]. Door de complexheid van de indirecte pathway, is het lastig om deze in vitro na te bootsen. Daarom is er weinig bekend met betrekking tot de contributie van T –cellen, die antigeen herkennen via de indirecte pathway, op transplantaat afstoting en in de ontwikkeling van tolerantie voor transplantaten. Verschillende technieken voor detectie van T-cellen met indirecte allospecificiteit zijn beschreven. Sommige studies gebruiken donor cel fragmenten om ontvanger APC te laden met allo-antigenen. Een voordeel is dat donor cel fragmenten theoretisch het gehele HLA-repertoire omvatten. Maar de HLA-specificiteit van de responderende T-cel is onbekend en er is bewijs dat intacte donor HLA-moleculen nog kunnen voorkomen in gefragmenteerde donor cel suspensies, welke tot gevolg kunnen hebben dat zij via de directe pathway gepresenteerd kunnen worden waardoor er T-cellen met directe allospecificiteit geïnduceerd kunnen worden bij dit soort in vitro stimulaties [66]. Andere studies gebruiken synthetische donor HLA peptiden om ontvanger APC te laden. Een nadeel van deze benadering is dat het onmogelijk is om voldoende verschillende peptiden toe te voegen welke het totale aantal gemismatchte donor HLA-moleculen bevatten. Omdat we ook niet weten welke donor HLA peptiden er in vitro gepresenteerd worden, zou het kunnen zijn dat gesynthetiseerde peptiden neo-epitopen bevatten welke in vivo niet voorkomen. Om deze redenen hebben wij een nieuwe benadering bestudeerd om T-cellen met indirecte allospecificiteit te meten, waarbij we ontvanger APC door middel van gen overdracht geladen hebben met donor HLA. Hiervoor hebben we een DNA construct gemaakt welke geschikt is voor in

vitro transcriptie en welke codeert voor het gehele HLA-A*0201 sequentie, behalve het transmembraan domein, waardoor er wordt voorkomen dat er intacte HLA-A*0201 tot expressie wordt gebracht. Electroporatie is gekozen als de techniek voor gen transfectie vanwege de hoge efficiëntie vergeleken met overige transfectie methoden. CD40 geactiveerde B-cellen werden gekozen als ontvanger APC, omdat deze cellen vanuit kleine aantallen PBMCs kunnen worden opgekweekt tot grote hoeveelheden. In dit hoofdstuk laten we zien dat in het beginsel deze benadering van antigeen laden veelbelovend is; drie verschillende CD4+ T cel klonen, waarbij ieder een HLA-A*0201 peptide herkent in de context van een HLA-DR*0101 molecuul, reageerden ieder afzonderlijk op HLA-A*0201-HLA-DR*0101+ CD40 B-cellen die met HLA-A*0201 mRNA geelectroporeerd waren. Door toevoeging van anti-MHC klasse II antilichaam aan deze reactie laat zien dat de CD4+ respons compleet teniet wordt gedaan, wat aantoont dat de activatie van deze T-cel klonen MHC-klasse II afhankelijk is. Tevens, na electroporatie van HLA-A*0201-CD40 B-cellen met HLA-A*0201 coderende mRNA waarvan het transmembraan gedeelte van het α3 domein ontbreekt, observeerden we dat HLA-A*0201 intracellulair gemeten kon worden en niet op het cel oppervlak. Dit toont aan dat directe presentatie van intacte HLA moleculen hiermee voorkomen wordt. Een accurate test voor het detecteren van indirecte T cel alloresponsen is essentieel ter bepaling van de kinetiek en klinische relevantie van deze T-cellen in transplantaat afstoting. Als deze T-cellen met indirecte allospecificiteit significant bijdragen aan afstoting, dan zou een robuuste test kunnen helpen bij het identificeren van patienten waarin immunosuppressie veilig afgebouwd kan worden. Verder onderzoek zou aan moeten tonen dat de benadering die wij beschrijven accuraat genoeg is om CD4+ T-cel responsen met indirecte allospecifciteit te kwantificeren.

De voornaamste resultaten van dit onderzoek zijn:

1. CTLA-4 +49A/+6230G haplotype is een co-dominant risico allel voor acute afstoting na levertransplantatie. Dit impliceert dat zelfs onder immunosuppressiva, CTLA-4 invloed heeft op de regulatie van humane immuun respons tegen allogene transplantaten.
2. Genetische variaties in CYP3A5 en ABCB1 genen, zijn geen significante risicofactoren voor de ontwikkeling van CKD na LT. Het genotyperen van LT ontvangers of diens donoren voor SNPs in deze genen zullen geen inzicht leveren ter preventie van CKD.
3. Door een nieuwe technische benadering hebben we voor de eerste keer aangetoond dat er kort na levertransplantatie een stijging van donorspecifieke T-cel frequenties ontstaat. Daarnaast hebben we laten zien dat met deze nieuwe benadering T-cellen met directe allospecificiteit nog na 1 jaar na transplantatie detecteerbaar zijn in bloed.
4. Patiënten met primaire CMV infectie ontwikkelen een donorspecifieke T-cel hyporesponsiviteit waardoor deze patiënten beschermd zijn voor het ontwikkelen van

late acute afstoting. Dit zou een teken kunnen zijn dat CMV infectie tolerantie voor allogene levertransplantaten in mensen kan bevorderen.

5. We beschrijven nieuwe methode om T-cellen met indirecte allospecificiteit te meten waarbij CD40 B-cellen worden geelectroporeerd met mRNA van gemismatchte donor HLA-genen wat resulteert in succesvolle specifieke respons van deze T-cellen.

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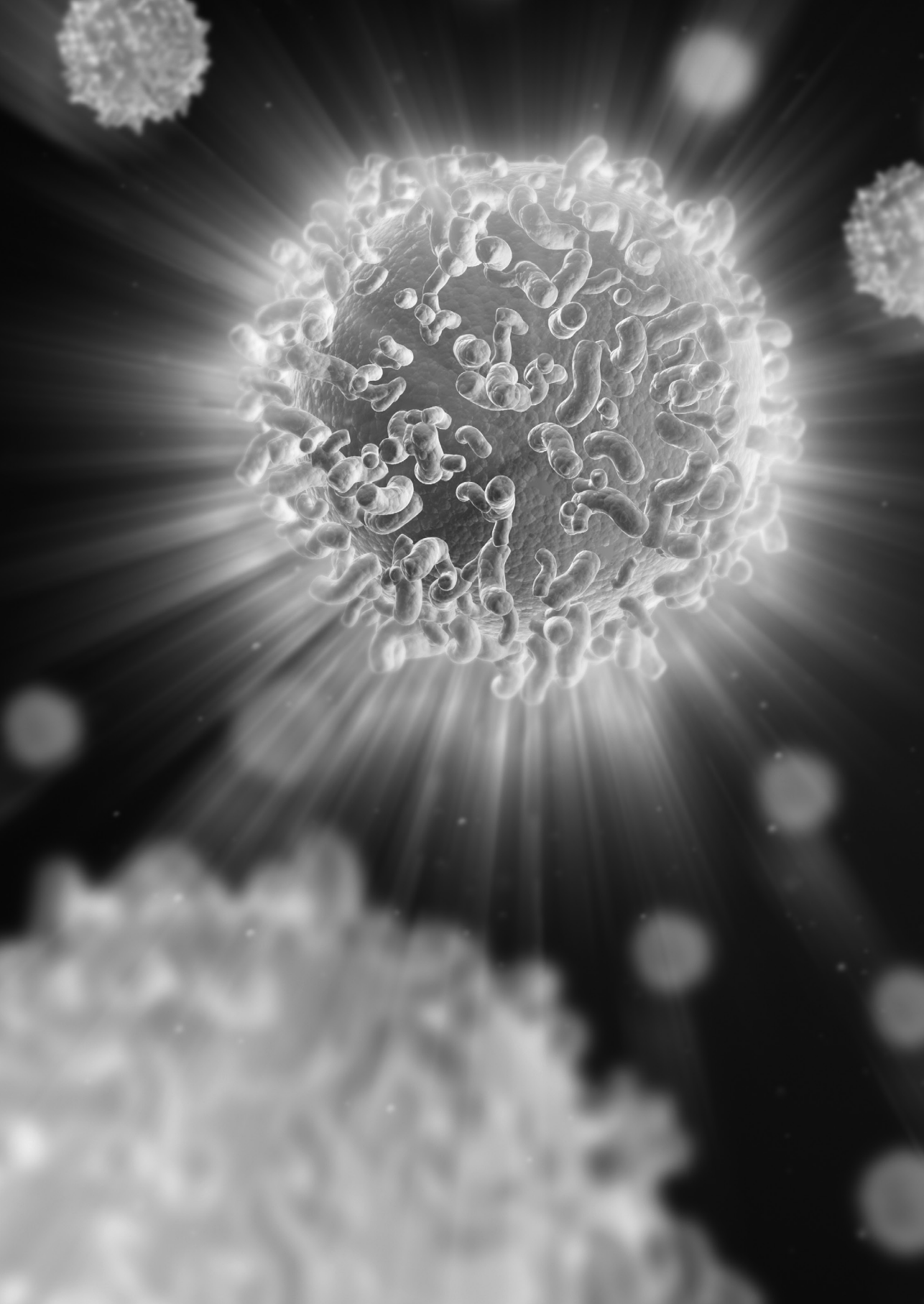

Chapter 9

Appendix

Acknowledgements/Dankwoord
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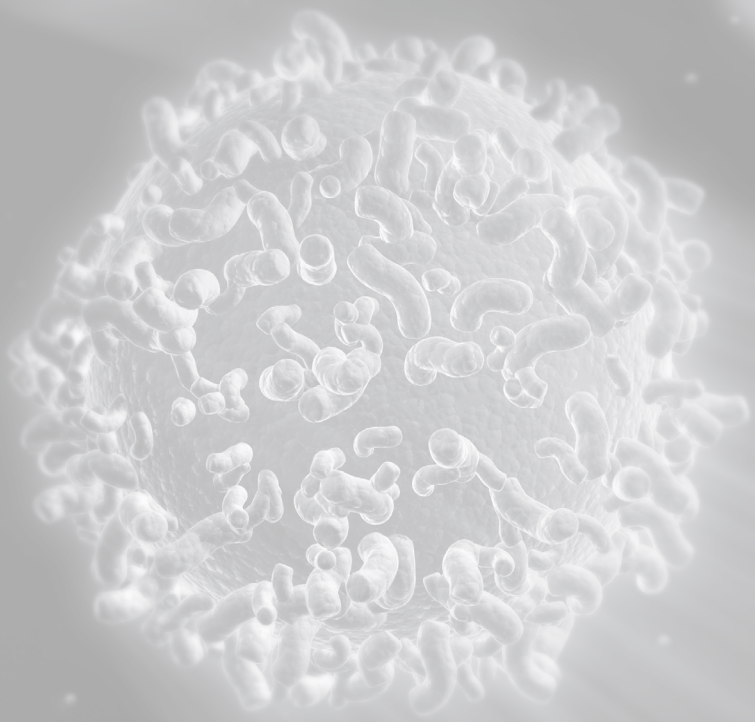

Acknowledgements/dankwoord

Dit proefschrift zou niet tot stand zijn gekomen zonder de hulp van vele mensen die ik graag zou willen bedanken.
Prof. Dr. Metselaar. Beste professor, dank u voor de kansen die u mij heeft gegeven. Uw klinische visie op dit basaal wetenschappelijk onderzoek heeft tot doelgerichte aanpak geleid. Ik wil u bedanken voor uw geduld en begeleiding bij dit proefschrift.
Dr. Kwekkeboom, beste Jaap, ik heb kennis met je gemaakt toen ik als student in het levertransplantatieteam kwam werken om biopten te verzamelen voor labonderzoek. Jouw wetenschappelijke enthousiasme heeft ertoe geleid dat ik zowel mijn afstudeer- als mijn promotie-onderzoek in de basale wetenschap heb verricht. Ik wil je ontzettend bedanken voor je geduld en je begeleiding bij dit proefschrift.
Prof. dr. Tilanus, beste professor, dank u voor uw altijd positieve en geïnteresseerde houding in mijn onderzoek.
Prof. dr. Van Gelder, prof. dr. Baan en prof. dr. Claas, ik dank jullie voor het beoordelen van dit proefschrift en jullie bereidheid om zitting te nemen in de commissie.
Dr. Van der Laan, beste Luc, dank voor je kritische commentaar en suggesties tijdens de werkbesprekingen en op mijn manuscripten.
Prof. dr. Peppelenbosch, beste Maikel, dank voor je adviezen en suggesties bij het afronden van dit proefschrift.
Dr. Hesselink, beste Dennis, dank je voor je hulp bij het schrijven van hoofdstuk 3. Ik sta nog steeds versteld van het feit dat je zelfs tijdens je vakantie in het buitenland vanuit een oude boerderij met slechte internetverbinding, binnen no time het manuscript had gelezen en kritisch had becommentarieerd.
Prof. dr. Weimar, beste professor Weimar, dank u dat u bereid bent zitting te nemen in de commissie. Het is een eer om u als commissielid te hebben.
Prof. dr. Thielemans, beste Kris, dank voor uw gastvrijheid en begeleiding op het laboratorium in Brussel. Ik bewonder uw kennis, inzicht en passie voor de wetenschap.
NWO wil ik graag bedanken voor het toekennen van de Mozaikbeurs, waarmee het tot stand komen van dit proefschrift mogelijk is gemaakt.
Mijn collega`s van de levertransplantatiegroep. Dear Viviana, I`m glad that I`ve known you. Even though, I didn`t keep in touch with you after you moved to Greece, I often think back to the times we have spent together, in which we had long conversations about life, sport, goals, work, happiness. And not to forget: food! Angela, jij bent mij enkele weken geleden vooraf gegaan, goed gedaan. Ik heb prettig met je samengewerkt, dank voor je gezelligheid op het werk en tijdens congressen. Emmeloes, in de laatste fase van mijn onderzoek heb ik je leren kennen, toen begon jij net aan je promotie. Veel succes met het afronden van jouw proefschrift. Xiaolei, you continued some work on the “indirect pathway”. Good luck with your PhD. Abdullah, I admire your scientific

knowledge and insight. Thank you for your help. Suomi, dank voor je gezelligheid op het lab en tijdens borrels en feesten. Petra, analist, promovendus, kunsthistoricus en nu celliste in wording, inspirerend... Veda, thank you for your conversations and company during trips and lunchbreaks. Scott, thank you for your help in understanding vector design.

Kamergenoten, MC Anthonie, dank voor de gezellige sfeer en de goede muziek die we in onze kamer hadden dankzij jouw zeer uitgebreide internationale collectie. Paula, dank voor je bereidheid tot hulp en gezelligheid. Mooie posters hebben we weten te ontwerpen mede dankzij jouw photoshop ervaringen. Dowty and Yi thank you for your company, sometimes till late at night in the lab. Thanya, we hebben kort een kamer gedeeld, dank voor je opgewektheid en enthousiasme voor de wetenschap.

Dan andere MDL lab collega`s; Lianne, Anouk, Bisheng, Aniek, Kim, Duygu, Arjan, Nadine, Mark, Eric, Jin, Jasper, Werner, Kim, Gertine, Jan, Greta, Martine, Buddy, Elvira, Wendy, Hanneke, Ayala dank voor jullie samenwerking op het lab en gezelligheid tijdens de lunchpauzes en labuitjes.

Andre, Andrea en Ron, dank voor jullie hulp en kritische vragen op het onderzoek.

Leonie en Raymond, het secretariaat en onmisbaar voor het draaiende houden van het lab. Joanne, dank voor je inzet tijdens het studentenproject.

Dames van het levertransplantatie secretariaat, dank voor jullie goede zorg, attentheid bij het verzamelen van patiëntenmateriaal. Marion, dank je voor je administratieve ondersteuning in de laatste fase.

Onderzoeksgroep in Brussel. Carlo, dank voor je begeleiding en het maken van ingewikkelde genetische constructen. Karine, Aude, Elsy, dank voor jullie samenwerking en begeleiding tijdens mijn verblijf in Brussel.

Onderzoeksgroepen te Leiden; dr. Miriam Heemskerk, dr.Illias Doxiadis, dr. Avital Amir, dr. Dave Roelen, dank jullie wel voor jullie medwerking en suggesties.

Medewerkers van het transplantatielab; Meindert, Martin, Ramin, Rens, Marieke, dankjulliewel voor jullie hulp en gezelligheid bij uitjes en congressen. Jeroen Gerritsen, dank je wel voor je begeleiding bij het inzetten van de Elispot. Nicole van Besouw en Nicolle Litjens, dank jullie wel voor jullie laagdrempelige bereidheid tot hulp.

Mijn collega`s in het St. Elisabeth Ziekenhuis en in het Tweesteden Ziekenhuis. Dank voor jullie interesse en goede werksfeer. Het is geen makkelijk jaar geweest. Ik hoop nog veel van jullie te mogen leren.

Mijn paranimfen Patrick en Shanta, ik ben ontzettend blij dat jullie op deze bijzondere dag naast mij staan. Patrick, voor een tijd ook mijn kamergenoot. Ik heb ontzettend veel van je geleerd. Onze ochtendstart begon met het samen doornemen van de Telegraaf en het becommentariëren van het nieuws. Ontzettend veel dank voor je vele hulp en steun tijdens het werk. Je was overal voor in, ook voor het inzetten van ellelange proeven buiten werktijd. Maar ook voor vrijdagmiddagborrels en congressen. Shanta, analist

en kloppend hart van het levertransplantatieteam. Dank je voor je hulp bij het inzetten van vele proeven. Het was een fijne samenwerking.
Mijn vriendinnen Linda en Tessa, dank jullie voor jullie morele steun. Wij kennen elkaar vanaf de tweede klas van het middelbaar onderwijs en hebben samen al veel meegemaakt. Hoewel we de afgelopen jaren elkaar niet meer zo vaak zien, is het altijd weer gezellig en als 'vanouds' als we elkaar ontmoeten. We moeten snel weer afspreken (en vaker, ik woon weer in de buurt :))
Vriendinnen en collega`s Özlem en Hatice, dank jullie wel voor jullie gezelligheid en de steun. Jullie ontvangen mij altijd met open armen en zijn altijd bereid om mij een luisterend oor te bieden, maakt niet uit hoe laat het is. Onze koffiemomentjes waren en zijn nog steeds erg waardevol, inspirerend en motiverend. Ik hoop dat er nog vele mogen volgen.
Nazli, vriendin en collega, dankje voor je interesse en steun. Ik vergeet nooit het moment dat je in mijn studentenkamer mijn CD collectie bekeek en zeer verrast was dat, naast jijzelf, ook ik, als één van de weinige 'Turken', albums van Jeff Buckley en Tori Amos in haar collectie had. Op cultureel vlak hebben wij veel gemeen, hopende nog vele inspirerende uitstapjes te mogen maken.
Aria, mijn ex-carpoolmaatje, collega, vriendin, dank je voor je hulp en steun. Ik mis iedere ochtend en avond onze gesprekken in de auto.

Mijn familie…
Woorden schieten tekort als ik mijn waardering voor jullie onvoorwaardelijke en steun en hulp moet beschrijven.
Ooms, tantes, neefjes en nichtjes; tijdens mijn verblijf in Rotterdam heb ik dankzij jullie nooit heimwee naar "thuis" gehad. Ik was ten alle tijden welkom voor gezelligheid of voor een maaltijd. Dank jullie wel voor jullie goede zorgen, steun en interesse.
Mijn zusje Gülgün, dankjewel voor je luisterend oor en het feit dat je altijd bereid bent om te helpen, hoe ver weg ik ook van je ben. Ik wens jou, Hasan Hüseyin, Yusuf Emin, Sara-on the way- en misschien wel nog één (of twee?), een hele mooie toekomst samen.
Mijn broertje, Hacı Osman, dankjewel voor je steun en motivatie. Je kalme uitstraling brengt mij vaak tot rust als ik weer eens chaotisch ben in mijn hoofd. Ik wens jou en Nazmiye veel geluk samen. Mijn broer, Senol abi, a.k.a. de stropdassenman, boekhouder, evernote watcher, kettlebell fanaat, blogger. Mijn tegenpool, ik introvert, jij extravert. Ik bewonder je creativiteit en het enthousiasme waarmee jij over je werk en hobby's vertelt en je doorzettingsvermogen om verder te studeren en steeds maar weer nieuwe examens af te leggen. Ik wens jou, Filiz yenge, Hamdi jr. en Mikail al het goede toe.
Annem ve babam, sizin desteğiniz ve dualarınız olmasaydı, ben asla bugünkü vardığım yere varamazdım. Bana bütün istediğim imkanları tanıdığınız için gönülden çok teşekkür ederim. Sadece bir teşekkür az, fakat bu içimdeki duyguyu nasıl dile getirebileceğimi

bilemiyorum, kelimeler eksik geliyor. Allah sizlerden razı olsun, sizleri başımızdan eksik etmesin.

Özlem

List of publication

Ö. Tapirdamaz, V. Pravica, H.J. Metselaar, B.E. Hansen, L. Moons, J.B.J. van Meurs, I.V. Hutchinson, J. Shaw, K. Agarwal, D.H. Adams, C.P. Day, and J. Kwekkeboom
Polymorphisms in the T cell regulatory gene cytotoxic T lymphocyte antigen 4 influence the rate of acute rejection after liver transplantation
Gut 2006, Jun;55(6):863-8. Epub 2005 Nov 18

Ö. Tapirdamaz , S. Mancham, L.J.W. van der Laan, G. Kazemier, K. Thielemans, H.J. Metselaar,
J. Kwekkeboom
Detailed kinetics of the direct allo-response in human liver transplant recipients: new insights from an optimized assay
PlosOne 2010 Dec 29;5(12):e14452

Ö. Tapirdamaz, D.A. Hesselink, S. el Bouazzaoui, M. Azimpour, B. Hansen, L.W.J. van der Laan, W. Polak, J. Kwekkeboom, R.H.N. van Schaik, T. van Gelder, H.J. Metselaar
Genetic variance in ABCB1 and CYP3A5 does not contribute to the development of chronic kidney disease after liver transplantation
Pharmacogenetics and Genomics 2014 Sep;24(9):427-35

X. Shi, E.L.D. de Mare-Bredemeijer, **Ö. Tapirdamaz**, B.E. Hansen, R. van Gent, M. J.H. van Campenhout, S. Mancham, N.H.R. Litjens, M. Betjes, A. A. van der Eijk, Q. Xia, L.J.W. van der Laan, J. de Jonge, H.J. Metselaar, J. Kwekkeboom
CMV primary infection is associated with donor-specific T-cell hyporesponsiveness and fewer late acute rejections after liver transplantation
Submitted for publication

PhD Portfolio

Courses (Erasmus University Medical Center):

Basic Immunology (2008)
Biostatistics for Clinicians (2008)
Regression analysis for Clinicians (2008)
Stralingshygiëne, deskundigheidsniveau 5B (2009)
English Biomedical Writing and Communication (2010)

Presentations at conferences:

The American Transplantation Congress, Seattle, USA (oral presentation, 2006)
Bootcongres, Zeewolde, The Netherlands (oral presentation, 2009)
European Society of Organ Transplantation, Paris, France (poster presentation, 2009)
European Immunology Congress, Berlin, Germany (oral presentation, 2009)
Dutch Experimental Gastroenterology & Hepatology, Veldhoven, The Netherlands (poster presentation, 2010)
Annual meeting of the Transplantation Society, Vancouver, Canada (oral presentation, 2010)

Scientific awards and grants:

NWO Mosaic (2006)
Travel grant Dutch transplantation society (2009)
Travel grant Trustfonds (2009)
International basic science Mentor-Mentee travel award of the Transplantation Society (2010)

About the author

Özlem Tapirdamaz was born on the 20th of January in Eindhoven. In 1999 she started her medical school at the Erasmus University in Rotterdam. In 2004 she performed her graduation research project on "CTLA-4 polymorhisms and acute rejection after liver transplantation" under supervision of dr. J. Kwekkeboom and in collaboration with prof. I. Hutchinson. For this project she performed the experiments at the department of Immunology and molecular sciences at the School for Biological Sciences at Manchester, UK, where she got interested in basic science. The results of this project were presented in 2005 at the annual meeting of the Dutch organization of Gastroenterology and Hepatology, where she received the student award for best oral presentation. In 2006 she received the Mosaic grant of the Netherlands Organization for Scientific Research (NWO) and started her PhD in 2007 under supervision of prof. dr. H.J. Metselaar and dr. J. Kwekkeboom. From 2011 till 2013 she worked as a medical resident at the department of internal medicine at Albert Schweitzer Hospital, Dordrecht.

Currently she`s working as a medical resident at the Emergency departments of the St. Elisabeth and Tweesteden Hospital, Tilburg, where she will start her education in 2015 to become an emergency physician.