NEW INSIGTHS IN IMMUNE REGULATION AFTER LIVER TRANSPLANTATION

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Colophon

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TABLE OF CONTENTS

CHAPTER 1

General introduction and outline of the thesis

Liver Transplantation

Liver transplantation is a highly successful treatment for patients with malignant and non-malignant end-stage liver diseases. About 6000 liver transplant procedures are performed each year in Europe and in the United States[1], with a 5-year survival of more than 70% in most centers compared with 20% in the mid-1980s[2]. One of the keys to such success is the improvement of post-transplant immunosuppression strategies to prevent graft rejection. Before the advent of cyclosporine, the rate of acute rejection was as high as 80% at day 5-7 after liver transplantation and was in some cases a life-threating event[3]. The introduction of cyclosporine-based immunosuppression protocol decreased the acute rejection risk to 30-40% and chronic rejection risk to 10-15% in the early 1990s. With the current immunosuppression protocols, which mainly involve tacrolimus, mycophenolate mofetil (MMF) and corticosteroids, the rate of acute rejection is only 20% and a rate of chronic rejection 5%. However the long-term use of immunosuppression regimens leads to complications such as infections, *de novo* cancer, cardiovascular diseases, metabolic syndrome and impaired renal function[4-12]. Therefore immunosuppression minimization or complete withdrawal is advocated to improve the long-term outcomes of transplanted patients[13]. Unlike other transplanted organs, the liver has a tolerogeneic nature. For example, liver grafts can be accepted without the need of immunosuppression in pigs, and in some rat and mice strains[14-16]. In humans, transplantation of liver is possible without matching of major histocompatibility complex (MHC) antigens, and even across ABO blood groups between recipient and donor[17, 18]. Furthermore transplantation of liver can protect the other transplanted organs derived from the same donor from rejection[19]. and about 20 % of stable liver patients can be weaned of immunosuppression without losing the allograft[20-23]. Unfortunately, no accurate parameters are available to discriminate tolerant from non-tolerant liver transplant patients. Therefore it is crucial to understand the regulation of allogeneic immune responses with regard of liver transplantation in humans, in order to facilitate the individualization of patient management, and to contribute to a better long-term outcome.

Allorecognition by T lymphocytes

The immune system of humans has evolved to distinguish self from non-self. Transplantation of tissues or organs between genetic disparate individuals of the same species exerts strong and complicated immune responses targeting the allogeneic antigens, which are mainly major histocompatibility (MHC) molecules. T lymphocytes (T cells) are the main instigators of allogeneic immune responses, which can be categorized into cytotoxic T cells ($CD8^+$ T cells) and T helper cells ($CD4^+$ T cells). It has been known for more than three decades that there are mainly two pathways of allorecognition involved in activation of T cells against MHC molecules: the direct pathway and the

indirect pathway[24, 25] (Figure 1). The direct pathway describes the recognition of intact allogeneic MHC molecules expressed on donor cells by recipient T cells, while the indirect recognition pathway describes a more "natural" process of T-cell activation, in which recipient APCs take up alloantigens from the transplanted organ and present them as peptides to recipient $CD4^+$ T cells. Recently, another semi-direct pathway of alloantigen recognition has been suggested, in which recipient T cells recognize intact donor MHC molecules taken up and presented by recipient antigen-presenting cells (APC)[26, 27]. It has been hypothesized that the direct pathway dominates the allogeneic immune responses early after transplantation and is the main cause of early acute rejection. In contrast, the indirect pathway takes over later after transplantation and contributes to the occurrence of chronic rejection[28-32]. Therefore, to promote a better long-term patient outcome, the understanding and monitoring of indirect allogeneic immune responses is absolutely crucial and may help to personalize immunosuppressive medication. For this purpose, an assay to detect indirect T cells with high sensitivity and specificity is needed.

Infection and transplant rejection

Infections with bacteria and virusses are common after organ transplantation due the compromised immune system of the patients. Exposure to microbes before transplantation and infection after transplantation are accompanied by changes of both the innate and adaptive immune systems of the body, and can thereby directly or indirectly influence allogeneic immune responses and outcome after transplantation. Unlike experimental animals raised in a pathogen-free environment, humans are exposed to large numbers of microbial species, generating pools of memory T cells which may be cross-reactive to allogeneic antigens, a phenomena termed as "heterologous immunity"[33, 34] (Figure 2). As cross-reactive memory T cells are more resistant to immunosuppression than naive T cells[33, 35], heterologous immunity following infections has been hypothesized to be a significant barrier to the induction of transplantation tolerance. In addition, infections at the time of or after transplantation, can result in the engagement of pattern-recognition receptors (PRRs) on antigen-presenting cells (APCs) and thereby induce various signals and cytokines, resulting in enhanced alloreactivity of T cells[36, 37]. Infections occurring late after transplantation can also provide various pro-inflammatory signals that re-activate tolerant T cells, enabling their escape from immunoregulatory mechanisms of tolerance, and finally leading to allograft rejection[38, 39]. Indeed in clinical practice, correlations between increased incidence of infections and acute rejection have been observed[40], however it is difficult to define a causal relationship[38].

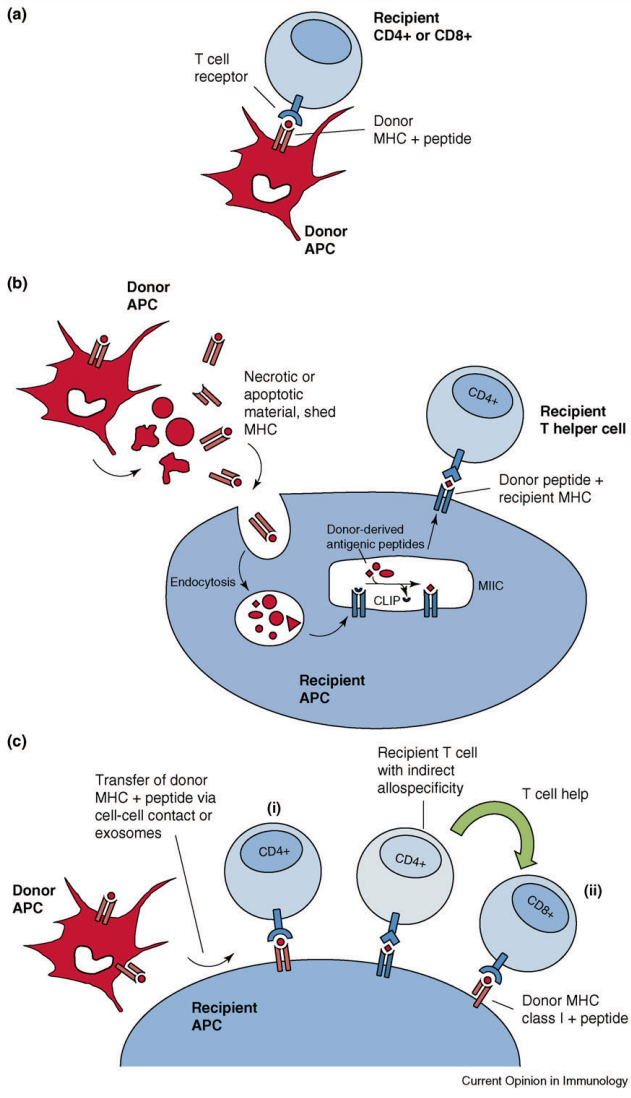

Figure 1. Pathways of allorecognition. In the direct pathway (a), intact donor MHC:peptide complexes on donor APCs are recognized by recipient T cells, with $CD4^+$ cells recognizing MHC class II, and $CD8^+$ cells recognizing class I molecules. The indirect pathway (b) involves the processing and presentation in peptide form of donor allogeneic MHC by recipient APCs. Allogeneic material is taken up by recipient APCs by endocytosis, and processed to form peptide fragments. In the MHC class-II-enriched compartment (MIIC), donor-derived peptide displaces class-II-associated invariant chain peptide (CLIP) on the surface of recipient MHC class II molecules. The complex of recipient MHC and donor peptide is then expressed on the cell surface, where it is recognized by self MHC-restricted $CD4^+$ T helper cells. The semi-direct pathway (c) results from the transfer of intact donor MHC-peptide complexes from donor to recipient APCs, either through cell–cell contact or exosomes. $CD4^+$ T helper cells can recognize donor MHC class II displayed in this way on recipient APCs (i); the uptake of intact donor MHC class I molecules can allow the activation of $CD8^+$ T cells and $CD4^+$ T cells with indirect or direct allospecificity on the surface of the same APC (ii). **Adapted from** ***Gökmen et al. Curr Opin Immunol 2008***

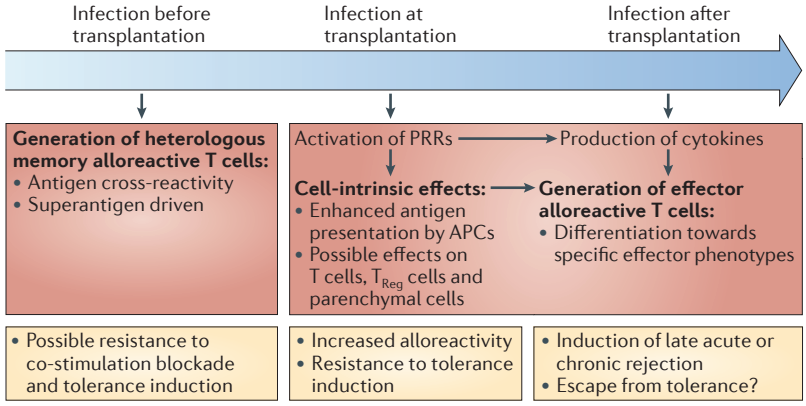

Figure 2. Possible effects of infections before, at and after transplantation. Some T cells specific for microbial peptides presented by self MHC molecules can cross-react with allogeneic MHC molecules, and bacterial superantigens can directly activate large populations of T cells. Therefore, infections experienced before transplantation can give rise to heterologous memory alloreactive T cells that may be more resistant to immunosuppression than naive T cells. The engagement of pattern-recognition receptors (PRRs) on antigen-presenting cells (APCs), T cells and/or parenchymal cells at the time of or after transplantation can induce various signals and cytokines. This can result in enhanced priming, survival and expansion of alloreactive T cells, as well as dictating the phenotype of differentiating alloreactive T cells. Infections occurring late after transplantation may elicit pro-inflammatory signals that activate tolerant T cells by enabling their escape from immunosuppression and/or peripheral mechanisms of tolerance, thereby precipitating rejection. **Adapted from *Chong et al. Nat Rev Immunol 2012***

A good example of this relationship is human cytomegalovirus (CMV). CMV, also known as human herpesvirus-5 (HHV-5), is a member of the β-herpesvirus subfamily, and one of the most common viruses among humans with an extremely high worldwide prevalence[41, 42]. The infection of CMV is generally subclinical, except in immunocompromised individuals, such as patients after organ transplantation. CMV infection after organ transplantation can cause a febrile syndrome with leukopenia and/or thrombocytopenia (CMV syndrome) as well as organ involvement, and is putatively associated with other opportunistic infections, malignancies and graft rejection[43]. Therefore, antiviral prophylaxis or preemptive therapies are widely used to prevent and control CMV infection in organ transplant patients[44, 45]. Evidence from experimental animal models show that infection or re-activation of CMV abrogates the establishment of transplant tolerance[46, 47], however evidence in humans have been less consistent[48]. Despite that, infection with CMV may have a systemic immunosuppressive effects in humans[49-51]. How CMV infection alters the immune responses towards a transplanted organ in humans is not known.

Co-stimulatory and co-inhibitory regulation of T-cell function

T cells are the key players and regulators of the adaptive immune system, the regulation of which is crucial to maintain the equilibrium between pathogen elimination and self-tolerance. Three signals are need to fully activate T cells. The first signal is the recognition of antigenic peptides presented by major histocompatibility complex (MHC) molecules by the T-cell receptor (TCR)[52]. The second is the antigen-independent co-stimulatory signals provided by APCs, which mainly involves the interaction between CD28 on the T cell and CD80/CD86 molecules on the APCs[53, 54]. Without signal two, T-cells will become anergic even in the presence of signal one[55]. The third signal is provided a number of cytokines produced by APCs which enables T-cell proliferation and differentiation[56]. The discovery of co-inhibitory receptors in recent years complicated our understanding of this model of T-cell activation[57]. In contrast to co-stimulatory receptors, co-inhibitory receptors negatively regulate T-cell functions, such as PD-1, CTLA-4, TIM-3, LAG-3, 2B4 (CD244), and CD160 (Figure 3). In some settings, such as chronic infections and malignancies, co-inhibitory receptors expressed on antigen-specific T cells restrict effective immune responses that are needed to clear the pathogens or tumor cells[58-60]. Monoclonal antibodies targeting PD-1, or its ligand PD-L1, have been shown to be effective in treatment of several types of cancer, and have been recently approved by the U.S. Food and Drug Administration (FDA)[61-63]. However in other settings, such as autoimmune diseases and organ transplantation, potent co-inhibitory regulatory pathways might help to limit the detrimental immune responses. For example, PD-1/PD-L1 pathway has been shown to be critical in the maintenance of liver transplant tolerance in animal models[64, 65], and agonistic targeting of this pathway which might have therapeutic potential[66, 67]. However pre-clinical evidence supporting the relevance of PD-1/PD-L1 pathway in the human transplant setting is still lacking.

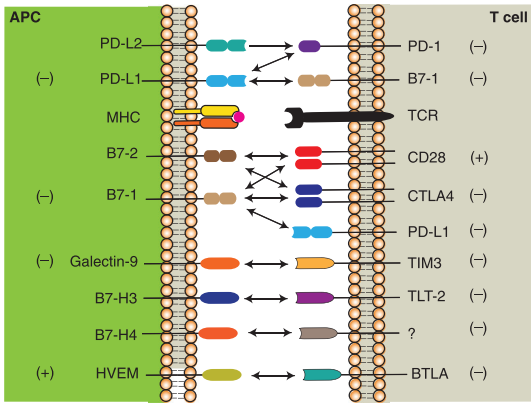

Figure 3. Co-inhibitory signals between antigen-presenting cells (APCs) and T cells. Representative illustration of the main co-inhibitory interactions with details about direction of signal and binding partners. **Adapted from *Murakami et al. Transplantation 2014***

Mixed hematopoietic chimerism

Chimerism can be defined as a phenomenon in which cells from one individual are present in another genetically disparate individual, which may occur after organ and hematopoietic stem cell transplantation. Several types of chimerism after liver transplantation have been described, including (1) recipient-derived cells in the transplanted organ[78, 79]; (2) donor-derived hematopoietic cells in the recipient peripheral blood[80-82]; and (3) donor-derived cells in the skin and lymph nodes of recipient[83]. The clinical relevance of the development of chimerism is not fully understood. It has been hypothesized that achieving chimerism might lead to a tolerogenic immune status against allografts, and therefore it has been considered as a strategy to achieve transplant tolerance in the clinical setting[84]. Indeed, successful tolerance induction has been reported in HLA-matched or mismatched renal transplant patients by inducing transient or durable mixed chimerism[85]. However other studies suggest that chimerism does not necessary correlate with graft tolerance[86]. In a recent study Wang et al. studied the blood chimerism in a large cohort liver transplant patients and analyzed the putative hematopoietic stem/progenitor cells (HSPCs) in adult human livers[87]. The authors concluded that there are two types of chimerisms in LT patients: transient chimerism resulting from mature donor leucocytes, and long-term chimerism derived from putative donor-derived HSPCs in the liver graft, and suggested that liver is a good ectopic niche for extra-marrow hematopoiesis. Still, the possibility of long-lived hematopoietic chimerism in the liver allograft has not been examined.

Aim of the thesis

The transplantation of human livers to save patients with end-stage liver diseases is always accompanied by immune responses trying to eliminate the transplanted organ, which are shaped and regulated by several environmental and genetic factors. Therefore, a better knowledge of the immune regulation in patients after liver transplantation is crucial to facilitate developing interventions to achieve a better long-term patient outcome. In this thesis, we study environmental and genetic factors that influence allo-reactivity in liver transplant patients, investigate their impact on clinical outcomes, and suggest novel perspectives that may help to personalize patient immunosuppressive management to minimize adverse effects of life-long standard immunosuppressive medication.

In chapters 2 and 3, we studied the impact of cytomegalovirus infection after liver transplantation on the allogeneic T-cell responses and graft rejection. Next in chapter 4, we summarize and discuss the recent evidence supporting beneficial aspects of cytomegalovirus infection on human disease and health. Chapter 5 studies the influence of genetic variations in the co-inhibitory PD-1/PD-L1 pathway in regulation of allogeneic

T-cell responses and graft rejection in human liver transplant recipients. Chapter 6 describes a novel assay which may help monitoring T cells that indirectly recognize major allo-antigens in transplanted patients, using messenger RNA electroporation. In chapter 7, we examine the phenomenon of hematopoietic chimerism within human liver allograft. Finally in chapter 8, we summarize and discuss in depth the work of this thesis.

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CHAPTER 2

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

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ABSTRACT

Viral infections, including cytomegalovirus (CMV), abrogate transplantation tolerance in animal models. Whether this also occurs in humans remains elusive. We investigated how CMV affects T cells and rejection episodes after liver transplantation (LT). Phenotype and alloreactivity of peripheral and allograft-infiltrating T cells from LT patients with different CMV status were analyzed by flow cytometry. The association of CMV status with early and late acute rejection was retrospectively analyzed in a cohort of 639 LT patients. CMV-positivity was associated with expansion of peripheral effector memory T-cell subsets after LT. Patients with CMV primary infection showed donor-specific $CD8^+$ T-cell hyporesponsiveness. While terminally differentiated effector memory cells comprised the majority of peripheral donor-specific $CD8^+$ T cells in CMV primary infection patients, they were rarely present in liver allografts. Retrospective analysis showed that R^-D^+ serostatus was an independent protective factor for late acute rejection by multivariate Cox regression analysis (hazard ratio=0.18, 95%CI=0.04-0.86, *P*=0.015). Additionally, CMV primary infection patients showed the highest Vδ1/Vδ2 γδ T-cell ratio, which has been shown to be associated with operational tolerance after LT. In conclusion, our data suggest that CMV primary infection may promote tolerance to liver allografts, and CMV status should be considered when tapering or withdrawing immunosuppression.

INTRODUCTION

Cytomegalovirus (CMV) is a prevalent β-herpesvirus that establishes 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 inconsistent results[6]. No consensus has been made on the effect of CMV infection on graft rejection or tolerance after liver transplantation (LT).

Acute rejection (AR) is primarily initiated by recipient T lymphocytes (T cells) that recognize nonself 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 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[16-18]. In addition, we performed a retrospective study in a cohort of 639 LT patients, to analyze the association 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 between 2009 and 2012 at Erasmus MC, The Netherlands (Supplemental Table 1). Liver allograft biopsies were obtained from explants of 10 patients that underwent re-LT (Supplemental Table 2), and 9 heathy donor livers prior to LT. 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 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. Immunosuppression therapies are described in Supplemental Materials and Methods.

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 described in Supplemental Materials and Methods.

Quantification of alloreactive T-cell frequencies

Alloreactive T cells were analyzed by determination of activation-induced CD137 expression after allogeneic stimulation[19, 20](Supplemental Figure 1). Protocol is described in Supplemental Materials and Methods.

CMV diagnostics, treatment, and patient groups

CMV serostatus of patients and donors was determined as part of the standard diagnostic routine. CMV viremia was determined by CMV-DNA polymerase chain reaction (PCR) assay, and 50 copies/mL was considered as the threshold for positive result. Patients included in the *ex vivo* T-cell analysis were grouped as: CMV-negative (R^-D^-), primary infection (R^-D^+), and R^+ patients. CMV primary infection was confirmed by detection of viremia or IgG seroconversion after LT, and CMV-negative was defined as no detection of viremia nor IgG seroconversion prior to blood collection (Supplemental Table 1). No differences in baseline characteristics were observed between groups in all experiments. Detailed CMV diagnostics and treatment strategies are available in Supplemental Materials and Methods.

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

Variable	Total n=639	R^-D^- n=127	R^-D^+ n=122	R^+ n=390	*P*-value
Recipient age, years	50 (16-71)	47 (17-68)	48 (16-71)	51 (16-67)	0.121
Recipient, female	264 (41%)	46 (36%)	54 (44%)	164 (42%)	0.039
Recipient BMI, kg/m^2	25 (16-43)	24 (17-39)	24 (17-37)	25 (16-43)	0.299
Primary liver diseases					< 0.001
AHF	119 (19%)	23 (18%)	25 (20%)	71 (18%)	
HCC	64 (10%)	10 (8%)	10 (8%)	44 (11%)	
PBC/PSC/AIH	149 (23%)	40 (31%)	37 (30%)	72 (18%)	
HBV/HCV	84 (13%)	5 (4%)	2 (2%)	77 (20%)	
Alcoholic cirrhosis	71 (11%)	14 (11%)	17 (14%)	40 (10%)	
Cryptogenic cirrhosis	41 (6%)	10 (8%)	8 (7%)	23 (6%)	
Others	111 (17%)	25 (20%)	23 (19%)	63 (16%)	
Donor age, years	46 (8-78)	43 (13-73)	45 (12-72)	46 (8-78)	0.149
Donor, female	333 (52%)	49 (39%)	61 (50%)	223 (57%)	0.001
DCD donor	49 (8%)	8 (6%)	9 (7%)	32 (8%)	0.775
Cold ischemia time, minutes	470 (114-1099)	476 (120-1099)	479 (114-988)	455 (133-913)	0.441
Warm ischemia time, minutes	36 (14-143)	34 (14-106)	35 (17-143)	37 (16-129)	0.121
Re-LT	75 (12%)	15 (12%)	9 (7%)	51 (13%)	0.233
Basiliximab as induction immunosuppression	375 (59%)	82 (65%)	72 (59%)	221 (57%)	0.290
Calcineurin inhibitor					0.645
Cyclosporin A	249 (39%)	55 (43%)	46 (38%)	148 (38%)	
Tacrolimus	367 (57%)	70 (55%)	68 (58%)	229 (59%)	
CMV prophylaxis	104 (16%)	-	104 (85%)	-	< 0.001
CMV viremia	145 (23%)	4 (3%)	59 (48%)	82 (21%)	< 0.001
Timing of CMV viremia detection after LT, days	35 (2-2502)	30 (17-35)	45 (22-288)	31 (2-2502)	0.001
Timing of first CMV viremia after LT < 180 days	134 (92%)	4 (100%)	54 (92%)	76 (93%)	0.703
Peak CMV-DNA copy number, copies/mL*	2.8 (1.8-6.8)	5.0 (4.0-6.8)	2.7 (1.8-5.3)	2.8 (1.9-6.5)	0.031
CMV IgG seroconversion**	97 (20%)	14 (14%)	83 (86%)	-	< 0.001
Early acute rejection	144 (23%)	26 (20%)	29 (24%)	89 (23%)	0.805
Timing of early acute rejection, days	9 (2-166)	8 (2-155)	9 (3-147)	8 (3-166)	0.999
Late acute rejection	41 (6%)	11 (9%)	2 (2%)	28 (7%)	0.048
Timing of late acute rejection, days	487 (186-6368)	997 (208-2967)	353 (186-521)	379 (206-6368)	0.138

* Log_{10} transformed
** Only patients with follow-up ≥ 180 days were taken into account (R^-D^- n=101, R^-D^+ n=97, R^+ n=299; total n=497), and from 9 patients serology data after LT were not available (R^-D^- n=5, R^-D^+ n=4).

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 EAR was defined as rejection occurring within 180 days after LT, 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

Patient baseline characteristics were summarized using median with range for continuous variables and percentage for discrete variables. Differences between groups were compared by Pearson Chi-Square test or one-way Kruskal-Wallis test. Experimental data were analyzed using nonparametric Mann-Whitney U test (unpaired) or Wilcoxon matched pairs test (paired) when comparing two groups, and one-way Kruskal-Wallis test (unpaired) or Friedman test (paired) with Dunn's multiple comparison test when comparing three groups. In the retrospective analysis, EAR and LAR were used as separate endpoints. The cumulative incidences of EAR and LAR were estimated using the Kaplan-Meier method with log-rank test. Analysis of risk factors for EAR and LAR was performed using Cox proportional-hazards regression model with likelihood ratio test. 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-positivity 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 with distinct CMV status 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 (Figure 1A)[10]. Patients were grouped based on CMV status (R^-D^- n=7, R^-D^+ n=10, R^+ n=17), and distribution of $CD8^+$ (Figure 1B) and $CD4^+$ (Figure 1C) T-cell subsets at three time points were compared.

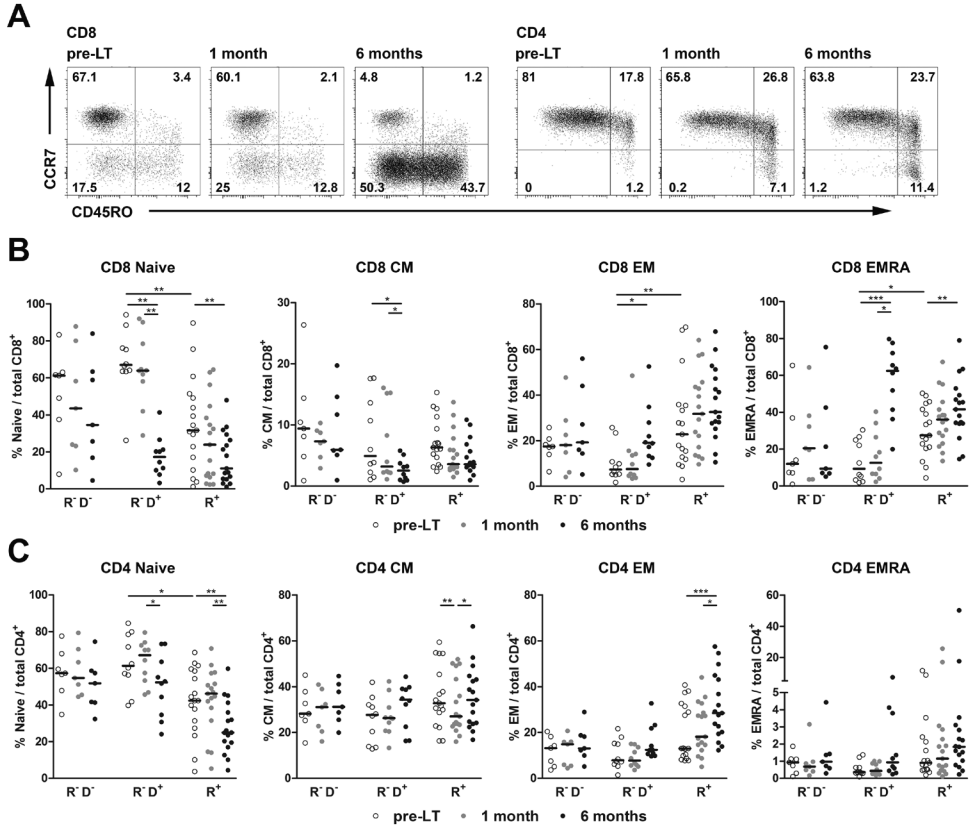

Figure 1. Changes of circulating CD4⁺ and CD8⁺ T-cell subsets after LT in relation to patient CMV status. (A) T-cell subsets were defined as T_{Naive} (CCR7⁺CD45RO⁻), T_{CM} (CCR7⁺CD45RO⁺), T_{EM} (CCR7⁻CD45RO⁺), and T_{EMRA} (CCR7⁻CD45RO⁻), shown as representative FACS plots from one patient. Patients were grouped based on CMV status (R^-D^- n=7, R^-D^+ n=10, R^+ n=17). Distribution of (B) CD8⁺ and (C) CD4⁺ T-cell subsets before (white), at 1 month (grey) and at 6 months (black) after LT were compared. Horizontal lines indicate median values. **P*<0.05, ***P*<0.01, ****P*<0.001.

The proportion of both CD4⁺ and CD8⁺ T-cell subsets remained stable in CMV-negative patients (R^-D^-). However in CMV primary infection (R^-D^+) and R^+ patients, the proportion of CD8⁺ T_{Naive} and T_{CM} decreased continuously within the first 6 months, while the percentages of CD8⁺ T_{EM} and T_{EMRA} increased. Particularly CD8⁺ T-cell subsets from R^-D^+ patients underwent the most dramatic changes, characterized by rapidly increasing percentage of T_{EMRA} (pre-LT 9%, 6 months 62%; median values). When dividing all patients at increased risk for CMV-replication (R^-D^+ and R^+) by proven viremia, increasing percentage of CD8⁺ T_{EMRA} were observed in both groups (Supplemental Figure 2), suggesting that the T_{EMRA} increase was a result of CMV-positivity, rather than CMV-replication. However it was likely that not all viremia episodes were detected in

R^+ patients. The change in $CD8^+$ T-cell subset distribution was a manifestation of effector memory T-cell expansion rather than a selective disappearance of naive T cells, as absolute numbers of T_{EM} and T_{EMRA} increased in R^-D^+ and R^+ patients, while the absolute numbers of T_{Naive} remained relatively stable (Supplemental Figure 3). As for the $CD4^+$ compartment, the percentages of T_{Naive} also decreased within 6 months in CMV primary infection and R^+ patients, which was mainly compensated by an increased percentage of T_{EM}. Together, these data show that CMV-positivity 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 (Supplemental Figure 1A, B). The numbers of donor-recipient and third party-recipient HLA-mismatches were similar between groups (Supplemental Figure 4).

Overall $CD8^+$ T cells showed donor-specific hyporesponsiveness (Figure 2A, left panel). We did not observe higher frequencies of alloreactive T cells in CMV primary infection or R^+ patients than in CMV-negative patients. Unexpectedly, patients with primary infection showed prominent donor-specific hyporesponsiveness in $CD8^+$ T cells (Figure 2B, left panel), which was evident in T_{Naive}, T_{CM} and T_{EM} (Figure 2C). In contrast, no donor-specific hyporesponsiveness was observed in CMV-negative patients, while in R^+ patients significant donor-specific hyporesponsiveness was only observed in T_{EM}. $CD4^+$ T cells did not show any significant donor-specific hyporesponsiveness (Figure 2A, right panel), and the frequencies of alloreactive $CD4^+$ T cells were also similar in patients with distinct CMV status (Figure 2B, right panel).

When focusing on patients with proven viremia, donor-specific hyporesponsiveness was only seen in R^-D^+ patients, and the frequency of donor-specific $CD8^+$ T cells in R^-D^+ patients were lower than that in R^+ patients (Supplemental Figure 5), indicating that the donor-specific hyporesponsiveness was not due to CMV-replication as such. However, the peak CMV-DNA copy number tended to be negatively associated with the frequency of donor-specific $CD8^+$ T cells (P=0.06), but not with third-party reactive $CD8^+$ T cells or alloreactive $CD4^+$ T cells (Supplemental Figure 6).

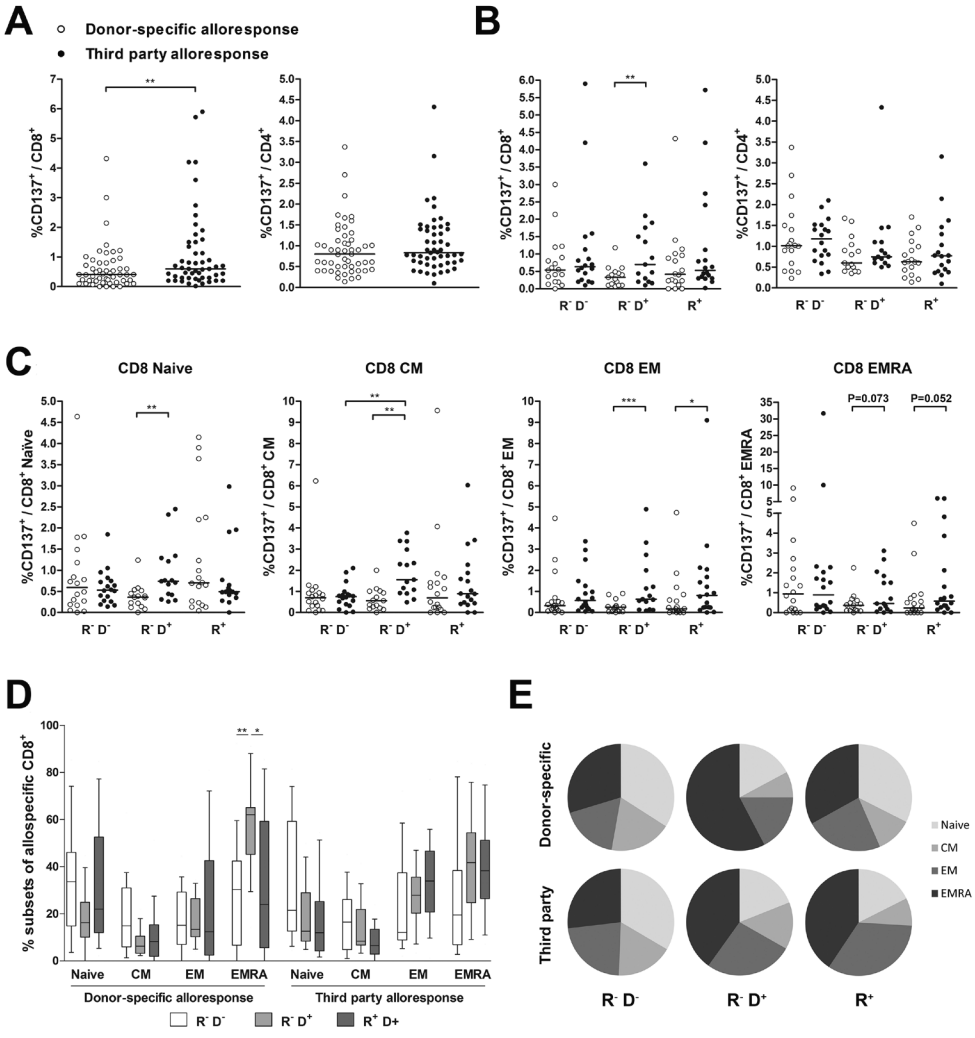

Figure 2. CMV primary infection is associated with the development of donor-specific CD8⁺ T-cell hyporesponsiveness after LT. The frequencies of peripheral alloreactive CD8⁺ and CD4⁺ T cells were analyzed by flow cytometric determination of CD137 expression on T cells after allogeneic stimulation. (A)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

Furthermore, we assessed the subset composition of alloreactive $CD8^+$ T cells ($CD8^+CD137^+$ T cells) (Supplemental Figure 1B). Compared to CMV-negative and R^+ patients, donor-specific T cells from patients with CMV primary infection were predominantly T_{EMRA} (R^-D^- 29.7%, R^-D^+ 57.7%, R^+ 33.1%; mean values) (Figure 2D, E), which is possibly due to the robust donor-specific hyporesponsiveness in other subsets except for T_{EMRA}.

$CD8^+$ T_{EMRA} are a minor T-cell population infiltrating liver allografts

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 patients with CMV primary infection, we wondered whether $CD8^+$ T_{EMRA} are abundantly present in liver allografts and thereby contribute to allograft rejection. To investigate this, we isolated intrahepatic lymphocytes (IHLs) from liver allografts which were explanted during re-LT (n=10; Supplemental Table 2). IHLs isolated from heathy donor livers prior to LT were used as healthy control. IHLs contained mainly $CD4^+$ and $CD8^+$ T_{EM} and hardly any T_{Naive} and T_{CM} (Figure 3A, B). $CD8^+$ T_{EMRA} were present in the liver allografts but accounted for a significantly smaller proportion than in paired PBMC samples and IHLs of healthy donor livers (Figure 3B). The ratio of $CD8^+$ T_{EM} and T_{EMRA} in liver allografts was 5-fold higher than that in peripheral blood, and 1.7-fold higher than that in healthy donor livers (median values, Figure 3C). Explant allografts with AR activity tended to have lower percentages of $CD8^+$ T_{EMRA} and a higher $CD8^+T_{EM}/T_{EMRA}$ ratio (Supplemental Figure 7). Sufficient amounts of IHLs were isolated from two of the liver explants to measure their allogeneic responses (Figure 3D). $CD8^+$ T_{EM} were enriched for donor-specific T cells, as 20% and 33.9% of $CD8^+$ T_{EM} from these 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 stimulation were subtracted). In contrast, $CD8^+$ T_{EMRA} contained less alloreactive cells than T_{EM}. Altogether, these data indicate that T_{EM} preferentially infiltrate liver allografts, while $CD8^+$ T_{EMRA} largely remain in the circulation.

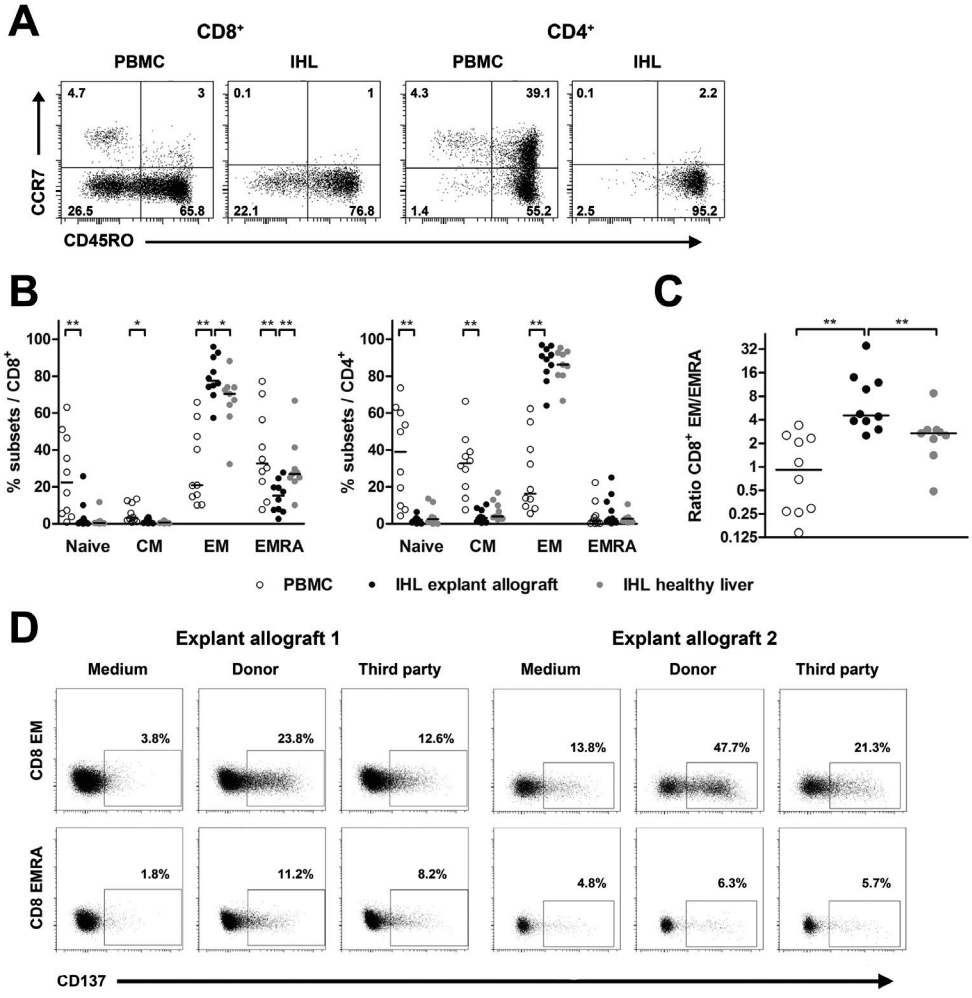

Figure 3. CD8⁺ T_{EMRA} are a minor T-cell population infiltrating liver allografts. Intrahepatic lymphocytes (IHLs) were isolated from explant liver allografts to study the subset composition of graft infiltrating T cells. CD8⁺ and CD4⁺ T-cell subsets of IHLs of explant allografts (n=10) were compared to those of paired PBMCs, and IHLs of healthy donor livers (n=9), and are shown (A) as representative FACS plots (PBMCs and paired explant IHLs) from one patient and (B) are summarized. (C) Ratios CD8⁺ T_{EM} and T_{EMRA} were also compared between groups. 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 both allografts. *P<0.05, **P<0.01.

CMV R^-D^+ status is associated with the protection against late acute rejection

To study whether the donor-specific T-cell hyporesponsiveness that we observed in CMV primary infection patients has any clinical impact, 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 have been variably used in literature as the cut-off to define EAR and LAR[21]. The first episodes of CMV viremia were detected at median 35 days after LT, 92% 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; range 2-166 days), and 41 (6.4%) developed LAR (median 487 days; range 186-6368 days) (Table 1).

EAR and LAR were set as separate endpoints for risk factor analysis. CMV serostatus was not associated with the cumulative incidence of EAR (P=0.77) (Figure 4A), while the incidence of LAR in R^-D^+ patients was lower than in R^-D^- patients (*P*=0.014) and R^+ patients (*P*=0.017) (Figure 4B). In univariate Cox regression analysis, recipient age (*P*=0.007), recipient BMI (*P*=0.026), female donor (*P*=0.034), warm ischemia time (*P*<0.001), basiliximab induction (*P*<0.001), tacrolimus as CNI (*P*<0.001), and CMV viremia (*P*=0.008) were significantly associated with EAR (Supplemental Table 3). Meanwhile, CMV serostatus (*P*=0.015) was the only factor associated with LAR (Table 2).

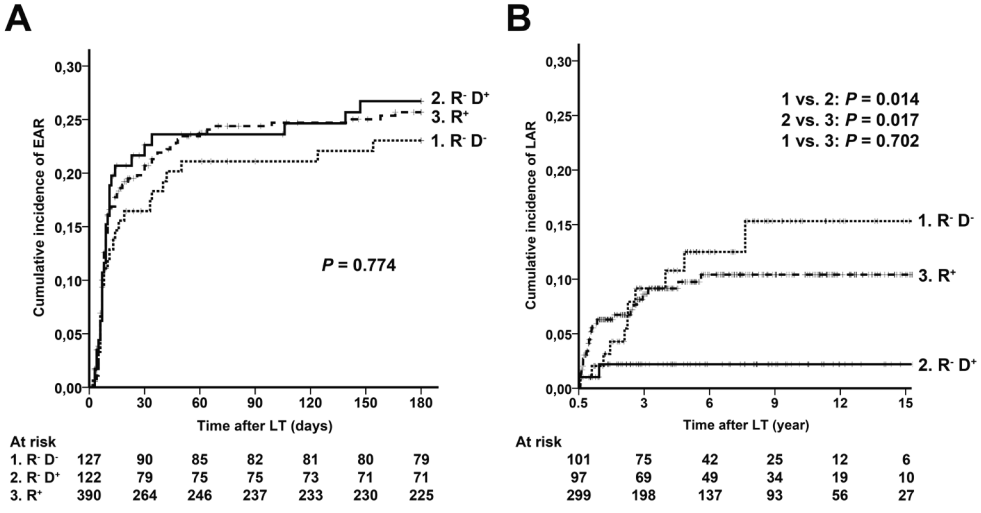

Figure 4. CMV R^-D^+ status is associated with the protection against late acute rejection. Patients were grouped based on CMV serostatus before LT as follows: Group 1: R^-D^-; Group 2: R^-D^+; Group 3: R^+. The cumulative incidences of (A) early acute rejection (EAR) and (B) late acute rejection (LAR) were estimated using the Kaplan-Meier method and differences between curves were analyzed using the 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			0.217
HBV/HCV (Ref)	1.00			1.00		
AHF	1.45	(0.49-4.34)		1.75	(0.57-5.44)	
HCC	0.29	(0.03-2.50)		0.35	(0.04-2.99)	
PBC/PSC/AIH	1.54	(0.56-4.29)		1.90	(0.67-5.43)	
Alcoholic cirrhosis	0.49	(0.10-2.54)		0.61	(0.12-3.16)	
Cryptogenic cirrhosis	2.04	(0.59-7.07)		2.22	(0.63-7.87)	
Others	0.81	(0.24-2.81)		1.05	(0.30-3.72)	
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	0.69	(0.36-1.31)	0.258
Early acute rejection	0.90	(0.44-1.86)	0.781			
CMV serostatus			0.015			0.015
$R^- D^-$ (Ref)	1.00			1.00		
$R^- D^+$	0.18	(0.04-0.81)		0.18	(0.04-0.86)	
R^+	0.87	(0.43-1.76)		0.99	(0.47-2.05)	
CMV viremia	0.87	(0.42-1.83)	0.711	1.10	(0.50-2.43)	0.813
Peak copy number during viremia	1.07	(0.55-2.09)	0.846			

In the multivariate Cox regression analysis, recipient age (*P*=0.046, hazard ratio [HR]=0.99), female donor (*P*=0.020, HR=0.67), basiliximab induction (*P*=0.015, HR=0.59), tacrolimus as CNI (*P*<0.001, HR=0.47), and CMV viremia (*P*=0.018, HR=1.62) were considered as independent factors associated with EAR (Supplemental Table 3). In contrast, CMV serostatus was the only independent factor associated with LAR (*P*=0.015; HR for R^-D^+=0.18; HR for R^+=0.99) (Table 2). CMV seroconversion and the use of CMV prophylaxis were not included in the analysis as they largely overlapped with R^-D^+ status, and yielded the same association as R^-D^+ status (data not shown). Altogether, these data indicate that CMV primary infection after LT protects patients against the occurrence of LAR, corroborating the *ex vivo* data on donor-specific T-cell hyporesponsiveness that we observed in CMV primary infection patients.

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

To further investigate whether patients with CMV primary infection show signs of tolerance, we measured peripheral Vδ1 and Vδ2 γδ T-cell subsets in patients at minimum 6 months after LT, and calculated the Vδ1/Vδ2 γδ T-cell ratio, which has been shown to be associated with operational tolerance after LT[16-18]. CMV primary infection patients contained the highest percentage of Vδ1 γδ T cells within peripheral $CD3^+$ cells, while CMV-negative patients contained the lowest (R^-D^- 0.73%, R^-D^+ 5.36%, R^+ 2.29%; median values) (Figure 5A). In contrast, the percentages of Vδ2 γδ T cells were similar (R^-D^- 0.56%, R^-D^+ 0.27%, R^+ 0.39%; median values) (Figure 5B). As a result, patients with CMV primary infection showed higher Vδ1/Vδ2 γδ T-cell ratio than the other two groups (R^-D^- 1.39, R^-D^+ 10.81, R^+ 6.31; median values) (Figure 5C).

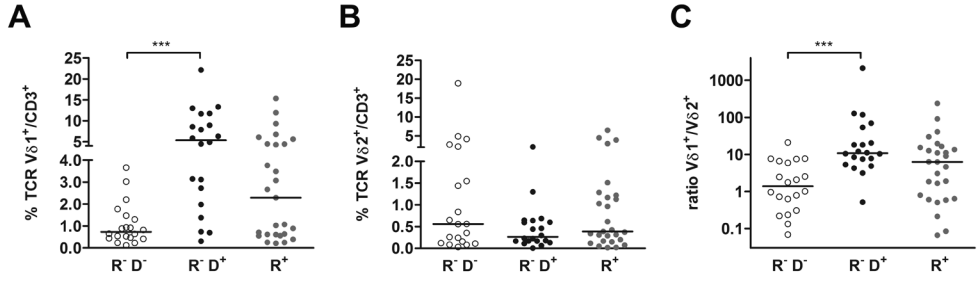

Figure 5. CMV primary infection patients show the highest peripheral Vδ1/ Vδ2 γδ T cell ratio after LT. Peripheral blood Vδ1 and Vδ2 γδ T-cell subsets were analyzed by flow cytometry in LT patients. The percentages of (A) Vδ1 and (B) Vδ2 γδ T cells within total $CD3^+$ T cells, and (C) the subsequent Vδ1/ Vδ2 γδ T-cell ratio were compared between patients with different CMV status (R^-D^- n=20, R^-D^+ n=20, R^+ n=27). Horizontal lines indicate median values. ***P<0.001.

DISCUSSION

Immune responses resulting from viral infections are proposed to promote allograft rejection and prevent the establishment of tolerance[14]. Cross-reactive viral-specific memory T cells are common in humans. Approximately 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 either R^+ patients or patients who developed CMV primary infection. In contrast, we found a robust donor-specific $CD8^+$ T-cell hyporesponsiveness in patients with CMV primary infection. In accordance with the donor-specific hyporesponsiveness, retrospective analysis showed that LT patients with R^-D^+ serostatus had a significantly lower risk to develop LAR. Moreover, CMV primary infection patients showed the highest ratio of peripheral Vδ1/Vδ2 γδ T cells, which has shown to be

associated with operational tolerance after LT. This is the first study showing that CMV primary infection remarkably reduces donor-specific T-cell reactivity and is a protective factor against the occurrence of LAR, suggesting a prominent role for CMV infection in transplant tolerance to liver allografts in humans.

We first found that CMV-positivity, particularly CMV primary infection, is associated with the expansion of $CD8^+$ 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[22]. Nonetheless we do not support a detrimental role of $CD8^+$ T_{EMRA} for LT patients, as our data suggest that $CD8^+$ T_{EMRA} rarely infiltrate liver allograft but largely remain in circulation. A recent study shows that increased numbers of circulating $CD8^+$ T_{EMRA} before kidney transplantation are associated with a reduced incidence of AR[23]. Similarly, few $CD8^+$ T_{EMRA} were found in rejecting kidney allografts[24]. However we cannot rule out the possibility that T_{EMRA} may change their phenotype into T_{EM} upon infiltration.

An intriguing question is how CMV infection induces donor-specific T-cell hyporesponsiveness. CMV infection is known to drive immunosenescence, which has been suggested to promote kidney allograft acceptance in elderly recipients[25]. Immunosenescence driven by CMV is 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[26, 27]. Indeed both in humans and mice CMV infection causes impaired T-cell immunity to other pathogens[28, 29], and CMV infection after organ transplantation is associated with a higher incidence of opportunistic infections[30]. 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 after kidney transplantation[31], and that high CMV-specific $CD4^+$ T-cell responses correlate with protection from cardiac allograft rejection[32]. We hypothesize that the donor-specific T-cell hyporesponsiveness observed in CMV primary infection patients might be related to the multifaceted immune evasion capacity used by CMV to establish latency, in particular its capacity to modulate antigen presentation[33]. Alexander et al. reported the development of hematopoietic chimerism and donor-specific hyporesponsiveness in a patient with severe CMV disease early after LT[34]. We also reported three cases of long-term hematopoietic chimerism within liver allografts[35], and interestingly all three 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 graft are the main instigators of T-cell immunity against the graft[36, 37], but CMV infected dendritic cells are impaired in their ability to stimulate allogeneic lymphocytes[38]. The immunomodulatory effect of primary infection has also been suggested, shown by higher bacterial and fungal infection-related mortality in R^-D^+ patients after allogeneic hematopoietic stem cell transplantation, which is independent from CMV-replication, diseases and treatments[39]. We hypothesize that the rapidly inflated anti-CMV immunity following primary infection in an immunocompromised environment, may contribute to its immunomodulatory effect. 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 latency[40]. Heavy immunosuppression during EAR treatment may also increase the risk of CMV viremia. In the multivariate analysis older patient age decreased the risk of EAR, which could be explained by the senescence of immune system[25, 41]. The use of basiliximab induction and tacrolimus both decreased the risk of EAR, which is in line with previous findings[42, 43]. Patients with female donors had a decreased incidence of EAR. However this could be a finding by chance, as previous studies showed that donor gender does not influence AR incidences. In contrast, we found that CMV R^-D^+ serostatus was an independent protective factor against LAR. Despite antiviral prophylaxis, the rate of seroconversion in R^-D^+ patients was 86%, in agreement with previous study[44], indicating that almost all R^-D^+ patients get primarily infected eventually. LAR was not associated with viremia or peak CMV-DNA copy number, suggesting that the lower incidence of LAR was not due to CMV-replication as such. We cannot exclude an effect of prophylaxis, however it is unlikely since higher dosages of valGCV or GCV were administrated in case of CMV viremia, but viremia was not associated with lower incidence of LAR. As there is no indication that in R^-D^- or R^+ patients immunosuppression was prescribed differently hence affecting the occurrence of LAR, the lower incidence of LAR is probably a reflection of the pro-tolerogeneic status of the patients with CMV primary infection. The discrepancy of EAR and LAR in relation to CMV infection indicates that CMV during active replication or in a quiescent state may have differential effects on graft rejection. Careful distinguishing CMV primary infection from non-primary infection, and between EAR and LAR in our analysis, may be possible reasons why this association has never been reported before.

Immunosuppression can be completely discontinued in more than 40% of stable, adult LT patients[45, 46]. Whether CMV infection plays a role in achieving operational tolerance 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 even been used as a surrogate marker to predict operational tolerance[16-18]. Interestingly, expansion of Vδ1 γδ T cells is also a feature that is observed upon CMV infection[47, 48], arguing in favour of a potential association between CMV infection and liver graft tolerance. In addition to previous findings, we found that patients with CMV primary infection showed the highest Vδ1/Vδ2 ratio. This finding corroborates the lower incidence of LAR in our retrospective analysis and suggests that these patients may have the highest chance to achieve operational tolerance after immunosuppression discontinuance. Since we do not minimize immunosuppression routinely, we are not able to demonstrate a direct link between CMV infection and operational tolerance.

There are limitations of this study that need to be acknowledged. First, we focused on allogeneic T-cell responses in LT patients with different CMV status, however anti-CMV responses were not studied, which could help understanding the interplay between allogeneic and anti-viral immune responses[31]. Second, we were not able to demonstrate the direct link between the donor-specific T-cell hyporesponsiveness and the lower incidence of LAR in primary infection patients, as the overall incidence of LAR was low and we did not have enough PBMC samples available from LAR patients. A case-control study comparing LAR cases to patients with stable graft function, or a large-scale prospective study with long follow-up, may reveal the direct relationship between T-cell responses and LAR.

To conclude, the primary findings of this study are that patients with CMV primary infection after LT show donor-specific $CD8^+$ T-cell hyporesponsiveness, are protected from the occurrence of LAR, and show signs of operational tolerance. Further investigation into the role of CMV infection in the development of operational tolerance is necessary. Since CMV status is easily measured without additional effects or costs, this parameter can be taken into account by physicians when selecting patients for tapering or withdrawing immunosuppressive therapy in LT patients. Altogether, for the first time to our knowledge, we show evidences that CMV primary infection may promote immunological tolerance towards allogeneic liver graft in humans.

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SUPPLEMENTAL 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. Tacrolimus was initiated within the first 5 days after transplantation in a dose of 1-2 mg/kg body weight/day. The target trough level was 10-15 ng/ml in the first month, 8-12 ng/ml between 1 to 6 months, 5-10 ng/ml between 6 to 12 months, and 4-8 ng/ml after 1 year. Cyclosporine was initiated within 24 hours post-reperfusion in a dose of 10-15 mg/kg body weight/day, and the dosage was adjusted to trough levels according to a range from 200-400 ng/mL during the first 3 months and thereafter 100-200 ng/mL. Acute rejection episodes were treated with high dose of methylprednisolone intravenously, and standard levels of immunosuppression were applied again after the rejection resolved. Immunosuppressive regimens were similar in patients from whom blood samples were collected, consisting of corticosteroids, MMF, tacrolimus and induction with basiliximab (Supplemental Table 1).

Cell isolation

Peripheral blood mononuclear cells (PBMCs) were isolated by standard Ficoll gradient centrifugation, and were cryopreserved for phenotyping and functional experiments. To isolate intrahepatic lymphocytes (IHLs), biopsies of explant allografts and pre-LT healthy donor livers were collected in University of Wisconsin (UW) preservation solution. Fresh tissue was cut into small pieces and digested with 0.5 mg/mL collagenase IV (Sigma-Aldrich, St. Louis, MO) and 0.1 mg/mL DNase I (Roche, Indianapolis, IN) for 30 minutes at 37°C. Cell suspensions were filtered through 70μm cell strainers and IHLs were obtained by Ficoll density gradient centrifugation. Phenotyping and functional experiments were performed immediately after IHLs isolation. Human splenocytes were isolated from splenic tissue derived from liver donors. Splenic samples were cut into small pieces and forced through 74 μm netwell filters (Costar, Corning International, NY) to obtain single cell suspensions. Mononuclear cells were isolated by standard Ficoll gradient centrifugation, and were cryopreserved for future experiments.

Flow cytometry and antibodies

The following antibodies were used: CD3-HorizonV500, CD4-APC-H7, CD8-Pacific Blue (BD Biosciences, San Diego, CA); TCR-Vδ1-FITC (Thermo Scientific, Waltham, MA);

CD3-Pacific Blue, TCR-Vδ2-PE (BD Pharmingen, Erembodegem, Belgium); CD45RA-PE-Vio770 (Miltenyi Biotec, Bergisch Gladbach, Germany); CCR7-FITC (R&D System, Minneapolis, MN); CD45RO-PerCP-Cy5.5, CD137-APC (Biolegend, San Diego, CA); CD3-PE-Cy7, TCR-αβ-APC (eBioscience, San Diego, CA); CD3-FITC (Beckman Coulter, Marseille, France). Non-viable cells were excluded using 7-AAD (BD Biosciences, San Diego, CA) or LIVE/DEAD Fixable Aqua Dead Cell Stain Kit (Invitrogen, Carlsbad, CA). Flow cytometry was performed on FACSCanto II flow cytometer (BD Biosciences, San Diego, CA). Data were analyzed with BD FACSDiva software version 6.1.1.

Quantification of alloreactive T-cell frequencies

Alloreactive T cells were analyzed by determination of activation-induced CD137 expression on T cells, as previously described with minor modifications[1]. In brief: $2.5x10^6$ PBMCs or intra-hepatic lymphocytes (IHLs) were co-cultured with donor or third-party (mismatched at HLA-A, B and DR loci with both donor and recipient) splenocytes at a 1:1 ratio in polypropylene tubes (BD Pharmingen, Erembodegem, Belgium) in duplicate, in 1 mL IMDM (Lonza, Breda, The Netherlands) supplemented with 10% heat-inactivated human serum and 1% Penicilline/Streptomycine. Co-stimulaton was provided by addition of 1 μg/mL anti-CD49d (BD Pharmingen, Erembodegem, Belgium) and 1 μg/mL anti-CD28 (Serotec, Kidlington, Oxford, UK). Prior to co-culture, the splenocytes were labeled with PKH26 (Sigma-Aldrich, St. Louis, MO) according to the manufacturer's instructions, to discriminate between stimulator splenocytes and responder PBMCs or IHLs during FACS analysis. As a control, cells were cultured in the presence of anti-CD49d and anti-CD28 mAb only, without allogeneic stimulation. After 24 hours, cells were harvested for FACS analysis. Alloreactive $CD4^+$ and $CD8^+$ T cells were identified by the upregulation of CD137 (Supplemental Figure 1). Background expression of CD137 in conditions without allogeneic stimulation was subtracted during analysis. This assay has been used in the identification and isolation of viral, tumor, and allospecific T cells regardless of their differentiation stage or cytokine production profile[1, 2].

CMV diagnostics and treatment strategies

CMV-DNA PCR measurements were only performed on a weekly basis in high risk patients (R^-D^+) until 90 days post-LT. For all other patients, PCR was performed in case of clinical suspicion of CMV infection. Due to the non-protocolized CMV-DNA PCR monitoring, the incidence of CMV reactivation/reinfection in R^+ patients was probably underestimated. Low-dose (450 mg once daily) valganciclovir (valGCV) prophylaxis was administered to high risk patients, starting at day 7 and continuing up to day 90 post-LT. A therapeutic GCV-based regime, either intravenous GCV 5 mg/kg twice daily or valGCV 900 mg twice daily, was given to patients with positive PCR results. The

regimen was given for 10 to 14 days, and PCR results were negative before withdrawing GCV therapy. Adjustments of immunosuppressive therapy were made if necessary, and normal levels of immunosuppression were applied again after the clearance of viremia.

SUPPLEMENTAL REFERENCES

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Supplemental Table 1. Characteristics of patients included for peripheral blood T-cell analysis

Total: 75 patients	$R^- D^-$ (n=20)	$R^- D^+$ (n=20)	R^+ (n=35)	*P* value
Recipient				
Age (median, range), years	54 (33-65)	48 (21-64)	54 (19-68)	0.190
Sex, female	7 (35%)	10 (50%)	14 (40%)	0.614
Donor				
Age (median, range), years	48 (16-64)	58 (22-73)	51 (13-78)	0.118
Sex, female	8 (40%)	13 (65%)	15 (43%)	0.202
Primary liver disease*				0.831
AHF	1 (5%)	4 (20%)	4 (11%)	
HBV/HCV	1 (5%)	1 (5%)	3 (9%)	
HCC	3 (15%)	3 (15%)	9 (26%)	
Metabolic diseases	2 (10%)	1 (5%)	3 (9%)	
PSC/PBC/AIH	9 (45%)	6 (30%)	8 (23%)	
other	4 (20%)	5 (25%)	8 (23%)	
Initial immunosuppressive regimen				
Basiliximab	16 (80%)	19 (95%)	29 (83%)	0.347
Tacrolimus	20 (100%)	20 (100%)	33 (94%)	0.309
Mycophenolate mofetyl	16 (80%)	13 (65%)	25 (71%)	0.569
Early acute rejection	4 (20%)	3 (15%)	6 (17%)	0.916
Late acute rejection	1 (5%)	1 (5%)	1 (3%)	0.894
CMV viremia	0 (0%)	17 (85%)	10 (29%)	0.000
Timing of viremia (median, range), days	-	79 (23-288)	27 (12-60)	0.003
Peak CMV-DNA copy number, copies/mL**	-	2.9 (1.9-5.0)	2.5 (2.1-5.5)	0.628
CMV IgG seroconversion	0 (0%)	20 (100%)	-	0.000

*AHF, acute hepatic failure; HCC, hepatocellular carcinoma; HBV, hepatitis B virus infection; HCV, hepatitis C virus infection; PBC, primary biliary cirrhosis; PSC, primary sclerosing cholangitis; AIH, autoimmune hepatitis.
**Log_{10} transformed

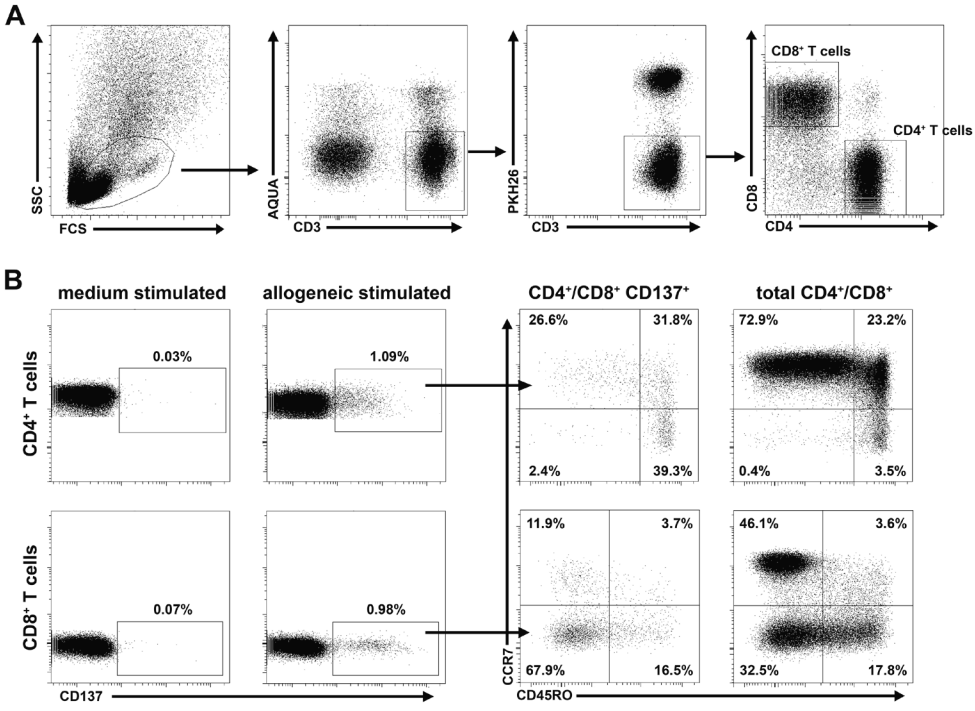
Supplemental Table 2. Characteristics of patients studied for liver allograft infiltrating T cells

Patient no.	Age	Sex	Time since first LT	Re-LT indication	RAI score	CMV status
1	66	M	7 months	Hepatic artery thrombosis and HCV recurrence	7	$R^+ D^-$
2	40	M	6 years	PSC recurrence	4	$R^- D^-$
3	67	M	3 years	Chronic rejection	3-4	$R^+ D^+$
4	59	M	8 years	HCV recurrence	-	$R^+ D^-$
5*	56	M	1 year	Chronic rejection	-	$R^+ D^+$
6	56	F	1 year	Ischemic cholangiopathy	-	$R^- D^+$
7	38	M	24 years	Chronic rejection	4	$R^- D^-$
8*	29	M	6 years	Chronic rejection	-	$R^+ D^+$
9	56	M	2 years	Chronic rejection	3-4	$R^+ D^-$
10	50	M	2 years	PSC recurrence	-	$R^+ D^-$

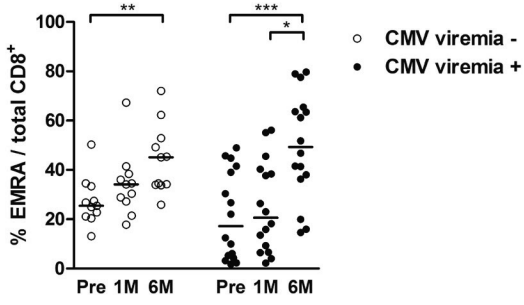
* Large amount of IHLs were isolated for functional experiments

Supplemental Table 3. Risk factor analysis for early acute rejection following liver transplantation

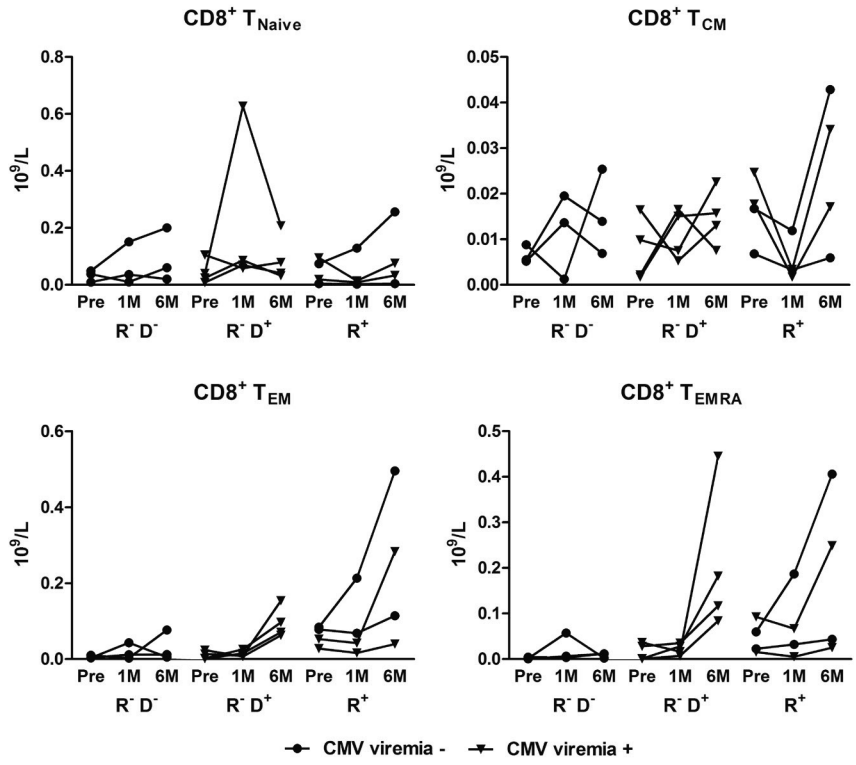
	Univariate analysis			Multivariate analysis		
Variable	HR	95% CI	*P* Value	HR	95% CI	*P* Value
Recipient age, year	0.98	(0.97-0.99)	0.007	0.99	(0.97-1.00)	0.046
Recipient, female	1.26	(0.91-1.76)	0.164	1.14	(0.81-1.60)	0.448
Recipient BMI, kg/m^2	0.96	(0.92-1.00)	0.026	0.98	(0.94-1.02)	0.375
Primary liver disease			0.415			
HBV/HCV (Ref)	1.00					
AHF	1.40	(0.78-2.53)				
HCC	0.95	(0.46-1.96)				
PBC/PSC/AIH	1.27	(0.72-2.24)				
Alcoholic cirrhosis	0.82	(0.39-1.72)				
Cryptogenic cirrhosis	0.64	(0.25-1.63)				
Others	1.10	(0.60-2.03)				
Donor age, year	0.99	(0.98-1.00)	0.192	1.00	(0.99-1.01)	0.904
Donor, female	0.70	(0.50-0.98)	0.034	0.67	(0.47-0.94)	0.020
DCD donor	0.50	(0.22-1.14)	0.065	0.91	(0.39-2.11)	0.820
Cold ischemia time, 10 min	1.01	(1.00-1.02)	0.067	1.00	(0.99-1.01)	0.354
Warm ischemia time, 10 min	1.19	(1.12-1.26)	0.000	1.05	(0.97-1.15)	0.249
Re-LT	0.85	(0.50-1.45)	0.535			
Basiliximab induction	0.39	(0.28-0.55)	0.000	0.59	(0.39-0.90)	0.015
Calcineurin inhibitor, Tacrolimus	0.36	(0.26-0.50)	0.000	0.47	(0.32-0.71)	0.000
CMV serostatus			0.773			0.856
R^-/D^- (Ref)	1.00			1.00		
R^-/D^+	1.20	(0.71-2.04)		0.92	(0.51-1.67)	
R^+	1.14	(0.74-1.76)		1.05	(0.66-1.66)	
CMV viremia	1.62	(1.14-2.28)	0.008	1.62	(1.09-2.39)	0.018
Peak CMV-DNA copy number	1.17	(0.85-1.63)	0.352			



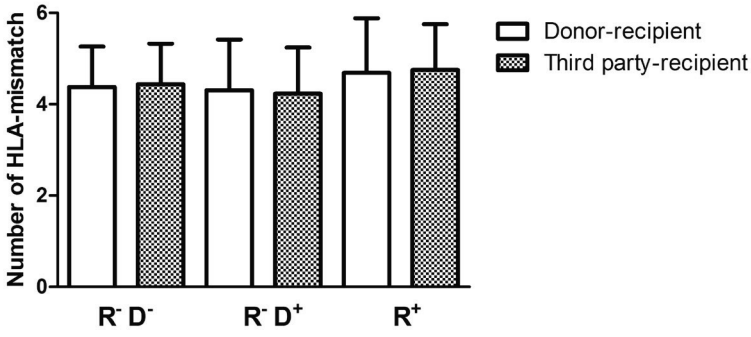
Supplemental Figure 1. Determination of allogeneic activation-induced CD137 expression on CD4⁺ and CD8⁺ T cells to study the frequencies and subset composition of alloreactive T cells by flow cytometry. (A) AQUA staining was used to exclude dead cells, and stimulatory splenocytes were labeled by PKH26 and were excluded from the analysis. (B) Alloreactive CD4⁺ and CD8⁺ T cells were identified by the up-regulation of CD137 after 24 hours of co-culture. Subset composition of alloreactive T cells was analyzed by measuring the expression of CCR7 and CD45RO on CD137⁺ T cells, and was defined as follows: T_{Naive} ($CCR7^+CD45RO^-$), T_{CM} ($CCR7^+CD45RO^+$), T_{EM} ($CCR7^-CD45RO^+$), and T_{EMRA} ($CCR7^-CD45RO^-$). All events in culture were recorded to ensure the detection of alloreactive T cells. Background expression of CD137 in conditions without allogeneic stimulation was subtracted during analysis.



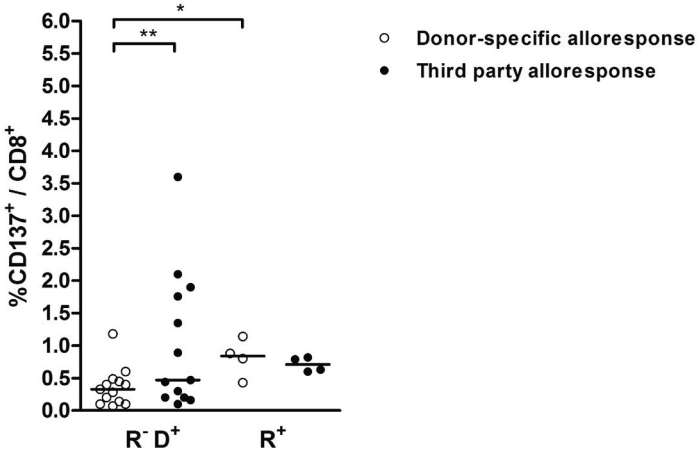
Supplemental Figure 2. Comparison of $CD8^+$ T_{EMRA} changes in CMV-positive patients with or without proven viremia. Viremia was detected in 16 of 27 CMV-positive patients (R^-D^+ and R^+). We compared the percentages of $CD8^+$ T_{EMRA} before (Pre), at 1 month (1M) and at 6 months (6M) after LT (one-way Friedman test) in both groups. Horizontal lines indicate median values. *P<0.05, **P<0.01, ***P<0.001.



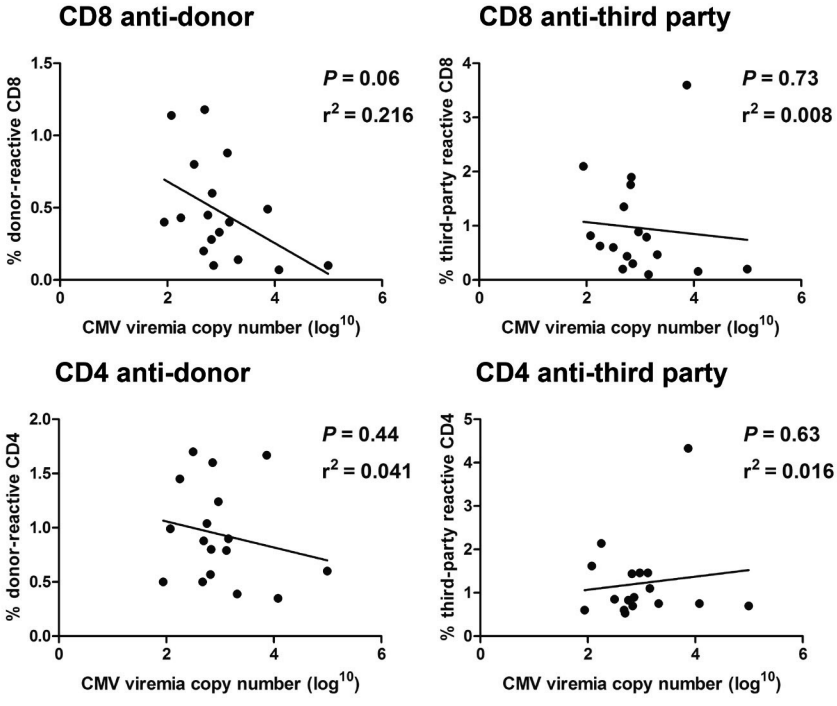
Supplemental Figure 3. Changes in absolute numbers of CD8$^+$ T-cell subsets after LT in relation to CMV status. Absolute numbers of CD8$^+$ T-cell subsets were measured longitudinally in 11 patients (R^-D^- n=3, R^-D^+ n=4, R^+ n=4) before (Pre), at 1 month (1M) and at 6 months (6M) after LT. Black dots represent patients without detected viremia, and black triangles represent patients with detected CMV viremia after LT. Absolute numbers are shown on the Y-axis.



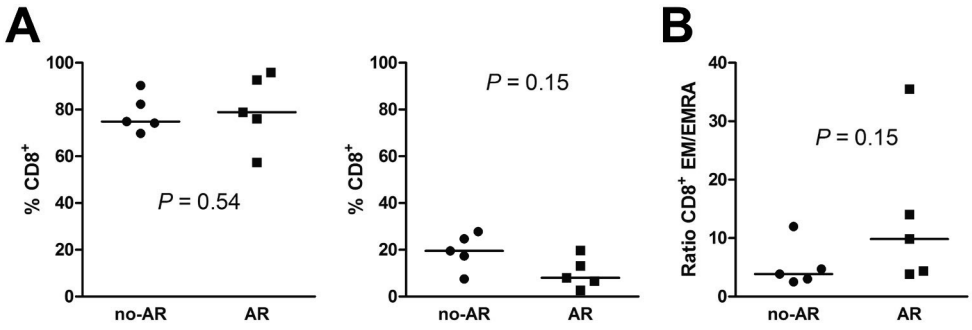
Supplemental Figure 4. Numbers of HLA-mismatches between donor-recipient and third party-recipient in *ex vivo* allogeneic stimulations. The total numbers of HLA-mismatches at HLA-A, B, DR loci between donor-recipient and third party-recipient in the *ex vivo* allogeneic stimulation experiments are shown as mean with standard deviation, and were compared between the three experimental groups. No statistically significant difference was observed.



Supplemental Figure 5. Alloreactive CD8⁺ T-cell frequencies in patients with proven viremia. The frequencies of donor-reactive and third party-reactive CD8⁺ T cells were compared between R^-D^+ patients with viremia and R^+ patients with viremia. Horizontal lines indicate median values. *P<0.05, **P<0.01.



Supplemental Figure 6. Correlation of alloreactive T-cell frequencies with CMV viremia level. In patients with proven viremia, the correlation between alloreactive T-cell frequencies and the peak CMV-DNA copy number during viremia was analyzed by linear regression. The CMV-DNA copy number was $\log_{10}$-transformed.



Supplemental Figure 7. Comparison of CD8⁺ T-cell subsets in explant allografts with or without acute rejection activity. Five explant allografts presented acute rejection activity (AR, versus no-AR). (A) Percentages of intragraft CD8⁺ T_{EM}, T_{EMRA}, and (B) the subsequent ratio were compared (Mann-Whitney U test). Horizontal lines indicate median values.

CHAPTER 3

CMV-induced expression of CD244 after liver transplantation is associated with CD8+ T-cell hyporesponsiveness to allo-antigen

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ABSTRACT

Chronic presence of viral antigens can induce T-cell exhaustion, characterized by upregulation of co-inhibitory receptors and loss of T-cell function. We studied whether a similar phenomenon occurs after liver transplantation (LTx), when there is continuous exposure to allo-antigen. Expression of co-inhibitory receptors on circulating $CD4^+$ and $CD8^+$ T cells was longitudinally analyzed in 19 patients until 6 months after LTx, and cross-sectionally in 38 patients late (1-12 years) after LTx. Expression of the co-inhibitory receptors CD160 and CD244 on circulating $CD8^+$ T cells was already 6 months after LTx higher than pre-LTx, and the elevated expression was sustained late after LTx, with CD244 showing the most prominent increase. The strongest upregulation of CD244 on circulating $CD8^+$ T cells was observed in patients who experienced cytomegalovirus (CMV) infection after LTx. CMV infection was also associated with reduced $CD8^+$ T-cell proliferation and cytotoxic degranulation in response to allo-antigen late after LTx. Purified $CD244^+$ $CD8^+$ T cells of LTx patients showed lower proliferative responses to allo-antigen as well as to polyclonal stimulation than their $CD244^-$ counterparts. In addition, the $CD244^+$ $CD8^+$ T-cell population contained the majority of CMV-peptide-loaded MHC class I tetramer-binding cells. In conclusion, CMV infection after LTx, rather than persistence of allo-antigen, induces accumulation of dysfunctional $CD8^+CD244^+$ T cells in the circulation which persist on long-term, resulting in reduced frequencies of circulating allo-reactive $CD8^+$ T cells. These results suggest that CMV infection restrains $CD8^+$ T-cell allo-responses after LTx.

INTRODUCTION

After liver transplantation (LTx), most patients need lifelong immunosuppression to prevent rejection of the allograft, but some patients develop spontaneously immunological tolerance to their liver graft and can be completely withdrawn from all immunosuppression[1]. This phenomenon occurs more frequently after LTx than in any other organ transplant setting[2], suggesting that the immunological alloresponse is skewed towards tolerance. Allograft rejection is mainly mediated by T cells of the recipient that respond to allogeneic donor antigens[3]. In the transplantation setting, activation of allogeneic T cells via their T-cell receptor (TCR) is triggered by specific recognition of donor-derived allo-antigenic peptides presented by recipient major histocompatibility complex (MHC) molecules or by direct interaction of the TCR with allogeneic MHC molecules. However, T cells can also express co-stimulatory and co-inhibitory receptors that affect outcome of T-cell responses[4]. Hence, these receptors might also influence the outcome of T-cell responses towards the allograft.

Expression of co-inhibitory receptors is upregulated on T cells in patients with chronic viral infections, such as hepatitis C virus (HCV) and HIV, and in patients with cancer[5-12]. High expression of co-inhibitory receptors is associated with T-cell dysfunction, or "exhaustion", and in these patients virus-specific or tumor-specific T-cell responses are therefore impaired. T-cell exhaustion can be defined as a state of antigen-specific T-cell dysfunction in response to chronic persistence of high antigenic load[5]. Exhausted T cells have poor proliferative and effector function, show sustained expression of co-inhibitory receptors and their transcriptional state is distinct from that of functional effector or memory T cells[5]. Several co-inhibitory receptors have been described to be important in inhibiting T-cell responses, among which the most well-studied are: Programmed Death 1 (PD1), Lymphocyte-Activation Gene 3 (LAG3), T cell immunoglobulin mucin 3 (TIM3), CD160 and CD244[5, 6]. PD1 has two ligands: PD-L1 and PD-L2[7, 13]. PD-L1 is expressed on both hematopoietic cells (mainly on dendritic cells (DC) and macrophages) and non-hematopoietic cells (parenchymal cells of many organs including liver; and endothelial cells)[7, 14]. PD-L2 expression is restricted to DC and macrophages. Binding of PD1 to its ligand(s) negatively regulates T-cell responses[13, 15]. LAG3 is a protein closely related to CD4, and mediates negative regulation of T-cell functions through interactions with its ligand MHC class II to which it binds with higher affinity than CD4[16, 17]. TIM3 inhibits $CD8^+$ T-cell responses by interaction with its ligand galectin-9[8, 9]. CD160 is a glycosylphosphatidylinositol-anchored receptor that inhibits T-cell responses upon binding to its ligand herpes virus entry mediator (HVEM) that is expressed on both hematopoietic and non-hematopoietic cells (parenchymal cells)[18]. CD244, also called 2B4,

can mediate both activating and inhibitory signals upon binding with its ligand CD48. High levels of CD244 expression on T cells were found to be associated with inhibitory receptor function[10, 14, 19].

After organ transplantation, numbers of circulating T cells that react to donor allo-antigen decrease over time in a majority of patients[20-22]. The mechanism underlying this phenomenon is as yet unclear. Whether chronic stimulation by the persistence of a high allo-antigenic load induces upregulation of co-inhibitory receptors and exhaustion of donor-specific T cells, as observed during chronic viral infections, is unknown. However, various experimental animal studies have shown enhanced rejection and/or decreased graft survival after blockade of co-inhibitory receptors in organ transplanted mice. This implies that co-inhibitory receptors are involved in suppressing allograft rejection in mice[23-26]. However, the role of T-cell exhaustion and co-inhibitory receptor-ligand interactions in human solid organ transplantation has not yet been widely studied[25].

The aim of this study was to determine whether the expression of co-inhibitory receptors on circulating T cells is upregulated after LTx in humans, to analyze which clinical factors influence such upregulation, and to assess whether co-inhibitory receptors impair allogeneic T-cell responses after LTx. We hypothesized that long-term persistence of a high load of allo-antigens after LTx may induce exhaustion of allogeneic T-cells, characterized by upregulation of co-inhibitory receptors and hyporesponsiveness of $CD8^+$ T cells to allo-antigens.

MATERIALS AND METHODS

Study design and patients

Heparinized blood was collected at 1 and 6 months after transplantation from 19 primary liver transplant recipients who were transplanted in the Erasmus Medical Centre Rotterdam (The Netherlands) (early post-LTx cohort). In addition, blood was collected during a single regular visit at the outpatient clinic from 38 stable primary liver transplant recipients 1 to 12 years after transplantation in the Erasmus Medical Centre Rotterdam (The Netherlands) (late post-LTx cohort). Multi-organ transplantation patients were excluded. The occurrence of CMV infection (either primary infection or reactivation) after transplantation was determined either by CMV DNA polymerase chain reaction (PCR) > 50 copies/ml or by CMV IgG seroconversion. All patients gave informed consent, and the study was approved by the Medical Ethics Committee of the Erasmus MC. Nineteen clinically healthy blood bank donors were used as healthy controls and were age-matched with the late post-LTx cohort.

Cell culture

Peripheral blood mononuclear cells (PBMC) from patients and healthy individuals were isolated using Ficoll Hypaque density gradient centrifugation. From the same patients, cryopreserved PBMC collected before LTx (pre-LTx), that were available in our bio bank, were used for baseline measurements. Cryopreserved splenocytes, isolated according to standard procedures [27] from splenic tissue of liver transplant donors, were available in our bio bank as well. CD40-activated B cells were expanded from donor splenocytes, as described previously [27], and used as stimulator cells in allogeneic T-cell stimulation assays. Only B cells containing <1% $CD3^+$ T cells were used. PBMC and expanded B cells were cryopreserved at -135°C until further use.

Flow cytometry

Flow cytometry was performed to determine T-cell subsets and co-inhibitory receptor expression. For analysis of $CD4^+$ and $CD8^+$ T cells, isolated PBMC were stained with anti-CD3-horizonV500 (UCHT1, BD Biosciences, Erembodegem, Belgium), anti-CD4-APC-H7 (SK3, BD Biosciences), and anti-CD8-efluor450 (RPA-T8, eBioscience, Vienna, Austria). To distinguish naive and memory T-cell subsets, cells were stained with anti-CCR7-FITC (150503, R&D systems, Abingdon, United Kingdom) and anti-CD45RO-PerCP-Cy5.5 (UCHL1, Biolegend, London, United Kingdom). Naive T cells (Tn) were defined as $CD45RO^-CCR7^+$ T cells; central memory T cells (Tcm) as $CD45RO^+CCR7^+$; effector memory T cells (Tem) as $CD45RO^+CCR7^-$ and terminally differentiated T cells (Temra) as $CD45RO^-CCR7^-$ [28]. Surface expression of co-inhibitory receptors was determined by staining cells with anti-CD279(PD1)-PECy7 (J105, eBioscience), anti-CD223(LAG3)-PE (polyclonal, R&D systems), anti-CD160-PE (688327, R&D systems) and anti-CD244-APC (eBioDM244, eBioscience). Flow cytometry was performed using a FACS Canto II (BD Biosciences). Gates for PD1 expression were set using an isotype-matched control antibody and gates for LAG3, CD160 and CD244 expression were set on distinct positive populations. For analysis FACS Diva software was used (BD Biosciences).

Allogeneic T-cell stimulation using PBMC

To quantify allo-reactive T-cell responses, PBMC were labeled with 0.5 μM CFSE (Invitrogen, Paisley, United Kingdom) and $1*10^5$ recipient PBMC were stimulated with $2*10^5$ irradiated (30 Gy) donor-derived or third party-derived CD40-B cells. Third party CD40-B cells were expanded from splenocytes of an individual having the same number of HLA mismatches with the patient as the number of mismatches between patient and donor, but completely mismatched with the donor on HLA-A, B, and DR [27]. Co-cultures were performed in 96-wells U-bottom plates in a final volume of 200 µl B-cell medium (IMDM + 10% human serum + 1% Penicillin/Streptomycin (Gibco) + 1% Insulin/

Transferrin/Selenium (Gibco)) [27]. In addition, to determine responses to polyclonal stimulation, PBMC were stimulated with PHA (5μg/ml, Murex, Paris, France). Each assay was performed in duplicate. Flow cytometric analysis was performed after 5 days of culture at 37°C and 5% CO_2. Cells were washed with PBS (Lonza) and staining for cell viability was performed using LIVE/DEAD® Fixable Dead Cell Stain Kit (Invitrogen), according to the manufacturer's protocol. Cells were then stained with anti-CD3-PerCP-Cy5.5 (UCHT1, BD Biosciences), anti-CD4-APC-H7 (SK3, BD Biosciences), anti-CD8-efluor450 (RPA-T8, eBioscience) to distinguish T cells, and anti-CD19-horizonV500 (HIB19, BD Biosciences) to exclude B cells. Cytotoxic degranulation was detected using CD107a-APC (eBioscience), added during the last 15 hours of the co-cultures. Cells were analyzed for proliferation using CFSE-dilution patterns, and for phenotype on a BD FACS Canto II Flow cytometer (BD Biosciences, San Jose, CA). For analysis of phenotypic markers we used FACS Diva software (BD Biosciences). Precursor frequencies (PF), which is the proportion of the cells that respond to the stimulus, of allo-reactive $CD4^+$ and $CD8^+$ T cells were calculated using ModFit LT® software (Verity Software House, USA), as previously described [27]. From duplicate assays, average PF were calculated.

Allogeneic T-cell stimulations of sorted T cells

To compare the proliferative capacities of $CD8^+CD244^+$ and $CD8^+CD244^-$ T cells, post-LTx PBMC of patients of the late cohort were thawed and labeled with 0.5 μM CFSE. CFSE-labeled PBMC were stained with anti-CD3-PerCP-Cy5.5 (UCHT1, BD Biosciences), anti-CD4-APC-H7 (SK3, BD Biosciences), anti-CD8-efluor450 (RPA-T8, eBioscience) and anti-CD244-APC (eBioDM244, eBioscience), and $CD8^+CD244^+$, $CD8^+CD244^-$, and $CD4^+$ T cells were purified by flowcytometric sorting, using a FacsAria Cell Sorter (BD Biosciences). Only cells with purity >95% were used. Purified $CD8^+CD244^+$ or $CD8^+CD244^-$ T cells ($2*10^4$) together with purified autologous $CD4^+$ T cells ($2*10^4$) were stimulated with $1.6*10^5$ irradiated (30 Gy) donor CD40-B cells or third party CD40-B cells, as described above. To study the role of co-inhibitory receptor-ligand interactions in allogeneic T-cell responses, ligands of co-inhibitory receptors were blocked by addition of neutralizing anti-CD270 (HVEM/TNFRSF14) (Clone 94801, R&D systems [29]), or anti-CD48 (eBio156-4H9, eBioscience [10, 11]) antibodies, either alone or in combination to selected allogeneic T-cells stimulations. After 5 days of culturing, cells were stained and ModFit analyses were performed as described above.

Determination of CMV-specific T cells

To determine frequencies of CMV-specific $CD8^+$ T cells in PBMC and to assess whether these cells expressed CD244, $1*10^6$ PBMC were stained with a mixture of HLA-A*01:01, A*02:01, A*11:01, A*24:02, B*07:02, B*08:01, and B*35:01 MHC class I tetramers loaded

with Pp50-derived, Pp65-derived and IE-derived peptides (Department of Hematology, Leiden University Medical Center, The Netherlands), depending on the HLA-types of the patient. The following peptides were used: Pp50: VTEHDTLLY (HLA-A0101); Pp65: YSEHPTFTSQY (HLA-A0101), NLVPMVATV (HLA-A0201), ATVQGQNLK (HLA-A1101), AYAQKIFKIL (HLA-A2402), RPHERNGFTVL (HLA-B0702), TPRVTGGGAM (HLA-B0702), and IPSINVHHY (HLA-B3501); IE1: QIKVRVDMV (HLA-B0801) and ELRRKMMYM (HLA-B0801). In addition, cells were stained with anti-CD4-PerCP (clone Leu3A SK7, BD Biosciences), anti-CD8-Pacific Blue (clone RPA-T8, BD Biosciences) and anti-CD244-APC (eBioDM244, eBioscience). Flow cytometry was performed using a LSRII (BD Bioscience) and data were analyzed using FACS Diva software.

Statistical analysis

All data are presented as means + SEM. All data sets were tested for normal Gaussian distribution using the Shapiro-Wilk normality test. Significance of differences between paired observations was tested using the paired *t*-test for normally distributed data or the Wilcoxon signed rank test for non-normally distributed data. Differences between unrelated groups were tested using the Mann-Whitney U test. Statistical analyses were performed using GraphPad Prism (Version 5.01, GraphPad Software Inc, San Diego, CA). Multivariate analysis was performed using linear regression in SPSS for Windows (version 21.0 software package). A p-value <0.05 was considered statistically significant.

RESULTS

Rapid and sustained increase of CD244 and CD160 expression on circulating T cells after LTx

To investigate whether expression of co-inhibitory receptors on T cells changed after LTx, we first analyzed the longitudinal course of the expression of 5 well-known co-inhibitory receptors, namely PD1, LAG3, TIM3, CD160 and CD244 [5], on circulating $CD4^+$ and $CD8^+$ T cells in 19 patients during the first 6 months after LTx. Patient characteristics are depicted in Table I and described in Materials and Methods. Because TIM3 was hardly expressed on T cells in any of the individuals, we excluded TIM3 from further analyses (data not shown). Representative FACS plots are shown in Figure 1A.

Table 1. Demographic and clinical characteristics of patients included in the early post-LTx and late post-LTx cohorts

Early Post-LTx Cohort	Total: 19 Patients
Recipient age (y; median [range])	43 (25–63)
Recipient gender, female (*n* [%])	10 (53)
Underlying disease (*n* [%])	
AHF	4 (21)
HCC	0 (0)
PBC/PSC/AIH/SBC	6 (32)
HBV/HCV	4 (21)
Alcoholic cirrhosis	2 (10)
Other	3 (16)
Donor age (y; median [range])	44 (15–77)
Donor gender, female (*n* [%])	10 (53)
Basiliximab as induction immunosuppression (*n* [%])	10 (53)
Immunosuppressive treatment (*n* [%]); trough level at 6 mo (μg/l; median [range])	
Cyclosporin A	7 (37) 175 (25–300)
Tacrolimus	11 (58) 9.1 (4.5–25)
Everolimus	2 (10) 11.7 (6.6–16.8)[a]
Mycophenolate mofetil	1 (5) 2.96[b]
No immunosuppression	0 (0)
Late Post-LTx Cohort	**Total: 38 Patients**
Recipient age (y; median [range])	46 (20–64)
Recipient gender, female (*n* [%])	16 (42)
Underlying disease (*n* [%])	
AHF	1 (3)
HCC	1 (3)
PBC/PSC/AIH	17 (45)
HBV/HCV	1 (3)
Alcoholic cirrhosis	8 (21)
Other	10 (26)
Donor age (y; median [range])	43 (12–77)
Time after LTx (y; median [range])	7 (1–12)
Donor gender, female (*n*, %)	18 (47)
Basiliximab as induction immunosuppression (*n* [%])	27 (71)
Immunosuppressive treatment (*n*, %, trough level at time of post-LTx blood collection (median, range) μg/l)	
Cyclosporin A	2 (5) levels unknown
Tacrolimus	28 (74) 4.4 (0–9.8)
Everolimus	5[c] (13) 12.6 (10–15.7)
Rapamycin	1[d] (3) 1.14
Mycophenolate mofetil	4[b] (11) levels unknown
No immunosuppression	3 (8)
CMV infection between LTx and collection of post-LTx blood sample (*n* [%])	
Yes	20 (53)
No	18 (47)

[a]One patient in the early post-LTx cohort received a combination of tacrolimus and everolimus.
[b]One patient in the early post-LTx cohort received a combination of tacrolimus and mycophenolate mofetil; four patients in the late post-LTx cohort received mycophenolate mofetil (two in combination with tacrolimus).
[c]Five patients in the late post-LTx cohort received everolimus (two in combination with tacrolimus).
[d]One patient in the late post-LTx cohort received a combination of rapamycin and tacrolimus.
AHF, acute hepatic failure; AIH, autoimmune hepatitis; HCC, hepatocellular carcinoma; PBC, primary biliary cirrhosis; PSC, primary sclerosing cholangitis; SBC secondary biliary cirrhosis.

Already 1 month after LTx a slight rise in expression of PD1 on $CD4^+$ T cells and CD244 on $CD8^+$ T cells was observed (Figure 1B). At 6 months post-LTx, expression of PD1 on $CD4^+$ T cells had returned to baseline level, while CD244 expression on $CD8^+$ T cells was further increased. In addition, expression of CD244 on $CD4^+$ T cells and of CD160 on $CD8^+$ T cells were significantly increased at 6 months post-LTx. Longitudinal LAG3 expression levels tended to rise at 1 month post-LTx on both $CD4^+$ ($p=0.145$) and $CD8^+$ ($p=0.138$) T cells, but at 6 months post-LTx returned to levels similar to pre-LTx (Figure 1B).

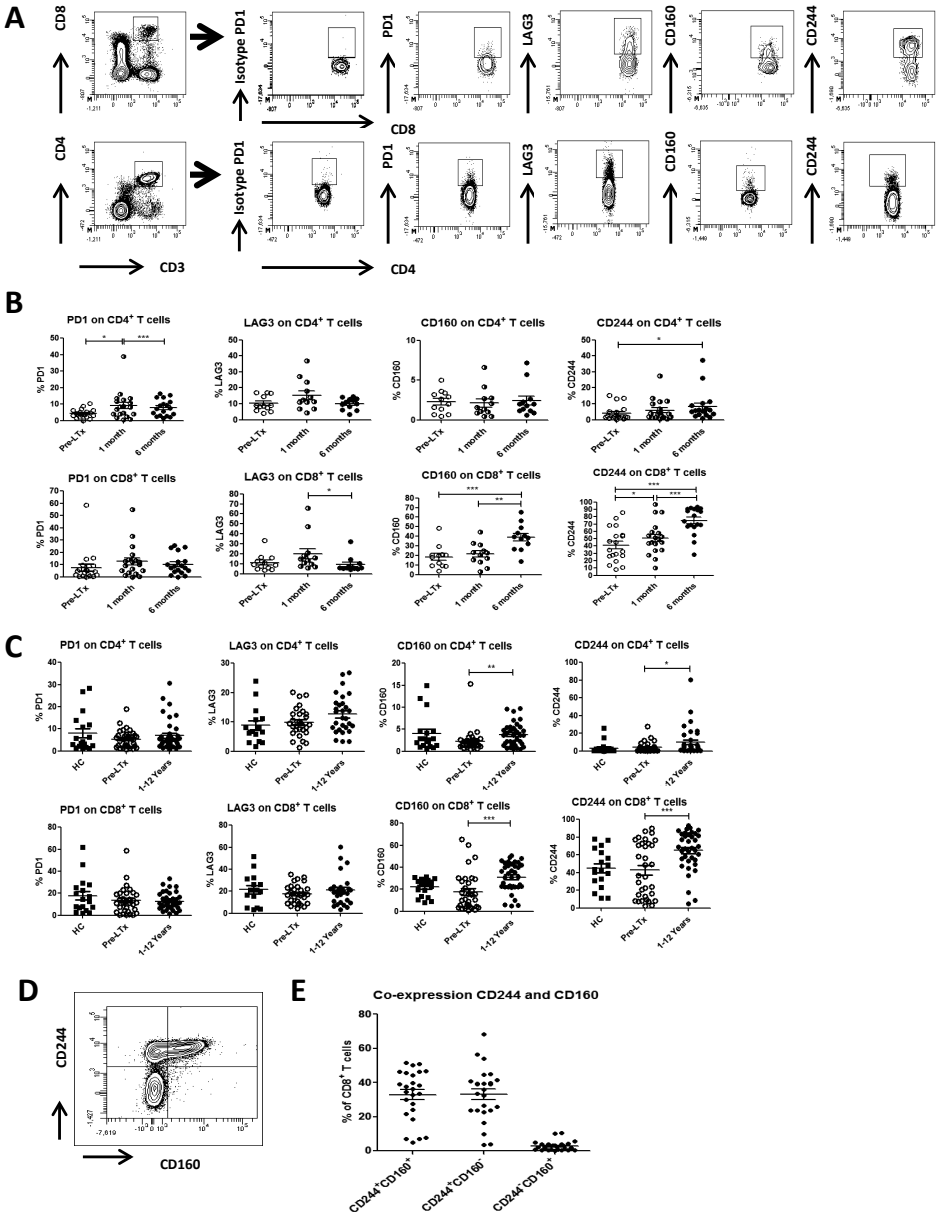

Figure 1 Expression of co-inhibitory receptors on circulating T cells after LTx. (A) Representative FACS plots showing analysis of co-inhibitory receptors on $CD4^+$ and $CD8^+$ T cells (B) Expression of co-inhibitory receptors on circulating $CD4^+$ and $CD8^+$ T cells of 19 liver transplant recipients before (pre-LTx) and early (1-6 months) post-LTx. (C) Expression of co-inhibitory receptors on circulating $CD4^+$ and $CD8^+$ T cells of healthy controls (HC) and 38 liver transplant recipients before (pre-LTx) and 1-12 years post-LTx. (D) Representative FACS plot showing co-expression of CD244 and CD160 on circulating $CD8^+$ T cells (E) Co-expression of CD244 and CD160 on circulating $CD8^+$ T cells in patients late after LTx (n=25). Each dot represents 1 patient, and lines indicate mean with standard error of the mean. *p<0.05, **p<0.005, ***p<0.0005

To establish whether the changes observed in the first 6 months after LTx were sustained later after LTx, we assessed co-inhibitory receptor expression on circulating T cells in blood samples collected from 38 patients 1 to 12 years after LTx and compared it with expression before LTx. Patient characteristics are depicted in Table I. We found no significant differences in PD1 and LAG3 expression on both $CD4^+$ and $CD8^+$ T cells between pre-LTx and post-LTX samples. However, CD160 and CD244 expression were increased late after LTx on both $CD4^+$ and $CD8^+$ T cells (Figure 1C). In addition, we found that CD160 and CD244 were strongly co-expressed on $CD8^+$ T cells late after LTx: CD160 was mainly expressed on $CD244^+$ $CD8^+$ T cells and almost no $CD160^+CD244^-$ T cells were observed (Figure 1D-E). Interestingly, expression of co-inhibitory receptors on T cells did not differ between patients pre-LTx and healthy age-matched controls (Figure 1C), indicating that liver disease had no influence on expression levels of co-inhibitory receptors.

Collectively, early after LTx a slight rise in PD1 expression on $CD4^+$ T cells was found, which was not sustained, while the early increase of CD160 and CD244 expression on both $CD4^+$ and $CD8^+$ T cells was sustained late after LTx. We therefore further focused on these two co-inhibitory receptors.

Enhanced expression of CD244 and CD160 on circulating T-cell subsets after LTx

Since CD244 and CD160 expression is low or absent on naive T cells and increases progressively with memory differentiation state of T cells [11, 30], we assessed whether the distribution of circulating naive and memory T-cell subsets changed after LTx (Figure 2A; Materials and Methods). After LTx, a significant reduction of $CD4^+$ and $CD8^+$ Tn was observed, while $CD4^+$ Tem and Temra and $CD8^+$ Temra significantly increased (Supplemental Figure 1). Therefore, to establish whether the upregulation of CD160 and CD244 after LTx was due to the changes in T-cell subset distribution, we determined the expression of these co-inhibitory receptors on each individual T-cell subset.

In the early post-LTx cohort, we observed a significant increase in CD160 expression on $CD8^+$ Tcm and Tem 6 months post-LTx (Figure 2B). In the late post-LTx cohort, CD160 expression significantly increased after LTx on $CD4^+$ Tem and $CD8^+$ Tcm, Tem and Temra (Figure 2C).

On $CD4^+$ Tem and Temra and on all $CD8^+$ T-cell subsets we found increasing expression of CD244 during the first 6 months after LTx in the early post-LTx cohort (Figure 2B). In the late post-LTx cohort, CD244 expression on $CD4^+$ and $CD8^+$ Tem and Temra increased significantly after LTx (Figure 2C).

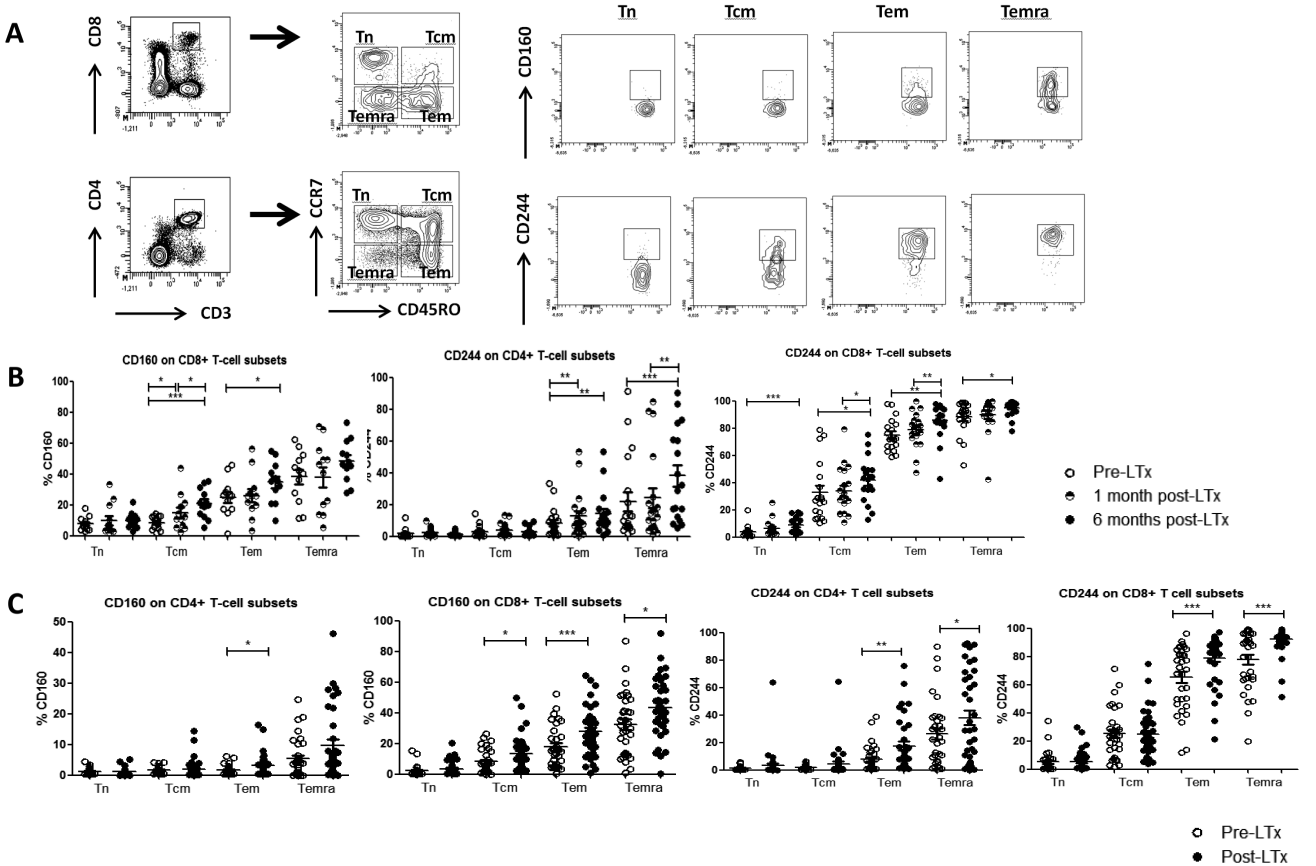

Figure 2 Expression of co-inhibitory receptors on circulating CD4⁺ and CD8⁺ T-cell subsets before and after LTx. (A) Representative FACS plots showing the gating strategy of naive T cells (Tn), central memory T cells (Tcm), effector memory T cells (Tem), and terminally differentiated T cells (Temra) and expression of CD160 and CD244 on circulating CD8⁺ T-cell subsets. (B) Expression of CD160 and CD244 on different T-cell subsets in patients of the early post-LTx cohort (C) Expression of CD160 and CD244 on different T-cell subsets in patients of the late post-LTx cohort. Each dot represents 1 patient, and lines indicate mean with standard error of the mean. *p<0.05, **p<0.005, ***p<0.0005

Taken together, these results show that the observed upregulation of CD160 and CD244 expression on circulating T cells after LTx was not only caused by a shift in T-cell subset distribution, but was also due to increased expression on the individual T-cell subsets.

Upregulation of CD244 on CD8$^+$ T cells after LTx is associated with CMV infection

Next, we focused on the expression of CD244 and CD160 in patients late after LTx. Since expression of CD244 and CD160 on T cells increases with age [31] and their expression can also be induced by viral infections, such as HBV, HCV and CMV [5, 10, 14, 32-34] we first asked whether the increasing expression of these co-inhibitory receptors after LTx was related to age of the patients, underlying disease (including chronic viral hepatitis), time after LTx, or CMV infection after LTx. Importantly, for this study CMV infection was only regarded as relevant when occurring between LTx and collection of the post-LTx blood sample. Multivariate linear regression analysis showed that the increases in CD160 and CD244 expression (delta expression = expression post-LTx minus expression pre-LTx) were not significantly associated with patient age, time after LTx, or underlying disease. However, upregulation of CD244, but not CD160, on CD8$^+$ T cells after LTx showed a significant positive association with CMV infection after LTx ($p=0.004$) (Table II). Although we focused on the expression of CD244 and CD160, we also analyzed the association between CMV infection and the expression of PD1 and LAG3, but no increase in their expression levels was found in CMV infected patients.

Figure 3A shows that the expression of CD244 on CD8$^+$ T cells was significantly upregulated after LTx in patients with CMV infection, but not in patients without CMV infection after LTx. The increases in CD244 expression observed in patients without CMV infection were small (on the average only 7%), while a significantly higher average increase of 38% was observed in patients with CMV infection (Figure 3B). We therefore conclude that CMV infection importantly contributes to the rise in CD244 expression on CD8$^+$ T cells after LTx. Together, our data suggest that strong CD244 expression is induced by CMV-infection early after LTx and that CD244-expression remains high, even many years after CMV infection is cleared. To verify this, we determined CD244 expression levels on CD8$^+$ T cells at 1 year post-LTx in a subgroup of CMV-infected patients (n=6) of the long-term cohort. As shown in Figure 3C, expression of CD244 was already increased in these patients at 1 year after LTx. Although expression levels showed a partial decrease in blood samples taken at 6-11 years post-LTx, they remained significantly higher than pre-LTx expression levels. These data indicate that CMV infection, which occurs predominantly in the first 9 months after transplantation, induces accumulation of CD8$^+$ T cells expressing CD244, and after the clearance of infection (latency) these CD8+CD244+ T cells persist.

Table 2. Associations of independent covariates with increasing (Δ) CD160 and CD244 expression on CD4⁺ and CD8⁺ T cells in multivariate linear regression analysis

Variable	β	*p* Value
ΔCD160 on CD4⁺ T Cells		
Age recipient	0.336	0.199
Time after LTx (y)	0.171	0.492
Underlying disease	0.047	0.842
CMV infection after LTx	0.357	0.165
ΔCD160 on CD8⁺ T Cells		
Age recipient	0.239	0.368
Time after LTx (y)	−0.287	0.269
Underlying disease	−0.048	0.843
CMV infection after LTx	0.434	0.106
ΔCD244 on CD4⁺ T cells		
Age recipient	0.469	0.069
Time after LTx (y)	0.180	0.450
Underlying disease	−0.066	0.769
CMV infection after LTx	0.232	0.337
ΔCD244 on CD8⁺ T cells		
Age recipient	−0.223	0.251
Time after LTx (y)	0.161	0.388
Underlying disease	−0.147	0.410
CMV infection after LTx	0.631	0.004

Δ expression, expression post-LTx − expression pre-LTx.

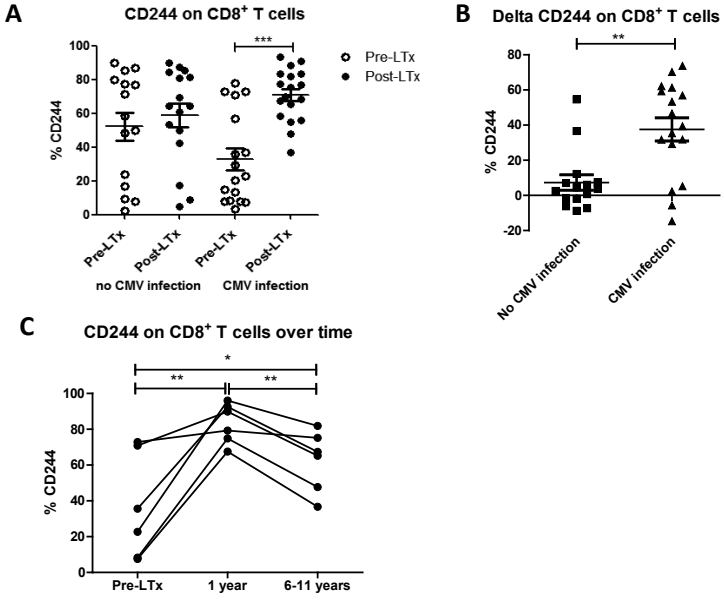

Figure 3 Expression of CD244 on CD8⁺ T cells before and after LTx in patients of the late post-LTx cohort with and without CMV infection after LTx. (A) Expression of CD244 on CD8⁺ T cells before and after LTx in patients of the late post-LTx cohort with and without CMV infection after LTx. (B) Increase of CD244 expression on CD8⁺ T cells, i.e. delta CD244 (= CD244 expression post-LTx minus pre-LTx expression) in patients with and without CMV-infection after LTx. (C) Expression of CD244 on CD8⁺ T cells before (pre-LTx), at 1 year, and at 6-11 years post-LTx in a subgroup of patients with CMV infection (n=6) of the long-term cohort. Each dot represents 1 patient, and lines indicate mean with standard error of the mean. *p<0.05, **p<0.005, ***p<0.0005

Reduced allogeneic CD8$^+$ T-cell responses in patients with CMV infection after LTx
Since we found that CMV infection after LTx was associated with upregulation of CD244 expression on CD8$^+$ T cells after LTx, we asked whether CMV infection also affected the allo-reactivity of these cells. We therefore determined post-LTx CD8$^+$ T-cell allo-responses in patients of the late post-LTx cohort with and without CMV infection, by co-culturing CFSE-labeled patient PBMC with CD40-activated B cells from their liver transplant donors or from an HLA-mismatched third party. After 5 days, proliferation and effector function of CD8$^+$ T cells were assessed. PF of proliferating cells were calculated using Modfit software [27], and representative examples of Modfit proliferation analyses are shown in Figure 4A. As depicted in Figure 4B, PF of CD8$^+$ T cells proliferating in response to donor allo-antigens were significantly lower in PBMC from patients with CMV infection than from patients without CMV infection after LTx, with the same trend ($p=0.213$) in the responses to third party allo-antigens.

To assess cytotoxic effector function of CD8$^+$ T cells in both patient categories, we determined their cytotoxic degranulation capacity by analyzing CD107a surface expression at the end of the co-cultures (Figure 4C). After stimulation with donor or third party allo-antigens, CD8$^+$ T cells of patients with CMV infection after LTx showed significantly lower levels of CD107a expression than CD8$^+$ T cells of patients without CMV infection (Figure 4D). More specifically, CD107a expression in non-proliferating cells was lowered in patients with CMV-infection, but not CD107a expression in proliferating CD8$^+$ T cells (Supplemental Figure 2). These data demonstrate the existence of circulating CD8$^+$ T cells that do not proliferate but are still capable of cytotoxic degranulation in response to allo-antigens, and show that the decreased allogeneic cytotoxic degranulation capacity in CMV-infected patients was confined to these non-proliferating cells.

Together, these data demonstrate a reduction of allogeneic CD8$^+$ T-cell proliferative and cytotoxic degranulation responses in LTx patients with CMV infection after LTx, and show that CMV-infection induces accumulation of a population of dysfunctional CD8$^+$ T cells which does neither proliferate, nor degranulate in response to allo-stimulation.

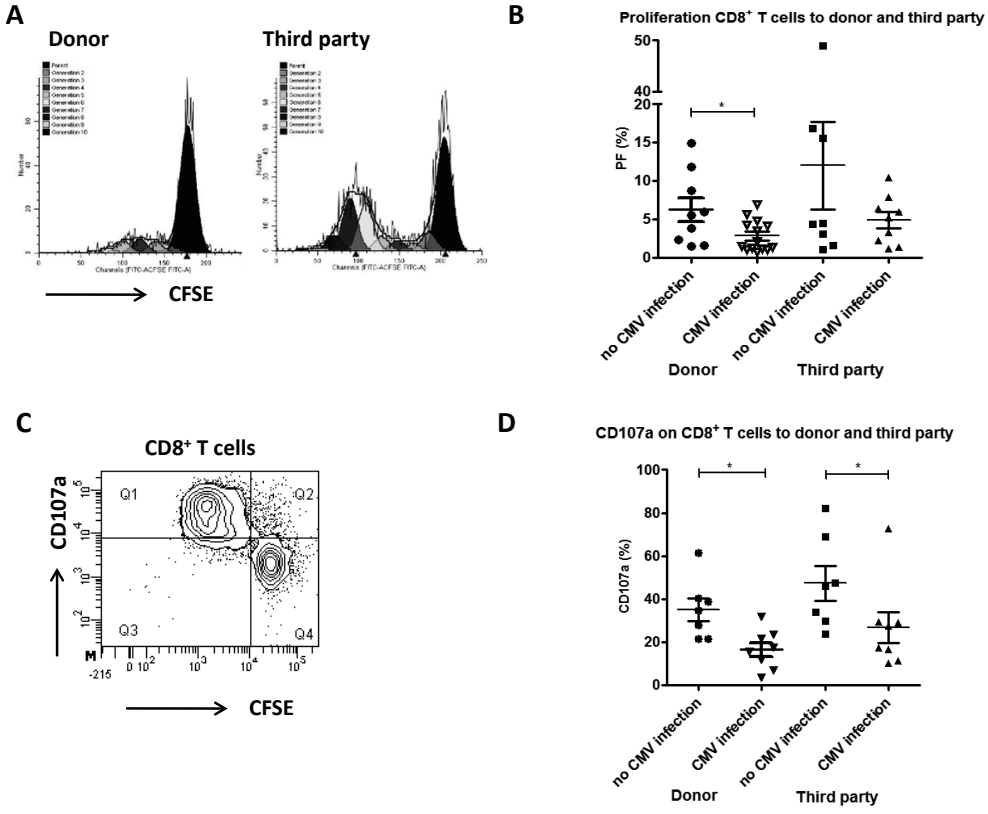

Figure 4 Allogeneic proliferative and cytotoxic degranulation responses of CD8⁺ T cells from patients with or without CMV infection after LTx. (A) Representative Modfit analysis plots showing CD8⁺ T-cell proliferation after 5 days of stimulation with donor-derived or 3rd party-derived CD40-activated B cells. (B) Precursor frequencies (PF) of proliferating CD8⁺ T cells in post-LTx PBMC of patients with or without CMV infection after LTx in response to donor (n=14 with CMV and n=9 without CMV) or third party (n=9 with CMV and n=8 without CMV) allo-antigens. Blood samples were collected 1-12 years after LTx (median 7 years). (C) Representative FACS plots showing CD107a expression on CFSE-labeled CD8⁺ T cells after 5 days of stimulation with donor-derived CD40-activated B cells. (D) CD107a expression on both proliferated and non-proliferated CD8⁺ T cells as percentage of all CD8⁺ T cells in post-LTx PBMC of patients with (n=8) or without (n=7) CMV infection after LTx in response to donor or third party allo-antigen. Each dot represents 1 patient, and lines indicate mean with standard error of the mean. *p<0.05

CD244⁺ CD8⁺ T cells show impaired proliferative responses to allogeneic stimulation

Since we found that CMV infection was associated with a strong rise in CD244 expression on circulating CD8⁺ T cells and with hyporesponsiveness of CD8⁺ T cells to allo-antigens after LTx, we wondered whether CD244 expression hallmarks a subpopulation of CD8⁺ T cells with reduced functionality. To test this hypothesis, CFSE-labeled CD244⁻ and CD244⁺ CD8⁺ T cells, as well as CD4⁺ T cells, were sorted from 17 LTx patients of the late post-LTx cohort. The sorted CD8⁺ T-cell subsets were co-cultured with autologous CD4⁺

T cells to provide CD4-help to the $CD8^+$ T cells, and stimulated with allogeneic CD40-activated B cells either derived from the donor or from an HLA-mismatched third party. In addition, both sorted subsets were stimulated with PHA. After 5 days of culture, cells were harvested and proliferation was measured. Significantly lower numbers of $CD244^+$ T cells than $CD244^-$ T cells proliferated in response to PHA (Figure 5A; p=0.0001). Similarly, significantly less $CD244^+$ T cells than $CD244^-$ T cells proliferated in response to allogeneic stimulations (p=0.002 for donor and p=0.023 for third party stimulation). The impaired proliferative responses of $CD244^+$ T cells were independent of the allo-antigenic source, as differences between $CD244^+$ and $CD244^-$ T cells were similar in response to donor and third party stimulations (Figure 5B). To assess whether blocking the interaction of CD244 with its ligand CD48 could restore the proliferative capacity of $CD244^+$ $CD8^+$ T cells, we repeated the above described experiments in a subgroup of patients in the presence of blocking antibodies directed against CD48. In addition, we studied the effect of blocking the interaction of CD160, which is co-expressed with CD244, with its ligand HVEM during culture. Both CD48 and HVEM were expressed on CD40-activated B cells (data not shown). After 5 days, we did not find any difference in allogeneic proliferation of both $CD244^+$ and $CD244^-$ $CD8^+$ T cells between conditions with and without blocking antibodies (data not shown). Collectively, these data suggest that CD244 expression marks a subset of dysfunctional $CD8^+$ T cells, but the receptor itself and the co-expressed CD160 receptor do not mediate the dysfunctionality.

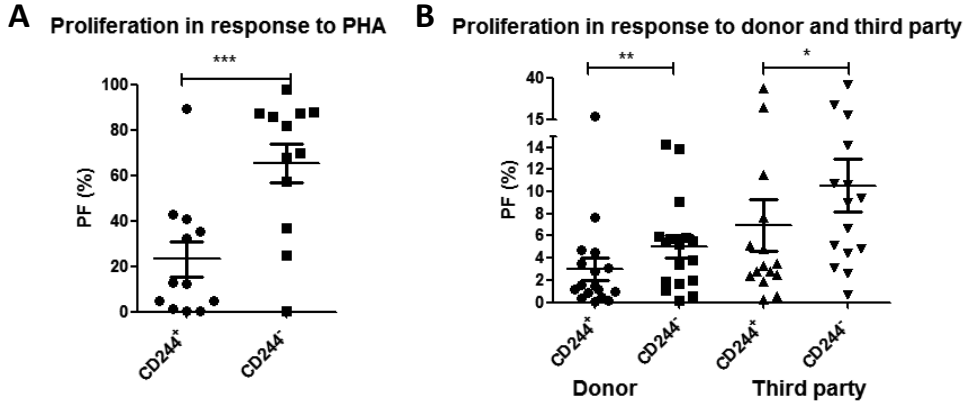

Figure 5 Proliferation of $CD244^+$ versus $CD244^-$ $CD8^+$ T cells in response to polyclonal and allogeneic stimulation. (A) Precursor frequencies (PF) of sorted $CD244^+$ versus $CD244^-$ $CD8^+$ T cells in response to polyclonal stimulation (PHA). (B) Precursor frequencies of $CD244^+$ versus $CD244^-$ $CD8^+$ T cells of patients post-LTx in response to donor and third party stimulation. Cells were sorted from PBMC collected from 17 patients 2-10 years after LTx (median 6.2 years). Each dot represents 1 patient, and lines indicate mean with standard error of the mean. *p<0.05, **p<0.005, ***p<0.0005

CD244$^+$ CD8$^+$ T cells contain the majority of CMV-specific cells

Since upregulation of CD244 on circulating CD8+ T cells was strongly associated with CMV infection after LTx and CD8$^+$CD244$^+$ T cells were dysfunctional, we analyzed whether CD244$^+$ CD8$^+$ T cells contained CMV-specific cells. We therefore co-stained PBMC from 5 CMV-experienced LTx patients from our study with MHC class I tetramers loaded with CMV-peptides and CD244 mAb, as described in Materials and Methods. A representative FACS plot is shown in Figure 6A. We found that the CD244$^+$ CD8$^+$ T-cell population contained the majority of CMV-tetramer positive cells; significantly more than the CD244$^-$ CD8$^+$ T-cell population (Figure 6B, p=0.033). These data support a causal relationship between CMV infection and the expansion of dysfunctional CD8$^+$ CD244$^+$ T cells after LTx.

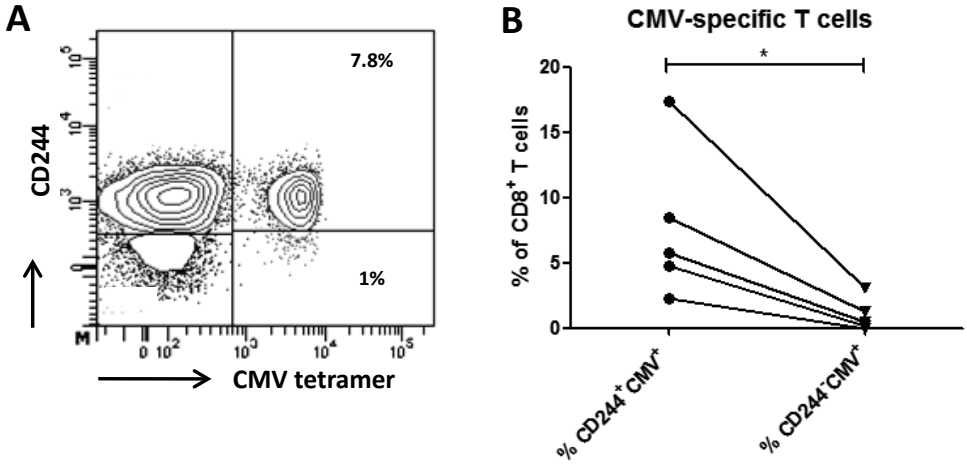

Figure 6 CMV-specific cells within CD244$^+$ and CD244$^-$ CD8$^+$ T-cell populations. (A) Representative FACS plots showing CD244$^+$ and CD244$^-$ CMV-tetramer positive cells CD8$^+$ T cells. (B) Percentage CD244$^+$ and CD244$^-$ CMV-tetramer positive cells of CD8$^+$ T cells of CMV-positive patients post-LTx (n=5).

DISCUSSION

In this study, we showed that the co-inhibitory receptor CD160 was upregulated on circulating memory CD8$^+$ T cells, while the co-inhibitory receptor CD244 was upregulated on both CD4$^+$ and CD8$^+$ memory T cells of patients early after LTx. In addition, we found that the increased expression of both receptors was sustained late after LTx. In contrast, PD1 was transiently upregulated on CD4$^+$ T cells 1 month after LTx, but its expression normalized already at 6 months after LTx. Our original hypothesis postulated that

long-term persistence of a high load of allo-antigens after LTx may induce exhaustion of allogeneic T-cells. In contrast to this hypothesis, impaired allogeneic $CD8^+$ T-cell responses and strong upregulation of CD244 on $CD8^+$ T cells after LTx were restricted to patients with CMV infection after LTx. Expression of CD244 on the majority of the CMV-specific $CD8^+$ cells in patients with CMV infection after LTx suggested a causal relationship between CMV infection and the observed expansion of $CD8^+CD244^+$ T cells. The observed lower proliferative capacity of $CD244^+$ $CD8^+$ T cells than $CD244^-CD8^+$ T cells in response to allogeneic stimulation suggested that the allogeneic $CD8^+$ T-cell hyporesponsiveness in LTx patients after CMV infection is caused by the accumulated $CD8^+CD244^+$ T cells. Together, these data suggest that CMV infection after LTx induces persistent accumulation of $CD8^+CD244^+$ T cells in the circulation, which display features of senescence or exhaustion, resulting in impaired peripheral $CD8^+$ T-cell responses to allo-antigens in these patients.

The observed association between accumulation of $CD8^+CD244^+$ T cells and CMV infection [11, 14, 30, 35] as well as the selective expression of CD160 and CD244 on memory T-cell subsets is consistent with previous studies [4, 11, 30, 36]. However, to our knowledge, this is the first study showing that CMV infection after organ transplantation induces sustained CD244 expression on memory $CD8^+$ T cells and that the resulting $CD8^+CD244^+$ T-cell subset is hyporesponsive to allo-antigens. It has been well-documented that CMV infection induces vast expansion in the circulation of a population of CMV-specific CD8+ T cells which are actively cycling. After establishment of CMV-latency the majority of CMV-specific CD8+ T cells become long-lived terminally differentiated resting T cells with poor proliferative capacity [37, 38]. Therefore, we hypothesize that expansion of these cells after LTx occurs during active CMV infection, while they differentiate into long-lived CD244-expressing terminally differentiated T cells with poor proliferative capacity after establishment of CMV-latency. Expression of CD244 is also induced on $CD8^+$ T cells by HIV and HCV infection, and results in impaired $CD8^+$ T-cell responses to viral antigens. However, the impairment of anti-viral $CD8^+$ T-cell responses in these patients can be abrogated by blocking the interaction between CD244 and its ligand CD48 [10, 11, 14]. In contrast, our data suggest that the observed allogeneic hyporesponsiveness of CMV-induced $CD244^+CD8^+$ T cells was not mediated by CD244-CD48 interaction, neither by interaction of the co-expressed inhibitory CD160 receptor with its ligand HVEM, since blocking CD48 or HVEM did not lead to abrogation of hyporesponsiveness of $CD8^+CD244^+$ T cells to allo-antigens in experiments with sorted $CD244^+$ T cells. The impaired response of this subset to allo-antigens may be related to its high content of CMV-specific T cells, resulting in lower proportions of T cells with other specificities, including allo-reactive T cells [39-41]. In addition, the limited TCR repertoire of CMV-specific cells [39, 42,

[43] accumulated in this subset may result in poor cross-reactivity to directly presented allo-antigens. However, these phenomena do not explain the impaired proliferation of the expanded $CD8^+CD244^+$ T cells to PHA (this study), or to CD3/CD28 stimulation in a previous study [30]. Interestingly, CMV-induced expansion of $CD8^+$ effector memory cells correlates with a decrease in T-cell telomere length, indicating T-cell senescence [44], and T-cell senescence has particularly been related to impaired proliferative capacity [45]. We therefore propose that the observed rise in CD244 expression in LTx patients with CMV infection marks expansion of a subset of highly differentiated but dysfunctional $CD8^+$ T cells, which shows features of senescence or exhaustion. However, its proliferative capacity is hampered by an as yet unknown mechanism.

The observed reduction in allo-reactive $CD8^+$ T-cell responses in LTx patients after CMV infection challenges the broadly accepted notion, based on experimental animal studies, that viral infections stimulate heterologous immunity resulting in increased frequencies of allo-reactive T-cells [46]. Indeed, reactivation of CMV infection as well as primary CMV infection abrogate transplant acceptance in mice and rat [47, 48]. However, several previously published observations in humans support our finding. First, CMV infection leads to T-cell senescence, and thereby impairs T-cell responses to other antigens and to vaccinations [34, 40, 49-51]. Interestingly, CMV infection after LTx is associated with an increased predisposition to develop opportunistic infections [52]. Secondly, immune senescence has been associated with improved kidney allograft survival [53]. Thirdly, the majority of $CD8^+CD244^+$ T cells in our patients belong to the Temra subset, and accumulation of circulating $CD8^+$ Temra has recently been shown to be associated with lower risk of acute rejection after kidney transplantation [41]. It was not feasible to investigate whether CMV infection or accumulation of CD8+CD244+ cells were associated with differences in clinical outcome such as graft or patient survival or acute rejection in our long-term study cohort, since all patients have stable graft function and are still alive. In addition, only 5 patients suffered from acute rejection. A larger prospective study is required to investigate associations between CMV infection or rise in CD244 and clinical outcomes.

The mechanism by which CMV infection induces expansion of $CD244^+CD8^+$ memory T cells is as yet unknown, but may be related to bystander effects of inflammatory responses caused by CMV or by its immune evasion strategies. A recent mouse study showed that CD244 is more highly upregulated during secondary than during primary $CD8^+$ T-cell responses, suggesting that T-cell reactivation is required for induction of high CD244 expression [54]. In addition, it has been shown that chronic lymphocytic choriomeningitis virus (LCMV) or Toxoplasm infections in mice impair memory T-cell responses against unrelated antigens due to generation of $CD8^+$ Temra. This was caused by increased

IFN-type signalling due to chronic inflammation caused by the persistent infections [55]. A similar mechanism may be driven by CMV infection after LTx, which also causes inflammation in the graft and in other organs [52], while CMV is able to induce IFN-α production [56]. A possible relation between inflammation and reduced allo-responses after LTx is supported by a recent study that showed that chronic HCV patients who are operationally tolerant after LTx over-express type I IFN and Interferon-Stimulated Genes in the liver graft [57]. A second explanation for the association between CMV infection and $CD8^+$ T-cell hyporesponsiveness may be that CMV produces viral IL-10 [58], which inhibits expansion of allo-reactive $CD8^+$ T cells. A third explanation may be that the immunological space of the recipient is occupied by large quantities of CMV-specific $CD8^+$ Temra that compete with and thus hamper the expansion of T cells with other specificities [59]. However, these explanations remain speculative, and further research is needed to decipher the mechanisms by which CMV infection induces expansion of $CD8^+CD244^+$ memory T cells, which is beyond the scope of the present study.

In contrast to the sustained increase in CD244 and CD160 expression on circulating T cells after LTx, we found that PD1 was only transiently upregulated following LTx. As PD1 can be upregulated by TCR-activation, this finding may be explained by the early and transient activation of donor-specific T cells after LTx that we observed previously [27]. However, the use of calcineurin inhibitors (CNI) by the majority of our patients may prevent sustained upregulation of PD1, since PD1 induction by TCR-ligation involves NFAT-signaling, which is inhibited by CNI [32]. PD1 upregulation is also prevented by mTOR inhibitors [60], immunosuppressive drugs used by a small group of patients in our cohorts. LAG3 and TIM3 expression did not show an increase after LTx, but we do not know whether this is related to the use of immunosuppressive drugs since no evidence exists on the effect of immunosuppressive drugs on the expression of these receptors.

A limitation of our study is that we were not able to link the $CD8^+$ T-cell hyporesponsiveness to an immunologically tolerant state towards the liver allograft. To investigate the clinical impact of the findings presented in our study, it will be interesting to determine the implications of CMV infection in LTx patients on the success rate of withdrawal of immunosuppressive drugs. A prospective study in which immunosuppressive drugs are weaned off is needed to investigate this.

In conclusion, in this study we showed that CMV infection after LTx was associated with the expansion of $CD8^+CD244^+$ T-cells with impaired proliferative capacity in response to allo-antigen, causing allogeneic $CD8^+$ T-cell hyporesponsiveness. These results suggest that CMV infection may hamper T-cell immunity and thereby promote immunological graft acceptance after LTx.

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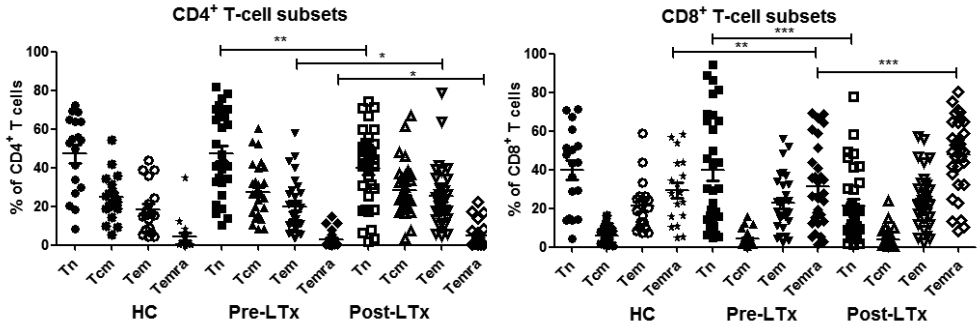
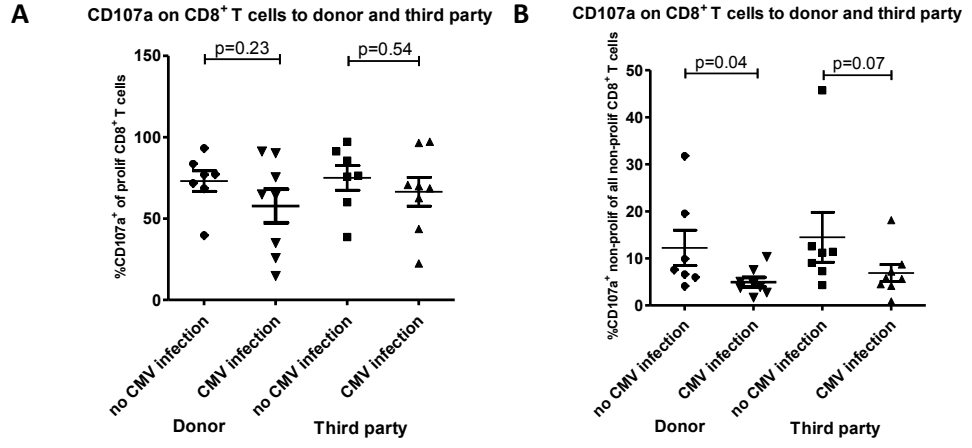


Supplemental Figure 1Subset distribution of circulate CD4⁺ and CD8⁺ T cells of healthy controls and patients before and late after LTx. Differentiation stages of T cells in LTx patients of the late post-LTx cohort (n=38) before and after LTx, and in healthy control subjects (HC) (n=18).



Supplemental Figure 2 Allogeneic cytotoxic degranulation responses of CD8+ T cells from patients with or without CMV infection after LTx. (A) CD107a expression on proliferated CD8+ T cells as percentage of all proliferated CD8+ T cells in post-LTx PBMC of patients with (n=8) or without (n=7) CMV infection after LTx in response to donor or third party allo-antigen. (B) CD107a expression on non-proliferated CD8+ T cells as percentage of all non-proliferated CD8+ T cells in post-LTx PBMC of patients with (n=8) or without (n=7) CMV infection after LTx in response to donor or third party allo-antigen. Each dot represents 1 patient, and lines indicate mean with standard error of the mean.

CHAPTER 4

Literature review

There is a silver lining in cytomegalovirus infection

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INTRODUCTION

Human cytomegalovirus (CMV), also known as human herpesvirus-5 (HHV-5), is extremely prevalent among humans. The seroprevalence of CMV in western countries is on average 60% and increases to >90% in the elderly ≥80 years old, and almost 100% in developing countries[1, 2]. Since its discovery in 1904, described by German scientists as large intranuclear inclusions in luetic fetus tissues[3, 4], substantial knowledge has been gained in the past century regarding its biology and pathogenesis, and the diagnostic and treatment options for CMV infection have considerably improved. CMV generally remains quiescent in healthy individuals, but can cause severe diseases in immunocompromised individuals, such as newborns, AIDS patients, and patients after hematopoietic stem cell transplantation (HSCT) or solid organ transplantation (SOT)[5]. Because of the global disease burden associated with CMV infection, increasing international efforts have been made to design vaccines against CMV with encouraging outcomes in recent years, aiming at a universal immunization[6, 7].

Nevertheless, every cloud has a silver lining. For the past three decades evidences have been emerging showing that CMV infection after HSCT is associated with a reduced incidence of hematological cancer recurrence. In recent years, evidences suggest that CMV infection could also exhibit anti-tumor effects after SOT, and promote transplant tolerance, and improve the immune response of childhood. Shadowed by the disease burden caused by CMV, these reports have not drawn much attention from the general medical and scientific community. The exact mechanisms behind these phenomena are not well understood, and the clinical implications are also not clear.

In this perspective review we summarize the accumulating evidences indicating beneficial effects of CMV infection, mainly focusing on its potential role in fighting malignancy, promoting transplant tolerance, and boosting childhood immunity, and discuss directions for future research and the potential impact of these findings on clinical practice.

MALIGNANCY

Leukemia

CMV infection is a frequent and major complication after HSCT, causing a variety of organ-specific diseases in patients receiving HSCT, including pneumonia and gastrointestinal disease. Prior to the age of prophylactic and pre-emptive treatment of CMV infection,

CMV pneumonia was the most common infectious cause of death after HSCT, with a mortality of 85%. Despite advances in diagnostic techniques and treatment strategies, CMV seropositivity remains to be associated with inferior outcome after HSCT[8]. However, growing evidences are supporting an anti-leukemic role of CMV infection after HSCT (Table 1). The earliest description of a "virus-versus-leukemia" effect dates back to 1986, when Lönnqvist et al. found in a small cohort of bone-marrow transplant recipients that acute leukemia patients with CMV infection were protected from relapse, which was independent from the effect of chronic graft-versus-host disease (GVHD)[9]. In a follow-up study, Jacobsen et al. found in a small acute myeloid leukemia (AML) patient cohort of four Nordic centers that the protective effect from relapse was associated with CMV positive donors, independent from GVHD and CMV infection[10]. Next, in a larger retrospective study from the Nordic Bone Marrow Transplantation Group, Jacobsen et al. confirmed the association between post-transplantation CMV infection and lower relapse risk of acute leukemia[11]. During this time period the diagnosis of post-transplant CMV infection still relied on viral culture, immunofluorescent CMV detection in biopsies, and changes in IgM/IgG titers, and there was no effective treatment available for CMV infection. Thus the effects of CMV-replication, anti-viral treatment and anti-viral immune response could not be established.

Shadowed by the adverse impact of CMV infection after HSCT, a possible "virus-versus-leukemia" effect did not attract much attention. In the next two decades, there was much debate about the impact of donor and recipient CMV serostatus on HSCT outcomes, and the optimal strategy of donor selection regarding CMV serostatus, but studies on the effect of CMV serostatus on leukemic relapse were scarce. Nachbaur et al. found that donor CMV-positivity in bone-marrow transplants from HLA-identical sibling donors significantly reduced the incidence of relapse, resulting in an improved overall survival[12]. Interestingly the observed protective effect was restricted to HLA-A2 positive patients, suggesting cross-reactivity between donor-derived anti-CMV $CD8^+$ T cells and recipient minor histocompatibility antigens presented by HLA-A2. Nachbaur et al. reported a similar finding in a cohort of reduced-intensity HSCT patients. Donor CMV-positivity was associated with a significantly reduced risk for relapse, but it did not lead to improved survival due to higher rate of bacterial and fungal infection related mortality in CMV-seronegative recipients transplanted with seropositive donors[13]. Behrendt et al. reported that pediatric patients receiving HSCT for acute leukemia had reduced risk of relapse and superior relapse-free survival when recipient and/or donor was CMV-seropositive before transplantation[14]. However, in these three studies, the anti-leukemia effect was not associated with CMV infection but with seropositivity.

After two decades of relative radio silence, a landmark study brought people's attention back to the possible "virus-versus-leukemia" effect of CMV. In a homogeneous cohort of adult AML patients monitored by pp65 antigenemia assay and treated by preemptive anti-CMV therapy, Elmaagacli et al. showed that patients with early CMV-replication after allo-HSCT had a significantly reduced risk to develop relapse within 10 years after transplantation (cumulative incidence of relapse at 10 years: 9% versus 42% in patients without CMV-infection)[15]. After adjusting for disease stage and acute/chronic GVHD, which suggests a "graft-versus-leukemia" effect, the profound reduction of relapse risk by CMV-replication remained significant. The reduced risk for relapse also translated into improved overall survival. In a cohort of chronic myeloid leukemia (CML) patients, Ito et al. also found that early CMV-replication, monitored by pp65 antigenemia or CMV-DNA PCR, was an independent protective factor against CML-relapse[16]. Later on, in a large cohort of allo-HSCT patients treated for different hematologic malignancies, Green et al. confirmed the anti-leukemia effect of early CMV-replication in AML patients, with a significant decreased rate of relapse at 100 days and 1 year after transplantation[17]. However the observed decrease in relapse was less striking than that described by Elmaagacli et al., and no improvement in overall survival was observed, probably due to the increased non-relapse mortality rate related to CMV infection. The observation of reduced CML relapse described by Ito et al. was also not verified by the study of Green et al. In contrast, the association between early CMV-replication after HSCT and reduction of relapse risk in AML patients was further validated by two independent studies recently[18, 19].

Solid tumor and lymphoma

CMV has recently been suggested to promote several types of solid malignancies by providing mechanisms for oncogenic transformation, oncomodulation and tumor cell immune evasion[20]. However clinical evidence supporting this hypothesis merely indicate the presence of virus within tumor, but without demonstrating a causal effect on tumor occurrence or progression. Thus skepticism remains regarding the relationship between CMV and cancer. Unlike in hematopoietic malignancies, clinical evidence supporting an anti-tumor effect in solid cancer are scarce. Couzi et al. retrospectively analyzed the incidence of cancer in 131 kidney transplant recipients, and found that CMV-naive patients had a 5-fold higher risk of cancer compared with CMV-exposed patients during the 8 years of follow-up[21]. However, in a large cohort of 455 kidney transplant patients, an opposite correlation was observed between CMV exposure and cancer occurrence[22]. In a large multicenter retrospective study of 44828 kidney transplant recipients, the highest incidence of lymphoma is observed in CMV seronegative patients with CMV seronegative donors ($p=0.09$)[23]. A randomized clinical trial comparing

preemptive therapy with prophylaxis in kidney transplant recipients showed that, while CMV prophylaxis reduced the incidence of CMV infection compared to pre-emptive therapy, death associated with malignancy was more likely to occur in patients received prophylaxis, again suggesting protection from cancer by CMV-infection[24].

Possible mechanism of the anti-malignancy effect

What could be the mechanisms for the anti-malignancy effect of CMV infection? Several possible explanations for this phenomena have been suggested. The most likely explanation could be the crosstalk between immune responses triggered by CMV infection and cancer cells. Infection of CMV leaves a deep and life-long imprint on the human immune system. Several types of immune cells induced by CMV infection, for example natural killer (NK) cells and γδ T cells as first line of defense, and the following adaptive immune response mainly dominated by $CD8^+$ $TCR\alpha\beta^+$ T cells, are described to recognize cancer cells.

NK cells are an important compartment of the innate immune system controlling viral infections. CMV infection after allogeneic HSCT and solid organ transplantation induces a lasting expansion of a "memory NK-cell" population, which express the activating NK-cell receptor NKG2C and are potent producers of IFN-γ[25, 26]. It has been shown that CMV latency alone is sufficient for expansion of these cells in both HSCT patients and healthy individuals[27, 28]. CMV-infected fibroblasts also promote $NKG2C^+$ NK-cell outgrowth *in vitro*[29, 30]. NK-cell self-tolerance is mediated by inhibitory receptors, such as killer cell immunoglobulin-like receptors (KIR), recognizing self-MHC class I molecules, and activating receptors recognizing ligands primarily expressed on infected or transformed cells. Therefore, allogeneic or tumor cells missing self-HLA ligands are targeted by NK cells by cytotoxic killing, namely the "missing-self hypothesis"[31]. In AML patients receiving T-cell depleted HSCT from KIR-MHC class I mismatched donors, donor-derived NK cells were found to exert a potent anti-leukemic effect and can prevent leukemia relapse[32, 33]. Pre-transplant infusion of alloreactive NK cells also eradicates advanced human leukemia in mice[33]. Therefore, the expansion of $NKG2C^+$ NK cells by CMV infection after allogeneic HSTC could contribute to the elimination of residual leukemic blasts[25].

γδ T-cell population provides a first line of host immune defense to microbial pathogens and have also some adaptive features[34]. A strong and durable expansion of $V\delta2^{neg}$ γδ T cells that reactive to CMV infected cells is a hallmark of CMV infection after solid organ transplantation and HSCT[35-39]. γδ T cells express Vδ1, Vδ3 or Vδ5, but not Vδ2, are collectively designated as $V\delta2^{neg}$ γδ T cells, which reside mainly in intestinal and

skin epithelia, spleen, and liver[40]. Vδ2^{neg} γδ T cells infiltrate many types of tumors and exert strong cytotoxicity against tumor cells *ex vivo*[41, 42]. Adoptive transfer of expanded γδ T cell is currently under investigation as immunotherapy for cancer treatment[43]. In a human colon cancer xenograft mouse models, adoptive transfer of CMV-induced Vδ2^{neg} γδ T cells inhibited tumor growth and metastasis *in vivo*[44, 45]. It has been shown in kidney transplant patients that circulating Vδ2-negative γδ T-cell expansion, which was induced by pre-transplant or post-transplant CMV infection, was associated with a reduced cancer risk[21]. In addition, Vδ2^{neg} γδ T cells isolated from CMV-infected transplant recipients were found to cross-react to intestinal tumor epithelia cell lines and primary leukemic blasts *in vitro*[39, 46, 47]. Thus, the expansion of γδ T cells following CMV infection, and its cross-reactivity to malignant cells, could contribute to the observed protective effect of CMV infection against cancer.

As to the adaptive immunity, CMV also contributes to the reconstitution or repopulation of TCRαβ^{+} T cells after HSCT and organ transplantation[48, 49]. CMV is known to drive the expansion of differentiated effector memory T cells lacking costimulatory molecule CD28[50]. Poor CD8^{+} T cell recovery after allogeneic HSCT with diminished numbers of CD28^{-} CD8^{+} T cells is associated with a greater risk of subsequent relapse of hematological malignancies[51]. Cross-reactivity between viral-specific cytotoxic T cells (CTLs) and unrelated antigens such as HLA are common[52]. It could be that CMV-specific CTLs are cross-reactive to tumor-associated antigens or allo-HLA molecules expressed on the leukemic blasts. However in the latter case, the anti-leukemia effect of CMV is independent from acute and chronic graft-versus-host disease (GVHD) in several studies. Nevertheless, a positive correlation between anti-viral T-cell response and protection from leukemic relapse has been reported[53, 54].

Apart from inducing immune responses against malignant cells, a direct anti-cancer effect of CMV has also been proposed, as CMV replication can be cytolytic during acute infection at epithelial surface. Cancer cells could be reservoir for CMV. Large copy numbers of CMV are found in cancer cells of many AML, B-ALL and B-ALL patients[55], and the CMV presence has also been identified in several types of solid tumors[56-58]. Several types of viruses can exert oncolytic effects, and genetically engineered strains are under investigation as therapy targeting neoplastic cells in preclinical studies and human clinical trials with promising results[59]. CMV infection has been shown to inhibit proliferation and induce apoptosis of acute leukemia cell lines *in vitro*[60]. CMV reactivation in cancer cells may also induce changes cell phenotype and make them potential targets of cytotoxic immune cells[61].

CHILDHOOD IMMUNITY

In experimental animal models, latent infection of herpesviruses, including MCMV, alters the immune responses of the hosts and exerts a durable protection from unrelated pathogens, a process known as heterologous immunity[62-65]. However little is known about how CMV influences immune responses to other unrelated pathogens in immunocompetent humans. It has been reported that CMV-seropositive individuals are less responsive to influenza vaccines[66-68], which is suggested to be related to the CMV-driven immune senescence in the elderly[69]. But this observation was not confirmed in another study analyzing more than 700 older subjects[70]. Meanwhile, evidences are emerging in favor of a beneficial effect in young populations. For example, Miles et al. studied the immune responses to measles vaccination and the polyclonal T-cell responses to the staphylococcal enterotoxin B (SEB), and found stronger CD8 T-cell proliferation in response to SEB in CMV-positive infants, and that the antibody response to measles vaccines correlated with the IFN-γ response to CMV[71]. Pera et al. found that CMV-positivity is associated with higher levels of polyfunctional $CD8^+$ T cells in young and middle aged individuals[72]. More recently, Furman and coworkers applied a systemic approach to study the differences between CMV-positive and negative immunocompetent subjects, and found enhanced antibody responses to influenza vaccines, stronger $CD8^+$ T cell responses to cytokine stimuli, and elevated levels of circulating IFN-γ in CMV-positive young individuals, in contrast no such effects were observed in elderly individuals[73]. Furthermore, their murine experiments confirmed a significant cross-protection against influenza in young mice infected with MCMV, and this effect declined with aging of mice. Together, these data suggest that CMV may improve immune responses to unrelated pathogens in young individuals.

TRANSPLANT TOLERANCE

Viral infection, including CMV, has long been considered to be associated with allograft rejection after organ transplantation[74]. In experimental animal models, both acute infection and reactivation of the latent MCMV prevent graft acceptance[75, 76]. However the association between CMV infection and graft rejection in the clinical setting is less clear[74]. Several observations suggest that CMV infection has systemic immunosuppressive effects in the clinical transplant setting. Firstly, CMV infection after organ transplantation is often associated with opportunistic superinfections of fungi, bacteria and other viruses[77]. Secondly, CMV might drive senescence of immune system in elderly kidney transplant recipients, which is associated with fewer acute rejection episodes[78]. Thirdly, increased

numbers of CMV-IE-1-specific memory T cells are associated with lower numbers of alloreactive T cells and improved renal allograft function after kidney transplantation[79], and strong CMV-specific $CD4^+$ T-cell responses correlate with protection from cardiac allograft rejection[80]. In addition, we recently showed that, even though CMV drives memory T-cell expansion after liver transplantation, it does not lead to a higher frequency of alloreactive T cells. Instead, liver transplant patients with CMV primary infection develop donor-specific $CD8^+$ T-cell hyporesponsiveness, and are protected from the occurrence of acute rejection episodes late after transplantation[81].

As compared with other solid organ grafts, liver displays unique immunological features, and liver transplantation is the only setting in which a significant proportion of patients can eventually discontinue immunosuppressive medication without undergoing rejection, a phenomenon knowns as spontaneous operational tolerance[82, 83]. Recent prospective immunosuppression withdrawal studies showed that operational tolerance can be achieved in more than 40% of selected liver transplant patients, including patients with chronic hepatitis C virus (HCV) infection[84, 85]. Studies that sought to identify biomarkers for operational tolerance have found expansion of peripheral Vδ1 γδ T cells, and an increased Vδ1/Vδ2 ratio has been observed in tolerant LT patients in several independent studies, which has even been used as a surrogate marker to predict operational tolerance after liver transplantation[85-88]. As mentioned earlier, expansion of circulating Vδ1 γδ T cells and an increased peripheral Vδ1/Vδ2 ratio is also a feature related to CMV infection. Increased numbers of highly differentiated and dysfunctional $CD8^+$ T cells might be another common feature shared by CMV infection and operational tolerance after liver transplantation, suggesting a possible association[81, 85].

How CMV restrains alloreactivity after liver transplantation remains elusive, and whether CMV-induced cell signatures play a functional role in promoting tolerance is as yet unknown. The well-established immune evasion strategies of CMV is a possible clue. For example, CMV produces viral IL-10 to establish latency, which may also have a systemic immunosuppressive effect[89, 90]. It is also associated with the inflation memory T-cell and immune senescence[69]. After liver transplantation, CMV infection induces the expansion of a $CD8^+$ T-cell subset expressing inhibitory receptor 2B4, with reduced proliferative and degranulative abilities in response to alloantigen (de Mare-Bredemeijer, J Immunol. 2015). Moreover, CMV has been recently shown to significantly modulate peripheral mixed T-cell chimerism after HSCT[91]. After organ transplantation, chimerism is rare in peripheral blood, but more commonly observed within grafted organ[92, 93]. It has been suggested in a case report that severe CMV disease early after LT contributed to the development of hematopoietic chimerism and subsequent donor-

specific hyporesponsiveness[94]. In addition, we hypothesize that inflammation coupled with CMV infection may contribute to the induction of liver allograft tolerance. CMV is known to drive a T helper 1 cell (Th1) polarization in peripheral. Within the human liver, CMV infection of sinusoidal endothelium triggers significant production of type I interferon by HSEC, and recruit Th1 effector memory T cells and regulatory T cells[95]. Th1 cells produce high amounts of pro-inflammatory cytokine IFN-γ, which is strikingly absolutely needed for liver transplant tolerance in animal models[96]. Recently, Morita et al. described a mesenchyme-mediated immune control (MMIC) mechanism utilized by liver allograft to eliminate effector T cells and maintain tolerance through IFN-γ and B7-H1[97]. The importance of type I interferon signalling has also been highlighted recently by Bohne et al., showing in an immunosuppression withdrawal trial of HCV-infected liver transplant patients, that operational tolerant patients overexpress type 1 interferon and interferon-stimulated genes (ISG) in liver[85]. To sum, accumulating evidences suggest that in humans, the immunomodulatory effects exerted by CMV could restrict alloimmune responses and promote transplant tolerance.

DIRECTIONS FOR FUTURE RESEARCH

Various questions remain to be answered by future research. One of the key questions is how CMV infection, directly or indirectly, is associated with beneficial clinical outcomes, and more often paradoxical effects. Large-scale longitudinal studies covering different phases of CMV infection are needed. A variety of outcomes should be monitored in different cohorts, namely immunocompetent and immunocompromised, young and old populations. Meanwhile, systemic approaches should be applied to closely monitor the CMV-related virological and immunological measures, the status of immune system, and the antigen-specific immune responses targeting both CMV and other unrelated antigens (such as pathogens, TAAs, allo-antigens). Once those benefits and the underlying mechanisms become clear, another important area for research is to develop approaches to skew the natural course of CMV infection, or the related immune responses, to achieve those beneficial effects, while limiting the established adverse effects of CMV infection. Clinical trials of antiviral therapies and immunotherapies targeting CMV infection should also pay more attention to outcomes not directly linked to CMV infection, such as recurrent/de novo cancer and unrelated infectious diseases in longer terms, to understand better how these therapies effect the virus-host interaction. The ideal therapies in the future should aim at decreasing CMV-related morbidity, while conveying or even enhancing those beneficial effects of natural CMV infection.

IMPLICATIONS FOR CMV VACCINE DEVELOPMENT

Due to the health burden associated with CMV, the development and licensure of an effective CMV vaccine has been on the schedule of government, industry and scientific community for two decades, however no licensed vaccine is available yet[7]. However, several clinical trials, of both modified virus vaccines (MVV) and individual antigen vaccines (IAV), are ongoing, with promising results in controlling congenital virus transmission and reducing viral replication and antiviral usage in SOT patients[98-101]. Despite all the difficulties in designing, effective CMV vaccines are likely to come available in the future. It is a matter of discussion which populations should be covered by CMV vaccines. It is advocated to add CMV vaccine to the childhood vaccination schedule to obtain universal immunization[6, 7]. However, the advantages and disadvantages of doing so should be carefully weighed. Firstly, the majority of the infant population is at very low risk to develop serious health problems caused by CMV. Secondly, given the extremely high prevalence of CMV, early childhood immunization is possibly only leading to delayed nature infection and increasing risk of infection late in life, which has been hypothesized to have extra negative effects[102]. Thus vaccinating the entire population would be ethically problematic. The ubiquity of CMV in humans suggests that CMV may convey certain mutual advantages to human beings through virus-host interactions, which possibly have been established and coevolved for over one hundred million years[103]. Actions disrupting this equilibrium should be taken with extra caution before a better understanding of it is achieved.

CONCLUSION REMARKS

While host-microbiota interaction has drawn increasing attention, and its impact on human diseases and health is becoming more and more clear in recent years, the interaction with life-long persistent virus remains a whole new world to explore[104-106]. The evidences summarized in this review may not be comprehensive, but rather provide examples showing that CMV, one of the most prevalent herpesvirus, can have paradoxical effects and benefit human health under certain circumstances, despite the diseases it causes in immunocompromised individuals. More research in different fields is definitely needed to better understand this paradigm, and such knowledge can be utilized to reduce the disease burden associated with CMV infection and improve human health from certain aspects. Meanwhile, the universal vaccination strategy advocated at this moment should be of concern and a matter of debate.

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CHAPTER 5

Interplay between donor PD-L1 and recipient PD-1 regulates acute graft rejection after liver transplantation

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ABSTRACT

Background & Aims: Co-inhibitory receptor-ligand interactions fine-tune immune responses by negatively regulating T-cell functions. Our aim is to examine the involvement of co-inhibitory receptor-ligand pair PD-1/PD-L1 in regulating acute rejection after liver transplantation (LT) in humans.

Methods: PD-L1/PD-1 expression in liver allograft was determined by immunohistochemistry or flow-cytometry, and the effect of blockade was studied using graft-infiltrating T cells *ex vivo*. Five single nucleotide polymorphisms within *PD-1* and *PD-L1* genes were genotyped in 528 LT recipients and 410 donors, and associations with both early (≤ 6 months) and late (> 6 months) acute rejection were analyzed using Cox proportional-hazards regression model. The effect of *PD-L1* rs4143815 on PD-L1 expression was analyzed using donor hepatic leukocytes.

Results: PD-L1 was expressed by hepatocytes, cholangiocytes and along the sinusoids in post-transplant liver allografts, and PD-1 was abundantly expressed on allograft-infiltrating T cells. PD-L1 blockade enhanced allogeneic proliferative responses of graft-infiltrating T cells. In the genetic association analysis, donor *PD-L1* rs4143815 (CC/CG versus GG; HR=0.230; p=0.002) and recipient *PD-1* rs11568821 (AA/AG versus GG; HR=3.739; p=0.004) were associated with acute rejection late after LT in multivariate analysis. Recipients carrying the *PD-1* rs11568821 A allele who were transplanted with liver grafts of *PD-L1* rs4143815 GG homozygous donors showed the highest risk for late acute rejection. *PD-L1* rs4143815 is associated with differential PD-L1 expression on donor hepatic dendritic cells upon IFN-γ stimulation.

Conclusion: Our data show that PD-1/PD-L1 pathway regulates allograft rejection in humans, and suggest a critical interplay between donor PD-L1 and recipient PD-1.

INTRODUCTION

T cells are the key drivers of the adaptive immune response, playing an important role in controlling infections by pathogens. T cells are also critically involved in graft rejection after allogeneic organ transplantation[1]. At the time of TCR ligation, costimulatory signals delivered by antigen presentation cells are required to fully activate T cells to proliferate and perform effector functions[2]. However to provide immune regulation and maintain tolerance, signaling through co-inhibitory pathways, such as PD-1/PD-L1, negatively regulates T cell functions and fine-tunes immune responses[3].

Programmed death 1 (PD-1) receptor and its ligand PD-L1 (also known as CD274 or B7-H1) are important for the induction and maintenance of allograft tolerance in several experimental animal models[4, 5]. However whether PD-1/PD-L1 pathway is involved in regulation of graft rejection in humans is unknown. PD-1 is inducibly expressed on T cells after TCR engagement or during antigen persistence[6]. PD-1 was first found to be expressed on hepatic T cells of patients with chronic HBV and HCV-infection, and regulates virus-specific T-cell responses [7, 8]. A recent study showed that T cells in healthy liver tissue also abundantly express PD-1, suggesting a role in maintaining the immune-tolerant microenvironment of liver[9]. PD-L1 is characterized by its broad expression, being not only expressed on hematopoietic cells, such as Kupffer cells in the liver, but also on non-hematopoietic cells such as sinusoidal endothelial cells and hepatocytes[10, 11], and is critically involved in the regulation of intrahepatic $CD8^+$ T cells accumulation and deletion[12]. Its expression in liver tissue is further upregulated by inflammatory stimuli, including ischemia/reperfusion injury [13, 14] However, whether PD-1 and PD-L1 are expressed in human liver grafts after transplantation is unknown.

Single nucleotide polymorphisms (SNPs) in *PD-L1* and *PD-1* have been found to be associated with the development of a variety of immunological disorders. We showed previously that SNPs in the co-inhibitory molecule cytotoxic T-lymphocyte antigen 4 (*CTLA-4*) gene are associated with the development of acute rejection after liver transplantation (LT), indicating that CTLA-4 is involved in the regulation of allogeneic immunity in humans[15, 16]. Two studies did not find an association between *PD-1* SNPs and graft rejection after LT, however both studies were limited by their small sample size[17, 18].

In the current study, we hypothesize that T-cell co-inhibition via PD-1/PD-L1 pathway is involved in the regulation of allograft rejection in humans. For this purpose, we first examined the expression of PD-1 and PD-L1 in human liver allografts, and tested the effect

of PD-1/PD-L1 blockade on *ex vivo* proliferation of graft-infiltrating T cells in response to alloantigens. Next we studied whether common genetic variations within *PD-1* and *PD-L1* genes affect the incidence of graft rejection in patients after LT. Genotyping was performed in a large and well-characterized cohort of LT patients, including 528 recipients and 410 donors. Associations with both early (≤ 6 months after LT) and late (> 6 months after LT) acute rejection were analyzed. Finally, the functional relevance of the selected SNP was analyzed.

PATIENTS AND METHODS

Study population

Patients that underwent orthotopic LT between 1992 and 2012 at Erasmus MC, The Netherlands, from whom recipient and/or donor DNA samples were available, were included in this study. In total 584 patients were included in the analysis. From 354 patients both recipient and donor DNA samples were available, from 174 patients only recipient DNA samples were available, and from 56 patients only donor DNA samples were available. Demographic 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 in May, 2013. Immunosuppressive therapy is described in Supplemental Materials and Methods. Acute rejection 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 was defined as rejection occurring within 180 days after LT, late acute rejections were defined as those occurring after 180 days after LT[19].

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

DNA extraction, selection and determination of gene polymorphisms

Detailed protocols are described in Supplemental Materials and Methods.

Cell isolation, culture, stimulation, flow cytometry, and immunohistochemistry

Detailed protocols are described in Supplemental Materials and Methods.

Statistics

Statistics are described in Supplemental Materials and Methods.

Table 1. Patient characteristics

Baseline characteristics	Total n=584
Recipient	
Age	50 (16-69)
Gender, female	230 (39.4%)
Recipient ethnicity	
Caucasian	500 (85.6%)
African	44 (7.5%)
Asian	40 (6.8%)
Donor	
Age	47 (11-82)
Gender, female	297 (50.9%)
Donor ethnicity	
Caucasian	410 (100%)
Primary liver diseases	
AHF	85 (14.6%)
HCC	69 (11.8%)
PBC/PSC/AIH	154 (26.4%)
HBV/HCV	71 (12.2%)
Alcoholic cirrhosis	60 (10.3%)
Cryptogenic	33 (5.7%)
Metabolic	20 (3.4%)
Others	92 (15.8%)
re-LTx	65 (11.1%)
Type of donor	
DBD	508 (87.0%)
DCD	76 (13.0%)
Basiliximab induction	413 (70.7%)
Tacrolimus as CNI	404 (69.2%)
Early acute rejection	125 (21.4%)
Late acute rejection	41 (7.0%)

AHF, acute hepatic failure; HCC, hepatocellular carcinoma; HBV, hepatitis B virus infection; HCV, hepatitis C virus infection; PBC, primary biliary cirrhosis; PSC, primary sclerosing cholangitis; AIH, autoimmune hepatitis; DBD, donor after brain death; DCD, donor after cardiac death; CNI, calcineurin inhibitor.

RESULTS

PD-L1 expression in human liver allografts

In liver grafts PD-L1 expression is up-regulated immediately after LT due to ischemia and reperfusion injury[14]. However, whether PD-L1 is expressed in liver allografts during acute rejection episodes is unknown. Therefore, we examined the expression of PD-L1 by immunohistochemistry in pre-LT donor liver biopsies obtained at the end of the cold ischemic period and in post-LT liver allograft biopsies taken during acute rejection episodes using a validated antibody (405.9A11). Second-trimester placental tissue was used as positive control, and we observed strong PD-L1 staining at the microvillous membrane and moderate staining in the cytoplasm of the villous syncytiotrophoblasts (**Fig.1A**), in agreement with a previous study[20]. Absence or low level PD-L1 expression was observed in the pre-LT donor biopsies (**Fig.1B**), but increased expression was found in post-LT rejection biopsies in both portal and lobular regions (**Fig.1C**). In portal regions, PD-L1 was expressed by cholangiocytes and infiltrating leukocytes (**Fig.1D, left**). In the lobular region, we observed PD-L1 expression by hepatocytes and along liver sinusoids (**Fig.1D, right**). While cholangiocytes, infiltrating leukocytes and sinusoidal cells showed both surface and cytoplasmic staining, PD-L1 expression by hepatocytes was restricted to cytoplasm. Immunohistochemistry staining using another validated anti-PD-L1 antibody (5H1) showed a similar PD-L1 expression pattern (**Supplementary Fig.1A**). The degree of PD-L1 staining was graded in parenchyma and in portal tract (**Fig.1E**). Both early and late rejection biopsies showed significantly higher PD-L1 expression by cholangiocytes, infiltrating leukocytes and cells along the liver sinusoids than healthy donor liver biopsies. The expression on hepatocytes also tended to be higher in rejection biopsies than in healthy donor biopsies, but the difference was not significant. No significant difference in PD-L1 expression was observed between early and late rejection biopsies. Nevertheless, hepatic PD-L1 is not exclusively induced in liver during graft rejection, for example liver with chronic HBV infection showed a similar expression pattern (**Supplementary Fig.1B**). This is in line with a previous study showing that hepatic PD-L1 expression is related to inflammation rather than to the etiology of liver damage[13].

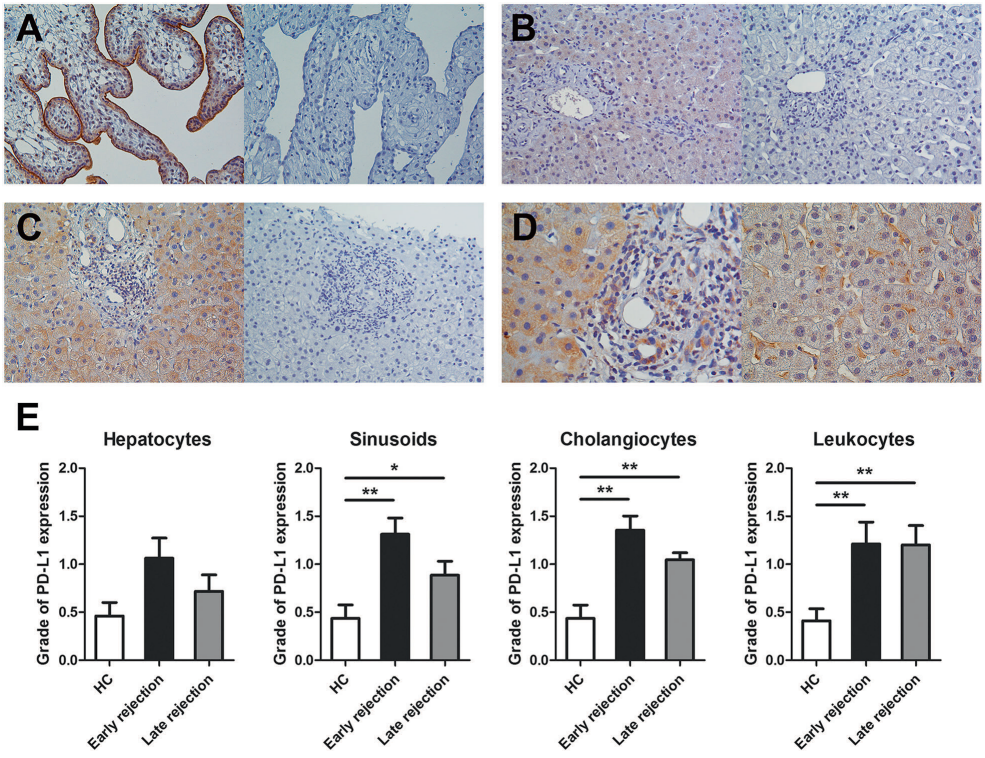

Fig.1. PD-L1 expression in liver allograft biopsies. PD-L1 expression was evaluated by immunohistochemistry. Representative images of (A) second-trimester placenta tissue, (B) pre-LT donor biopsies, and (C) post-LT biopsies with acute rejection, stained with anti-PD-L1 antibody (405.9A11, left) or negative control (right). Expression of PD-L1 in post-LT biopsies was observed in (D, left) hepatocytes, cholangiocytes, infiltrating leukocytes, and (D, right) along the liver sinusoids. (E) The PD-L1 expression level of different cell types was graded and compared between healthy donor biopsies (HC), early and late acute rejection biopsies (n=14, 8, 11 respectively). Original magnification ×200 (A, B, C) and ×400 (D). *P<0.05, **P<0.01.

PD-1 is highly expressed on graft infiltrating T cells, and PD-1/PD-L1 blockade enhances graft infiltrating T-cell proliferation in response to alloantigen

We studied PD-1 expression by flow cytometry on paired samples of peripheral blood T cells and allograft-infiltrating T cells isolated from transplanted liver tissues (n=19, **Supplementary Table 1**), and also hepatic T cells isolated from healthy donor liver perfusates (n=20). Compared to circulating T cells, PD-1 was expressed at higher levels on graft-infiltrating T cells, but no difference was observed between PD-1 expression on T cells isolated from transplanted liver and healthy liver perfusates (**Fig.2A**). There was also no difference in PD-1 expression between T cells isolated from rejection biopsies, non-rejection biopsies or liver explants due to re-LT (**Fig.2B**). Together with PD-1, the expression of other T-cell co-inhibitory receptors TIM-3, LAG3, CD160 and 2B4 was

determined. Compared to PD-1$^-$ T cells, their PD-1$^+$ counterparts expressed the other co-inhibitory receptors at higher levels (except for CD160 expression on CD8 T cells)(**Fig.2C**). To study whether the PD-1/PD-L1 expression is functionally relevant in regulating allogeneic responses of graft-infiltrating T cells, we used larger quantities of graft-infiltrating leukocytes that were isolated from re-LT liver explants (n=5, **Supplementary Table 1**). Allograft-infiltrating cells were labeled with carboxyfluorescein succinimidyl ester (CFSE), and were stimulated by either donor or third-party derived splenocytes. Co-cultures were performed in the presence of 10-μg/ml anti-PD-L1 antibody or matched isotype. After 5 days, T-cell proliferation was determined by CFSE dilution (**Fig.2D**).

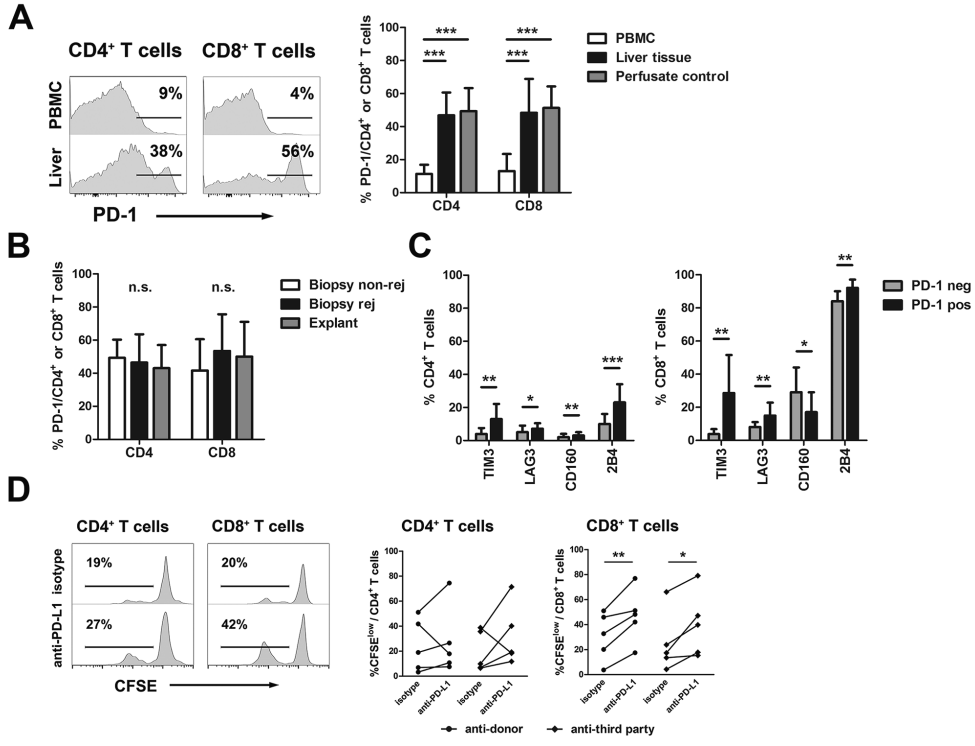

Fig.2. PD-1 expression on allograft-infiltrating T cells and the effect of blockade on T-cell proliferation. (A) PD-1 expression was measured by flow-cytometry on graft infiltrating T cells, and was compared to that on T cells in paired PBMC samples (n=19) and perfusates of healthy donor livers (n=20). (B) PD-1 expression on T cells isolated from rejection biopsies (n=7), non-rejection biopsies (n=7), and re-LT explants (n=5) was also comparted. (C) The expression of other T-cell co-inhibitory receptors was compared between PD-1$^+$ and PD-1$^-$ graft-infiltrating T cells (n=11). (D) Graft-infiltrating leukocytes isolated from explant biopsies were labeled by CFSE and were stimulated by donor or third-party splenocytes for 5 days. T-cell proliferation in the presence of anti-PD-L1 blocking antibody or isotype-matched control antibody was determined by CFSE dilution. *P<0.05, **P<0.01, ***P<0.001.

Proliferation of both $CD4^+$ and $CD8^+$ graft-infiltrating T cells in response to both donor and third-party stimulation were enhanced by PD-1/PD-L1 blockade (with only one exception in CD4 proliferation). These results prompted us to further investigate the involvement of the PD-1/PD-L1 pathway in the regulation of acute rejection after LT.

Genetic study population and genotyping

For this purpose, we studied the associations between SNPs within donor and recipient *PD-1* and *PD-L1* genes and acute rejection in a large and well-characterized cohort of LT patients. 584 patients that underwent LT between 1992 and 2012 were included in this study (**Table 1**). The majority of the recipients (85.6%, 500/584), and all donors, were Caucasians. The main indication for LT was autoimmune-related liver diseases (PBC, PSC and AIH; 26.4%, 154/584), the second was acute hepatic failure (AHF, 14.6%, 85/584), and the third was cirrhosis caused by viral hepatitis (12.2%, 71/584). The overall incidence of early (≤ 6 months after LT) acute rejection was 21.4% (125/584), which developed at median 10 (range 2-167) days after LT. Recipient age (HR=0.982, p=0.009), donation after cardiac death (DCD, HR=0.386, p=0.005), basiliximab as induction immunosuppression (HR=0.465, p<0.001), and use of tacrolimus as CNI (HR=0.402, p<0.001), were associated with early acute rejection (**Supplementary Table 2**). The incidence of late (> 6 months after LT) acute rejection was 7.0% (41/584), which developed at median 645 (range 186-6368) days after LT. Previous early acute rejection or re-transplantation did not predispose patients to the development of late acute rejection in our cohort (**Supplementary Table 2**). As late acute rejection is more likely to occur during the immunosuppression dose reduction over time after LT, we compared the blood levels of immunosuppressive drugs prior to the occurrence of late acute rejection to the target levels. Blood levels of immunosuppressive drugs of 22 (54%) of 41 patients who developed late acute rejection were lower than the target level, while 18 (44%) patients remained within normal range (**Supplementary Table 3**). However also in patients without late acute rejection, immunosuppression lower than target level is a very common phenomena during follow-up. The observed genotype frequencies of all five SNPs in both recipient and donor subjects were in agreement with HWE (p>0.05) (**Table 2**). All call rates were above 95%, ranging from 95.9% to 97.5%, assuring the genotyping accuracy. No strong linkage disequilibrium (LD) was observed between any of the SNPs that we studied (r^2<0.8).

Table 2. Distribution of genotype frequencies and Hardy–Weinberg Equilibrium (HWE)

Genotype	Recipient n=528							
	1	2	3	Total	MAF	χ^2	P	Call rate
PD-1	GG	GA	AA					
rs11568821	386	119	7	512	0.13	0.41	0.52	97.0%
PD-1	TT	TC	CC					
rs2227981	182	242	86	510	0.41	0.13	0.72	96.6%
PD-1	GG	GA	AA					
rs10204525	404	98	11	513	0.12	2.90	0.09	97.2%
PD-L1	GG	GA	AA					
rs1411262	261	201	53	515	0.30	2.33	0.13	97.5%
PD-L1	GG	GC	CC					
rs4143815	281	190	44	515	0.27	2.10	0.15	97.5%
Genotype	Donor n=410							
	1	2	3	Total	MAF	χ^2	P	Call rate
PD-1	GG	GA	AA					
rs11568821	291	104	4	399	0.14	2.56	0.11	97.3%
PD-1	TT	TC	CC					
rs2227981	131	189	73	393	0.43	0.11	0.74	95.9%
PD-1	GG	GA	AA					
rs10204525	320	69	4	393	0.10	0.02	0.90	95.9%
PD-L1	GG	GA	AA					
rs1411262	218	156	23	397	0.25	0.51	0.48	96.8%
PD-L1	GG	GC	CC					
rs4143815	199	165	34	398	0.29	0.00	0.98	97.1%

MAF, minor allele frequency

Univariate analysis of associations between donor and recipient SNPs and acute rejection

Early and late acute rejection were used as separate endpoints in the univariate analysis by Cox proportional-hazards regression model. Due to the small sample size of the minor allele homozygous groups, we compared patients or donors carrying minor alleles to those major allele homozygotes. We first analyzed the correlation between donor SNPs and acute rejection (**Fig.3A**). SNPs within donor *PD-1* gene were not associated with the occurrence of acute rejection. In addition, donor *PD-L1* SNPs were not significantly associated with the development of early acute rejection. However, patients receiving liver grafts obtained from donors carrying the C allele of *PD-L1* rs4143815 had a lower incidence of late acute rejection (HR=0.32; 95%CI=0.14-0.76; p=0.006). Next we analyzed the correlation between recipient SNPs and acute rejection (**Fig.3B**). Recipients carrying the A allele of *PD-1* rs11568821 had an increased risk to develop late acute rejection

(HR=2.21; 95%CI=1.16-4.17; p=0.019). This association was marginally non-significant if Bonferroni correction was applied (to a p-value<0.01), however it became more significant when only Caucasian recipients were taken into account (HR=2.48; 95%CI=1.26-4.92; p=0.011). None of the recipient *PD-L1* SNPs was significantly associated with the development of acute rejection.

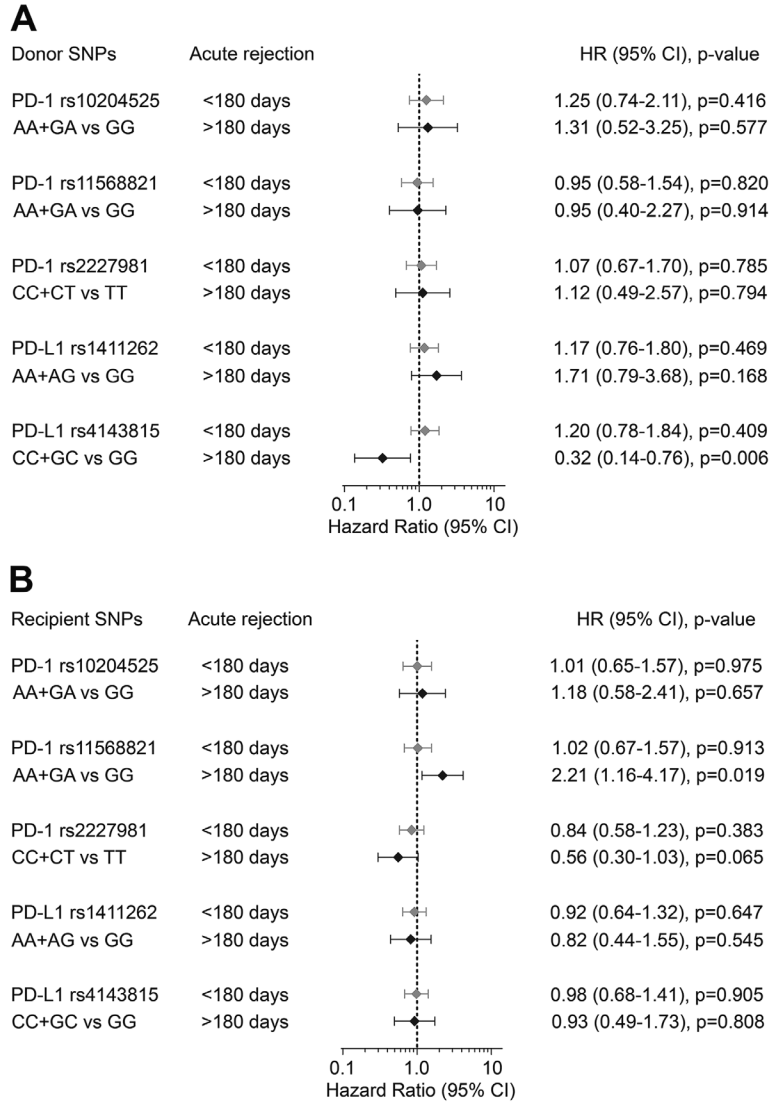

Fig.3. Univariate analysis of associations between SNPs and acute rejection after LT. The associations between (A) donor / (B) recipient SNPs and acute rejection were analyzed by Cox proportional-hazards regression model with a likelihood ratio test, and 180 days post-LT was used as the cut-off to define early and late acute rejection.

Multivariate analysis of associations of SNPs and baseline characteristics with acute rejection

Firstly, both recipient *PD-1* rs11568821 and donor *PD-L1* rs4143815 were analyzed in the multivariate Cox proportional-hazards regression model together with patient age (continuous), ethnicity, primary liver diseases, type of donation, basiliximab as induction immunosuppression, and the type of CNI. The year of transplantation (continuous) was also included in the model as patient management has been significantly improved overtime during our study period (**Table 3**). After adjusting for covariates, both donor *PD-L1* rs4143815 (HR=0.230; 95%CI=0.081-0.654; P=0.002)(**Fig.4A**) and recipient *PD-1* rs11568821 (HR=3.739; 95%CI=1.534-9.116; P=0.004)(**Fig.4B**) were found to be independent factors significantly associated with acute rejection late after LT. Next we stratified patients by combinations of recipient *PD-1* rs11568821 and donor *PD-L1* rs4143815 (**Table 3**). Recipient *PD-1* AA/AG and donor *PD-L1* GG combination showed the highest risk to develop acute rejection late after LT adjusted for covariates (compared to combination of recipient *PD-1* GG and donor *PD-L1* GG; HR=5.248; 95%CI=1.932-14.26; p=0.0003)(**Fig.4C**). Finally we analyzed the multiplicative interaction between recipient *PD-1* rs11568821 and donor *PD-L1* rs4143815 (**Table 3**), however the interaction was not significant (p=0.128).

Table 3. Multivariate analysis of recipient and donor SNPs adjusted for patient characteristics

Variables	HR	95% CI	P-value
Recipient *PD-1* rs11568821 AA+AG vs GG	3.739	(1.534-9.116)	0.004
Donor *PD-L1* rs4143815 CC+CG vs GG	0.230	(0.081-0.654)	0.002
Combinations of recipient *PD-1* rs11568821 and donor *PD-L1* rs4143815			0.0003
Recipient *PD-1* GG / Donor *PD-L1* GG	1.000		
Recipient *PD-1* GG / Donor *PD-L1* CC+GC	0.441	(0.125-1.552)	
Recipient *PD-1* AA+AG / Donor *PD-L1* GG	5.248	(1.932-14.26)	
Recipient *PD-1* AA+AG / Donor *PD-L1* CC+GC	0.389	(0.047-3.231)	
Multiplicative interaction of recipient *PD-1* rs11568821 and donor *PD-L1* rs4143815			
Recipient *PD-1* rs11568821 AA+AG vs GG	5.248	(1.932-14.26)	0.001
Donor *PD-L1* rs4143815 CC+CG vs GG	0.441	(0.125-1.552)	0.185
Recipient *PD-1* rs11568821 × Donor *PD-L1* rs4143815	0.168	(0.014-2.035)	0.128

Adjusted for patient ethnicity, age, primary liver diseases, types of donor, induction immunosuppression, CNI, and year of transplantation.

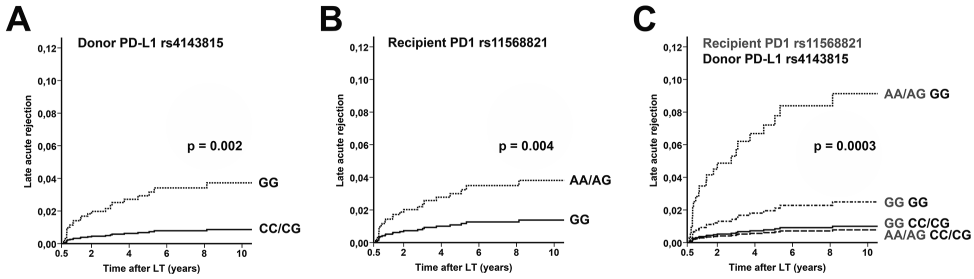

Fig.4. Multivariate analysis of the associations between donor and recipient SNPs and acute rejection late after LT. Cox proportional hazards model survival functions of the relationship between (A) donor PD-L1 rs4143815, (B) recipient PD-1 rs11568821, (C) combination of recipient PD-1 rs11568821 and donor PD-L1 rs4143815 with acute rejection late after LT, adjusted for recipient age, ethnicity, primary liver diseases, type of donor, types of induction immunosuppression and CNI, and the year of transplantation.

PD-L1 rs4143815 is associated with differential PD-L1 expression by hepatic dendritic cells upon IFN-γ stimulation

Whether *PD-L1* rs4143815 alters PD-L1 expression was unknown, thus we aimed to determine PD-L1 expression on donor liver cells of different genotypes. Due to the unavailability of viable liver parenchymal cells with known genotypes, we determined PD-L1 expression on hepatic leukocytes derived from donor liver perfusates as an alternative. PD-L1 expression on hepatic T cells, B cells, NK cells, NKT cells, macrophages/monocytes or BDCA1$^+$ dendritic cells (DC) did not differ between donors with different rs4143815 genotypes (*PD-L1* rs1411262 genotypes were matched between groups). When we used IFN-γ to induce PD-L1 expression, after 24 hours PD-L1 expression on macrophages/monocytes and DCs was strongly enhanced in a dose-dependent manner, but not on T cells, B cells, NK cells nor NKT cells (**Fig.5A**). Interestingly, hepatic BDCA1$^+$ DCs with rs4143815 CC genotype showed higher PD-L1 expression than those with GG genotype after IFN-γ stimulation (**Fig.5B**). No difference was observed in PD-L1 expression on macrophages/monocytes from donors with different rs4143815 genotypes (**Fig.5C**).

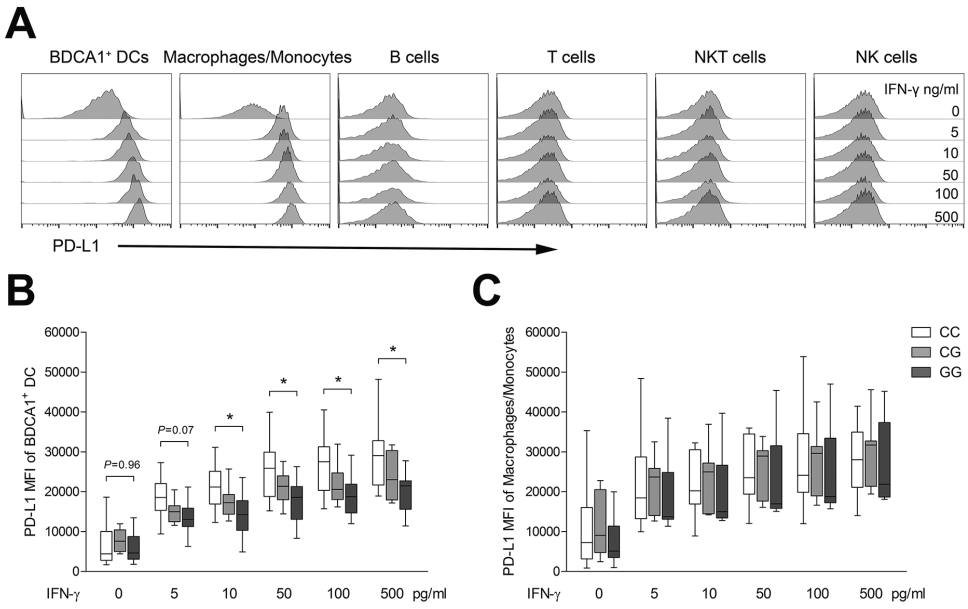

Fig.5. PD-L1 rs4143815 is associated with differential IFN-γ-induced PD-L1 expression on hepatic BDCA1⁺ dendritic cells. (A) Representative histograms showing the effects of different concentrations of IFN-γ on PD-L1 expression on hepatic leukocytes isolated from liver allograft perfusates. PD-L1 expression on (B) hepatic dendritic cells (DCs) and (C) macrophages/monocytes were compared between rs4143815 genotypes, shown by mean fluorescence intensity (MFI) (CC n=10, CG n=8, GG n=9). *P<0.05.

DISCUSSION

Although several studies have shown the involvement of PD-1/PD-L1 co-inhibitory pathway in modulation of graft tolerance in animal models, evidence for a role in regulating alloimmune responses and graft rejection in humans is still lacking. In the present study, we first showed that PD-L1 and PD-1 are abundantly expressed in liver allografts, and that PD-1/PD-L1 blockade leads to enhanced intragraft T-cell proliferation to allostimulation. Next, in the genetic association analysis, we found that recipient *PD-1* rs11568821 and donor *PD-L1* rs4143815 are associated with acute rejection late after transplantation, both in univariate and multivariate analysis. Finally, we showed that *PD-L1* rs4143815 is associated with differential PD-L1 expression on hepatic BDCA1⁺ DCs upon IFN-γ stimulation. To the best of our knowledge, this is the first study showing that PD-L1/PD-1 interaction is regulating graft rejection after organ transplantation in humans, and our data support an interplay between donor PD-L1 and recipient PD-1.

Intrahepatic PD-L1 expression was up-regulated in rejection biopsies compared to healthy donor liver, similar to what has been observed in inflamed livers of different etiologies[13]. Up-regulation of PD-L1 in liver has been shown to be driven by IFN-γ[11], and we conclude that it represents a counter-regulatory mechanism to dampen ongoing intrahepatic T-cell alloreactivity. This is supported by a recent study describing a immune control mechanism utilized by murine liver allografts to eliminate effector T cells and maintain tolerance, which is triggered by IFN-γ induced PD-L1 expression in the graft[21]. Hepatocytes showed only cytoplasmic and no membrane expression. Although PD-L1 can exerts its inhibitory effect on T cell functions only when expressed on cell surface, cytoplasmic expression has been detected in many tissues[22-24], including inflamed liver, and also in cultured hepatocytes[13, 14]. It has recently been hypothesized that cytoplasmic expression may represent intracellular stores of PD-L1, which may be deployed to the cell surface depending upon appropriate stimulation[25]. Alternatively, cytoplasmic PD-L1 may be released from hepatocytes as a soluble form into the micro-environment and thereby exert suppressive effects[26]. Moreover we found that PD-1 is highly expressed on T cells infiltrating transplanted liver allografts irrespective from their graft damage etiology and on T cells in healthy livers, in agreement with the observations of Kroy *et al*[9], suggesting that the abundant presence of PD-1 in liver may be a reflection of its immunologically tolerogenic environment. And this PD-1/PD-L1 interaction is non-specifically limiting intrahepatic allogeneic T-cell responses rather than contributing to a donor-specific hypo-responsiveness.

PD-1 rs11568821 (also named as *PD-1.3*) is located in an enhancer-like structure in intron 4 of *PDCD1* gene, which is the binding site for transcription factor RUNX1[27]. The minor A allele of *PD-1* rs11568821 disrupts the binding of RUNX1 shown by electrophoretic mobility shift assays, leading to altered transcriptional activity of PD-1 mRNA, reduced PD-1 protein expression and impaired PD-1 inhibitory effect[27-30]. However in a luciferase reporter assay, the A allele did not alter the gene transcriptional activity, casting doubts on the functional relevance of rs11568821[31]. Despite this controversy, the *PD-1* rs11568821 A/G polymorphism is associated with a variety of immune-related diseases, such as systemic lupus erythematosus, rheumatoid arthritis, multiple sclerosis, primary biliary cirrhosis, type 1 diabetes, and also with cytomegalovirus infection and the sustained virological response of HCV patients in response to treatment. Two studies have analyzed the effect of *PD-1* rs11568821 on LT outcome, and no association with acute rejection was found[17, 18]. However both studies were hampered by their small sample size, and only rejections within 1 month or 6 months after LT, respectively, were analyzed. In agreement with these two studies, we did not find an association of recipient *PD-1* rs11568821 with acute rejection early after LT. However, it independently influenced the occurrence of acute rejection late after LT.

PD-L1 rs4143815 is located in the binding site of miR-570 to the 3'-UTR of PD-L1 gene[32]. Genetic variants in the 3'-UTR of target mRNA can affect gene expression by inducing an altered binding pattern between miRNA and mRNA[33]. The C allele of *PD-L1* rs4143815 resulted in an increased transcriptional activity in a luciferase reporter assay by interfering with miR-570 function, and was associated with increased gastric cancer risk, possibly as a result of suppression of immunological tumor surveillance by increased PD-L1 expression[32]. In addition, the G allele of *PD-L1* rs4143815 is more frequently observed in patients with type 1 diabetes, in whom also lower plasma PD-L1 concentrations were found[34]. We observed that patients receiving a donor liver carrying the C allele had an decreased risk to develop late acute rejection. No difference in the baseline expression of PD-L1 was found on donor hepatic leukocytes of distinct rs4143815 genotypes. PD-L1 can be induced by pro-inflammatory cytokines such as IFN-γ in various cell types[35]. We found that donor hepatic BDCA1$^+$ DCs carrying the C allele of rs4143815 expressed PD-L1 at higher levels in response to IFN-γ stimulation. On one hand, donor DCs are the main instigators of allogeneic responses after LT[36, 37]. On the other hand, they are important for the maintenance of a tolerogenic liver environment, which is partially mediated by PD-L1[38, 39]. Thus, donor-derived hepatic BDCA1$^+$ DCs carrying the C allele may counteract allogeneic T-cell responses after transplantation by increased expression of PD-L1. PD-L1 expression in other cell types within liver could also be regulated by this SNP. We showed that PD-L1 was also expressed by non-hematopoietic cells, such as hepatocytes and cholangiocytes in liver allografts. As donor-derived non-hematopoietic cells are less likely to be replaced by cells of recipient origin after LT than donor-derived hematopoietic cells, they may counter-regulate T-cell responses for a longer term after LT than donor-derived DCs. In addition, in experimental animal models, PD-L1 expressed on non-hematopoietic cells is better at limiting tissue immunopathology than that expressed on hematopoietic cells[40]. In agreement with heart transplantation studies in mice[41, 42], our results indicate that donor PD-L1 may be more important than recipient PD-L1 in the regulation of liver allograft rejection, and support a central role of PD-L1 in regulating hepatic immunity in response to inflammation[12].

Furthermore, it is very interesting that the association between both SNPs and acute rejection is only observed late after transplantation. It could be that the high levels of immunosuppressive medication given early after transplantation overrule the effects of these SNPs. As a result of reduction of immunosuppression over time, the PD-1/PD-L1 pathway might play a more dominant role in preventing rejection late after transplantation. In addition, high levels of immunosuppressive drugs, especially calcineurin inhibitors, can suppress the up-regulation of PD-1 upon T-cell activation, thereby inhibiting this immunoregulatory mechanism[43].

Our study has limitation that needs to be acknowledged. Even though this study is based on a large and unique cohort of LT patients containing both donor and recipient samples, the genetic associations should be validated in an independent cohort in the future. However, it would be even more interesting and clinically relevant to study the effects of *PD-1*/*PD-L1* SNPs in immunosuppression withdrawal or minimization studies, which may help identifying patients that are more tolerant to no or low level of immunosuppression.

To conclude, we provide the first evidence of the involvement of PD-1/PD-L1 pathway in allograft rejection in humans. Our data suggest a critical interplay between donor PD-L1 and recipient PD-1 in counter-regulating graft rejection, and that targeting of PD-1 by administration of an appropriate agonist, such as PD-L1 Ig fusion protein[44, 45], may be an interesting avenue to develop novel immunosuppression strategies.

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SUPPLEMENTARY MATERIALS AND METHODS

Immunosuppression therapy

The standard immunosuppressive therapy in our center consisted of prednisone, calcineurin inhibitor (CNI; cyclosporine, CsA or tacrolimus, Tac), with or without azathioprine or mycophenolate mofetil (MMF). Since 1998 basiliximab was introduced as induction immunosuppression, and since 1996 CsA was gradually replaced by Tac. After CsA was initiated within 24 hours post-reperfusion in a dose of 10-15 mg/kg body weight/day, the dosage was adjusted to trough levels of 200-400 ng/mL during the first 3 months and thereafter 100-200 ng/mL. Tac was initiated within the first 5 days after transplantation in a dose of 1-2 mg/kg body weight/day. The target trough level of Tac was 10–15 ng/ml in the first month, 8–12 ng/ml between 1 to 6 months, 5-10 ng/ml between 6 to 12 months, and 4-8 ng/ml after 1 year.

DNA extraction, selection and determination of gene polymorphisms

DNA was extracted from whole blood of recipients and from splenocytes of deceased donors using a DNA Purification kit (Promega, Madison, Wisconsin, USA) according to the manufacturer's instructions. SNPs were selected based on the literature, which were either functionally relevant or had been found to be associated with immune-related diseases, and had a minor allele frequency >10%[27-30, 32, 46-53]. Genotyping was performed by LGC (http://www.lgcgroup.com/) using a competitive allele-specific PCR (KASP™) technique. Pairwise linkage disequilibrium (LD) between the SNPs was calculated by SNAP pairwise LD tool (http://www.broadinstitute.org/mpg/snap/ldsearchpw.php)[54].

Cell isolation

Intragraft leukocytes were isolated from needle liver biopsies, and from liver tissue of explanted allografts obtained during re-LT. Liver tissues were digested with 0.5 mg/mL collagenase IV (Sigma-Aldrich, St. Louis, MO) and 0.1 mg/mL DNase I (Roche, Indianapolis, IN) for 30 minutes at 37°C. Tissues were smashed through 70 μm cell strainers, and cells which went through the strainers were collected. For explant tissues, additional Ficoll density gradient centrifugation was performed to purify mononuclear leukocytes. Isolation of PBMC from LT patients, and splenocytes from splenic tissue derived from liver donors, were performed as previously described[55]. Healthy liver graft perfusates were collected during the second back table flush. These perfusates contain donor liver leukocytes with minimal contamination of peripheral blood leukocytes[56]. Hepatic leukocytes from perfusates were isolated within 12 hours by Ficoll density gradient centrifugation, and were cryopreserved for future experiments.

Cell culture and stimulation

Graft infiltrating leukocytes isolated from explant tissues were labeled with 0.1 μM carboxyfluorescein succinimidyl ester (CFSE, Invitrogen, Carlsbad, CA) and co-cultured with either donor or third-party (mismatched at HLA-A, B and DR loci with both donor and recipient) splenocytes (from which $CD3^+$ cells were depleted using CD3 microbeads, Miltenyi Biotec, Bergisch Gladbach, Germany), at a ratio of 1:1 for 5 days in round-bottom 96-well plates. Co-culture was performed in the presence of blocking antibody for PD-L1 (clone 5H1, 10 μg/ml, kindly provided by Dr. Haidong Dong, Mayo Foundation for Medical Education and Research)[24, 57] or matched IgG1 isotype control antibody (10 μg/ml, eBioscience, San Diego, CA). T-cell proliferation was measured by determination of CFSE-dilution in $CD4^+$ and $CD8^+$ T cells on day 5 by flow cytometry.

Donor hepatic leukocytes were stimulated with different concentrations of recombinant human IFN-γ (Miltenyi Biotec, Bergisch Gladbach, Germany) in RPMI 1640 culture medium supplemented with 10% heat-inactivated fetal calf serum and 1% Penicilline/Streptomycine for 24 hours, and PD-L1 expression was measured by flow cytometry.

Flow cytometry and antibodies

The following antibodies were used: PD-L1-PE-Cy7, CD3-PE-Cy7, CD3-APC-eFluor780, CD4-APC-eFluor780, CD8-eFluor450, PD-1-APC, PD-1-PE-Cy7, 2B4-APC (eBioscience, San Diego, CA); CD19-HorizonV500 (BD Biosciences, San Diego, CA), CD56-APC (Beckman Coulter, Marseille, France), CD14-Pacific Blue (BD Pharmingen, Erembodegem, Belgium), BDCA1-PE (Miltenyi Biotec, Bergisch Gladbach, Germany), LAG-3-PE (R&D Systems, Minneapolis, MN), CD160-PerCP-Cy5.5, TIM3-Brilliant-Vio421 (Biolegend, San Diego, CA). Non-viable cells were excluded using 7-AAD (BD Biosciences, San Diego, CA) or LIVE/DEAD Fixable Aqua Dead Cell Stain Kit (Invitrogen, Carlsbad, CA). Phenotypes of donor hepatic leukocytes were defined as follows: T cells ($CD3^+CD56^-$), B cells ($CD19^+$), NK cells ($CD3^-CD56^+$), NKT cells ($CD3^+CD56^+$), macrophages/monocytes ($CD14^+$), dendritic cells (DCs, $CD19^-BDCA1^+$). Flow cytometry was performed using a FACSCanto II flow cytometer (BD Biosciences, San Diego, CA). Data were analyzed with FlowJo software (FlowJo, Ashland, OR, USA).

Immunohistochemistry

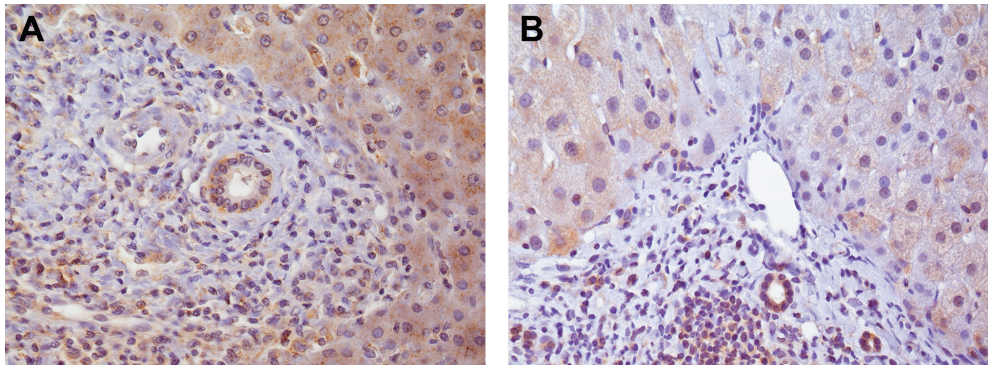
PD-L1 expression was evaluated by Immunohistochemistry (IHC) on formalin-fixed, paraffin-embedded sections (4μm) from second-trimester placental villi tissue (positive control), in liver allograft biopsies with early acute rejection (n=8), late acute rejection (n=11) and in biopsies taken from the donor livers at the end of the cold ischemic period (n=14). Early and late rejection biopsies showed similar rejection activity manifested by

similar RAI score. Antigen-retrieval was performed by heating in a microwave in Tris/ethylene diamine tetra-acetic acid (pH 9.0) buffer. Endogenous peroxidase blockage was performed by incubation for 15 minutes with 0.05% H_2O_2 in citric/phosphate buffer (pH 5.8). Tissue sections were labeled with an extensively validated mouse monoclonal anti-PD-L1 antibody (13 μg/mL, clone 405.9A11)[58, 59], kindly provided by Dr. Gordon Freeman, Dana-Farber Cancer Institute, Boston, MA. The EnVision system HRP labelled polymer goat-anti-mouse antibody (Dako, Glostrup, Denmark) was used to visualize PD-L1 expression. Counterstaining was performed using hematoxylin. In addition, a selection of biopsies were stained using another validated anti-PD-L1 antibody (5H1), according to a previously described protocol with minor modifications[60]. Scoring of immunohistochemical stainings was performed by two investigators and differences were resolved by mutual agreement. PD-L1 expression on biopsies was graded on a 0-2 scale. Mean values of at least 3 microscopic fields of parenchymal tissue and of 3 different portal tracts were calculated for each biopsy.

Statistics

Calculation of Hardy–Weinberg Equilibrium (HWE) was performed at http://www.oege.org/software/hwe-mr-calc.shtml[61]. Actual and expected genotype frequencies of both donors and recipients were compared using Chi-Square analysis and p-values of >0.05 were considered as no deviation from HWE.

Patient baseline characteristics were summarized using median with range for continuous variables and percentages for discrete variables. Early and late acute rejections were used as separate endpoints in analysis. The associations between acute rejections and patient characteristics, as well as SNPs, were analyzed using the Cox proportional-hazards regression model with a likelihood ratio test. We first performed univariate analysis for each SNP. SNPs with p-values <0.05 were included in the multivariate analysis together with recipient age, ethnicity, primary liver diseases, type of donation, use of basiliximab as induction immunosuppression, type of CNI, and the year of transplantation. Linearity of continuous variables and clinically relevant interactions were tested. Where multiple pairwise comparisons were made, a Bonferroni correction was applied, and p-values <0.01 were considered significant. SPSS v. 21 was used for statistical analysis.



Supplementary Fig.1. (A) Representative immunohistochemistry staining of PD-L1 on acute rejection biopsies (clone 5H1). (B) Representative immunohistochemistry staining of PD-L1 on biopsies with chronic HBV-infection (clone 405.9A11).

Supplementary Table 1. List of patients studied for liver allograft infiltrating T cells

Total n=19	Rejection biopsies n=7	Non-rejection biopsies n=7	Re-LT explants n=5
Primary liver diseases			
Viral	1	2	0
Autoimmune	4	1	2
Others	2	4	3
*Time after LT, months	6	11	57
Indications for re-LT			
PSC recurrence	-	-	2
Chronic rejection	-	-	2
ITBL	-	-	1
*AST, U/L	79	82	1394
*ALT, U/L	134	239	603
*RAI score	5	2	0

*Median values are displayed. PSC, primary sclerosing cholangitis; ITBL, ischemic-type biliary lesions; AST, aspartate aminotransferase; ALT, alanine aminotransferase.

Supplementary Table 2. Association between patient characteristics and acute rejection

Patient characteristics	Early acute rejection			Late acute rejection		
	HR	95%CI	P-value	HR	95%CI	P-value
Recipient age	0.982	(0.969-0.995)	0.009	0.982	(0.958-1.005)	0.137
Recipient gender, female	1.250	(0.878-1.780)	0.217	1.173	(0.631-2.179)	0.615
Donor age	0.999	(0.988-1.010)	0.812	0.984	(0.965-1.003)	0.099
Donor gender, female	0.904	(0.637-1.284)	0.573	1.034	(0.560-1.909)	0.914
Patient ethnicity			0.548			0.983
Caucasian	1.000			1.000		
African	1.051	(0.550-2.008)		0.905	(0.278-2.942)	
Asian	0.656	(0.288-1.492)		1.041	(0.320-3.390)	
Primary liver diseases			0.136			0.220
HBV/HCV	1.000			1.000		
AHF	1.746	(0.893-3.413)		1.261	(0.437-3.635)	
HCC	0.666	(0.285-1.557)		0.455	(0.091-2.264)	
PBC/PSC/AIH	1.398	(0.745-2.625)		1.495	(0.589-3.794)	
Alcoholic cirrhosis	0.960	(0.430-2.143)		0.437	(0.088-2.165)	
Cryptogenic	0.786	(0.280-2.206)		1.060	(0.265-4.240)	
Metabolic	0.750	(0.214-2.630)		0.628	(0.076-5.219)	
others	1.388	(0.695-2.772)		0.360	(0.073-1.785)	
re-LTx	1.116	(0.640-1.946)	0.703	0.855	(0.263-2.776)	0.789
Type of donor, DCD	0.386	(0.180-0.826)	0.005	0.560	(0.134-2.333)	0.384
Basiliximab induction	0.465	(0.327-0.663)	0.000	0.801	(0.417-1.539)	0.510
Tacrolimus as CNI	0.402	(0.283-0.572)	0.000	0.621	(0.324-1.188)	0.152
Early acute rejection	-	-	-	1.382	(0.713-2.676)	0.348

The associations between acute rejections and baseline characteristics were analyzed using the Cox proportional-hazards regression model. P-values were calculated with a likelihood ratio test.

Supplementary Table 3. The level of immunosuppressive drugs prior to the occurrence of late acute rejection.

Immunosuppression	Target level	n	%
CsA C0 n=13	<100 ng/mL	9	69%
	100-200 ng/mL	3	23%
	>200 ng/mL	1	8%
CsA C2 n=5	<400 ng/mL	2	40%
	400-600 ng/mL	3	60%
Tac C0 n=17	<4 ng/mL	9	53%
	4-8 ng/mL	6	35%
	>8 ng/mL	2	12%
MMF C0 n=3	<1 mg/L	1	33%
	1-3.5 mg/L	2	67%
Everolimus C0 n=2	<8	1	50%
	8-12	1	50%
All patients n=41	lower than target level	22	54%
	within normal range	18	44%
	unknown	1	2%

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CHAPTER 6

Ex vivo detection of T-cell indirect allorecognition in human transplant recipients using engineered HLA messenger RNA

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Submitted

ABSTRACT

Studies on indirect allorecognition in transplanted patients are hampered by the lack of a reliable *in vitro* method to present alloantigens indirectly to T cells. The aim of this study is to develop a novel approach enabling specific detection of human $CD4^+$ T cells with indirect allospecificity. By *in vitro* transcription we engineered mRNA encoding HLA-A*0201, but we excluded the transmembrane region from the DNA construct to prevent the possibility of direct allorecognition. HLA-A*0201^{neg}/HLA-DRB1*0101^{pos} peripheral B cells were expanded and differentiated into antigen presenting B-cell blasts (CD40-B), and transfected with HLA-A*0201-encoding mRNA. No surface expression of HLA-A2 was observed on transfected CD40-B, but an HLA-DRB1*0101 restricted $CD4^+$ T-cell clone recognizing an HLA-A*0201 derived peptide produced IFN-γ upon co-culture with the transfected CD40-B, which could be completely prevented by MHC class II blockade. Using autologous CD40-B transfected with HLA-A*0201 mRNA, $CD4^+$ T cells responding to indirectly presented HLA-A2 were detected in HLA-A2-negative liver transplant patients transplanted with livers of HLA-A2 positive donors. In conclusion, using engineered HLA mRNA, we developed a novel technique to specifically measure human T cells with indirect allospecificity, which may be a promising tool to study indirect allorecognition in human transplant recipients.

INTRODUCTION

T cells that recognize mismatched donor HLA-molecules are the major driving force of allograft rejection. Recipient T cells can recognize foreign HLA-molecules via two different mechanisms, the direct pathway and the indirect pathway[1]. The direct pathway describes the recognition of intact allogeneic HLA molecules expressed on donor cells by recipient T cells, while the indirect recognition pathway describes a more "natural" process of T-cell activation, in which recipient antigen-presenting cells (APCs) take up alloantigens from the transplanted organ, process and present them as peptides to recipient $CD4^+$ T cells. More recently, another semi-direct pathway of alloantigen recognition has been suggested, in which recipient T cells recognize intact donor MHC molecules taken up and presented as intact molecules by recipient antigen-presenting cells (APC)[2, 3].

It is believed that the direct pathway drives early acute allograft rejection, but that it subsides upon gradual depletion of donor-derived APC later after transplantation. Meanwhile the indirect pathway kicks in and remains active as long as the graft is present[4, 5]. Recipient T cells recognizing donor HLA via the indirect pathway are therefore thought to be related to long-term transplant outcomes, such as chronic rejection. Indeed, augmented T-cell responses to donor HLA-derived peptides presented by recipient MHC have been observed in transplanted patients with chronic rejection[6-15]. Experimental animal studies have shown that $CD4^+$ T cells which indirectly recognize alloantigens provide help to cytotoxic $CD8^+$ T cells and to donor-reactive B cells to produce donor-specific antibodies[16-20]. In addition, recent evidences suggest that regulatory T cells with indirect allospecificity are superior in promoting allograft tolerance than Treg with direct allospecificity, and constrain both cellular and humoral allogeneic immune responses[21-24].

However, studies on indirect allorecognition in humans are hampered by the lack of a reliable *ex vivo* assay to detect T cells with indirect allospecificity. Two different techniques have been mainly used to detect indirect T-cell allorecognition, which have been previously reviewed[25]. In the first approach, recipient APC are incubated with donor-derived cellular fragments, which has the advantage that it may result in the presentation of the full natural peptide repertoire of donor-derived alloantigens[8, 10, 11, 13]. However the major drawback of this technique is that the cellular fragments contain intact HLA molecules and costimulatory molecules, which may activate T cells via direct or semi-direct pathways. A recent study showed that the use of cellular fragments indeed induces T-cell responses via the semi-direct pathway, casting doubts on previous studies using this technique to study indirect alloreactivity[26]. Another common technique uses

synthetic peptides derived from mismatched donor HLA. Since little is known about the immunodominance of HLA-peptides, either randomly selected or overlapping peptides are used in such studies[6, 7, 12, 15, 17]. However, apart from the practical difficulty of testing *ex vivo* T-cell responses to a large library of different peptides to cover the complete potential peptidome of mismatched HLA, the generation of synthetic peptides involves an unnatural way of antigen processing, which may introduce neo-epitopes that do not exist *in vivo*. Indeed, by using synthetic HLA-peptides *ex vivo* T-cell responses to self HLA have been observed in patients awaiting renal transplantation, in healthy subjects, and even in cord blood[25, 27].

The aim of this study is to develop a novel *ex vivo* assay, which overcomes the disadvantages of the previous methods, to monitor T-cell responses with indirect allospecificity in patients after organ transplantation. Since $CD8^+$ T cells with indirect specificity are thought to be less relevant in organ transplantation[28], we focus on indirect alloantigen presentation to $CD4^+$ T cells. We introduce indirect alloantigen presentation by electroporation of autologous APC with *in vitro* transcribed messenger RNA (mRNA) encoding for mismatched donor HLA. The transfected autologous APC utilize their cellular machinery to synthesize and process mismatched donor HLA, and present a full repertoire of natural allo-HLA peptides to T cells. This technique has previously been demonstrated to be very effective in generating antigen-specific T-cell responses to tumor-associated antigens, however has not been yet applied in the transplant-immunology field[29-33].

MATERIALS & METHODS

Expansion of human B cells by stimulation with trimeric CD40-ligand

PBMC were isolated by Ficoll gradient centrifugation from HLA-typed buffy coats obtained from the Sanquin Blood Bank (Rotterdam, The Netherlands), or from peripheral blood of HLA-A2 negative liver transplant (LT) recipients transplanted with allografts from HLA-A2 positive donors (Erasmus MC, Rotterdam, The Netherlands). The study was approved by the Medical Ethics Committee of Erasmus MC, and written informed consent was obtained from all patients. On day 0, PBMC were seeded on 6-well plates (Costar, Cambridge, USA) at a concentration of $2\text{-}3\times10^6$ cells/ml in IMDM (Lonza, Breda, The Netherlands) with 10% heat inactivated human serum, 1% penicillin/streptomycin, 1% insulin-transferrin-selenium solution (Gibco-Invitrogen, Breda, the Netherlands), 40 IU/ml recombinant human interleukin-4 (rhIL-4, Strathmann Bioscience, Germany), and 1 μg/ml soluble human trimeric CD40 ligand (sCD40L, kindly provided by

Celldex Therapeutics, Hampton, NJ, USA). During the first 7 days of culture, 1 μg/ml cyclosporine A (CsA, Novartis, Basel, Switzerland) was also added to prevent T-cell expansion. $CD3^+$ cells were depleted by magnetic-activated cell sorting using CD3 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) on day 7. Since then cells were passed every 3-4 days at a concentration of 0.8×10^6 $CD19^+$ cells/ml in the presence of rhIL-4 and sCD40L but without CsA. The purity of CD40-stimulated B cells (CD40-B) was checked during every passage using flow-cytometry.

In vitro generation of engineered HLA messenger RNA

A schematic overview of the constructed vectors is depicted in Figure 1. The back bone vector pST1 was kindly provided by U. Sahin (Johannes-Gutenberg University, Mainz, Germany)[34]. The first vector, pST1-HLA-A*0201, contains the complete *HLA-A**0201 sequence, including the transmembrane and cytoplasmic parts. The second vector, pST1-HLA-A*0201-delTM-DCL, contains a codon-optimized insert that encodes the α1, α2 and α3 domains of the HLA-A*0201 protein except the transmembrane region, to prevent surface expression of HLA-molecules that might result in direct allorecognition. The extracellular part of HLA-A2 in this construct is directly followed by a sequence encoding the transmembrane and luminal regions of Dendritic Cell Lysosome Associated Membrane Protein (DCLamp), which is a targeting signal for the endo-lysosomal compartment, resulting in peptide loading in MHC-II[35-39]. Both HLA inserts were synthesized by GeneArt (Life Technologies, Gent, Belgium). Prior to *in vitro* transcription, the DNA plasmids were linearized with *SapI*. *In vitro* RNA transcription was performed using T7 RNA polymerase according to the manufacturer's instructions (Ambion mMESSAGE mMACHINE™ kit, Austin, TX, USA). The quality of the *in vitro* transcribed mRNA was confirmed by agarose gel electrophoresis and the mRNA concentration was measured by spectrophotometry.The obtained mRNA were stored in small aliquots at -20°C till use.

Messenger RNA electroporation

Before electroporation, CD40-B were washed twice, first with serum-free IMDM (Lonza, Breda, The Netherlands) and subsequently with Opti-MEM (Life technologies, Bleiswijk, The Netherlands). $8\text{-}10\times10^6$ CD40-B were resuspended in a final volume of 200 μL of Opti-MEM containing 20 μg of mRNA. Electroporation was performed in a 4 mm gap cuvette using a Gene Pulser Xcell™ electroporation system (Bio-Rad Laboratories, Hercules, CA). We used a square wave pulse of 600 V in a pulse time of 0.6 ms in all experiments, which was found to yield optimal CD40-B survival, HLA-A2 surface expression and T-cell activation (data not shown). Immediately after electroporation, CD40-B were transferred into IMDM without phenol red (Life Technologies, Bleiswijk,

The Netherlands) with 10% heat-inactivated human serum, 1% penicillin/streptomycin and 1% insulin-transferrin-selenium solution. Two hours after electroporation, cells were harvested for further experiments.

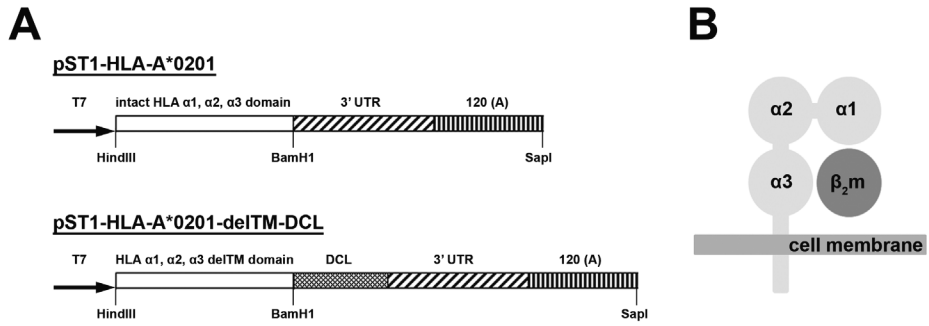

Figure 1. Schematic representation of the DNA constructs used for *in vitro* generation of HLA mRNA. (A) The T7 promotor, 3′ flanked untranslated region (3′UTR), 120bp-long poly-A tail, and the unique HindIII, BamH1 and SapI sites of the pST1 backbone vector are shown. The 3′ UTR and the 120 poly-A tail are attached to increase stability of the mRNA. The non-functional parts of the plasmids are not shown. pST1-HLA-A*0201 contains an insert that encodes the complete HLA-A*0201 protein. pST1-HLA-A*0201-delTM-DCL contains an insert that encodes the three α units of HLA-A*0201 protein except the transmembrane part, and also DCLamp (DCL). (B) Schematic overview of HLA class I protein, consisting of the MHC class I-encoded α-chain and β2-microglobulin, expressed on cell surface.

Flow cytometry and antibodies

The following antibodies were used: CD19-APC-eFluor780, HLA-DR-PE, CD83-FITC, CD27-APC (eBioscience, San Diego, CA); CD3-PerCP-Cy5.5 (BD Biosciences, San Diego, CA); HLA-A2-APC (BD Pharmingen, Erembodegem, Belgium); HLA-A,B,C-FITC, CD80-PE-Cy7, CD86-Pacific Blue, CD3-PE, CD40-PerCP-Cy5.5 (Biolegend, San Diego, CA); CD38-Pacific Blue (Exbio, Vestec, Czech Republic). Non-viable cells were excluded using 7-AAD (BD Biosciences, San Diego, CA) or LIVE/DEAD Fixable Aqua Dead Cell Stain Kit (Invitrogen, Carlsbad, CA). Flow cytometry was performed using a FACSCanto II flow cytometer (BD Biosciences, San Diego, CA). Data were analyzed with FlowJo software (FlowJo, Ashland, OR, USA).

CD4$^+$ T-cell clone

A previously described HLA-DRB1*0101 restricted HLA-A*0201-specific CD4$^+$ clone 4.43, generated from an HLA-A*0201 positive patient with severe graft-versus-host disease after hematopoietic stem cell transplantation and donor lymphocyte infusion from an HLA-A*0201 negative donor[40], was used as responder cells for testing indirect presentation of HLA-A*0201. Cells were expanded by non-specific stimulation with

2 μg/ml PHA and irradiated allogeneic third-party PBMC as feeder cells, in IMDM supplemented with 5% fetal calf serum, 5% human serum, 1% penicillin/streptomycin, L-glutamine, and 2 μg/mL IL-2. Every three days T cells were further expanded by splitting the cultures and replenishing fresh culture medium without feeder cells. At day 9, cells were harvested and cryopreserved till further use.

Indirect alloantigen presentation assay

To detect indirectly presented alloantigen, $1x10^4$ cells of the T-cell clone 4.43 were co-cultured with $5x10^4$ mRNA-transfected CD40-B from an HLA-A*0201 negative/HLA-DRB1*0101 positive blood bank donor, in triplicate for 24 hours in 96-well U-bottom microtiter plates in IMDM supplemented with 10% heat inactivated human serum, 1% penicillin/streptomycin and 1% insulin-transferrin-selenium. 4.43 co-cultured with CD40-B from an HLA-A*0201 positive/HLA-DRB1*0101 positive donor was used as positive control, and 4.43 co-cultured with non-transfected HLA-A2 negative/HLA-DRB1*0101 positive CD40-B, or 4.43 alone, were used as negative controls. After 24 hours 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 (Life technologies, Bleiswijk, The Netherlands). To check for MHC class II restriction, mouse anti-human MHC class II mAb (clone PdV5.2, Santa Cruz Biotechnology; 10 μg/ml) was added to the co-cultures.

Detection of patient $CD4^+$ T cells with indirect allospecificity by IFN-γ ELISPOT

$CD4^+$ T cells were isolated by MACS negative selection (Miltenyi Biotec, Bergisch Gladbach, Germany) from PBMC samples of HLA-A2 negative patients that were transplanted with livers from HLA-A2 positive donors. $CD4^+$ T cells were stimulated by pST1-HLA-A*0201-delTM-DCL transfected autologous CD40-B for 7 days in RPMI supplemented with 10% human serum, 2 mM L-glutamine, 20 μM β-mercaptoethanol and 1% penicillin/streptomycin. On day 7 $CD4^+$ T cells were harvested, B cells were depleted using CD19 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany), and $CD4^+$ T cells were re-stimulated by either pST1-HLA-A*0201-delTM-DCL transfected or non-transfected autologous CD40-B in IFN-γ-pre-coated enzyme-linked immunospot plates (ELISPOT, Mabtech, Sweden) overnight following manufacture's protocol. Depending on the yields, 2×10^4 to 1×10^5 $CD4^+$ T cells were co-cultured with 1×10^5 CD40-B per well in triplicate in 200 μl RPMI supplemented with 10% FCS and 1% penicillin/streptomycin for 24 hours. The numbers of spot-forming cells were counted using an automated ELISPOT reader system (AID, Strassberg, Germany), and numbers of triplicate wells were added together. The numbers of spots reacting to non-transfected autologous CD40-B were subtracted to determine the specific response to indirectly

presented HLA-A2, and fewer than 10 spots per 3 wells above background in co-cultures with non-transfected autologous CD40-B was considered as a negative result. Results were normalized to spots per million $CD4^+$ T cells.

RESULTS

Expansion of CD40-stimulated B cells as antigen-presenting cells using soluble trimeric CD40 ligand

Dendritic cells are the most common APC used in assays to detect antigen-specific T-cell responses *in vitro*, however only limited numbers can be isolated from human blood or differentiated *ex vivo* from circulating monocytes, and they lack proliferative capacity *in vitro*. B cells activated via CD40 stimulation are potent APC, which can be expanded vigorously from small numbers of both fresh and cryopreserved human PBMC[41]. Previously we used CD40L-transfected mouse fibroblasts to expand CD40-B, however the protocol was labor intensive and included xenogeneic cells which might result in stimulation of xenogeneic T-cell responses when these expanded B cells are used for T-cell stimulation assays[42]. Now we used a recombinant soluble human trimeric CD40L to stimulate and expand B cells from PBMC. The absolute numbers of B cells expanded 4×10^3-fold (Figure 2A, mean value, range 2×10^2-1×10^4) within 4 weeks of culture. The purity of CD40-B reached around 99% between day 10-14. For further experiments, CD40-B were harvested at day 17 or 21 with a purity >99%. The phenotype of CD40-B upon harvesting was determined by flow-cytometry. High levels of MHC-I, HLA-DR, and costimulatory molecule CD86 were consistently observed, suggesting potent antigen-presenting capacity. The costimulatory molecule CD80 was also expressed, but at a lower level. Part of the CD40-B expressed CD27 indicating that they were memory B cells. CD40 (costimulatory receptor), CD38 and CD83 (activation markers) were also highly expressed on CD40-B.

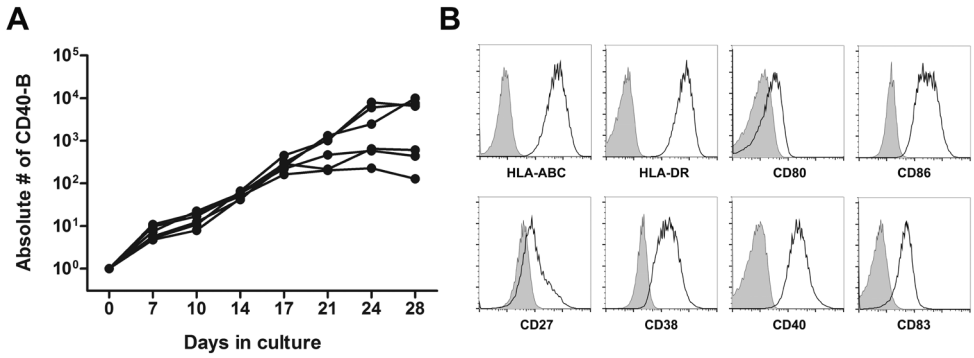

Figure 2. Expansion of CD40-stimulated B cells as antigen presenting cells using soluble trimeric CD40 ligand. PBMCs obtained from blood donors were cultured in the presence of IL-4 (40 IU/ml) and soluble human trimeric CD40 ligand (1 μg/ml). Cells were passed every 3-4 days, meanwhile the purity and numbers of cells were monitored (n=6 independent experiments). (A) The absolute numbers of B cells during a four-week expansion period is shown (the absolute number of B cells on day 0 was normalized to 1×10^6). (B) The phenotype of CD40-B was examined by the end of culture period using flow-cytometry analysis. Flow-cytometry histograms from a representative donor are shown.

Electroporation with mRNA encoding HLA-A2 without transmembrane part prevents surface expression of HLA-A2 on HLA-A2 negative CD40-B

We made two mRNA constructs to transfect HLA-A2 negative CD40-B with HLA-A2. pST1-HLA-A*0201 encoded for the intact α chain of HLA-A*0201 (α1, α2 and α3; Figure 1B), while pST1-HLA-A*0201-delTM-DCL encoded for the α chain without the transmembrane region. We hypothesized that deletion of the transmembrane region would completely eliminate the surface expression of the molecule, thereby preventing activation of T cells with direct or semi-direct allospecificity. Both mRNAs were used to transfect HLA-A2 negative CD40-B by electroporation. Surface expression of HLA-A2 was examined by flow-cytometry at 2, 8 and 24 hours after electroporation. Transfection using pST1-HLA-A*0201 led to rapid surface HLA-A2 expression on HLA-A2 negative CD40-B (Figure 3A, left panel); 38%, 82% and 89% of the CD40-B became HLA-A2 positive at 2, 8 and 24 hours after electroporation respectively (Figure 3B, mean values). Meanwhile, no surface HLA-A2 expression was detected on pST1-HLA-A*0201-delTM-DCL transfected CD40-B (Figure 3A, right panel).

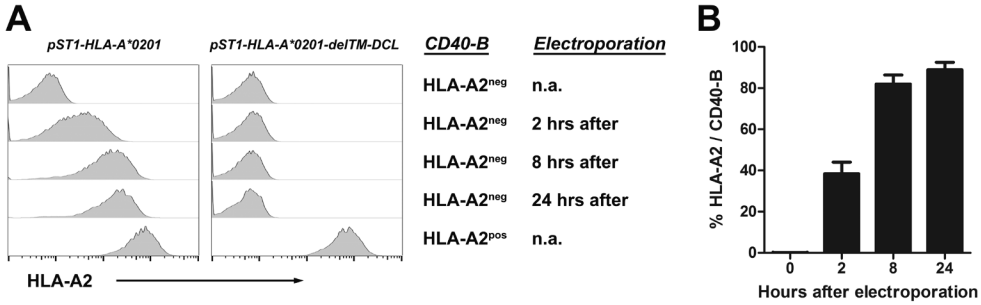

Figure 3. Electroporation of CD40-B with pST1-HLA-A*0201 mRNA leads to surface expression of HLA-A2 on HLA-A2 negative CD40-B but electroporation with pST1-HLA-A*0201-delTM-DCL mRNA not. Two mRNA constructs, pST1-HLA-A*0201 and HLA-A*0201-delTM-DCL, were used to transfect HLA-A2 negative CD40-B by electroporation. The surface expression of HLA-A2 was determined by flow-cytometry at 2, 8, 24 hours after electroporation. Non-electroporated HLA-A2 negative and HLA-A2 positive CD40-B were used as negative and positive controls, respectively. (A) Representative histograms from one experiment showing the surface expression of HLA-A2 after mRNA electroporation. (B) Pooled data summarizing the HLA-A2 expression after electroporation with pST1-HLA-A*0201 (n=3 independent experiments). Column bar graphs represent means with SEM.

CD40-B electroporated with pST1-HLA-A*0201-delTM-DCL mRNA activate CD4$^+$ T-cell clone specific for HLA-A2 presented by MHC class II

To test whether CD40-B transfected with pST1-HLA-A*0201 or pST1-HLA-A*0201-delTM-DCL mRNA were able to activate CD4$^+$ T cells which specifically recognize HLA-A*0201 via the indirect alloantigen presentation pathway, we utilized a previously described CD4$^+$ T-cell clone strictly recognizing HLA-A*0201 derived peptide presented by HLA-DRB1*0101 (referred to as 4.43) to establish a model for indirect allorecognition[40]. We expanded CD40-B from HLA-A2 negative/HLA-DRB1*0101 positive healthy blood donors. After electroporation with pST1-HLA-A*0201 or pST1-HLA-A*0201-delTM-DCL mRNA, the transfected CD40-B were co-cultured with T-cell clone 4.43 for 24 hours. As a positive control, 4.43 T cells were co-cultured with non-transfected CD40-B from an HLA-A*0201 positive/HLA-DRB1*0101 positive donor which are expected to present its native peptide ligand, while 4.43 co-cultured with non-transfected HLA-A2 negative/HLA-DRB1*0101 positive CD40-B, or 4.43 alone, served as negative controls. After 24 hours, culture supernatant was collected to measure the IFN-γ production by the T-cell clone. Both pST1-HLA-A*0201 transfected CD40-B and pST1-HLA-A*0201-delTM-DCL transfected CD40-B were able to activate T-cell clone 4.43, however the amount of IFN-γ production by 4.43 was 5-fold higher using pST1-HLA-A*0201-delTM-DCL transfected CD40-B (Figure 4A).When an anti-MHC-II blocking antibody was added to the co-culture, IFN-γ production was almost completely abrogated, illustrating that antigen presentation was restricted by MHC-II (Figure 4B). Thus we concluded that transfection of autologous CD40-B using pST1-HLA-A*0201-delTM-DCL was a suitable tool to study CD4$^+$ T-cell indirect HLA allorecognition.

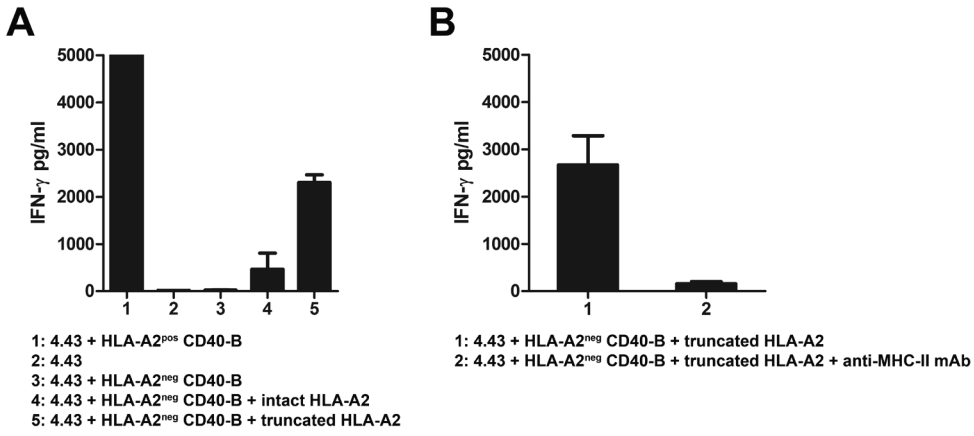

Figure 4. CD40-B electroporated with pST1-HLA-A*0201-delTM-DCL mRNA activate CD4+ T-cell clone specific for HLA-A2 presented by MHC class II. (A) HLA-A*0201^{neg}/HLA-DRB1*0101^{pos} CD40-B cells electroporated with pST1-HLA-A*0201 or HLA-A*0201-delTM-DCL mRNA were co-cultured with CD4+ T-cell clone 4.43. T-cell clone 4.43 co-cultured with HLA-A*0201^{pos}/HLA-DRB1*0101^{pos} CD40-B was used as positive control. T-cell clone 4.43 co-cultured with non-electroporated HLA-A*0201^{neg}/HLA-DRB1*0101^{pos} CD40-B, or T-cell clone 4.43 alone, served as negative controls. After 24 hours, supernatants were collected and the concentrations of IFN-γ were measured by ELISA (n=4 independent experiments). (B) HLA-A*0201^{neg}/HLA-DRB1*0101^{pos} CD40-B electroporated with HLA-A*0201-delTM-DCL mRNA were co-cultured with CD4+ T-cell clone 4.43 with or without a blocking anti-MHC-II monoclonal antibody (mAb) (n=5 independent experiments). Column bar graphs represent mean with SEM.

Detection of CD4+ T cells with indirect allospecificity for HLA-A2 in LT patients

We applied this technique to detect CD4+ T-cell responses with indirect allospecificity for HLA-A2 in 8 patients after LT by IFN-γ ELISPOT. All selected patients were HLA-A2 negative and had been transplanted with livers from HLA-A2 positive donors. Patient characteristics are described in Table 1. In patients 1-4, samples at pre-LT, 1 month, 6 months and 1 year after LT were studied, while in patients 5-8, samples at pre-LT, and between 5-11 years after LT were studied. Overall indirect HLA-A2-specific CD4+ T cells producing IFN-γ were detected in 4 patients (Figure 5). Of the 4 patients analyzed short-term after LT, only patient 4 exhibited a strong response at 6 months after LT, interestingly during the occurrence of chronic rejection. Of the 4 stable patients analyzed late after LT, HLA-A2-specific indirect responses were detected in 3 patients at 9-11 years after LT. Only patient 8 showed response prior to transplantation, which might be due to alloantigen exposure via previous blood transfusion or pregnancy.

Table 1. Patients studied for the CD4+ T-cell response to indirectly presented HLA-A2

#	Primary liver diseases	Sex	Acute rejection	Chronic rejection	Sample time-points
1	HCV	M	-	2 years after LT	pre-LT, 1, 6 months post-LT
2	PSC	M	2 years after LT	-	pre-LT, 1, 12 months post-LT
3	HCC	M	-	-	pre-LT, 1, 6, 12 months post-LT
4	NASH	M	-	6 months after LT	pre-LT, 6 months post-LT
5	Alcoholic cirrhosis	M	2 years after LT	-	pre-LT, 5 years post-LT
6	HCC	M	-	-	pre-LT, 9 years post-LT
7	HBV	M	-	-	pre-LT, 11 years post-LT
8	PSC	F	-	-	pre-LT, 9 years post-LT

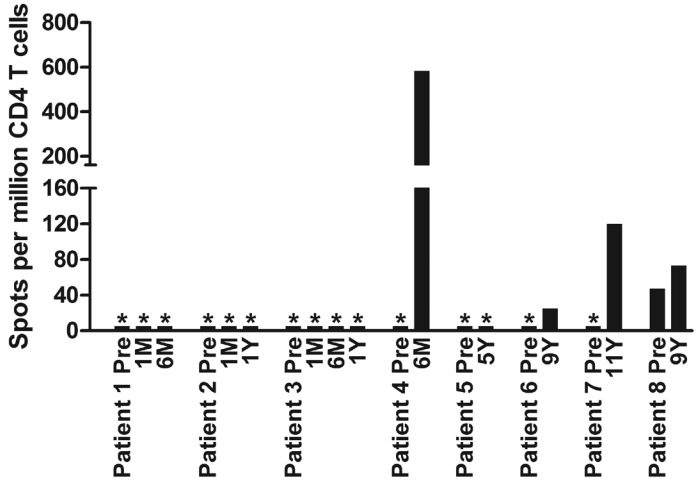

Figure 5. Detection of CD4+ T cells with indirect allospecificity for HLA-A2 by IFN-γ ELISpot in LT patients. CD4+ T cells were isolated from PBMC samples of 8 LT patients and co-cultured for 7 days with pST1-HLA-A*0201-delTM-DCL transfected autologous CD40-B. Thereafter, CD4+ T cells were re-stimulated by either pST1-HLA-A*0201-delTM-DCL transfected or mock-electroporated autologous CD40-B in IFN-γ-pre-coated ELISPOT plates. In patients 1-4, samples at pre-LT, 1 month, 6 months and 1 year after LT were examined, while in patients 5-8, samples at pre-LT, or between 5-11 years after LT were examined. The frequencies of IFN-γ producing CD4+ T cells in response to indirectly presented HLA-A2 after subtraction of frequencies of spots in response to mock-electroporated CD40-B, are plotted as spots per million CD4+ T cells (y-axis) for each time-point and patient (x-axis). * indicates that fewer than 10 spots per triplicate wells were observed above background, which was considered as a negative result.

DISCUSSION

The role of indirect allorecognition in the long-term transplant outcomes in humans is not well-understood, largely due to the lack of a proper *ex vivo* assay that specifically detects T cells responding to indirectly presented alloantigens. In this study we demonstrate that electroporation of CD40-B with *in vitro* transcribed mRNA that encodes a genetically disparate HLA-A molecule without the transmembrane region results in self-MHC class II restricted presentation of peptides derived from this molecule to $CD4^+$ T cells, while it does not lead to surface expression of HLA-A*0201 and therefore cannot be recognized by T cells via the direct pathway of allorecognition. We show that this technique enables detection of $CD4^+$ T cells responding to indirectly presented HLA-A2 in HLA-A2 mismatched patients after LT.

The way of alloantigen introduction is the key to develop a successful assay to measure *ex vivo* T-cell responses to indirectly presented alloantigens. Transfection of mRNA has been proven to be a potent technique to achieve antigen presentation to $CD4^+$ and $CD8^+$ T cells, with similar efficacy as lentiviral gene transduction[32, 39]. It is applicable for different cell types, such as PBMC[43], monocytes[44], moDC[29, 31, 32], and CD40-B[30, 41]. 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[29]. We used HLA-A2 as a model alloantigen because, in contrast to expression of donor MHC class II which extinguishes after disappearance of donor-derived APC from the graft, expression of donor MHC class I in organ graft is expected to be long-lived, and HLA-A2 is the most prevalent HLA-A allele in Caucasians. Deletion of the transmembrane region not only prevented surface expression, but also resulted in enhanced indirect $CD4^+$ T-cell activation compared to mRNA which encoded the intact HLA-A*0201. This could be due to increased protein degradation and presentation on MHC-II, as it does not lead to surface expression. In addition, the DCLamp signal included is expected to result in better targeting of the HLA-A2 protein to the endo-lysosomal system resulting in a more efficient antigen presentation on MHC-II[39].

The novel technique of indirect alloantigen presentation presented in this study has several advantages. Firstly, allorecognition via the direct or semi-direct pathways is completely excluded, since the sequence encoding the transmembrane region of the HLA-molecule is lacking, which prevents expression on the cell surface. Recently Breman et al. used synthetic HLA class I monomers to achieve indirect presentation to $CD4^+$ T cells[26]. 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. Secondly, compared to overlapping synthetic peptides, the technique presented here does not create neo-epitopes, as the alloantigen is processed by the natural processing machinery of CD40-B. Thirdly, compared to known HLA-epitopes, such as the HLA-A2 derived peptide 103-120, our technique conveys the full antigenic spectrum of HLA molecules and is not restricted by HLA-typing of the patients. Moreover, mRNAs encoding for different antigens can be co-electroporated together, enabling transfection with different HLA-encoding mRNAs in case of multiple HLA mismatches between donor and recipient[33]. Lastly, using CD40-B as a source of APC, which can be rapidly expanded to large amounts from a limited amount of blood, ensures availability of sufficient amounts of APC for repetitive patient monitoring. Although dendritic cells are considered to be the most professional APC of the immune system, obtaining sufficient numbers for repetitive *ex vivo* assays will be difficult in transplant patients in whom numbers of circulating immune cells are repressed due to immunosuppressive medication[45].

In future research it should be evaluated that whether this assay, either or not in combination with quantification of T cell responses to directly presented donor alloantigens, is associated with and/or can predict the risk of (chronic) allograft rejection and other long-term outcomes in transplant recipients, thereby enabling personalized immunosuppression strategies in clinical practice. It will be particularly relevant to determine whether operationally tolerant LT recipients can be identified using this technique. However, for this purpose a library of DNA constructs encoding a broad spectrum of prevalent human HLA molecules should first be constructed.

To conclude, we developed a novel *ex vivo* assay to measure $CD4^+$ T-cell responses to indirectly presented alloantigen in transplanted patients with high specificity, which may help to better understand the full spectrum of allogeneic T-cell responses in humans. In addition, this technique may be useful to guide personalized immunosuppression.

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DISCLOSURE

The authors of this manuscript have no conflicts of interest to disclose as described by the *American Journal of Transplantation*.

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CHAPTER 7

Correspondence

Long-lived intragraft donor leukocytes or relocated donor HSPCs can cause long-term hematopoietic chimerism after liver transplantation

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To the editor:

We read with great interest the article in HEPATOLOGY by Wang et al.[1], which characterized blood chimerism in liver transplant (LT) patients and showed that multipotent hematopoietic stem/progenitor cells (HSPCs) reside in adult human livers. The authors concluded that there are two types of chimerism after LT: transient chimerism resulting from migration of mature donor leukocytes from the liver graft, which usually disappears within 3 weeks after LT, and long-term chimerism derived from putative donor HSPCs in the liver graft. Finally, the authors asked whether donor HSPCs generate mature leukocytes inside the grafted liver or circulate to recipient bone marrow for hematopoiesis.

In a previous study on liver NK cell precursors[2], we observed that mature NK cells of donor origin were detectable in liver grafts up to 2 years after LT, while all donor-derived NK-cell precursors were replaced by recipient-derived precursors within 1 week after LT. To study whether other types of mature donor leukocytes remain present in liver grafts after LT, we now determined intragraft chimerism of $CD3^+$ T cells, $CD56^+$ T cells, and $CD14^+$ monocytes/Kupffer cells in leukocytes isolated from first liver grafts of 5 LT patients undergoing re-LT. We selected recipient/donor pairs that were mismatched for HLA-A2 or HLA-Bw4 during the first transplantation. Using flow-cytometry with mAb for HLA-A2 or HLA-Bw4 we could differentiate donor from recipient cells. In all 5 patients, we detected considerable percentages of donor-derived mature leukocytes in the first graft, even up to 2 years after transplantation (Table I). These data are not consistent with the hypothesis of Wang et al [1] that donor-derived leukocytes disappear within 3 weeks after LT, at least within the grafted liver, but demonstrate the possible existence of long-lived donor-derived leukocytes resident in the liver graft.

We also measured chimerism in lineage$^-$CD34$^+$ HSPCs (at least 2×10^6 events were recorded), which contain the multipotent lin$^-$CD34$^+$CD38$^-$CD90$^+$ HSPCs described in the article[1]. We found that all 5 explanted liver grafts contained only recipient-derived, but no donor-derived, HSPCs (Table I), indicating that donor-derived hepatic HSPCs are replaced by circulating HSPCs of recipient origin within the first week after transplantation.

Our data suggest that the long-term chimerism described in the paper of Wang is probably caused by long-lived donor leukocytes resident in liver grafts, and/or hematopoiesis of relocated donor HSPCs. The latter concept is supported by a study of Massberg et al. [3], which describes the liver as one of the peripheral organs in which HPSCs reside shortly before returning to the blood and re-migrating to the bone marrow. The relocation of HSPCs from transplanted liver remains to be investigated.

Table I: Percentages of donor-derived leukocytes in first liver grafts explanted during re-LT.
Numbers between parentheses indicate the percentages of donor-derived cells within each subset.

% of total intragraft leukocytes (% of donor-derived within each subset)					
	Patient 1 (7 days)	Patient 2 (16 days)	Patient 3 (1 year)	Patient 4 (2 years)	Patient 5 (2 years)
$CD56^+$ T cells	7.5 (6.8)	1.7 (18.2)	5.7 (1.6)	5.1 (3.7)	6.6 (16.3)
$CD3^+$ T cells	12.5 (2.1)	76.5 (6.6)	22.8 (0.8)	10.5 (16)	8.8 (18.6)
$CD14^+$ cells	15.4 (63.3)	15.9 (90.5)	7.8 (27.5)	7.2 (37.0)	22.2 (46.0)
Lin^-CD34^+ HSPCs	0.5 (0)	0.2 (0)	0.1 (0)	0.3 (0)	0.1 (0)

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CHAPTER 8

Summary and discussion

The transplantation of a liver from a genetic disparate donor is always accompanied by immune responses targeting the allograft. These immune responses are associated with post-transplant complications, such as graft rejection. Therefore the used of long-term immunosuppressive medication is a necessity to suppress the post-transplant alloimmune responses, which is associated with adverse effects affecting long-term outcome. The aim of this thesis is to improve our understanding of environmental and genetic factors shaping and regulating allogeneic T-cell responses and graft rejection in liver transplant patients, in order to facilitate personalized patient management and to achieve a better long-term patient outcome.

I. CMV infection restricts allogeneic CD8$^+$ T-cell responses and may promote tolerance in patients after liver transplantation

From experimental animal models we learned that viral infections after organ transplantation break transplant tolerance[1-3], not only caused by the excessive inflammation associated with infection but also due to the activation of pathogen-specific memory T cells that are cross-reactive to allo-antigens[4]. Clinical studies have provided evidence for a positive association between infections and organ transplant rejection in humans, however it is difficult to determine a causal relationship[5]. Therefore, in this thesis we studied the influence of CMV infection, one of the most common viral infections after organ transplantation, on T-cell alloreactivity after liver transplantation, and its correlation with allograft rejection and tolerance. In ***Chapter 2***, we find that, even though CMV primary infection after liver transplantation drives expansion of a memory T-cell pool, it is associated with the development of donor-specific hyporesponsiveness of CD8$^+$ T cells. Further analysis of a large cohort of liver transplant patients shows that, while CMV viremia is associated with an increased risk of early acute rejection, CMV primary infection is associated with a lower risk of late acute rejection. In addition, we showed that CMV primary infection is associated with the highest Vδ1/Vδ2 γδ T-cell ratio in the circulation, a cell-signature that is related to operational tolerance after liver transplantation, indicating that these patients probably have a higher chance to achieve operational tolerance after immunosuppression withdrawal. In ***Chapter 3***, we studied the expression of a series of co-inhibitory receptors on T cells after liver transplantation, of which the expression of CD160 and CD244 showed a magnificent and sustained up-regulation on CD8$^+$ T cells after transplantation. The long-lasting up-regulation of CD244 on CD8 T cells was found to be associated with CMV infection after transplantation. In vitro, purified CD244$^+$ CD8 T cells proliferated less in response to allogeneic stimulation compared to their CD244$^-$ counterparts, which could explain the

lower proliferative capacity of CD8 T cells in response to allostimulation in patients with CMV infection. In addition, we showed the majority of CMV-specific $CD8^+$ T cells are CD244 positive. In conclusion, CMV infection after LTx is associated with the expansion of a dysfunctional $CD244^+$ T-cell subset and development of systemic allogeneic CD8+ T-cell hyporesponsiveness. Taken together, results from these two chapters suggest that CMV infection restrains $CD8^+$ T-cell alloresponses, and may actually promote transplant tolerance after human liver transplantation.

The most surprising finding of our study is the paradoxical relationship that we observed between CMV infection and early versus late liver allograft rejection: while CMV viremia is associated with more early acute rejection episodes, CMV primary infection is associated with fewer late acute rejection episodes, donor-specific $CD8^+$ T-cell hyporesponsiveness, and a tolerance-related cell-signature. In the majority of the cases, early acute rejection occurs earlier than CMV infection. This is in line with the hypothesis that inflammation associated with allogeneic immune responses may trigger the replication of CMV from latency[6]. On the contrary, the lower risk of late acute rejection can be the consequence of the earlier primary infection of CMV, which usually occurs within the first six months after transplantation. Medication incompliance or insufficient amount of immunosuppressive medication are generally considered as the main causes of late acute rejection[7]. However, inadequate immunosuppression, or immunosuppression with a trough level below the target, is very common in patients late after transplantation. But not all these patients develop acute rejection, and thereby we interpret absence of rejection in the majority of under-immunosuppressed patients as a sign of (partial) tolerance to their liver graft. These paradoxical effects and bidirectional relationships between CMV infection and early versus late acute rejection may explain the inconsistency of previous clinical study results regarding the association between CMV infection and allograft rejection[5].

How CMV restrains $CD8^+$ T-cell alloreactivity after liver transplantation remains elusive, and whether CMV-induced γδ T-cell signatures play a functional role in promoting tolerance is as yet unknown. The well-established immune evasion strategies of CMV provide possible clues. For example, CMV produces viral IL-10 to establish latency, which may also have a systemic immunosuppressive effect[8, 9]. CMV infection is also associated with the sustained inflation of circulating memory T-cells and immune senescence[10]. 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[11, 12]. Moreover, CMV has been recently shown to significantly modulate

peripheral mixed T-cell chimerism after HSCT, facilitating the reconstitution of donor-derived cells[13]. In contrast to the common short-term hematopoietic chimerism observed after organ transplantation, long-term hematopoietic chimerism is rare in peripheral blood. However it is more commonly observed within grafted organ[14, 15]. It has been suggested in a case report that severe CMV disease early after LT contributed to the development of long-term hematopoietic chimerism and subsequent donor-specific T-cell hyporesponsiveness[16]. In addition, we hypothesize that inflammation provoked by CMV infection may contribute to the induction of liver allograft tolerance. CMV is known to drive T helper 1 cell (Th1) polarization. Within the human liver, CMV infection of sinusoidal endothelium triggers significant production of type I interferon by these cells, and recruits Th1 effector memory T cells and regulatory T cells[17]. Th1 cells produce high amounts of the pro-inflammatory cytokine IFN-γ, which is strikingly absolutely needed for liver transplant tolerance in animal models[18]. Recently, Morita et al. described a mesenchyme-mediated immune control (MMIC) mechanism utilized by liver allografts to eliminate effector T cells and maintain tolerance through IFN-γ and PD-L1[19]. The importance of type I interferon signalling has also been highlighted recently by Bohne et al., showing in an immunosuppression withdrawal trial of HCV-infected liver transplant patients, that operational tolerant patients overexpress type 1 interferon and interferon-stimulated genes (ISG) in their liver graft[20]. To sum, accumulating evidences suggest that in humans, infection of CMV does not necessarily preclude the establishment of transplant tolerance but, on the contrary, the immunomodulatory effects exerted by CMV could restrict alloimmune T-cell responses and promote transplant tolerance.

Inspired by our own finding that CMV infection may benefit liver transplant patients by promoting tolerance to their graft, we studied in depth scientific literature to find out whether CMV can benefit human health in other conditions, despite all the clinical problems that it cause. In ***Chapter 4***, we summarized the possible beneficial effects that CMV can convey to its human hosts. For example, emerging evidences are showing that CMV infection after HSCT may reduce the risk of cancer recurrence in humans, which has been validated in several studies, particularly for patients with AML[21-23]. Studies on childhood immunity suggest that CMV exposure may boost the immunity of children to unrelated pathogens[24]. The mechanisms contributing to these clinical associations are still not fully understood. They are most likely related to the NK cell, γδ T-cell, and T cell responses triggered by CMV infection, , which convey a cross protection towards unrelated antigens. Considering the potential benefits that CMV infection may provide, we propose that more research should be done to better understand the virus-host inaction between CMV and humans: how CMV infection, directly or indirectly, is associated with beneficial clinical outcomes; how to skew the natural course of CMV infection,

or the related immune responses, to achieve those beneficial effects while limiting the established adverse effects of CMV infection; whether a universal vaccination strategy should be advocated at this moment.

II. Genetic variations in the PD-L1/PD-1 co-inhibitory pathway influence graft rejection after liver transplantation in humans

T-cell co-inhibitory receptor-ligand interactions fine-tune immune responses by negatively regulating T-cell functions[25, 26]. We hypothesized that T-cell co-inhibitory pathways are involved in the regulation of allogeneic T-cell responses and graft rejection in liver transplant recipients. In ***Chapter 5***, we focused on the most prominent PD-1/PD-L1 pathway. We find that compared to peripheral blood, PD-1 is highly expressed on T cells infiltrating liver allografts. Meanwhile, its ligand, PD-L1 is also highly expressed in liver allografts undergoing rejection. Blockade of this interaction increased the proliferation of graft-derived T cells in response to allostimulation. Next, from the genetic perspective, we analyzed the correlation between SNPs within PD-1/PD-L1 genes and the incidence of graft rejection, and we foud that donor *PD-L1* rs4143815 and recipient *PD-1* rs11568821 are associated with the occurrence acute rejection late after transplantation, suggesting that this pathway is involved in the pathogenesis of graft rejection. Further we confirmed the functional relevance of the *PD-L1* rs4143815, which results in altered PD-L1 expression on dendritic cells following IFN-γ stimulation. These data show that PD-L1/PD-1 pathway is actively involved in the regulation of allogeneic immune response and graft rejection, and that genetic variations in donor PD-L1 and recipient PD-1 contribute to inter-individual susceptibility to graft rejection after liver transplantation.

It is very interesting that SNPs within PD-1/PD-L1 are only associated with the occurrence graft rejection late after transplantation but not those early episodes. Several possibilities might explain this observation. First of all, the functional relevance of the SNPs might not be as significant as other factors that are associated with early rejection, such as immunosuppressive therapies, which are more intensive early after transplantation. The allogeneic immune response driving acute rejection is also much stronger early after transplantation. Thereby the minor differences in co-inhibitory signaling caused by the SNPs may be over-shadowed by those more dominant factors. Secondly, early and late acute rejection might in fact have distinct pathogenesis. For instance, late graft rejection is more likely to occur during immunosuppression dose reduction, or during insufficient immunosuppression due to incompliance. PD-1/PD-L1 pathway might be more active in balancing the rejection versus tolerance in these situations with low level of immunosuppressive therapies.

Can we make use of these data in clinical practice. First of all, PD-1/PD-L1 pathway should be further studied in liver transplant patients that are operational tolerant, investigating whether patient/donor combinations with favorable n genetic variants provide advantages when discontinuing or minimizing immunosuppression. If this is the case, then SNPs within this pathway, together with other characteristics, such as circulating γδ T cells[20, 27-29], can be used to identify operationally tolerant patients. Secondly, targeting of PD-1 by administration of an appropriate agonist, such as PD-L1 Ig fusion protein[30, 31], may be an interesting avenue to develop novel immunosuppression strategies. Considering the high expression of PD-1 on intrahepatic T cells, targeting PD-1 might selectively inhibit graft infiltrating T cells while leaving the peripheral T cells functionally intact.

III. In vitro transcribed messenger RNA can be used to monitor indirect allorecognition in human transplant recipients

After organ transplantation two pathways are mainly responsible for the activation of allogeneic T cells, namely the direct and the indirect pathway[32]. It has been hypothesized that the direct pathway is dominant early after transplantation and is responsible for the graft rejection early after transplantation. In contrast indirect pathway gradually takes over and is associated with immunological graft damage late after transplantation[33, 34], such as chronic rejection. However current techniques detecting indirectly activated allogeneic T cells in human transplant recipients are flawed, as reviewed by Waanders et al.[35], hampering interpretation of data regarding indirect pathway in human transplant recipients. In ***Chapter 6***, we described a novel technique based on transfection of autologous APC with engineered HLA-encoding messenger RNA electroporation to detect $CD4^+$ T cells with indirect allospecificity *ex vivo*. Compared to earlier methods, our technique has several advantages. Most importantly, this technique is highly specific to detect indirectly activated $CD4^+$ T cells. We engineered a DNA construct from which HLA-A2 mRNA lacking the sequence encoding the transmembrane part can be transcribed *in vitro*. Transfection with this mRNA completely prevents surface expression of HLA-A2 after protein translation, which excludes the possibility of activating T cells with direct or semi-direct allo-specificity. Secondly, compared to stimulation with short peptides containing one of a few known epitopes recognized by T cells, such as the HLA-A2 derived peptide 103-120, this technique conveys the full antigenic spectrum of HLA molecules and is not restricted by HLA-typing of the patients. Thirdly, we use CD40-B as APC. CD40-B can be expanded into large quantities from very limited amounts of human blood samples, which makes them a source providing sufficient APC for repetitive assays in patients. We applied this technique to measure $CD4^+$ T-cell responses to indirectly

presented donor HLA in a small number of HLA-A2 negative patients transplanted with liver from HLA-A2 donors, and indirect $CD4^+$ T-cell responses towards HLA-A2 could be detected in 4 patients out of 8 patients studied. Most interestingly, a very strong response was detected in a patient at the time of chronic rejection, which eventually caused the loss of graft. This is in line with previous studies suggesting that the T-cell indirect allo-activation pathway is participating in the process of chronic rejection[36-45], and suggests that alternative therapies targeting indirect allo-antigen presentation might be useful in treating chronic rejection. Future research is required to obtain a better understanding of the clinical relevance of the indirect allo-antigen presentation pathway by measuring indirect T-cell responses during a variety of post-transplant complications. Then efforts can be made to develop novel immunosuppressive therapies targeting the indirect pathway, or selecting therapies better at limiting indirect alloresponse to achieve a better long-term graft outcome. In addition, the basis of our technique can be used for the generation of regulatory T cells with indirect allospecificity as an alternative immunosuppressive cell-therapy[46, 47]. Due to the long-term complications associated with conventional immunosuppressive medication, Treg-based immunoregulatory therapy holds promise for tolerance induction and improving long-term outcomes, with several ongoing clinical trials[48, 49]. Current studies focus on ex vivo expansion of polyclonal Tregs, or generation of donor-specific Tregs with direct alloreactivity. However, evidences from animal models suggest that Tregs involved in transplant tolerance are restricted to the indirect pathway[50-52], and that Tregs with indirect allospecificity might be more crucial in the induction of tolerance than Tregs with direct allospecificity[46, 47]. A protocol generating donor-specific Tregs that are specific for indirectly presented alloantigens *ex vivo* is not available yet, and the present study may pave the way for developing such protocol. Lastly, the indirect alloresponse should also be determined in immunosuppression withdrawal or minimization studies, to establish whether it can contribute to identification of patients from whom immunosuppression can be safely withdrawn or minimized, in order to reduce the risk of immunosuppression related complications in the long-term.

IV. Long-term hematopoietic chimerism is common within liver allograft

The clinical relevance of hematopoietic chimerism after liver transplantation is still not well-understood. Recently, Wang et al. studied the blood chimerism in a large cohort liver transplant patients and analyzed the putative hematopoietic stem/progenitor cells (HSPCs) in adult human livers[53]. The authors concluded that there are two types of chimerisms in LT patients: transient chimerism resulting from mature donor leucocytes, and long-term chimerism derived from putative donor HSPCs residing in the liver graft, and suggested that liver is a good ectopic niche for extra-marrow hematopoiesis. To

comment on their work, we studied the hematopoietic chimerism within transplanted liver allografts in ***Chapter 7***. We find that leukocytes within allograft are composed of cells from both donor and recipient origin up to 2 years after transplantation, showing that long-term hematopoietic chimerism is common within transplanted allograft. In contrast, hepatic HSPCs after LTx are rapidly replaced by HSPCs of recipient origin. Our results suggest that the long-term chimerism described in the paper of Wang is probably caused by long-lived donor leukocytes resident in liver grafts, and/or hematopoiesis of relocated donor HSPCs. However it is also debatable whether intrahepatic leukocytes can be long-lived without repopulation by hematopoiesis from donor HSPCs. For example, NK cells possess features of memory cells and adaptive immunity has been described in literature[54]. However it is not yet known whether such memory NK cell population is present in liver and is able to undergo homeostatic proliferation without NK cell precursor. Kupffer cells are also known to have a short life-span[55]. Without extrahepatic hematopoiesis, the local precursor cells in liver might be responsible for the homeostatic proliferation of donor Kupffer cells. Therefore, it would be interesting to investigate the maintenance of donor leukocytes pool within the transplanted liver in future studies, how do they maintain their homeostatic proliferation, and how do they phenotypically and functionally differ from recipient derived leukocytes.

OVERALL CONCLUSIONS

In this thesis, we first show that CMV infection restricts allogeneic $CD8^+$ T-cell responses and may promote tolerance in patients after liver transplantation. Next we provide evidences for the involvement of PD-1/PD-L1 pathway, including genetic variants in donor PD-L1 and recipient PD-1, in the regulation of graft rejection late after transplantation. In addition, we develop a novel technique enabling the determination of $CD4^+$ T cells responding to indirectly presented allo-antigens. Finallyr, the existence of long-term hematopoietic chimerism within human liver allografts is described. Overall, this thesis provides suggestions for new clues that may be useful in patient selection strategies for immunosuppression withdrawal. In Table I the main findings of this thesis are summarized, including strengths and limitations of our studies, a summary of possible applications of the findings in clinical practice is provided, as well as suggestions for future study.

Table I. Summary and discussion of the main findings of this thesis Chapter

Chapter	Main findings	Strength	Limitations	Clinical significance	Suggestions for future research
2	- CMV primary infection is associated with $CD8^+$ T-cell donor-specific hyporesponsiveness - Patients with CMV primary infection have a lower risk for late acute rejection - CMV primary infection is associated with a marker for operational tolerance: high circulating Vδ1/Vδ2 γδ T-cell ratio	- Conclusion is supported by both in vitro and clinical data	- No functional assessment of T cells in relation to CMV infection - Correlation between donor-specific T-cell hyporesponsiveness and reduced incidence of late rejection was not studied	Consider CMV status when tapering/ withdrawing immunosuppressive drugs in LTx patients	Study the correlation between CMV infection and operational tolerance in a prospective weaning trial
3	CMV infection is associated with upregulation of CD244 on $CD8^+$ T cells and $CD8^+$ T-cell dysfunction after LTx	- Data on events both early and late post-LTx - First study investigating inhibitory receptors in patients after LTx	Whether CD244 is causative for CD8 T-cell dysfunction is not studied	Consider CMV status when tapering/ withdrawing immunosuppressive drugs in LTx patients	Study the correlation between CMV infection and operational tolerance in a prospective weaning trial
4	Review describing potential beneficial effects of CMV infection in humans	Describing the generally overlooked beneficial effects of CMV infection to humans in several different fields	How the effects of CMV infection can be paradoxical is not discussed	- CMV infection can be utilized in certain ways to benefit human health - The advantages and disadvantages of applying a universal CMV vaccine should be carefully weighed	- Understand the mechanisms behind CMV infection and those beneficial outcomes - Inducing immune responses similar to those evoked by CMV to benefic human health
5	- PD-1 is highly expressed on allograft infiltrating T cells - PD-L1 is abundantly present in rejecting liver allografts - SNPs within recipient PD-1 and donor PD-L1 genes are associated with late acute rejection - PD-L1 rs4143815 is associated with differential PD-L1 expression on donor hepatic dendritic cells upon IFN-γ stimulation	- Conclusion is supported by both in vitro and clinical data - The functional significance of the SNPs are confirmed - This study highlights a interaction between donor and recipient derived genetic traits	Association with operational tolerance after LT is not studied	PD-1 and PD-L1 SNPs can be considered when tapering/ withdrawing immunosuppressive drugs in LTx patients	Develop an appropriate agonist to target PD-1/ PD-L1 pathway as a novel immunosuppressive strategy

6	Using an engineered HLA messenger RNA based technique to detect CD4 T cells with indirect allospecificity is feasible	This technique is highly specific to detect T cells reactive to indirect presented HLA antigens	- Small numbers of patients studied - Sensitivity of the assay is not studied	This technique enables the monitoring of indirect CD4 T-cell alloresponses in transplanted patients	- Study the correlation between indirect pathway and complications/tolerance in patients after LTx - Generate regulatory T cells with indirect allospecificity based on this technique
7	- Leukocytes within allograft are composed of cells from both donor and recipient origin - Hepatic HSPCs after LTx are replaced by HSPCs of recipient origin	This is a unique study on intrahepatic hematopoietic chimerism after LTx	Small numbers of patients studied	Clinical significance of intrahepatic hematopoietic chimerism is not clear	Study the correlation between intragraft hematopoietic chimerism and complications/tolerance in patients after LTx

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CHAPTER 9

Appendix

Samenvatting / Dutch summary
Dankwoord / Acknowledgements
List of publications
PhD portfolio
Curriculum Vitae

DUTCH SUMMARY / NEDERLANDSE SAMENVATTING

Transplantatie van een lever van een genetisch niet-identieke donor gaat altijd gepaard met immuunresponsen die de donor lever aanvallen. Deze immuunresponsen (zogenaamde "allogene T-cel responsen") gaan gepaard met complicaties na de levertransplantatie, zoals afstoting van de donor lever. Transplantatiepatiënten moeten daarom levenslang afweeronderdrukkende (immunosuppressieve) medicijnen gebruiken om de immuunresponsen te onderdrukken, maar deze medicijnen hebben bijwerkingen die de lange termijn uitkomst van de transplantatie verslechteren. Het doel van dit proefschrift is om beter te begrijpen welke omgevingsfactoren en genetische factoren invloed hebben op de allogene T-celresponsen en daarmee op afstoting van de lever in transplantatiepatiënten. Deze inzichten moeten leiden tot een meer op de individuele patiënt afgemeten behandeling en een betere lange termijn uitkomst van de patiënt na levertransplantatie.

I. CMV infectie vermindert allogene CD8+ T-cel responsen en kan tolerantie in levertransplantatiepatiënten bevorderen

Uit studies met experimentele diermodellen weten we dat virale infecties na orgaantransplantatie tolerantie na transplantatie verhinderen. Dit wordt onder anderen veroorzaakt door inflammatie die met de infectie gepaard gaat en door activatie van geheugen T-cellen die gaan kruisreageren tegen allo-antigenen van het donor orgaan. Ook klinische studies hebben een relatie laten zien tussen infecties en transplantaat afsoting in mensen, maar een causaal verband is niet duidelijk aangetoond. In dit proefschrift hebben we daarom onderzocht wat het effect van CMV infectie - een veelvoorkomende virale infectie na levertransplantatie – is op T cel responsen, afstoting en tolerantie na levertranplantatie. In **Hoofdstuk 2** laten we zien dat, hoewel primaire CMV infectie na levertransplantatie toename van geheugen T cellen veroorzaakt, deze infectie juist geassocieerd is met donor-specifieke hyporesponsiviteit van CD8+ T cellen. Verdere analyse van dit verband in een groot cohort van levertransplantatie patiënten laat zien dat CMV viremie is geassocieerd met een verhoogd risico op vroege acute afstoting, maar primaire CMV infectie is geassocieerd met een lager risico op late acute afstoting. Verder toont dit hoofdstuk dat primaire CMV Infectie geassocieerd is met een hoge Vδ1/Vδ1 γδ T-cel ratio in de circulatie van deze patiënten, een kenmerk dat in eerdere studies is gerelateerd aan tolerantie na levertransplantatie. In **Hoofdstuk 3** bestuderen we de expressie van een aantal co-inhibitoire receptoren op T cellen van levertransplantatiepatiënten, waarbij we vinden dat de expressie van CD160 en CD244 langdurig verhoogd zijn op CD8+ T cellen na transplantatie. Deze verhoogde expressie

is geassocieerd met CMV infectie na transplantatie. *In vitro* delen gesorteerde CD244+ CD8+ T cellen minder goed dan CD244- CD8+ T cellen in reactie op stimulatie met alloantigen, hetgeen een verklaring kan zijn voor de verminderde reactie van CD8+ T cellen in patiënten met CMV infectie. Daarnaast tonen we aan dat de meerderheid van de CMV-specifeke CD8+ T cellen CD244+ zijn. In conclusie, CMV infectie na levertransplantatie is geassocieerd met de expansie van een dysfunctionele CD244+ T cel subset en met systemische hyporesponsiviteit van de CD8+ T cellen. Gezamenlijk laten deze twee hoofdstukken zien dat CMV infectie juist CD8+ T cel responsen vermindert en daarmee tolerantie na levertransplantatie kan bevorderen.

De meest verrassende bevinding van onze studie is het tegenstrijdige verband dat we vinden tussen CMV infectie en vroege versus late afstoting: terwijl CMV viremie is geassocieerd met meer vroege afstotingen, is primaire CMV infectie geassocieerd met minder late acute afstotingen, hyporesponsiviteit van donor-specifieke CD8+ T cellen en in het perifere bloed tekenen van tolerantie. In de meerderheid van de gevallen vindt vroege afstoting plaats vóór de CMV infectie. Dit is in lijn met de hypothese dat inflammatie die gepaard gaat met de allogene immuunrespons aanzet geeft tot replicatie van het latent aanwezige CMV. Anderzijds kan het lagere risico op late acute afstoting ook het gevolg zijn van de primaire CMV infectie, welke meestal optreedt in de eerste zes maanden na transplantatie. In deze patiënten wordt mogelijk om die reden de dosering van de immunosuppressieve medicatie verlaagd, wat in de literatuur beschreven is als één van de belangrijkste redenen voor late acute afstoting. Bovengenoemde factoren waarbij CMV infectie en afstoting een wisselwerking kunnen zijn van elkaars aanwezigheid kunnen mogelijk verklaren dat wij een omgekeerde relatie tussen CMV en afstoting vinden dan in sommige eerdere studies beschreven is.

Hoe CMV immuunrespons van CD8+ T cellen vermindert, is nog niet precies bekend. Wel zijn in eerdere studies immunomudulatoire mechanismen beschreven waardoor CMV een latent aanwezig virus wordt, zoals: de productie van viraal IL-10, leidend tot een systemisch immunosuppressief effect; CMV is geassocieerd met verzwakte respons van immuuncellen; grote hoeveelheid geheugen T cellen gericht tegen CMV voorkomen dat er meer naieve T cellen bijkomen waardoor de diversiteit van T cellen en de respons tegen andere pathogenen afneemt; CMV leidt tot lange termijn chimerisme en daarmee donor-specifieke T cel hyporesponsiviteit; CMV induceert toename van Th1 cellen en zet cellen in de lever aan tot het maken van type I Interferon, waardoor Th1 cellen en regulatoire T cellen worden aangetrokken; Th1 cellen maken daarnaast grote hoeveelheden IFN-γ, waarvan in muismodellen is aangetoond dat dit bijdraagt aan tolerantie na transplantatie. Bovengenoemde immunomudulatoire mechanismen van CMV kunnen na levertransplantatie bijdragen aan tolerantie voor de donor lever.

Naar aanleiding van onze bevinding dat CMV infectie een gunstig effect zou kunnen hebben in lever transplantatie patiënten hebben we in **Hoofdstuk 4** de literatuur betreffende dit onderwerp samengevat, waarin de gunstige effecten van CMV infectie in de mens beschreven worden. Een voorbeelden hiervan is dat CMV infectie na hematopoietische stamceltransplantatie het risico op terugkeer van kanker vermindert. Ons inziens zou meer onderzoek nodig zijn om de mechanismen te ontrafelen waarmee CMV zijn gunstige effecten op de mens uitoefent; om in de toekomst deze effecten te kunnen laten toenemen en ook de mogelijkheden en voordelen van een CMV vaccinatie strategie te onderzoeken.

II. Genetische variaties in de PD-L1/PD-1 co-inhibitoire pathway beïnvloeden afstoting na levertransplantatie in mensen

Co-inhibitoire receptoren op T cellen die interactie aangaan met hun liganden op diverse cellen zorgen ervoor dat immuunresponsen van T cellen geremd worden. Onze hypothese was dat deze co-inhibitoire pathways ook betrokken zijn bij het afremmen/reguleren van allogene T cel responsen en bij afstoting in liver transplantatie patiënten. In **Hoofdstuk 5** onderzoeken we de meest bekende co-inhibitoire pathway, namelijk de PD-1/PD-L1 pathway. We vinden dat de receptor PD-1 hoog tot expressie wordt gebracht op T cellen die de donor lever infiltreren en dat de ligand van PD-1 (PD-L1) hoog tot expressie komt in getransplanteerde levers waarin een afstoting plaatsvond. Wanneer we deze pathway blokkeren, vertonen T cellen afkomstig van deze levers meer celdeling in reactie op stimulatie met donor antigenen. Daarnaast vinden we een associatie tussen genetische variaties in deze pathway en het optreden van late acute afstotingen na lever transplantatie. Deze bevindingen laten ons inziens de rol zien van deze pathway in het optreden van afstoting na lever transplantatie. Daarnaast kunnen deze genetische variaties tussen patiënten bijdragen aan het verschil in vatbaarheid voor afstoting dat er is tussen patiënten.

Het is interessant dat de genetische variaties in de PD-1/PD-L1 pathway alleen geassocieerd zijn met het optreden van late acute afstotingen. Een verklaring hiervoor kan zijn dat het optreden van vroege acute afstoting sterk gerelateerd is aan het gebruik van immunosuppressieve medicatie, welke vroeg na transplantatie in hogere dosering gegeven wordt. Ook de allogene immuunrespons is vroeg na transplantatie sterker dan laat na transplantatie. In die vroege periode worden de kleine variaties in co-inhibitoire pathways waarschijnlijk overschaduwd door deze sterkere factoren, terwijl later na transplantatie de rol van de PD-1/PD-L1 pathway wel zichtbaarder kan worden en voor verschillen in vatbaarheid voor afstoting kan zorgen.

Voordat genetische verschillen in de PD-1/PD-L1 pathway in de klinische praktijk toegepast kunnen worden, is eerst meer onderzoek nodig. Allereerst zou de rol van deze pathway moeten worden onderzocht in levertransplantatie patiënten van wie bekend is dat ze operationeel tolerant zijn. Er zou onderzocht moeten worden of bepaalde donor/ontvanger combinaties met gunstige genetische varianten de succeskans bij het afbouwen van immunosuppressieve medicatie kunnen verhogen. Als dit zo is, moet bekeken worden of deze genetische karakteristieken, tezamen met andere karakteristieken zoals γδT-cellen, kunnen worden gebruikt om tolerante patiënten te identificeren. Daarnaast zou PD-1 een doelwit kunnen zijn voor nieuwe immunosuppresieve medicatie, waarbij de remmende werking van PD-1 in transplantatie patiënten wordt gestimuleerd. Gezien de hoge expressie van PD-1 in de lever, zou het stimuleren van PD-1 in transplantatie patiënten heel gericht de lever infiltrerende T cellen moeten remmen, terwijl de werking van tegen pathogenen gerichte T cellen intact blijft.

III. *In vitro* getranscribeerd messenger RNA kan worden gebruikt voor monitoring van indirecte allo-herkenning in menselijke levertransplantatie patiënten

Na levertransplantatie spelen twee pathways een rol bij het activeren van T cellen die reageren tegen donor antigenen, namelijk de directe en de indirecte pathway. De directe pathway is vermoedelijk dominant vroeg na transplantatie, en vroege afstoting wordt dus waarschijnlijk met name geïnduceerd door deze pathway. De indirecte pathway neemt het later na transplantatie geleidelijk van de directe pathway over en wordt geassocieerd met de latere schade aan het donororgaan, zoals chronische afstoting. Echter, op dit moment schieten technieken nog te kort om de T cellen te detecteren die via de indirecte pathway geactiveerd worden. In **Hoofdstuk 6** beschrijven we hier een nieuwe techniek voor, die gebaseerd is op het door middel van electroporatie inbrengen van allogeen messenger RNA in autologe antigeen presenterende cellen (APC). Hiermee kunnen CD4+ T cellen die via de indirecte pathway tegen allo-antigenen reageren gedecteerd worden. Onze nieuwe techniek heeft een aantal voordelen. Allereerst reageren via deze methode de CD4+ T cellen erg specifiek op alleen *indirect* gepresenteerd allo-antigen, omdat oppervlakte expressie van het allogene HLA wordt vookómen. Ten tweede zorgt deze techniek er, in tegenstelling tot het gebruik van slechts een aantal peptiden, voor dat het volledige antigen spectrum van de geëlectroporeerde HLA moleculen gebruikt wordt, waardoor de techniek niet beperkt wordt door HLA-typering van de patiënten. Ten derde gebruiken we CD40B-cellen als APC, welke in grote hoeveelheden kunnen worden geëxpandeerd vanuit kleine hoeveelheden bloedmonsters.

Wanneer we in acht levertransplantatiepatiënten deze assay toepassen, vinden we in vier van hen CD4+ T cellen die tegen allo-antigen reageren via de indirecte pathway. Het meest interessant is dat in één van deze vier patiënten een zeer sterke respons te meten is en dat deze patiënt op dat moment een chronische afstoting doormaakt die zelfs geleid heeft tot verlies van het donor orgaan. Dit is in lijn met de hierboven beschreven hypothese dat de indirecte pathway waarschijnlijk een rol speelt bij chronische afstoting. Er is verder onderzoek nodig om beter te begrijpen bij welke post-transplantatie complicaties de indirecte pathway betrokken is en ook om de klinische relevantie ervan aan te tonen. Hier kunnen vervolgens immunosuppressieve therapieën beter op afgestemd worden om daarmee de lange termijn uitkomsten van levertransplantatie te verbeteren. Ook kan onze techniek gebruikt worden voor het genereren van regulatoire T cellen (Tregs) met indirecte allospecificiteit als alternatieve immunosuppressieve celtherapie. Op dit moment worden verschillende klinische trials uitgevoerd met Tregs als nieuwe immunosuppressieve therapie, maar deze studies gebruiken Tregs met directe allospecificiteit. In muismodellen is echter laten zien dat Tregs die geassocieerd zijn met tolerantie na transplantatie juist via de indirecte pathway reageren en dat Tregs met indirecte allospecificiteit meer cruciaal zijn in het induceren van tolerantie dan Tregs met directe allospecificiteit. Ten slotte, de indirecte allorespons moet ook worden bepaald in studies waarin patiënten geïdentificeerd worden die geschikt zijn voor het afbouwen van de immunosuppressieve medicatie, om te bepalen of de indirecte pathway ook in deze selectie een belangrijke factor is. Op deze manier kunnen we het risico op lange-termijn complicaties van immunosuppressie zo veel mogelijk terugdringen.

IV. Lange termijn hematopoietisch chimerisme komt veel voor in getransplanteerde levers

De klinische relevantie van hematopoietisch chimerisme na levertransplantatie is nog niet precies bekend. Een onderzoek van Wang et al. liet zien dat er twee vormen van chimerisme zijn in levertransplantatie patiënten: 1. Tijdelijk chimerisme ten gevolge van rijpe donor leucocyten en 2. Lange-termijn chimerisme ten gevolge van donor hematopietische stamcellen afkomstig van de donor lever, waarbij gesuggereerd werd dat de lever een goede locatie is voor hematopoiese buiten het beenmerg. In **Hoofdstuk 7** beschrijven we een commentaar op de studie van Wang et al., waarbij we chimerisme binnen getransplanteerde levers onderzoeken. We vinden dat leucocyten in deze levers zowel van donor als ontvanger afkomstig zijn, zelfs tot twee jaar na transplantatie. Dit laat zien dat lange-termijn chimerisme veel voorkomt in getransplanteerde levers. In tegenstelling tot de leucocyten zijn de hematopietische stamcellen van de donor in de donorlevers kort na transplantatie al vervangen door hematopietische stamcellen van

de ontvanger. Deze resultaten suggereren dat het lange-termijn chimerisme dat Wang et al. beschreef mogelijk veroorzaakt wordt door langlevende donor leucocyten in het transplantaat en/of door hematopoiese van hematopoietische donor stamcellen die opnieuw naar de lever zijn gegaan. Het is echter niet bekend of intrahepatische leucocyten zo lang kunnen leven zonder de aanwezigheid van hematopietische donor stamcellen. Verder onderzoek is nodig om te weten te komen hoe deze in de lever aanwezige cellen zo lang in leven kunnen blijven en welk type cellen dit precies zijn.

CONCLUSIE

In dit proefschrift laten we allereerst zien dat CMV infectie allogene CD8+ T cel responsen vermindert en tolerantie in levertransplantatiepatiënten kan bevorderen. Daarnaast laten onze resultaten zien dat de PD-L1/PD-1 co-inhibitoire pathway betrokken is bij het reguleren van afstoting na levertransplantatie, namelijk genetische variaties in donor PD-L1 en ontvanger PD-1. Verder ontwikkelen we een nieuwe techniek die het mogelijk maakt om CD4+ T cellen te meten die reageren tegen indirect gepresenteerde allo-antigenen. Ten slotte beschrijven we het vóórkomen van lange termijn hematopoietisch chimerisme in humane levertransplantaten. In conclusie geeft dit proefschrift nieuwe aanknopingspunten voor het ontwikkelen van behandelingsstrategieën waarbij patiënten kunnen worden geselecteerd/geïdentificeerd bij wie afbouwen van immunosuppresieve medicijnen succesvol kan zijn.

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LIST OF PUBLICATIONS

1. **Shi XL**, Mancham S, Hansen BE, de Knegt RJ, de Jonge J, van der Laan LJW, et al. Interplay between donor PD-L1 and recipient PD-1 regulates acute graft rejection after liver transplantation. Journal of Hepatology. Accepted.
2. **Shi XL**, de Mare-Bredemeijer EL, Tapirdamaz O, Hansen BE, van Gent R, van Campenhout MJ, et al. CMV primary infection is associated with donor-specific T cell hyporesponsiveness and fewer late acute rejections after liver transplantation. Am J Transplant. 2015. Sep;15(9):2431-42.
3. de Mare-Bredemeijer ELD, **Shi XL**, Mancham S, van Gent R, van der Heide-Mulder SA, de Boer R, et al. CMV-induced expression of CD244 after liver transplantation is associated with CD8+ T-cell hyporesponsiveness to allo-antigen. J Immunol. 2015;195:1838-1848.
4. **Shi XL**, Moroso V, Metselaar HJ, Kwekkeboom J. Long-lived intragraft donor leukocytes or relocated donor hematopoietic stem/progenitor cells can cause long-term hematopoietic chimerism after liver transplantation. Hepatology. 2013; 57(6): 2542.
5. **Shi XL**, Chen XX, Jin R, Li H, Xue P, Yao YF, et al. Polyclonal antibodies preparation of the Escherichia coli type VI secretion system related proteins and detection of secreted proteins. Journal of Shanghai Jiaotong University(Medical Science). 2011; 31(1): 118-22.
6. **Shi XL**, Tapirdamaz Ö, Heirman C, de Mare-Bredemeijer ELD, Mancham S, Heemskerk MHM, et al. Ex vivo detection of T-cell indirect allorecognition in human transplant recipients using engineered HLA messenger RNA. Submitted.

PHD PORTFOLIO

Name PhD student	Xiaolei Shi
Erasmus MC Department	Gastroenterology and Hepatology
PhD period	August 2011 – August 2015
Promoter	Prof. dr. H.J. Metselaar
Co-promotor	Dr. J. Kwekkeboom

General Courses

2011 Study Design
2011 Genetics for Dummies
2011 SPSS
2011 Biomedical English Writing
2011 Pubmed
2011 Endnote
2011 Biomedical Research Techniques
2012 Survival Analysis
2012 Research Management for PhD student
2012 Presenting Skills for PhD student
2012 Workshop Writing Successful Grant Proposals
2013 Workshop Photoshop & Illustrator
2013 SNPs and Human Diseases
2013 Course on Molecular Immunology
2015 Clinical Trials

Conferences

2012 24th International Congress of The Transplantation Society, Berlin, Germany (attendance)
2013 ESOT/TTS Basic Science Meeting, Paris, France (two poster presentations)
2014 Bootcongres, Leiden, The Netherlands (two oral presentations)
2014 World Transplant Congress, San Francisco, USA (two poster presentations)
2014 NVVI 50th Anniversary Congress, Efteling, The Netherlands (poster presentation)
2015 Joint British Transplantation Society & Nederlandse Transplantatie Vereniging Congress, Bournemouth, UK (oral presentation)
2015 8th Dutch Experimental Gastroenterology and Hepatology (DEGH) meeting. Veldhoven, The Netherlands (two oral presentations)
2015 4th European Congress of Immunology, Vienna, Austria (oral presentation)

Scientific Awards and Grants

2011 China Scholarship Council (CSC) Scholarship (File No.2011623039)
2015 Jon J. van Rood Award, by Nederlandse Transplantatie Vereniging

Travel Grants

2012 Erasmus MC, Research Master Infection & Immunity Backpack Fund
2013 Erasmus MC, Research Master I&I Fund
2014 Erasmus MC, Research Master I&I Fund
2015 Erasmus Trustfonds travel grant
2015 Dutch Society for Immunology travel grant

Ad Hoc Manuscript Reviewer

Journal of Hepatology, Scientific Reports

Other Activities

Member of Erasmus University Medical Center PhD committee and Erasmus University Medical Center Post-Graduate School Molecular Medicine PhD committee

CURRICULUM VITAE

Xiaolei Shi was born in Cixi, Zhejiang Province, China, on 15th September 1987. He was raised by his beloved parents Hongyue Fan and Yuesheng Shi. In 2006, he graduated from high school and started medical training at Shanghai Jiao Tong University School of Medicine. He conducted his clinical rotation in Shanghai Ruijin Hospital in 2011. During his medical training, he also performed research at the Laboratory of Bacterial Pathogenesis, under the supervision of Prof. Yufeng Yao, and he was granted a research fund for undergraduates. In 2010, he performed research at the Department of Internal Medicine, The University of Michigan Medical School, Ann Arbor, as exchange student, under the supervision of Prof.dr. Eric Fearon. In 2011, he was awarded the "China Scholarship Council Grant", with which he conducted his PhD fellowship at the Department of Gastroenterology and Hepatology, Erasmus University Medical Center Rotterdam, the Netherlands, under the supervision of Prof.dr. H.J. Metselaar and Dr. J. Kwekkeboom. During these four years, he conducted several studies regarding the immune regulation after liver transplantation. Some of his works are included in this thesis. After finishing his PhD, Xiaolei is going to start his surgical residency at the Renji Hospital, Shanghai Jiao Tong University in 2016.