Immunomodulation after Liver Transplantation
A Role for Dendritic Cells

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Een rol voor dendritische cellen

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General introduction and outline of this thesis

INTRODUCTION

The liver is the largest organ in the body with essential metabolic activities. In the liver large amounts of oxygenated blood carried by the hepatic artery are used to mobilize energy from dietary components. Toxic compounds, derived from food are transported from the bowel via the portal vein and detoxified in the liver. In order to avoid unwarranted responses to harmless dietary proteins and components of the commensal intestinal flora, tight control of the local immune response in the liver is mandatory. However as a consequence the liver is a target for chronic viral and parasitic infections and metastasis of malignant diseases develop relatively easily in the liver (1). To meet these specific requirements, the repertoire of immune cells present in the liver differs dramatically from those in other non-lymphoid tissues of the body, and is considered to play a specialized role in hepatic immune responses (2).

The liver hosts various unique resident cell types with immune potential, such as a lymphoid population selectively enriched for $CD8^+$ T cells, NK cells and NKT cells, and cells with accessory immunologic functions such as dendritic cells (DC), Kupffer cells, sinusoidal endothelial cells and biliary epithelial cells. It is hypothesized that DC, the most potent antigen presenting cells, are the key players in maintaining the fine balance between immune responsiveness and unresponsiveness in the liver (3). Under normal circumstances, most peripheral tissues, such as the liver, contain immature DC, whose function is uptake and processing of antigen. Under steady state conditions there is a continuous migration of DC towards the draining lymph nodes (LN) and this process is accelerated upon antigenic stimulation. During this migration DC mature and acquire a strong T cell stimulatory capacity (4-6).

Donor-derived immune cells after liver transplantation

Liver grafts differ from other solid organs in that the immunological rejection of a transplanted allogeneic liver is frequently impaired. In healthy animals hepatic tolerance is manifested as long-term survival of the liver allograft in the absence of immunosuppression even across MHC barriers (7-9). In humans, liver transplants are less susceptible to antibody mediated rejection compared to other organ grafts, and liver graft survival is usually not affected in antibody cross-match-positive patients (10). Liver transplants do not require MHC matching of donor and recipient, and in contrast to recipients of heart or kidney transplants it is estimated that about one third of liver transplant recipients with stable function can be totally withdrawn from immunosuppression (11-14). Liver transplants have also been reported to protect kidney transplants from the same donor from humoral and cellular rejection (15, 16).

The transfer of donor leukocytes into recipients after organ transplantation, resulting in leukocyte chimerism, has been associated with donor-specific tolerance. In an experimen-

tal liver transplantation model depletion of passenger leukocytes from liver grafts led to rejection in spontaneously tolerant recipients, whereas reconstitution of donor leukocytes by i.v. injection recovered graft acceptance (17, 18). Furthermore higher levels of chimerism after human liver transplantation have been associated with reduced incidence of acute rejection and better initial graft acceptance (19, 20). The role of chimerism in graft acceptance is most likely of greatest magnitude after liver transplantation, since the liver contains very high numbers of leukocytes which can migrate into the recipient after transplantation. This implies a crucial role for donor liver-derived cells, including DC and lymphocytes, in regulating the balance between tolerance and rejection.

There is no uniform concept regarding the role of donor DC after organ transplantation. On the one hand, it is generally accepted that donor DC are responsible for the occurrence of acute rejection after solid organ- and tissue transplantation due to their potent capacity to stimulate recipient T cell responses against the graft. In experimental animal studies it has been shown that DC from the graft, expressing donor allo-antigens, migrate into the recipient's lymph nodes and spleen where they activate recipient T cells against the graft (Figure 1). This hypothesis is supported by the fact that in a murine heart and liver transplant model augmentation of donor DC induced rejection (21). However, there is currently no evidence that graft-derived DC migrate into recipients after organ transplantation in humans. On the other hand, there are several animal studies showing

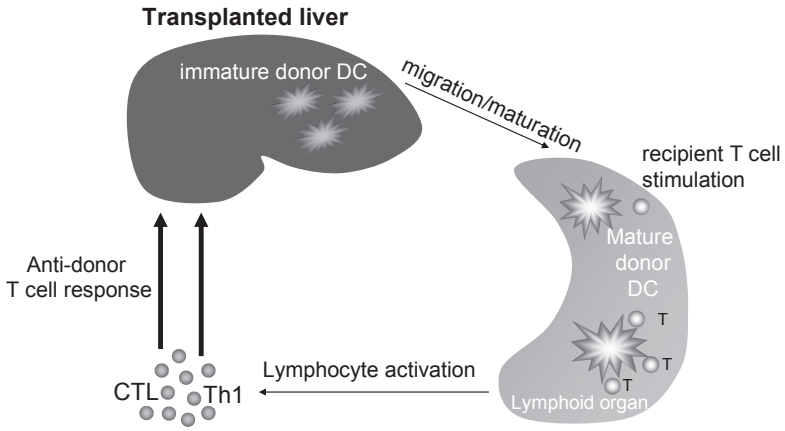

Figure 1. Mechanism of liver allograft rejection. An ordered sequence of events is thought to lead to rejection: Immature donor DC migrate from the transplanted liver into the recipient's lymphoid organs, including spleen and lymph nodes. In the lymphoid organs, donor DC present donor allo-antigens directly to the recipients T cells leading to a vigorous anti-donor T cell response.

that infusion of donor DC can prolong graft survival (22, 23). Therefore, donor-derived DC are thought to play a dualistic role after organ transplantation. It still remains to be elucidated under which conditions donor-derived DC promote or counteract rejection. Probably this is dependent on the type of donor DC that enters the recipient.
Within the T cell compartment there is a subpopulation of cells, the $CD4^+CD25^+$ regulatory T cells (Treg), that play a critical role in various models of transplant tolerance (24, 25). However, whether graft-derived donor Treg play a role in chimerism-associated tolerance still remains to be determined. Moreover, the mechanisms by which leukocyte chimerism contributes to tolerance are not fully understood, although clonal exhaustion or deletion, T cell anergy, and active suppression have been proposed as possible mechanisms.

Immunotherapy with tolerogenic DC for prevention of rejection after organ transplantation

Since the introduction of immunosuppressive drugs in the 1960s clinical transplantation has become very successful. However, the prolonged use of these immunosuppressants is accompanied with serious side effects like renal failure, high risk for malignancies and high susceptibility for infections. To prevent these side effects it is mandatory to develop a therapy which has no toxic side effects and induces donor-specific tolerance. Since DC play a central role in regulating immune responses, creating tolerogenic DC for use in cellular immunotherapy could be a promising alternative for the current immunosuppressive regimens. In humans, immunization with immature DC resulted in down regulation of $CD8^+$ T cell response to recall antigens and induction of IL-10 producing suppressive T cells, indicating the feasibility of DC-based immunotherapy to induce immunological hyporesponsiveness to a certain antigen (26, 27). Furthermore, modified DC have already been safely used to treat patients with advanced metastatic melanoma, in which injection of mature DC could elicit a tumor-specific immune response (28). Numerous publications have already emerged on establishing tolerogenic DC by *in vitro* treatment with corticosteroids (29-32), vitamin D derivatives (33-35), aspirin (36, 37) or other inhibitors of NFκB translocation (38). The resulting DC induced T cell hyporesponsiveness or regulatory T cells *in vitro* (35, 39). Moreover, a few studies demonstrated induction of transplant tolerance in experimental animals upon transfer of donor-derived tolerogenic DC *in vivo* (31, 32). However before introducing tolerance-inducing DC therapy in humans, the challenge is to create DC with stable tolerance-inducing properties that can not convert into immunogenic DC *in vivo*. This is mandatory since humans are constantly exposed to stimulatory agents such as inflammatory stimuli from pathogens and other environmental danger signals that can activate DC.

AIM AND OUTLINE OF THE THESIS

This thesis is focused on the immunomodulatory effects of DC and Treg after human liver transplantation. In particular, the objective was to study whether donor liver derived myeloid DC (MDC) and Treg can contribute to the relatively low immunogenicity of liver grafts. Furthermore the suitability of human blood MDC modified with corticosteroids for therapeutic use to induce transplant tolerance was investigated. This information could give insight into the mechanisms of how the balance between tolerance and rejection is maintained after liver transplantation. This knowledge could subsequently be utilized to develop donor-specific therapy for liver transplant recipients.

In **chapter two** it was investigated whether hepatic DC undergo *in vivo* an alternative maturation program leading to tolerogenic effector DC in the hepatic lymph nodes (LN). Immunophenotypical and functional characteristics of human hepatic LN DC were compared with skin/muscle draining LN DC. Skin/muscle draining LN DC were used for comparison because of the major differences in immune response between liver and skin.

In **chapter three** the presence of Treg in the donor liver was studied. The phenotype and suppressive capacity of donor liver $CD4^+CD25^+$ regulatory T cells, migrated from the liver graft into the perfusion solution shortly before transplantation, were investigated. Furthermore the presence of these donor-derived Treg in recipient blood was monitored.

In **chapter four** DC present in human liver grafts were characterized. MDC in the donor liver were visualized by immunohistochemistry and immunophenotyped in donor liver mononuclear cells. Moreover large numbers of viable MDC were detected in perfusates of the liver grafts obtained by *ex vivo* vascular perfusion pretransplantation. These perfusate MDC were used for further immunophenotypic and functional characterization of human donor liver MDC.

In **chapter five** additional immunophenotypical and functional characterization of perfusate MDC is described and the presence of donor MDC in the circulation of the recipient after liver transplantation is confirmed and compared with kidney transplantation.

In **chapter six** the impact of the immunosuppressive regimen on MDC in the circulation of liver transplant recipients during the first year after transplantation was monitored. The numbers of MDC were quantified and the maturation status of circulating MDC was determined along with their *ex vivo* capacity to mature and subsequently stimulate allogeneic T cells.

In **chapter seven** is described whether pre-treatment of immature human blood MDC with dexamethasone (dex) or combined treatment with dex and Toll-like Receptor-4 agonist lipopolysaccharide, leads to the induction of stable tolerogenic MDC.

Finally the research described in this thesis is summarized and discussed in **chapter eight**.

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2

Myeloid dendritic cells in hepatic lymph nodes are exhausted and have a poor T cell stimulatory capacity

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submitted

ABSTRACT

The liver is an immune-privileged organ, in which immune responses against food antigens and components of the commensal gut flora are tightly regulated. We investigated whether an alternative maturation program of hepatic dendritic cells (DC) leads to tolerogenic effector DC. As the draining lymph node (LN) is the main site where DC regulate T cell responses, we compared maturation-related characteristics of DC from hepatic LN with DC from skin/muscle draining LN. Hepatic LN were obtained from multiorgan donors (MOD). Skin/muscle draining LN were obtained from multiorgan donors (iliacal LN) or from kidney transplant recipients (inguinal LN). DC were immunophenotyped by flowcytometry, or isolated by positive immunomagnetic selection, and tested for their capacity to stimulate allogeneic T cell proliferation and produce cytokines.

Myeloid DC (MDC) from hepatic LN had a twofold reduced capacity to stimulate allogeneic T cell proliferation compared to MDC from inguinal LN, whereas plasmacytoid DC (PDC) from both tissues had a similar T cell stimulatory capacity. Despite the lower T cell stimulatory capacity, MDC from hepatic LN proved to be highly mature MDC, with significantly higher expressions of the co-stimulatory molecules (CD40, CD80 and CD86) and CD83 and CCR7 as compared to MDC from skin/muscle draining LN. The reduced T cell stimulatory capacity of MDC from hepatic LN may be related to their inability to produce cytokines. In contrast to MDC from inguinal LN, MDC from hepatic LN produced almost no cytokines upon stimulation with poly (I:C) and IFN-γ, *Staphyloccocus aureus* or CD40L and IFN-γ.

In conclusion, hepatic MDC mature *in vivo* into effector MDC that that are highly mature, but are unable to produce cytokines and have a poor allogeneic T cell stimulatory capacity. We postulate that MDC in the liver environment undergo hyper-maturation, due to continuous stimulation with gut-derived components, resulting in exhausted MDC in hepatic lymph nodes.

INTRODUCTION

The liver is an immune-privileged organ that favors induction of peripheral tolerance rather than induction of immunity. The liver is continuously exposed to food antigens and bacterial products that enter the liver from the gastrointestinal tract via the portal vein. The development of liver inflammation in response to these gut-derived components is undesired, and therefore tight control of the local immune responses in the liver is mandatory. However, as a consequence the liver is prone to chronic viral and parasitic infections, and metastases of malignant diseases develop relatively easily in the liver. The majority of hepatitis C virus infected patients develop chronic infections of the liver as a result of insufficient local immune responses (1), and the liver is the site of persistence of the malarial parasite (2).

Furthermore, liver grafts are unique in that indefinite survival in absence of immunosuppressive therapy can be achieved in pigs, rats and mice (3-5). Mouse liver allografts can even be accepted across MHC barriers and induce donor-specific tolerance without anti-rejection therapy (5). In humans, liver allografts have a lower susceptibility to chronic rejection compared to kidney grafts, and have the capacity to resist poor HLA-matching, ABO-incompatibility and positive cross-matches (6). Moreover, it is estimated that complete withdrawal of immunosuppression is feasible in about one third of liver transplant recipients (7-10).

It is hypothesized that dendritic cells (DC) are key players in maintaining the fine balance between immune responsiveness and unresponsiveness in the liver (11). DC are the most highly specialized antigen-presenting cells and they play a critical role in the initiation and direction of immune responses (12, 13). Immature myeloid DC (MDC) are located throughout most body tissues and are specialized in the uptake and processing of antigens. Under steady-state conditions there is a continuous migration of MDC towards the draining lymph nodes (LN) and this process is accelerated upon antigenic stimulation. During migration to the lymph nodes MDC complete their maturation and acquire a T cell stimulatory capacity (12-14).

As with all non-lymphoid tissue MDC (15), freshly isolated human and murine liver MDC are predominantly immature, expressing surface MHC but few co-stimulatory molecules (11, 16-20). However liver MDC have some unique characteristics. We and others showed that human liver MDC produce high amounts of IL-10 whereas blood or skin MDC fail to secrete IL-10 even after stimulation with different stimuli (18, 21). Liver MDC are also less efficient in stimulating allogeneic T cell proliferation compared with splenic or skin MDC (20-23). MDC propagated from murine liver progenitors even have the capacity to induce donor-specific hyporeponsiveness and prolong graft survival when injected prior to allogeneic organ or tissue transplantation (22, 24, 25).

However, in general T cell responses are not initiated in parenchymal organs, but in secondary lymphoid organs. Liver lymph drains to the hepatoduodenal LN at the hilus and

along the hepatic artery and portal vein. Indeed, T cells specific for viral antigens encoded in the liver are preferentially located in hepatic LN and not in the liver itself (26). In addition, immunological tolerance to antigens delivered into the portal vein coincides with induction of hyporesponsiveness in T cells in hepatic LN (27). Despite the importance of hepatic LN in the regulation of T cell responses to antigens expressed in the liver, very little is known about DC in liver-draining LN. We previously showed that in human subjects with a healthy liver hepatic LN contain comparable numbers of myeloid DC (MDC) compared skin/muscle draining LN, but fewer plasmacytoid DC (PDC) (28). However, in patients with hepatocellular carcinoma numbers of MDC in liver LN are reduced and numbers of PDC are increased (29), indicating the DC in liver-draining LN are influenced by processes ongoing in the liver itself. Since MDC are much more efficient than PDC in antigen presentation, while the main function of PDC is to contribute to the innate defense against viral infections by production of IFN-α, we focused in this study on the T cell stimulatory capacity of MDC in hepatic LN. We hypothesized that, in order to prevent immune reactivity of non-hazardous gut-derived antigens, hepatic MDC undergo *in vivo* an alternative maturation program leading to the presence of more tolerogenic effector MDC in the liver draining LN.

The aim of this study was to compare immunophenotypical and functional characteristics of MDC from hepatic LN with MDC from skin/muscle draining LN. The immunophenotype, cytokine production profile, and T cell stimulatory capacity of MDC from hepatic LN and from skin/muscle draining LN were studied in detail.

MATERIALS AND METHODS

Collection of lymph nodes

Hepatic LN (n=33) were obtained from the hilus and along the hepatic artery and portal vein from multi-organ donors. Iliacal LN (n=8) were also obtained from multi-organ donors. Eight hepatic LN were derived from the same multi-organ donors as the seven iliacal LN, enabling comparison of DC in paired LN. Inguinal LN (n=26) were obtained from kidney transplant recipients. Iliacal and inguinal LN both are skin/muscle draining lymph nodes. The Ethics Committee of the Erasmus MC approved the study protocol and informed consent of each patient was obtained.

Antibodies

The following mAbs were used: IgG1-FITC, IgG1-PE, IgG1-APC, CD20-PerCP, HLA-DR-FITC, CD86-APC, CD40-APC and streptavidin-APC from BD Biosciences, Heidelberg, Germany; CD80-FITC from Beckman Coulter Immunotech, Marseille, France; anti-BDCA1-PE, anti-BDCA1-FITC, anti-BDCA4-PE, CD19-microbeads and anti-PE-microbeads

from Miltenyi Biotec, Bergisch Gladbach, Germany; biotinylated anti-PD-L1 and anti-PD-L2, and purified neutralizing anti-PD-L1 (clone MIH1) (30) and anti-PD-L2 mAb (clone MIH18) (31) from eBioscience, San Diego, USA; CD83-PE from Caltag, Burlingame, USA; anti-CCR7-PE from R&D systems, Abingdon, United Kingdom.

Isolation of LN mononuclear cells (MNC)

LN were cut into small pieces and passed over a nylon mesh filter (200 µm pore diameter) to obtain a single cell suspension. MNC were obtained from single cell suspensions of LN by Ficoll-Paque (Amersham Biosciences, Roosendaal, the Netherlands) density centrifugation. Cell-viability was determined using trypan blue staining.

Isolation of LN DC

Flowcytometric analysis was used to immunophenotype the LN DC. MDC were defined as BDCA-1^{+}CD20^{-} cells and PDC as BDCA-4^{+}CD123^{+} cells. To determine the immunophenotype of MDC, LN MNC were labeled with following antibody-combinations: anti-BDCA1-PE and CD20-PerCP in combination with anti-HLA-DR-FITC, CD80-FITC, CD86-APC, or biotinylated anti-PD-L1, or biotinylated anti-PD-L2 with as secondary reagent streptavidin-APC; or anti-BDCA1-FITC and CD20-PerCP in combination with anti-CCR7-PE, CD83-PE , or CD40-APC Appropriate isotype-matched control antibodies were used. Optimal dilutions of all antibodies were established in preliminary experiments. The data were analyzed on a FACScalibur using Cellquest pro software.

Flowcytometry

MDC were isolated by labeling LN MNC with anti-BDCA1-PE and CD19-microbeads and depleting B cells by separation over a Large Depletion column using a MidiMACS separation device (Miltenyi Biotec, Bergisch Gladbach, Germany). Subsequently, the non-adherent cells were labeled with anti-PE-microbeads and MDC were isolated by two rounds of separation over MS columns using MiniMACS separation device (Miltenyi Biotec, Bergisch Gladbach, Germany). Purities of the isolated MDC as determined by flowcytometry was 82±3% for MDC from hepatic LN and 83±4% for MDC from inguinal LN. For isolation of PDC, LN MNC were labeled with anti-BDCA-4-PE. Subsequently, the MNC were incubated with anti-PE-microbeads and PDC were isolated by two rounds of separation over MS columns. Purities of PDC isolated from hepatic LN was 49±5% and of PDC isolated from inguinal LN 63±13%.

Allogeneic T cell stimulatory capacity of purified DC

Purified MDC or PDC were co-cultured at different concentrations (10, 5, 2.5, 1.25 x10^3 cells/200 µl) in flat bottom Costar culture plate (Costar Cambridge, MA) in RPMI supplemented with 10% FCS (Hyclone, Logan, UT, USA), pencillin (100U/ml) and streptomycin

(100 ug/ml; Gibco BRL life technologies, Breda) with 1.5 x10^5 nylon wool-enriched allogeneic T cells from blood of a healthy volunteer. In all experiments, T cells from the same individual were used. For blocking of PD-L1 and PD-L2, graded numbers of MDC (10, 5 x10^3 cells/200 µl) were first incubated with 10 µg/ml anti-PD-L1 and 10 µg/ml anti-PD-L2 mAb for 15 min at 37°C, and subsequently 1.5 x10^5 allogeneic purified T cells were added.

After five days, cell proliferation was assessed by measuring the incorporation of [^{3}H]-thymidine (Radiochemical Centre, Amersham, Little Chalfont, UK). A 0.5 µCi was added per well and cultures were harvested 18h later. Phytohemagglutinin (PHA; 5 µg/ml, Murex, Paris, France) was added to the T cells as a positive control for the proliferative potential.

Cytokine production by MDC

Purified MDC were cultured at a concentration of 4 x10^4 cells/200 µl in 96-wells flat bottom culture plate in RPMI supplemented with 10% FCS , pencillin, streptomycin , and GM-CSF (500 U/ml; Leucomax, Novartis Pharma, Arnhem, The Netherlands) and stimulated with either 20 ng/ml synthetic double-stranded RNA (polyriboinosinic-polyribocytidylic acid; poly I:C; Sigma-Aldrich, St. Louis, MO) and 1000U/ml IFN–γ (Strathmann Biotech), or 75 µg/ml *Staphyloccocus aureus* Cowan strain I (SAC; Calbiochem, San Diego, CA), or CD40L-transfected J558 plasmacytoma cells (4 x10^4 J558 cells) and 1000U/ml IFN–γ for 24 hours at 37° C. After 24 h, supernatants were harvested and levels of IL-12, IL-10, IL-6 and TNFα in the supernatants were determined by standard enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions (eBioscience, San Diego, CA, USA).

Statistical analysis

The Mann-Whitney test was used to test whether differences between unrelated groups were statistically significant. The Wilcoxon Signed Ranks test was used to analyze whether differences between groups of paired samples were statistically significant. Statistical analyses were performed using SPSS version 11.0. A p-value <0.05 was considered significant. All data are presented as means ± standard error of the mean (SEM).

RESULTS

T cell stimulatory capacity of hepatic LN DC compared to skin/muscle draining LN DC

To study whether *in vivo* liver MDC undergo an alternative maturation program leading to MDC with a reduced T cell stimulatory capacity, a comparison was made between the T cell stimulatory capacity of MDC purified from hepatic LN and from inguinal skin/

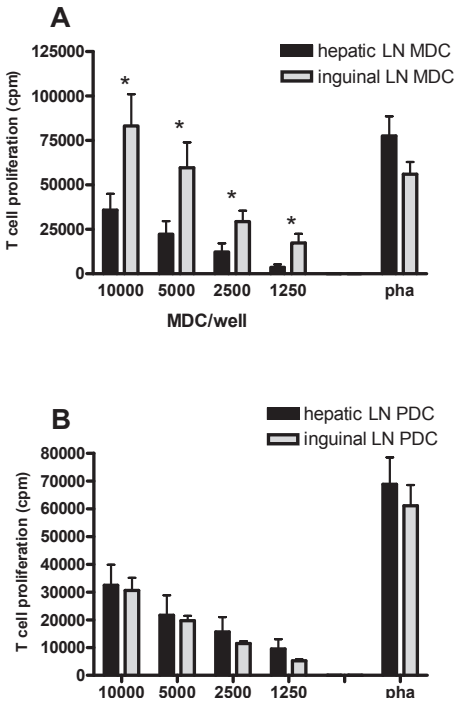

Figure 1. T cell stimulatory capacity of hepatic LN DC compared to skin/muscle draining LN DC. Graded numbers of hepatic LN and inguinal LN DC were co-cultured with allogeneic T cells (from one batch) and T cell proliferation was assessed after 5 days by [^{3}H]-thymidine incorporation. As a control for their proliferative capacity, in each experiments T cells were stimulated in the absence of DC with PHA. (A) T cell stimulatory capacity of MDC from hepatic LN (n=8) and inguinal LN (n=8). *P<0.05 (B) T cell stimulatory capacity of PDC from hepatic LN (n=6) and inguinal LN (n=5). Data represent the mean with SEM.

muscle draining LN. Hepatic LN MDC had a significantly weaker allogeneic T cell stimulatory capacity compared to inguinal LN MDC (Figure 1A). This was not due to variation between experiments, since PHA-stimulated T cells had a similar proliferation level in both sets of experiments (Figure 1A). For comparison, we determined the allogeneic T cell stimulatory capacity of PDC enriched from hepatic and inguinal LN. In contrast to MDC, there was no difference in the T cell stimulatory capacity of PDC obtained from hepatic and inguinal LN (Figure 1B). PDC from inguinal LN showed a much lower T cell stimulatory capacity compared to MDC from the same LN, while the T cell stimulatory capacities of MDC and PDC from hepatic LN were comparable.

Hepatic LN MDC have a more mature immunophenotype than skin/muscle draining LN MDC

To study whether the relatively low T cell stimulatory capacity of MDC in hepatic LN was due to low expression of T cell stimulatory surface molecules, expression of HLA-

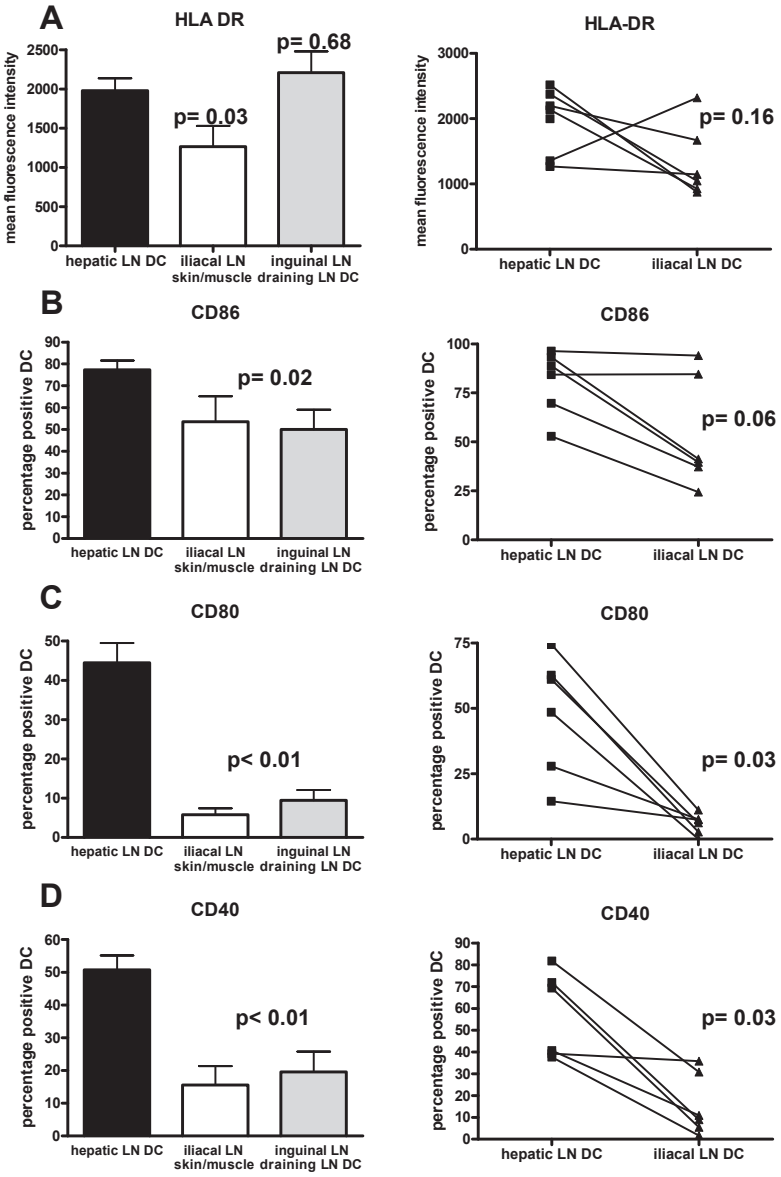

Figure 2. Co-stimulatory molecules and HLA-DR on MDC derived from hepatic LN compared with skin/muscle draining LN. The expression of HLA-DR and co-stimulatory molecules on LN MDC was determined by flowcytometric analysis of MNC. MDC were defined as BDCA-1^{+}CD20^{-} cells. Bar diagrams depict the percentages of MDC expressing co-stimulatory molecules, and the mean fluorescence intensities of HLA-DR expression on hepatic LN MDC (n=15), iliacal LN MDC (n=7) and inguinal LN MDC (n=8). Line diagrams show expressions on MDC in paired hepatic- and iliacal LN from the same multi-organ donors (n=6). (A) Expression of HLA-DR, (B) Expression of CD86, (C) Expression of CD80, (D) Expression of CD40. Data represent the mean with SEM.

DR and co-stimulatory molecules on MDC derived from hepatic LN was compared with expression on MDC from skin/muscle draining LN. Two types of skin/muscle draining LN were used: iliacal LN from multi-organ donors that were collected together with hepatic LN, and inguinal LN derived from kidney transplant recipients. Expression of HLA-DR on MDC in hepatic LN and inguinal LN was similar, while MDC in iliacal LN showed a slightly reduced expression level (Figure 2A). There was no significant difference in the expression of co-stimulatory molecules between MDC in iliacal- and inguinal LN. Therefore, expression of co-stimulatory molecules on MDC in hepatic LN was compared with that on MDC in the total group of skin/muscle draining LN MDC. Bar diagrams in Figures 2 B, C, and D show that significantly higher numbers of hepatic LN MDC express the co-stimulatory molecules CD86, CD80 and CD40 compared to MDC from skin/muscle draining LN. To exclude possible differences between multi-organ donors and kidney transplant recipients as a confounder paired comparisons of MDC from hepatic and iliacal LN derived from the same multi-organ donors was performed. The higher expressions of CD86, CD80 and CD40 on hepatic LN MDC compared to MDC from skin/muscle-draining LN

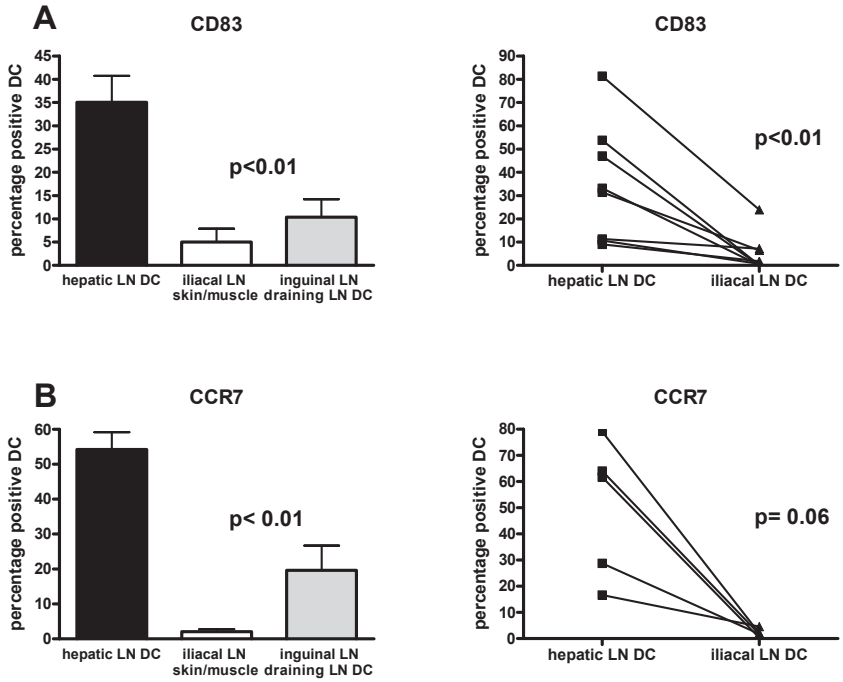

Figure 3. Expression of CD83 and CCR7 on MDC derived from hepatic LN compared with skin/muscle draining LN. Bar diagrams depict the percentages of MDC expressing CD83 (A) and CCR-7 (B) on hepatic LN MDC (n=15), iliacal LN MDC (n=7) and inguinal LN MDC (n=8). Line diagrams show expressions on MDC in paired hepatic- and iliacal LN from the same multi-organ donors (n=7). Data represent the mean with SEM.

were confirmed in these paired comparisons (line diagrams in Figure 2B, C and D). Also the expressions of co-stimulatory molecules on individual MDC, as determined by fluorescence intensity, were higher on MDC from hepatic LN compared to MDC from skin/muscle draining LN (data not shown).

To investigate whether the differences in co-stimulatory molecule expression were related to a further maturation state of hepatic LN MDC, the expressions of the maturation marker CD83 and chemokine receptor CCR7 were measured. MDC in hepatic LN indeed had higher expressions of CD83 and CCR7 compared to skin/muscle draining LN, and this was also confirmed in paired comparisons of hepatic and iliacal LN from the same multi-organ donors (Figure 3A and B). Together, these data show that MDC in hepatic LN are highly mature, with high expressions of MHC class II and co-stimulatory molecules. Their poor ability to stimulate allogeneic T cells is therefore not due to an immature immunophenotype.

Hepatic LN MDC express higher levels of programmed death ligands 1 and 2 (PD-L1 and PD-L2) in comparison with skin/muscle draining LN MDC

Ligation of programmed death receptor (PD-1) on T cells by one of it's ligands PD-L1 and PD-L2 inhibits T cell proliferation (32). Since PD-L1 and PD-L2 can be expressed on MDC, the impaired T cell stimulatory capacity of hepatic LN MDC could be the result of expression of these inhibitory molecules. Flowcytometric analysis of the expression of PD-L1 and PD-L2 indeed revealed that significantly more hepatic LN MDC expressed these inhibitory molecules compared to skin/muscle draining LN (Figure 4A and B). Paired comparison of MDC from hepatic and iliacal LN confirmed this differential expression (Figure 4A and B). However, blocking PD-L1 and PD-L2 on the hepatic LN MDC with neutralizing mAb did not increase their allogeneic T cell stimulatory capacity (Figure 4C). Thus, although expression of PD-L1 and PD-L2 are significantly higher on MDC from hepatic LN compared to MDC from skin/muscle draining LN, this does not explain the observed poor T cell stimulatory capacity of hepatic LN MDC.

Hepatic LN MDC hardly produce cytokines upon stimulation with different stimuli

To realize a strong T cell response, MDC have to produce cytokines (signal 3) together with the presentation of antigens on their MHC-molecules (signal 1) and co-stimulatory signals (signal 2). For that reason we determined the ability of MDC isolated from hepatic LN and skin/muscle draining LN to produce cytokines upon stimulation with different stimuli. Overall, skin/muscle draining LN MDC produced a wide variety of cytokines upon stimulation, whereas hepatic LN MDC hardly produced any cytokines (Figure 5). Skin/muscle draining LN MDC were capable to secrete the pro-inflammatory cytokines IL-12, TNFα and IL-6 and the immune-regulatory cytokine IL-10. Hepatic LN MDC only produced biologically relevant levels of TNFα, although in significantly lower amounts than skin/muscle LN MDC did.

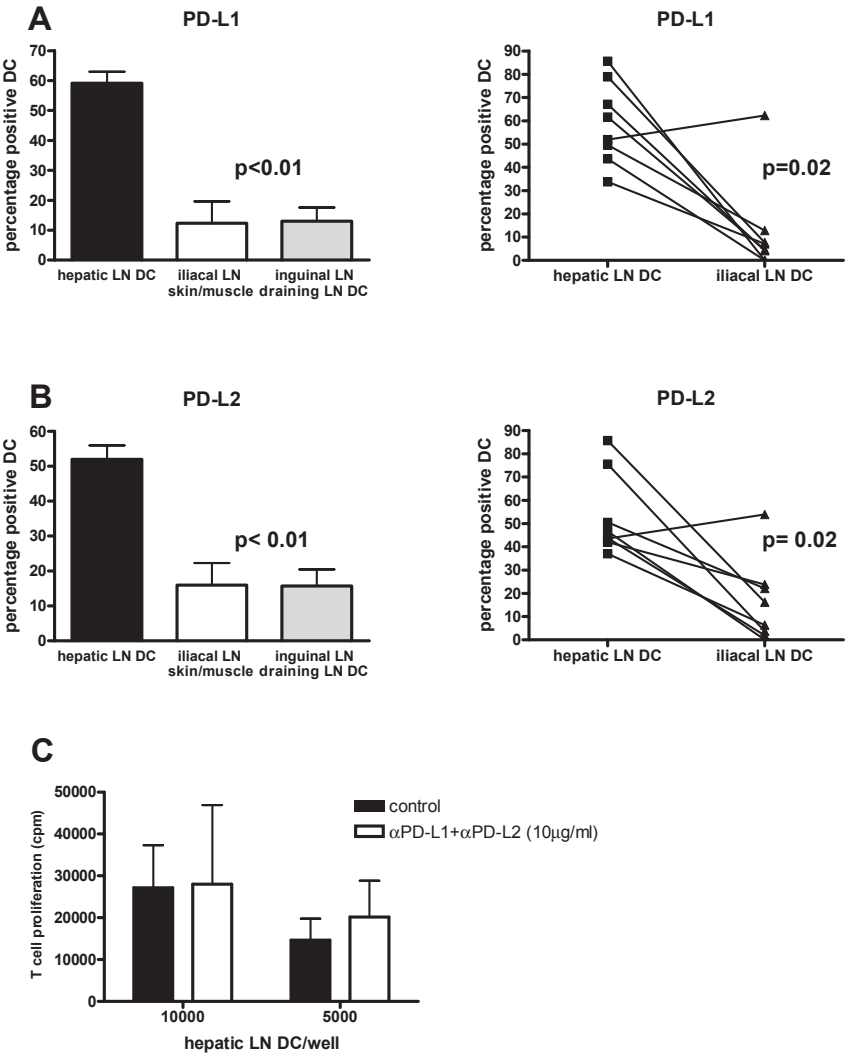

Figure 4. Expression and function of PD-L1 and PD-L2 on MDC derived from hepatic LN compared with skin/muscle draining LN. The expression of the programmed death ligands on LN MDC was determined by flowcytometric analysis of MNC. MDC were defined as BDCA-1^{+}CD20^{-} cells. Bar diagrams depict the percentages of MDC expressing PD-L1 (A) and PD-L2 (B) on hepatic LN MDC (n=15), iliacal LN MDC (n=7) and inguinal LN MDC (n=8). Line diagrams show expressions on MDC in paired hepatic- and iliacal LN from the same multi-organ donors (n=8). (C) Blocking of PD-L1 and PD-L2 on MDC from hepatic LN did not restore their T cell stimulatory capacity. Graded numbers of MDC isolated from hepatic LN MDC were incubated with PD-L1 and PD-L2 blocking antibodies (10 μg/ml of each) for 15 min at 37°C after which allogeneic T cells were added. Allogeneic T cell proliferation was assessed after 5 days by [^{3}H] thymidine incorporation. Data represent the mean with SEM from 4 different experiments.

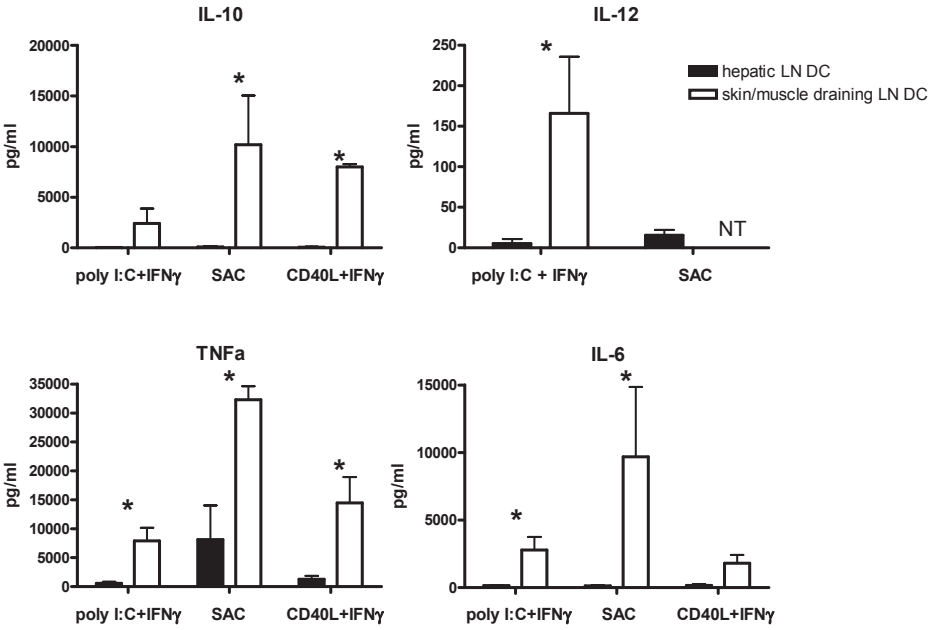

Figure 5. Hepatic LN MDC produce almost no cytokines compared to skin/muscle draining LN MDC. LN MDC were cultured at a concentration of 4 x10^4 cells/200 µl flat bottom culture plates, and stimulated with either 20 ng/ml poly I:C and 1000 U/ml IFN–γ , or with 75 µg/ml SAC, or with CD40L-transfected plasmacytoma cells (4 x10^4 J558 cells) and 1000U/ml IFN–γ, for 24 hours at 37° C. After 24 h, supernatants were harvested and levels of IL-10, IL-12, TNFα and IL-6 were determined. Data represent the mean with SEM. *$p<0.03$ for comparison of hepatic LN MDC (n=8) with skin/muscle draining LN MDC (n=5).

DISCUSSION

The liver is an immune-privileged organ, in which immune responses against food antigens and components of the commensal gut flora are tightly regulated. MDC, the most potent antigen presenting cells, play a central role in maintaining a fine balance between immune responsiveness and unresponsiveness of the liver. Therefore hepatic LN most likely contain a unique type of liver-derived DC. Skin/muscle draining LN DC were used for comparison because of major differences in immune responses between skin and liver. The skin is a major barrier against the external environment and disturbance of this barrier will cause a strong immune response. Furthermore skin allografts are more rapidly rejected than liver allografts (33).

The present study showed that hepatic LN MDC have a lower T cell stimulatory capacity than skin/muscle draining LN MDC, whereas PDC of both origins have a similar T cell stimulatory capacity. Furthermore hepatic LN MDC had a more mature immunopheno-

type than skin/muscle draining LN MDC, as shown by higher expressions of co-stimulatory molecules and CD83 and CCR7. Also PD-L1 and PD-L2 were highly expressed on hepatic LN MDC, but blocking of these ligands did not enhance the T cell stimulatory capacity of the hepatic LN MDC. Upon stimulation with various stimuli hepatic LN MDC hardly produced any cytokines while skin/muscle draining LN MDC produced IL-10, IL-12, IL-6 and TNFα.

The twofold reduced capacity of hepatic LN MDC to stimulate allogeneic T cell fits with the current hypothesis that the liver is an immune-privileged organ and that as a result MDC derived from this organ most likely have less immunogenic properties. The similar T cell stimulatory capacity of PDC derived from hepatic LN and inguinal LN could be anticipated since PDC are more associated with the innate defense in fighting infections than playing a major role in immunogenicity.

The observed lower T cell stimulatory capacity of hepatic LN MDC can not be explained by the immunophenotype of the hepatic LN MDC, since surprisingly these MDC had a more mature phenotype than skin/muscle draining LN MDC. The co-stimulatory molecules CD86, CD80 and CD40 and maturation markers CD83 and CCR7 were much higher expressed on hepatic LN MDC than on skin/muscle draining LN MDC. This difference was also confirmed in a paired analysis of hepatic and iliacal LN MDC derived from the same MOD. Hepatic and skin/muscle draining LN MDC had a similar expression of HLA-DR indicating that they had a similar ability to present antigens to T cells. The mature phenotype of human hepatic LN MDC is comparable to the mature phenotype of MDC present in rat liver lymph (34).

PD-L1 and PD-L2 belong to the B7 family and have been shown to downregulate T cell activation through ligation with their mutual receptor programmed death receptor (PD-1) (32). Hepatic LN MDC also have a higher expression of PD-L1 and PD-L2 compared to skin/muscle draining LN MDC. The higher expressions of PD-L1 and PD-L2 could be an explanation for the observed lower T cell stimulatory capacity. However blocking of these programmed death ligands on hepatic LN MDC did not result in a restoration of the T cell stimulatory capacity of hepatic LN MDC. A possible explanation why blocking of PD-L1 and PD-L2 has failed to restore the T cell stimulatory capacity of hepatic LN MDC is that blocking of these ligands is only effective when immature or semi-mature MDC are utilized. This hypothesis is supported by the fact that it has been shown that blocking of PD-L1 resulted in a higher T cell proliferation when immature DC were used, but that this effect was much less profound when mature MCD were used (35).

Besides the expression of co-stimulatory molecules or inhibitory molecules, the capacity of MDC to produce certain cytokines is also important for the T cell stimulatory capacity. To measure the ability of hepatic and skin/muscle draining LN MDC to produce cytokines, we stimulated these MDC with different stimuli for MDC; poly I:C and IFN-γ, SAC or CD40L and IFN-γ. It is known that human blood MDC produce significant levels of IL-10,

IL-12, IL-6 and TNFα upon stimulation with poly I:C and IFN-γ or SAC (36). Overall the hepatic LN MDC produced very little cytokines; Almost no detectable IL-10, IL-12 and IL-6 and very little TNFα compared to skin/muscle draining LN MDC. The lack of cytokine production could be a result of over stimulation of MDC by food antigens and components of the intestinal bacterial flora in the liver environment, leading to MDC that are exhausted and as a consequence have lost their ability to produce cytokines in response to a stimulus. DC exhaustion, defined by the lack of cytokine production, is known to be induced by exposure of MDC to LPS, poly I:C or TNFα and IL1β and is not prevented by IFN-γ (37). The liver is continuously exposed to food components and bacterial products that enter the liver from the gastrointestinal tract via the portal vein. These maturation stimuli are permanently present in the liver (38, 39) and could play a role in making hepatic MDC exhausted during migration to the hepatic LN. Since MDC from hepatic LN are deficient in producing cytokines and thus lack this important signal in the process of T cell stimulation, this could be an explanation for the observed lower T cell activating potential of hepatic LN MDC.

In conclusion, hepatic MDC mature *in vivo* into a type of effector MDC that have a high expression of co-stimulatory molecules, but produce almost no cytokines and have a poor allogeneic T cell stimulatory capacity. We postulate that MDC in the liver environment undergo hyper-maturation, due to continuous stimulation with gut-derived components, resulting in exhausted MDC in hepatic lymph nodes.

ACKNOWLEDGEMENTS

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3

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

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ABSTRACT

Organ transplantatio egrin expression and had a reversed CD4:CD8 ratio compared to control blood of healthy individuals. This indicates that perfusate cells are of liver origin and not derived from residual donor blood. Further characterization of perfusate mononuclear cells showed an increased proportion of $CD4^+CD25^+CTLA\text{-}4^+$ T cells compared to healthy control blood. Increased percentages of $Foxp3^+$ cells, which were negative for CD127, confirmed the enrichment of Treg in perfusates. In a mixed-leukocyte reaction, $CD4^+CD25^+$ T cells from perfusates suppressed proliferation and IFN-γ production of donor and recipient T cells. *In vivo*, within the first weeks after Tx up to 5% of $CD4^+CD25^+CTLA4^+$ T cells in recipient blood were derived from the donor liver.

In conclusion, a substantial numbers of donor Treg detach from the liver graft during perfusion and continue to migrate into the recipient after Tx. These donor Treg suppress the direct pathway alloresponses and may *in vivo* contribute to chimerism-associated tolerance early after liver Tx.

INTRODUCTION

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

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

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

the perfusates has an immature phenotype identical to DC isolated from liver tissue and produced higher amounts of IL-10 compared to blood DC (24).
In the current study we determined the migration and suppressive capacity of donor $CD4^+CD25^+$ Treg from the liver graft into the perfusate and the recipient after Tx. We show that the lymphocyte population that migrates from the human liver is enriched for $CD4^+CD25^+FoxP3^+$ cells and that these cells suppress proliferation and IFN-γ production of recipient T cells *in vitro*. In addition, we demonstrate that donor-derived cells with a regulatory phenotype can be detected in substantial numbers in circulation of recipients after liver Tx.

MATERIALS AND METHODS

Perfusate and peripheral blood collection

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

Monoclonal antibodies

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

Flow cytometric analysis

After thawing, liver perfusate mononuclear cells (LPMC) and PBMC were washed twice with phosphate-buffered saline (PBS) containing 0.3% bovine serum albumin (BSA) followed by staining with primary monoclonal antibodies CD3, CD4, CD8, CD25 and CD127 in PBS/0.3%BSA (30 minutes at 4ºC). Following primary incubation cells were washed, and for staining of intracellular CTLA-4 or FoxP3, the cells were fixed and permeabilized using the IntraPrep Reagents (Immunotech, Marseille, France) or fixation/permeabilization agents supplied by eBiosciences, respectively. HLA-A2 positive cells were determined by incubation with anti-HLA-A2 antibody followed by staining with FITC conjugated anti-mouse IgG. Expression of leukocyte function-associated antigen-1 (LFA-1, CD11a) was determined in a similar manner by primary staining for CD11a followed by secondary staining with FITC conjugated anti-mouse IgG. Flowcytomteric analysis was performed using FACS Calibur and CELLQuest Pro software (Becton Dickinson, San Jose, CA).

$CD4^+CD25^+$ T cell isolation

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

Mixed Leukocyte Reaction (MLR) and suppressor activity assays

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

Statistical analysis

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

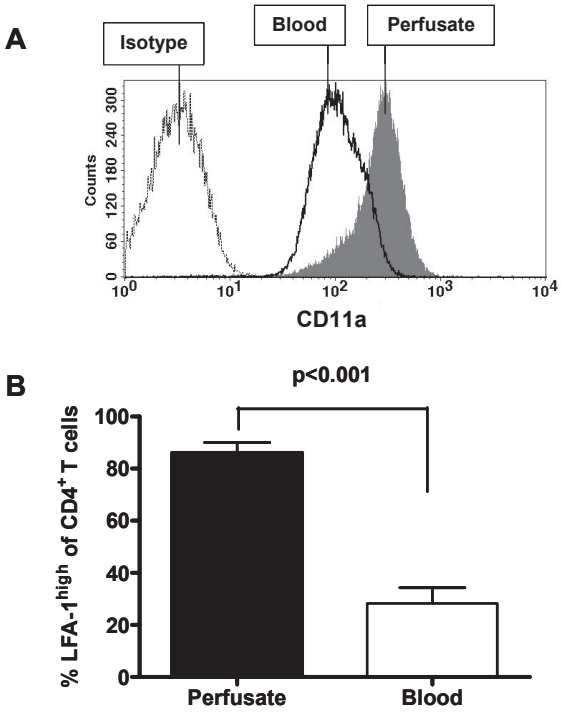

Figure 1. High LFA-1 expression on $CD4^+$ T cells from perfusates. (A) Assessment of LFA-1 expression on $CD4^+$ T cells in LPMC and PBMC. Representative histogram showing higher LFA-1 (CD11a) expression on $CD4^+$ T cells in LPMC compared to $CD4^+$ T cells in PBMC. (B) The increased proportion of LFA-1high$CD4^+$ T cells in perfusates suggests that these cells are of liver origin and not derived from residual donor blood. In perfusates (n=9) on average 81% of $CD4^+$ T cells are LFA-1high compared to 28% in PBMC of healthy controls (n=10). Shown are mean percentages ± SEM.

RESULTS

LPMC are of liver origin

Perfusates were collected during the pre-Tx albumin perfusion of donor livers on the bench. With a mean ischemia time of 7±2 hours, the perfusates contained on average $91x10^6$ mononuclear cells (range $10\text{-}500x10^6$). Viability as determined by trypan blue exclusion showed that 98 ± 2% of the LPMC were vital. Liver lymphocytes are known to express higher levels of LFA-1 than lymphocytes in peripheral blood (25, 26). Flowcytometric characterization of LPMC and PBMC showed that within the perfusates a significantly greater proportion of lymphocytes had high LFA-1 expression (Figure 1). The CD4:CD8 ratio in perfusates was 1:2.4 (n=22) and was significantly distinct from the 2:1 ratio in

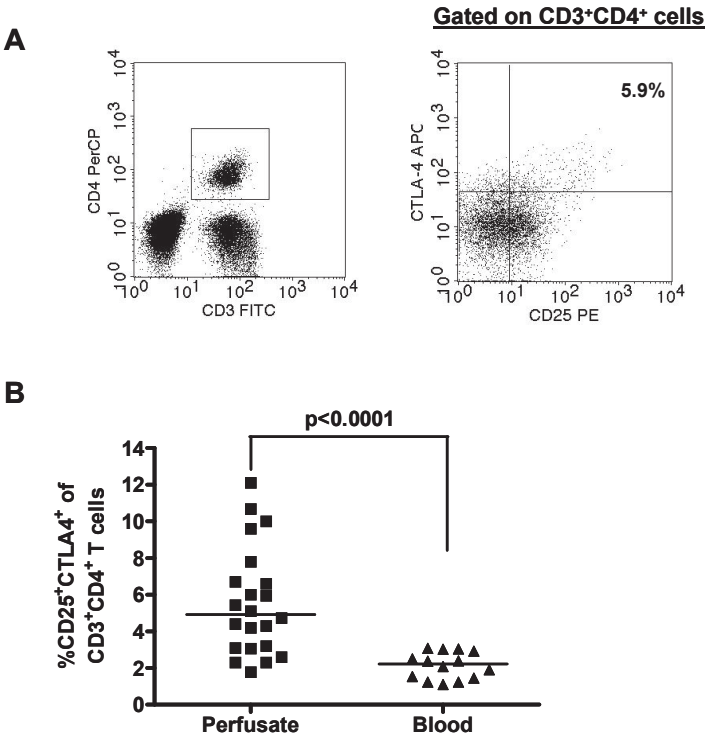

Figure 2. LPMC $CD4^+$ T cells contain a higher $CD25^+CTLA\text{-}4^+$ cell fraction compared to PBMC. (A) FACS profile of $CD3^+CD4^+CD25^+CTLA\text{-}4^+$ cells. Within the $CD3^+CD4^+$ T cell population the proportion of cells expressing membrane CD25 and intracellular CTLA-4 was determined. Shown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

blood (n=14, p<0.001). These findings indicate that leukocytes present in perfusates are predominantly liver derived and not derived from residual blood.

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

In order to determine the presence of T cells with regulatory phenotype, CD25, CTLA-4 and Foxp3 expression within $CD3^+CD4^+$ T cells was assessed by flowcytometry. Figure 2A is a FACS profile of $CD3^+CD4^+CD25^+CTLA\text{-}4^+$ T cells. As shown in Figure 2B, LPMC contained a median of 4.9% $CD4^+CD25^+CTLA\text{-}4^+$ T cells (range 1.8-12.1%), significantly higher compared to 2.2% in PBMC of healthy controls (range 1.1-3.1%). A subset of perfusates was analyzed for Foxp3 expression. In Figure 3A a representative staining for

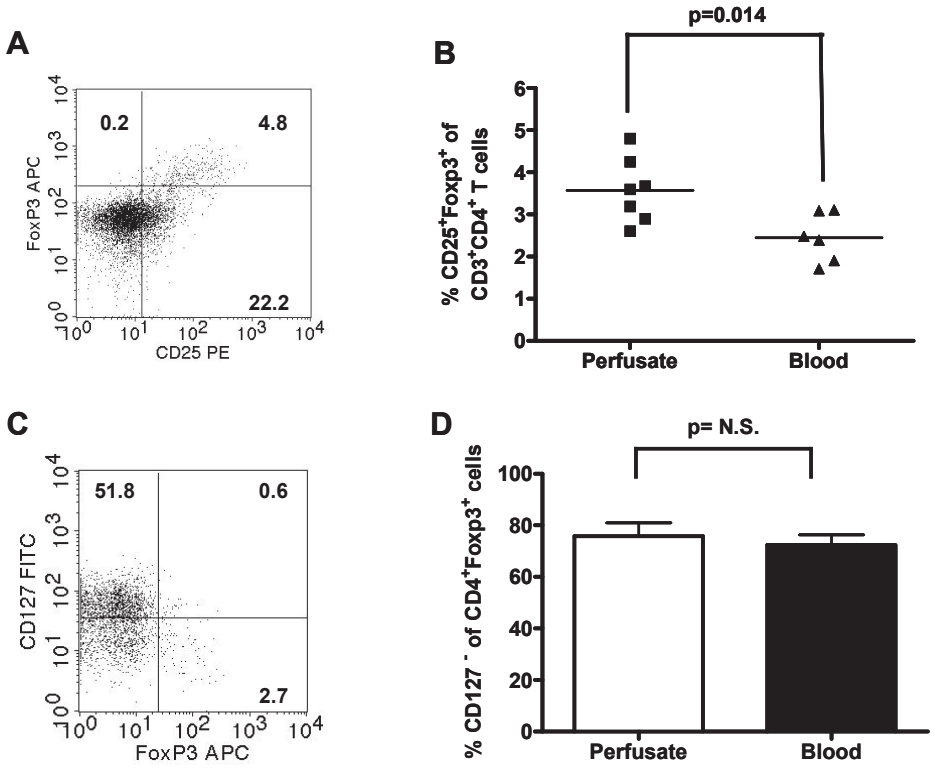

Figure 3. Increased percentage of $CD4^+CD25^+Foxp3^+$ cells in LPMC compared to PBMC. Assessment of $CD4^+CD25^+Foxp3^+$ T cells in perfusate and PBMC. Within the $CD3^+CD4^+$ T cell population the proportion of cells expressing CD25 and Foxp3 was determined. (A) Representative dot plot of perfusate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

Foxp3 expression in LPMC and PBMC is shown. Comparison of perfusate and peripheral blood revealed increased proportions of $CD4^+CD25^+Foxp3^+$ T cells in perfusates (Figure 3B). RT-PCR confirmed the presence of $Foxp3^+$ T cells by analysis of Foxp3 transcript levels in perfusates (data not shown). Analysis of CD127, the IL-7 receptor, showed that the majority of perfusate $Foxp3^+$ cells were negative for CD127 identical to $Foxp3^+$ cells

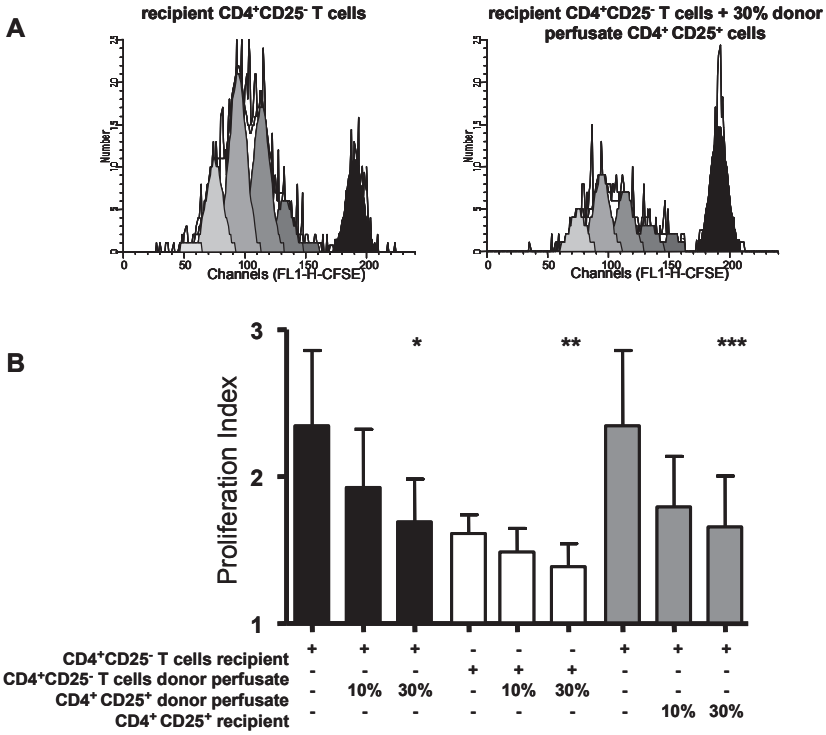

Figure 4. Perfusate $CD4^+CD25^+$ T cells inhibit proliferation of both recipient and donor responder T cells. (A) Representative MLR showing CFSE staining of control recipient $CD4^+CD25^-$ T cells (1×10^5) in the absence (left) and presence of perfusate $CD4^+CD25^+$ T cells (3×10^4) (right) upon stimulation with irradiated donor and recipient mononuclear cells. The number of cells in the daughter generations are significantly lowered in the presence of perfusate $CD4^+CD25^+$ T cells. (B) Effect of perfusate and recipient $CD4^+CD25^+$ T cells on proliferation of recipient and donor $CD4^+CD25^-$ T cells. Proliferation of recipient (1×10^5) and perfusate responder cells (1×10^5) was significantly (* p=0.034 and ** p=0.032, respectively) inhibited by perfusate $CD4^+CD25^+$ T cells (black and white bars, respectively). Responder cells were stimulated with irradiated donor (5×10^4) and recipient mononuclear cells (5×10^4). As a positive control the suppressive activity of recipient $CD4^+CD25^+$ T cells was also determined (gray bars, *** p=0.003). Significance was tested by analysis of the logarithmic transformation of the dependent variable with random intercept and random slope. Shown is the mean ± SEM of 5 experiments.

in blood (Figure 3C and 3D). In sum, these data indicate that a considerable proportion of helper T cells that detach from the liver during perfusion have a regulatory phenotype.

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

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

Next we tested the suppressive activity of perfusate $CD4^+CD25^+$ T cells in an allogeneic setting. As antigen-presenting cells, both irradiated PBMC (5×10^4) and LPMC (5×10^4) were used together to simultaneously activate recipient (responder) and donor (suppressor) T cells. Both perfusate and recipient $CD4^+CD25^+$ cells showed no proliferation in response to allogeneic stimulation, indicating a state of anergy (data not shown). When $CD4^+CD25^+$ T cells from LPMC were added to recipient $CD4^+CD25^-$ responder cells, we observed significant suppression of proliferation comparable to the inhibition observed in the autologous setting (Figure 4). Consistently, when looking at the IFN-γ production in

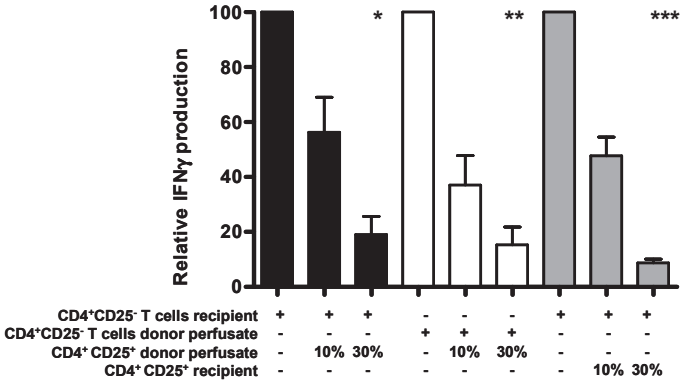

Figure 5. Inhibition of IFN-γ production by perfusate $CD4^+CD25^+$ T cells. $CD4^+CD25^+$ T cells inhibit IFN-γ production by effector $CD4^+CD25^-$ T cells. Shown are the mean relative inhibition of IFN-γ production (±SEM) for three experiments, as determined by ELISA. Perfusate $CD4^+CD25^+$ T cells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

these MLR analyses, similar results were found showing dose-dependent inhibition by donor CD4+CD25+ T cells of donor and recipient IFN-γ production (Figure 5). The suppression of both proliferation and IFN-γ production suggests that CD4+CD25+ T cells from the liver are able to suppress the direct-pathway alloresponse *in vitro*.

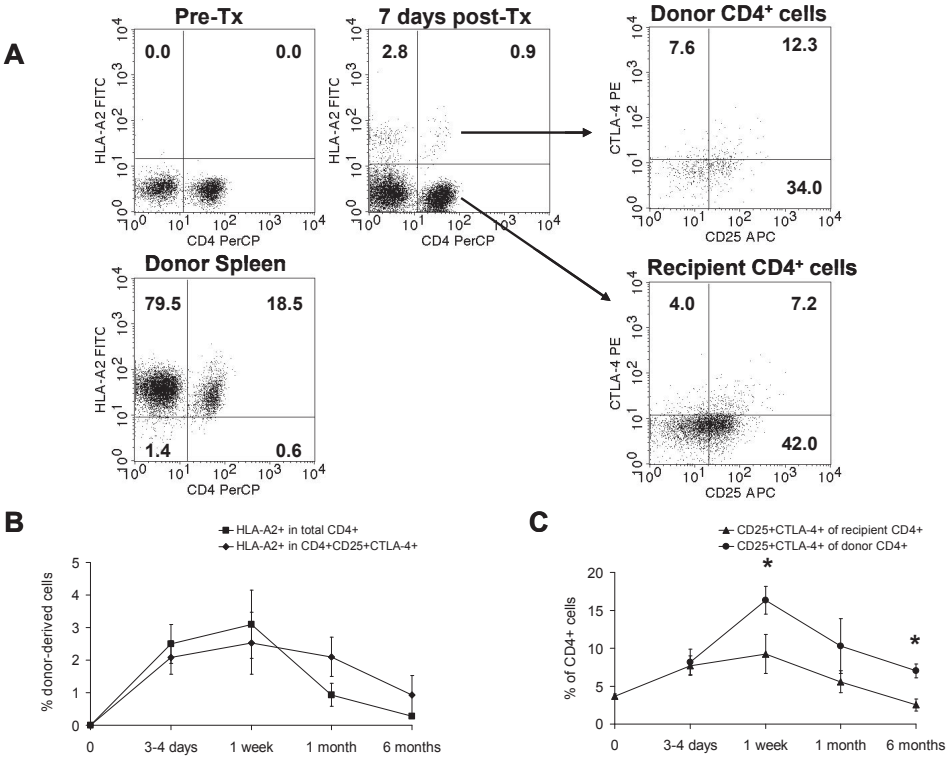

Figure 6. Donor CD4+CD25+CTLA-4+ T cells circulate in recipients after liver Tx. (A) Representative dot plots showing donor HLA-A2+ cells in recipient blood one week after Tx. Pre-Tx recipient PBMC and donor spleen cells serve as negative and positive control, respectively. The expression of CD25 and CTLA-4 of HLA-A2 positive and negative CD4+ cells is shown. (B) Proportion of HLA-A2+ cells within the CD4+ cells and within CD4+CD25+CTLA-4+ cells is the highest at one week (n=6) and gradually decrease at one (n=4) and six months after Tx (n=4). (C) One week and six months after Tx the proportion of donor CD4+CD25+CTLA-4+ cells within the donor CD4+ cells was increased compared to the proportions of CD4+CD25+CTLA-4+ cells within recipient CD4+ cells (* $p<0.05$). Both recipient and donor CD4+CD25+CTLA-4+ cells decrease at one and six months after Tx, although not reaching statistical significance due to small sample size.

$CD4^+CD25^+CTLA\text{-}4^+$ T cells detach from the liver graft after Tx and circulate in recipients

In order to determine whether donor T cells with a regulatory phenotype can be detected in recipients after liver Tx, we performed flowcytometric analysis of PBMC from HLA-A2 negative recipients who received an HLA-A2 positive liver graft. Determination of HLA class 1-A2 allele has previously been shown to be useful to distinguish between donor and recipients cells (27, 28). A representative staining is shown in Figure 6A. In the first week after Tx 3.1± 1.0% SEM of the total $CD4^+$ T cells and 2.5 ± 1.0% SEM of the $CD4^+CD25^+CTLA\text{-}4^+$ cells were found to be of donor origin (Figure 6B). These percentages of donor cells increased initially and gradually declined at one and six months after Tx. Similar results were found when analyzing the percentage of $HLA\text{-}A2^+$ cells within the $CD4^+CD25^+Foxp3^+$ fraction (n=4, data not shown). The proportion of $CD25^+CTLA\text{-}4^+$ cells within $CD4^+$ cells was significantly higher in donor-derived than in recipient cells (Figure 6C). Also at 6 months there was a difference although the overall percentage of $CD4^+CD25^+CTLA\text{-}4^+$ cells was declined at this time point. These data indicate that donor T cells with a regulatory phenotype migrate from the liver graft into the circulation of recipients.

DISCUSSION

The unique immunological properties of the liver have partly been attributed to the resident leukocyte population. Following liver Tx, donor leukocytes present in the graft are transferred and persist in recipients, a condition referred to as chimerism. In this study we aimed to investigate the presence of Treg in liver graft perfusion solution and in recipient blood after Tx. Within perfusate $CD3^+CD4^+$ T cells an increased proportion of $CD25^+CTLA\text{-}4^+$ and $CD25^+Foxp3^+$ T cells were found as compared to peripheral blood of healthy controls (Figures 2 and 3). FOXP3 mRNA expression in LPMC confirmed the presence of Treg (data not shown). Leukocytes in recipient circulation were shown to contain substantial numbers of donor $CD4^+CD25^+CTLA\text{-}4^+$ T cells indicative of a regulatory signature (Figure 6). The functionality of donor Treg was studied in MLR, showing inhibition of proliferation of self- and recipient-responder $CD4^+CD25^-$ T cells upon stimulation with donor and recipient mononuclear cells (Figure 4). Furthermore, the cytokine production was significantly inhibited, confirming the suppressive activity of $CD4^+CD25^+$ T cells within the LPMC (Figure 5).

The number of mononuclear cells obtained from perfusates showed a wide range, but did not correlate with ischemia time or perfusate volume (not shown). Immunophenotypic characterization of LPMC shows clearly differences compared to PBMC, yet comparable with leukocyte subsets obtained from liver tissue (23, 24). Our results show significantly lower proportions of $CD4^+$ T cells and higher proportions of $CD8^+$ within total LPMC T

cell population compared to peripheral blood. Previously, it was shown that liver infiltrating T cells have high expression levels of the β2-integrin, LFA-1 (22, 25). Therefore we determined the expression of LFA-1 α-chain (CD11a), which is expressed exclusively on leukocytes, and is involved in migration into tissues. The $CD4^+$ T cells in perfusates have a strong LFA-1 expression, which is significantly higher compared to $CD4^+$ T cells in PBMC (Figure 1). The strong adhesion molecule expression and the reversed CD4:CD8 ratio indicates that these cells originate from the liver tissue and have a pre-activated phenotype (22, 29).

Within the $CD4^+$ T cell population obtained from perfusates, a considerable proportion of T cells with a regulatory phenotype was observed. Treg characterized by CD4, CD25, CTLA-4 and Foxp3 expression were significantly increased compared to peripheral blood levels in healthy controls (Figures 2 and 3). Recently, the absence of the IL-7 receptor expression, CD127, has been suggested as a discriminating factor of $Foxp3^+$ Treg (30, 31). Our analysis showed that the majority of liver-derived $Foxp3^+$ cells were CD127 negative, which was comparable to control blood (Figure 3D). To our knowledge this is the first study to show the presence of $CD4^+CD25^+Foxp3^+$ T cells as passenger leukocytes in liver grafts. Following liver Tx, a substantial proportion of circulating leukocytes are of donor origin (28) and even higher in case of graft-versus-host disease (32). In the first week after Tx, we have shown that up to 5% of total $CD4^+CD25^+CTLA\text{-}4^+$ T cell population were of donor origin (Figure 6B), equivalent to approximately 5 to 15 $x10^6$ cells. The proportion of $CD4^+$ cells which expressed CD25 and CTLA-4 was higher in donor cells than in recipient cells at one and six months after Tx (Figure 6C). Both donor and recipient $CD4^+CD25^+CTLA\text{-}4^+$ cells decreased in time after Tx. This general drop of $CD4^+CD25^+CTLA\text{-}4^+$ Tregs was consistent with our previous studies (33, 34).

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

DC, which may explain the unique hepatic microenvironment that is known to promote tolerance.

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

Donor leukocyte migration and chimerism are associated with transplant tolerance both in the clinical and experimental setting (1, 2, 47). Depletion of donor leukocytes or failure to develop chimerism were shown to result in prompt rejection, but chimerism by itself is not sufficient to prevent rejection. In clinical Tx, the beneficial effect of donor leukocytes was clearly shown by pre-transplant blood transfusions leading to enhanced graft survival (48, 49). It is evident that donor leukocytes are important during the first weeks post-Tx in which maximal donor migration and interaction with host leukocytes occur. The exact mechanism in achieving tolerance through chimerism, however, has not been elucidated. Several mechanisms have been proposed in this process including clonal exhaustion or deletion, T cell anergy and active suppression (8, 10). Although the cell interactions are indisputably complex, tolerance to skin transplants following bone marrow Tx was $CD4^+CD25^+$ Treg dependent mostly so early after Tx (19). Conversely, in bone marrow Tx an important role for $CD4^+$ Treg has recently been identified in the induction of chimerism-associated tolerance, which was CD25 independent but CTLA-4 dependent (20). Whether graft-derived donor Treg influence the balance between the immunogenicity and tolerogenicity of organ allografts remains to be determined.

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

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4

Characterization of human liver dendritic cells in liver grafts and perfusates

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ABSTRACT

It is generally accepted that donor myeloid dendritic cells (MDC) are the main instigators of acute rejection after organ transplantation. The aim of the present study was to characterize MDC in human donor livers using liver grafts and perfusates as a source. Perfusates were collected during *ex vivo* vascular perfusion of liver grafts pretransplantation. MDC, visualized in wedge biopsies by immunohistochemistry with anti-BDCA-1 mAb, were predominantly observed in the portal fields. Liver MDC, isolated from liver wedge biopsies, had an immature phenotype with a low expression of CD80 and CD83. Perfusates were collected from 20 grafts; Perfusate mononuclear cells contained 1.5% (range 0.3-6.6%) MDC with a vitality of 97±2%. Perfusates were a rich source of hepatic MDC since 0.9 $x10^6$ (range 0.11-4.5 $x10^6$) MDC detached from donor livers during vascular perfusion pretransplantation. Perfusate MDC were used to further characterize hepatic MDC. Perfusate MDC expressed less DC-LAMP (p=0.000), CD80 (p=0.000) and CD86 (p=0.003) and CCR7 (p=0.014) compared to mature hepatic lymph node MDC, similar CD86 (p=0.140) and CCR7 (p=0.262) and more DC-LAMP (p=0.007) and CD80 (p=0.002) compared to immature blood MDC. Perfusate MDC differed from blood MDC in producing significantly higher amounts of IL-10 in response to lipopolysaccharide, and in being able to stimulate allogeneic T cell proliferation.
In conclusion, human donor livers contain exclusively immature MDC that detach in high numbers from the liver graft during pretransplantation perfusion. These viable MDC have the capacity to stimulate allogeneic T cells and thus may represent a major player in the induction of acute rejection.

INTRODUCTION

Myeloid dendritic cells (MDC) are amongst the most potent antigen-presenting cells and as such play a critical role in the initiation and direction of immune responses (1, 2). Under normal circumstances most peripheral tissues contain immature MDC whose function is uptake and processing of antigens. Upon antigenic stimulation they migrate towards the paracortex of the draining lymph nodes (LN) and spleen. During this migration MDC mature, and acquire a strong T cell stimulatory capacity (1-3). Animal experiments indicate that after transplantation donor DC migrate from the graft into the recipient's regional LN and spleen (4-7). In a murine liver transplantation model donor DC were even traced in the recipient's spleen seven months after transplantation (8).

It is generally accepted that donor MDC are the main instigators of acute rejection after solid organ and tissue transplantation due to their potent capacity to stimulate recipient T cell responses against the graft (4, 5, 7). Unlike experimental mouse heart or skin allografts, mouse liver allografts are accepted across MHC barriers and induce donor-specific tolerance without antirejection therapy (8). Interstitial DC of rodent liver are comparatively rare cells that are located predominantly in portal areas, and very occasionally, in sinusoids (9, 10). Freshly isolated murine liver DC are predominantly immature, expressing surface MHC but few co-stimulatory molecules (11-13). In addition, DC generated *in vitro* from murine liver leucocytes exhibited an immature phenotype (13-15). Injecting these *in vitro* propagated liver-derived DC pretransplantation has been shown to induce donor-specific hyporeponsiveness (16, 17). However, a marked increase of DC numbers and maturation state in liver allografts by donor pretreatment with the haematopoietic growth factor Flt3-ligand results in acute rejection (17, 18). This implies a crucial function for liver DC in regulating the balance between rejection and tolerance to liver grafts.

In spite of the ample knowledge on the properties of rodent liver DC, less is known about DC in human livers. Scarceness of human liver tissue, the relative low frequency of DC within human liver-tissue and until recently the paucity of DC-specific reagents which recognize all maturation stages of human DC, have hampered their investigation. DC-like cells have been detected immunohistochemically in the portal triads of the human liver (19). Recently it has been shown that MDC migrating from human liver tissue *in vitro* have a relative immature phenotype. These MDC have a high expression of MHC Class II and CD86, but a relative low expression of CD80 and CD83 (20). Nevertheless hepatic MDC have the capacity to mature in vivo, since MDC from hepatic LN have a mature phenotype, with higher expressions of these molecules compared to MDC from skin/muscle draining LN (21, 22).

Until now no data on freshly isolated human resident liver DC are available since it is difficult to obtain sufficient healthy human liver tissue for characterization of scarce cells like hepatic DC. It has been shown that substantial numbers of liver leukocytes readily detach

from the donor liver during vascular perfusion of liver grafts before transplantation (23). If such perfusates contain substantial numbers of DC, these could be used to study human donor liver DC.

The aim of this study was to characterize MDC in human liver grafts. Since the second type of DC, plasmacytoid DC (PDC), are less efficient in antigen presentation and recent data on their role in transplantation suggests that they facilitate organ graft survival (24), we have focused on the classical MDC. MDC were visualized by immunohistochemistry on liver-cryo-sections using the anti-BDCA-1 mAb, which is expressed on both immature and mature MDC. Donor liver mononuclear cells (MNC) were used for a limited analysis of the maturational status of liver MDC by flowcytometry. In addition, we found that large numbers of viable MDC were present in perfusates of human liver grafts obtained during *ex vivo* vascular perfusion pretransplantation. Perfusate MDC were used for further immunophenotypic and functional characterization of human donor liver MDC.

MATERIALS AND METHODS

Collection of materials

Wedge biopsies were obtained prior to transplantation from ten liver grafts and used for isolation of liver leucocytes (n=10) or were snap frozen for immunohistochemistry (n=7). Perfusates were collected from 20 liver grafts that immediately after arrival in the Erasmus MC Transplant Centre during the backtable procedure were perfused through the portal vein with 1 L to 2 L of University of Wisconson solution to remove residual blood from the vasculature. Immediately before transplantation the donor liver was again perfused *ex vivo* through the portal vein with 200 up to 500 ml of human albumin-solution under hydrostatic pressure and the perfusate was collected from the vena cava. Hepatic lymph nodes (LN) (n=12) were obtained from the hepatoduodenal ligament of donor livers during preparation of the liver pretransplantation. Blood samples were collected from three of the multiorgan donors and from twelve healthy volunteers. Nine hepatic LN were obtained from the same donors as perfusates, five out of ten liver wedge biopsies were obtained from the same donors as perfusates. The multiorgan donor blood samples were obtained from the same donors as both perfusates and hepatic LN. The Ethics Committee of the ErasmusMC approved the study protocol and informed consent of each patient was obtained.

Antibodies

The following mAbs were used: IgG1-FITC, IgG1-PE, IgG1-APC, IgG1-PerCP-cy5.5, IgG2a-FITC, CD4-PerCP, CD19-FITC, CD20-PerCP, CD14-FITC, HLA-DR-FITC, CD86-APC, and streptavidin-APC from Becton and Dickinson, Heidelberg, Germany; CD45-

FITC, CD3-PE, CD56-APC, CD80-FITC and anti-DC-LAMP-PE from Beckman Coulter Immunotech, Marseille, France; anti-BDCA-1 pure (=CD1c), anti-BDCA1-PE, anti-BDCA1-FITC, anti-BDCA2-FITC, CD19-microbeads and anti-PE-microbeads from Miltenyi Biotec, Bergisch Gladbach, Germany; CD8-APC and CD45-RPE-Cy5 from Dako, Glostrup, Denmark; CD83-APC from Caltag, Burlingame, USA; anti-CCR7-PE from R&D systems, Abingdon, United Kingdom.

Immunohistochemistry

To identify the location of MDC in donor livers pretransplantation, 5µm cryostat sections from donor liver biopsies were stained with anti-BDCA-1 mAb. Optimal dilutions of mAbs were established in preliminary experiments by titration on human tonsil cryosections. Briefly cryosections were fixed in acetone (10 min), after which endogenous peroxidase (PO) was blocked by incubation in citric acid/phosphate buffer-solution (pH=5.8) with 0.05% H_2O_2 and 0.2% NaN_3 (15 min, 20°C). The slides were washed with Tris-Buffered Saline (TBS, pH=7.4) twice, after which anti-BDCA-1 or IgG2a isotype matched control, were applied in optimal concentrations in TBS (pH 7.4) supplemented with 0.01% Normal Human Plasma for 18 h at 4°C. Binding of anti-BDCA-1 mAb was detected by incubation with conjugated goat anti-mouse Envision-PO (Envision Tm, DAKO, Glostrup, Denmark) for 30 min at room temperature and visualized using 3-amino-9-ethylcarbazole (AEC). The sections were counterstained with Mayers Haematoxylin (blue) (Merck, Haarlem, the Netherlands).
Additionally, anti-BDCA-1 mAb was also used in double staining with CD19 mAb. Sections were first incubated with FITC-conjugated CD19 for 1 hour at room temperature and subsequently with alkaline phosphatase (AP)-conjugated rabbit anti-FITC immunoglobulins (DAKO). Than slides were incubated overnight at 4°C with anti-BDCA-1, and binding was detected with PO-Envision. Visualization of AP was performed by incubation in Fast Blue salt / naphtol AS-BI phosphate solution supplemented with levamisole, giving a blue precipitate. Revelation of PO was performed with AEC giving a red precipitate.
Six portal fields and six microscopic fields of 400x magnification in the parenchyma were analyzed, immunohistochemically positive cells were counted, and means with standard deviation were calculated.

Isolation of mononuclear cells (MNC)

Cells were harvested from the perfusion fluid by centrifugation and resuspended in 30 ml RPMI$^+$ (RPMI 1640 with L-glutamine, Cambrex Bio Science, Verviers, Belgium). For isolation of single cells the fresh liver tissue wedge biopsies were cut into small pieces which were incubated in RPMI$^+$ supplemented with collagenase type IV (0.5 mg/ml; Gibco, Breda, the Netherlands) and DNase type I (0.02 mg/ml; Roche Diagnostics, Manheim, Germany) for 40 minutes at 37° C. Subsequently the tissue pieces were passed over a nylon mesh

filter (200 µm pore diameter) to obtain a single cell suspension. To remove hepatocytes the suspension was centrifuged at 360 rpm for 2 minutes and supernatant was collected. LN were also cut into small pieces and passed over a nylon mesh filter to obtain a single cell suspension. MNC were obtained from perfusates, single cell suspensions of wedge biopsies and LN, and blood by Ficoll-Paque (Amersham Biosciences, Roosendaal, the Netherlands) density centrifugation. Cell viability was determined using trypan blue staining.

Maturation of perfusate MDC

Perfusate MNC were routinely cultured in RPMI$^+$ supplemented with 10% FCS (Hyclone, Logan, UT, USA), pencillin (100U/ml), and streptomycin (100 ug/ml; Gibco BRL life technologies, Breda) for 24 hours at 37° C in the presence of 100 ng/ml lipopolysaccharide (LPS) (Sigma, Zwijndrecht, the Netherlands) in 24 wells plates using 1 x10^6 MNC per well. After 24 hours the cells were harvested and expression of maturation antigens and co-stimulatory molecules on MDC was determined using flowcytometric analysis.

Isolation of MDC from blood and perfusate MNC

For isolation of MDC 80 µl PBS supplemented with 2mM EDTA and 5 mg/ml BSA, 100 µl CD19-microbeads and 20 µl anti-BDCA1-PE were added per 100 x10^6 MNC and the cells were incubated for 15 min at 4° C. Hereafter, B cells were depleted by separation over a Large Depletion column using a MidiMACS separation device (Miltenyi Biotec). The non-adherent cells were incubated for 15 min at 4° C with 50 µl anti-PE microbeads and separated over a MS column using a MiniMACS device, after which the adherent cells were washed out and enriched further by separation over a second MS column. Purity of the isolated MDC was determined by flowcytometry and viability by Trypan blue exclusion. Purity and viability of perfusate MDC were respectively 92±10% and 89±8% and of blood MDC respectively 86±10% and 83±13%.

Flowcytometry

Flow cytometric analysis was used to immunophenotype the MNC. MNC were resuspended in PBS with human IgG (Octagam 1.25 µg/ml; Octapharma, Langenfeld, Germany) to prevent aspecific binding of antibodies to Fc-receptors on DC. Per labeling 1 x10^6 MNC were incubated with antibodies. The following antibody-combinations were used to determine the different subsets of the MNC: CD45-FITC, CD3-PE, CD4-PerCP and CD8-APC; CD45-FITC, CD3-PE and CD56-APC; CD45-FITC, anti-BDCA1-PE and CD20-PerCP; or anti-BDCA2-FITC and CD45-RPE-Cy5. MDC were defined as BDCA-1$^+$ and CD20$^-$ cells and PDC as BDCA2$^+$ cells. To determine the phenotype of MDC MNC were labeled with following antibody-combinations: anti-BDCA1-PE and CD20-PerCP in combination with anti-HLA-DR-FITC, CD83-APC, CD80-FITC, CD86-APC; or anti-BDCA1-FITC and CD20-PerCP in combination with anti-CCR7-PE or in combination with intracellular

staining with anti-DC-LAMP-PE. For the intracellular staining MNC were first incubated with anti-BDCA1-FITC and CD20-PerCP, than permeabilized and fixed with IntraPrep Permeabilization Reagent according to the manufacturers protocol (Beckman Coulter Immunotech, Marseille, France) before adding the anti-DC-LAMP-PE. Cell death of the DC was determined using 7-AAD staining (BD Biosciences Pharmingen, San Diego, USA). Appropriate isotype-matched control antibodies were used. Optimal dilutions of all antibodies were established in preliminary experiments. The effects of collagenase and DNase treatment on cell surface expression of the above markers was tested on blood MDC and no alterations were observed (data not shown). The data were analyzed on a FACScalibur using Cellquest pro software.

Allogeneic T cell stimulatory capacity of purified MDC

Purified MDC were co-cultured at different concentrations (10, 5, 2.5, 1.25 $x10^3$ cells/200 µl) in flat bottom Costar culture plate (Costar Cambridge, MA) with 1.5 $x10^5$ purified T cells from blood of a healthy volunteer. After five days, cell proliferation was assessed by measuring the incorporation of [3^H]-thymidine (Radiochemical Centre, Amersham, Little Chalfont, UK). A 0.5 µCi was added per well and cultures were harvested 18 h later. Phytohemagglutinin (5 µg/ml, Murex, Paris, France) was added to the T cells as a positive control. T cells were purified by incubation of peripheral blood MNC with CD14-PE, anti-BDCA1-PE and CD19 microbeads and subsequently with anti-PE microbeads for 15 min at 4 °C. T cells were enriched by negative selection over a Large Seperation column using a MidiMACS separation device (Miltenyi Biotec) and contained 78% $CD3^+$ T cells and 16% $CD56^+$ cells.

IL-10 production

Purified MDC were cultured at a concentration of 4 $x10^4$ cells/200 µl in 96-wells flat bottom culture plate in $RPMI^+$ supplemented with 10% FCS (Hyclone, Logan, UT, USA), pencillin (100U/ml) , streptomycin (100 ug/ml; Gibco BRL life technologies, Breda), and GM-CSF (500 U/ml; Leucomax, Novartis Pharma, Arnhem, The Netherlands) in the presence or absence of 1 µg/ml LPS for 24 hours at 37° C. After 24 h, supernatants were harvested and the level of IL-10 was determined by specific sandwich ELISA, using a mAb and recombinant cytokine standard from Biosource International, Camarillo, CA, USA.

Statistical analysis

The Kruskal-Wallis, Mann-Whitney test from SPSS version 11.0 were used to test whether differences between groups were statistically significant. The Wilcoxon Signed Ranks test from SPSS version 11.0 was used to test whether differences between groups of paired samples were statistically significant. A $p < 0.05$ was considered significant. All data are presented as means ± standard deviation (SD).

RESULTS

MDC in human donor livers

MDC were visualized in wedge biopsies of donor livers (n=7) by immunohistochemistry with anti-BDCA-1 mAb. BDCA-1$^+$ cells were predominantly observed in the portal fields (average: 6.1±2.3 cells per portal field) and only a few resided in the parenchyma (average: 0.6±0.5 cells per microscopic field; p=0.001) (Figure 1A and B). Since BDCA-1 is also expressed on a subpopulation of B cells (25) additional double stains with anti-BDCA-1 and CD19 mAb were performed (Figure 1C and D). Portal fields contained on average 4.9±6.0 BDCA-1$^+$CD19$^-$ MDC per portal field, which is comparable to the number of BDCA-1$^+$ cells observed in the single-stains with anti-BDCA-1 mAb (p= 0.351). Both in portal tracts and parenchyma only few BDCA-1/CD19 double-positive B cells were observed.

To characterize the MDC present in donor livers, MNC were isolated from donor liver wedge biopsies (n=10). Liver MNC contained on average 1.0% (range 0.2-2.2%) BDCA-1$^+$CD20$^-$ MDC. The expression of CD83 and CD80 was determined on these DC (Figure 2A). Donor liver MDC had an immature phenotype characterized by a relatively low expression of the maturation marker CD83 as well as the co-stimulatory molecule CD80 (Figure 2B and C).

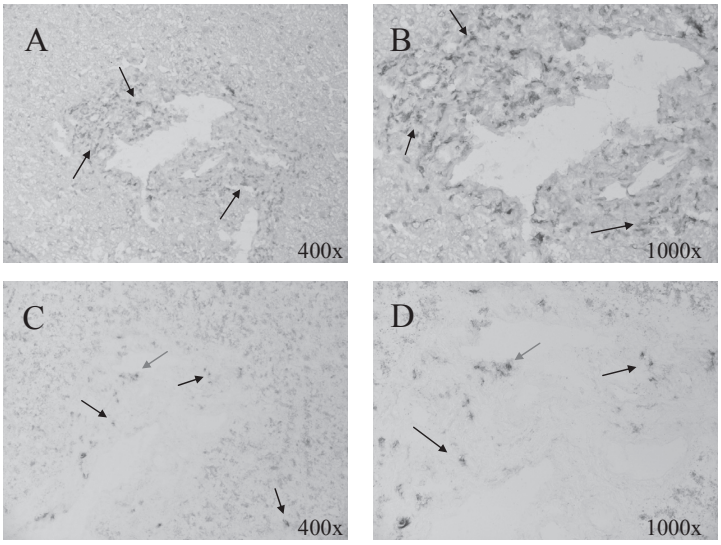

Figure 1. Localization of MDC in human donor livers. (A,B) MDC detected in a donor 5µm liver-cryosection by immunohistochemistry with anti-BDCA-1 mAb (red). The tissue was counterstained with haematoxylin (blue). (C,D) MDC detected in a donor 5µm liver-cryosection by immunohistochemistry with anti-BDCA-1 mAb (red) and CD19 (blue). The black arrows indicate BDCA-1$^+$ CD19$^-$ MDC. The red arrow indicates a BDCA-1$^+$ CD19$^+$ B cell.

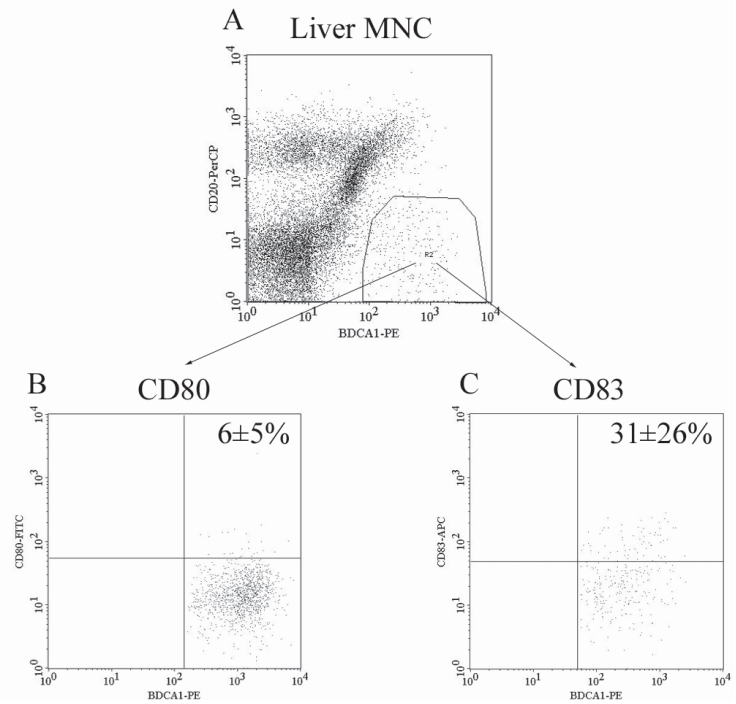

Figure 2. Characterization of MDC in human donor livers. (A) Liver MNC isolated from a donor liver wedge biopsy with MDC gated in R2. MDC were defined as BDCA-1$^+$ CD20$^-$ cells. A representative example of the expression of CD80 (B) and CD83 (C) on MDC in donor liver MNC. Data of MDC from ten donor livers are expressed as mean ± SD.

Composition of perfusate MNC in comparison with donor liver and blood

The perfusates collected during the pretransplantation *ex vivo* albumin perfusions of the donor livers had an average volume of 378±93 ml and contained 59±36x10^6 MNC. 99±2% of the MNC present in the perfusates were vital as determined by trypan blue staining. The CD4/CD8 ratio in perfusates (0.7±0.4) was identical to that in the donor liver MNC (0.6±0.1; p=1.000) and both perfusate (p=0.003) and donor liver MNC (p=0.014) differed significantly from blood (3.6±1.9) (Figure 3). Also the NK cell proportion, defined as the percentage of CD45$^+$ cells expressing CD56, in perfusate was similar to that in liver MNC (respectively 44±12% and 28±16%, p=0.413), but perfusate differed significantly from blood (13±5%, p=0.000). The observed similarity in CD4/CD8 ratio and percentage of NK cells between liver and perfusate and the significantly distinct ratios of these cells in blood strongly suggests that leukocytes present in perfusates are predominantly liver tissue derived.

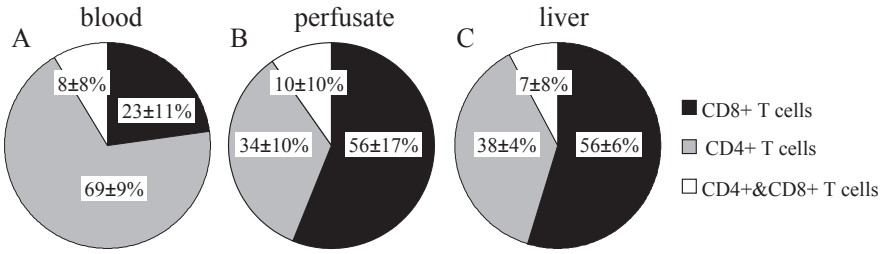

Figure 3. Composition of T cells in perfusate compared with blood and liver MNC. MNC were isolated from (A) blood (n=8), (B) perfusate (n=17) and (C) donor liver biopsies (n=10). MNC were incubated with CD45-FITC, CD3-PE, CD4-PerCP and CD8-APC mAbs and T cell subsets were analysed by flowcytometry. $CD4^+CD8^+$ T cells contained $CD4^{hi}CD8^{hi}$ as well as $CD4^{hi}CD8^{lo}$ subpopulations. Data are expressed as mean ± SD.

DC in perfusate, hepatic LN and blood

Perfusate MNC contained on average 1.5% (range 0.3-6.6%) MDC (=$BDCA1^+$ $CD20^-$ cells) with a vitality of 97±2% determined by 7-AAD staining. The total number of MDC that detached from donor livers during vascular perfusion pretransplantation was 0.9 $x10^6$ (range 0.11-4.5 $x10^6$). Perfusate MNC contained a significantly higher percentage of MDC in comparison with LN 0.6% (range 0.2-1.1%; p=0.03), and MDC percentages in perfusates tended to be higher than in blood 0.7% (range 0.2-1.3%; p=0.11). In addition, perfusate MNC contained on average 0.9 % (range 0.2-2.6%) PDC (=$BDCA2^+$ cells) similar to LN (0.3%; range 0.1-0.5%) and blood (0.5%; range 0.3-0.7%; p>0.10). The total number of PDC that detached from donor livers during vascular perfusion pretransplantation was 0.5 $x10^6$ (range 0.1-1.3 $x10^6$). The

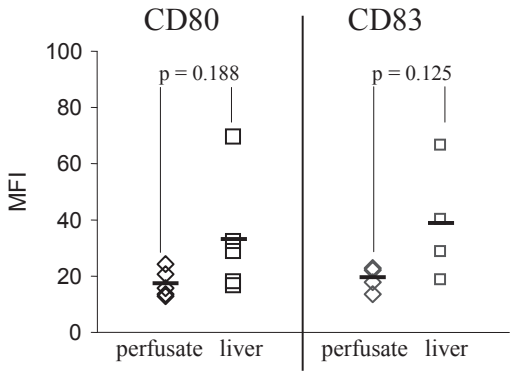

Figure 4. Expression of CD80 and CD83 on MDC from perfusate and liver MNC obtained from the same donor. The expression of the molecules on MDC was determined by flowcytometric analysis of MNC. MDC were defined as $BDCA-1^+$ and $CD20^-$ cells. Expression of CD80 (n=5) and CD83 (n=4) was similar on perfusate MDC and liver MNC MDC.

variation in volumes and numbers of MNC did not correlate with differences in cold ischaemic times (Bosma B.M., data not shown). Altogether these data indicate that a high number of DC detach during perfusion from donor livers pretransplantation.

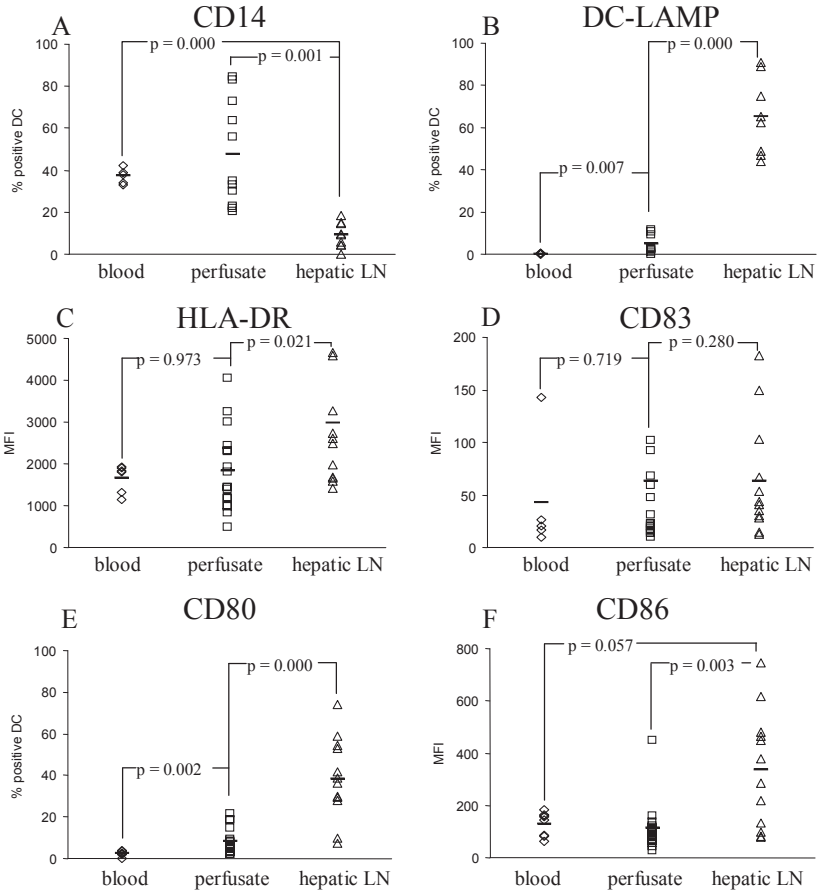

Figure 5. Maturation state of MDC derived from liver perfusate compared with blood and hepatic LN. The expression of maturation molecules on MDC was determined by flowcytometric analysis of MNC. MDC were defined as BDCA-1^{+} and CD20^{-} cells. (A) Expression of CD14 was significantly higher on perfusate (n=11) and blood MDC (n=5) compared to LN MDC (n=9). (B) DC-LAMP expression was lower on blood MDC (n=5), highest on LN MDC (n=8) and intermediate on perfusate MDC (n=9). (C) Perfusate DC had a significant lower expression of HLA-DR in comparison with LN MDC. (D) CD83 expression was not significantly different between blood MDC (n=6), perfusate MDC (n=17) and LN MDC (n=12). (E) CD80 expression was lower on blood MDC (n=8), highest on LN MDC (n=12) and intermediate on perfusate MDC (n=17). (F) CD86 expression on blood MDC (n=8) and perfusate MDC (n=17) was similar, but both groups differed significantly from LN MDC (n=12).

Maturation markers, co-stimulatory molecules and CCR7 on perfusate MDC in comparison with liver-, blood- and hepatic LN MDC

To determine whether MDC in liver perfusate are representative for resident liver MDC, the expression of CD80 and CD83 on perfusate and liver MDC from the same multiorgan donor were compared. The mean fluorescence intensities (MFI) (Figure 4) were similar. Additionally, a comparison of the expression of CD80 was made between liver, perfusate and blood MDC. Similar numbers of liver and perfusate MDC expressed CD80 (6.7±4.6% and 8.1±6.4% respectively; p= 1.0), but a significantly lower number of blood MDC expressed CD80 (2.4±1.1%; p<0.03). These data indicate that perfusate MDC are liver MDC. To further investigate the maturation state of liver MDC, we compared the expression of the maturation markers CD14, DC-LAMP, CD83 and HLA-DR on perfusate MDC with blood- and hepatic LN MDC. On average 48% of the perfusate MDC and blood MDC expressed CD14, while on LN MDC the monocyte marker was almost absent (Figure 5A). Expression of DC-LAMP was low on perfusate MDC, but intermediate between blood and LN MDC (Figure 5B). Perfusate MDC had a significant lower expression of HLA-DR in comparison with LN MDC (Figure 5C). There was no significant difference in CD83 expression between MDC derived from the different materials (Figure 5D).

Analysis of the expression of the co-stimulatory molecules and the LN homing receptor CCR7 on MDC revealed that the expression of CD80 was low on perfusate MDC, and intermediate between blood- and LN MDC (Figure 5E). CD86 expression on blood and perfusate MDC was similar, but the expression on LN MDC was significantly higher (figure 5F). The observed differences between MDC from perfusates and hepatic LN were confirmed in paired comparisons of MDC from the same multiorgan donor (n=9); CD80 (p=0.012), CD86 (p=0.004). CD14

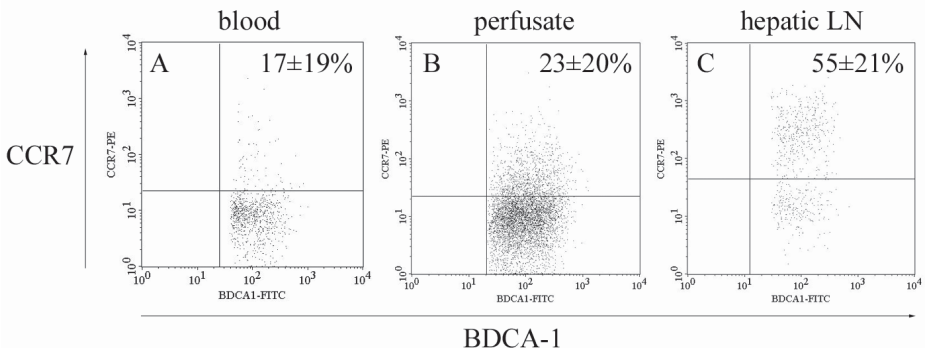

Figure 6. CCR7 expression on MDC from perfusate compared with blood and LN. CCR7 expression on MDC was determined by flowcytometric analysis of MNC. MDC were defined as BDCA-1$^+$ and CD20$^-$ cells. (A) Blood MDC (n=4) and (B) perfusate MDC (n=12) had a similar expression of CCR7, but this was significantly higher on (C) LN MDC (n=10) (p<0.014). Data are expressed as mean ± SD.

(p=0.016) and DC-LAMP (p= 0.063). MDC in blood and perfusate expressed significantly lower levels of CCR7 compared to LN MDC (p≤ 0.014) (Figure 6).

Maturation of perfusate MDC *in vitro*

To assess whether perfusate MDC are capable of maturing *in vitro* perfusate MNC were incubated with 100 ng/ml LPS for 24 hours (n=3). Twenty-four hours of incubation with LPS resulted in an increase of the expression of both maturation markers and co-stimulatory molecules on perfusate MDC (Figure 7). Thus MDC from liver grafts respond to a common bacterial danger signal.

IL-10 production and stimulation of allogeneic T cells by perfusate MDC compared with blood MDC

To determine whether perfusate MDC were able to stimulate allogeneic T cell proliferation, graded numbers of perfusate MDC were cultured with allogeneic T cells. The T cell stimulatory capacity of freshly isolated perfusate MDC was compared to immature blood MDC; Perfusate MDC stimulated allogeneic T cells whereas blood MDC were not able to induce T cell proliferation (Figure 8).

Recently Goddard et al (20) showed that human liver MDC produce high levels of IL-10 in comparison with skin DC. To ascertain that perfusate MDC are liver-derived, IL-10 pro-

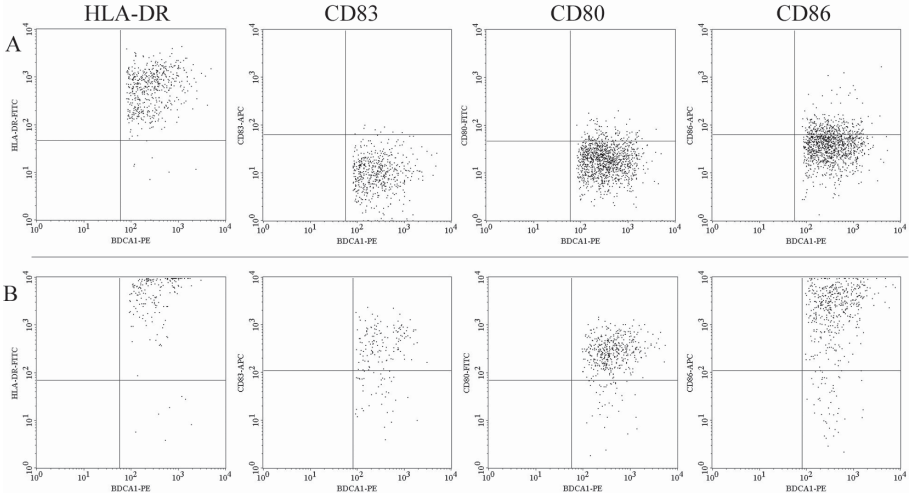

Figure 7. Phenotype of perfusate MDC after 24 hours maturation with LPS. Perfusate MNC were incubated with 100 ng/ml LPS at 37 °C for 24 hours. Flowcytometric analysis was used to determine the maturation state of perfusate MDC at t=0 and t=24h. MDC were defined as BDCA-1$^+$ and CD20$^-$ cells. (A) Expression of maturation and co-stimulatory molecules on perfusate MDC at t=0. (B) Elevated expression of maturation and co-stimulatory molecules on perfusate MDC at t=24h. Data shown are from 1 representative sample (n=3).

duction of perfusate MDC was compared with blood MDC. Both types of MDC did not produce IL-10 when cultured without a stimulus, however when MDC were stimulated with 1 µg/ml LPS perfusate MDC (n=3) produced significantly more IL-10 than blood MDC (n=7) (1800±1875 pg/ml and 137±45 pg/ml, respectively; p= 0.017).

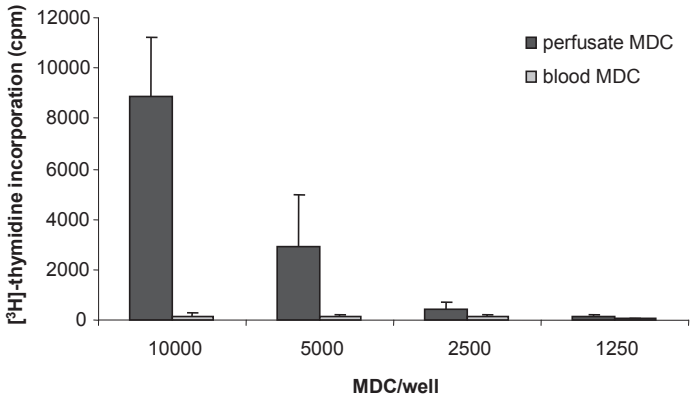

Figure 8. Allostimulatory capacity of perfusate MDC in comparison with blood MDC. Graded numbers of freshly isolated MDC were cultured with 1.5 x10^5 allogeneic T cells. After five days the cells were pulsed for another 18 hours with [^{3}H]-thymidine. Perfusate MDC (n=3) stimulated T cell proliferation, while blood MDC (n=3) did not. All data are expressed as mean ± SD.

DISCUSSION

To our knowledge this is the first study in which freshly isolated human liver MDC have been characterized by flowcytometric analysis. The obtained data indicate that MDC from human donor livers have a relatively low expression of the maturation marker CD83 and co-stimulatory molecule CD80, indicating that they are immature DC. These findings are consistent with data from murine studies showing that liver DC have a high expression of surface MHC class II, but few co-stimulatory molecules (11, 13). The immunochemical stains with anti-BDCA-1 mAb indicate that the majority of MDC reside in the portal fields of human donor livers.

The presence of liver specific immune cells, namely T cells, NK cells, monocytes and B cells, in perfusates of donor livers has been previously reported (23). Detection of DC was probably not attempted in the previous study due to the lack of suitable DC-specific markers at that time. The present study demonstrates that perfusates contained on average 0.9 x10^6 MDC and 0.5 x10^6 PDC. Since PDC are less efficient in antigen presentation and first data on their role in transplantation suggests that they do not induce rejection (24), we have focused on the classical MDC.

Our data show that MDC present in perfusates were liver MDC and not blood MDC: First perfusate MDC had a similar CD80 and CD83 expression compared to resident liver MDC. Furthermore CD80 expression was slightly higher on perfusate MDC in comparison with blood MDC. Second, perfusate MDC produced significantly higher amounts of IL-10 compared to blood MDC upon stimulation with LPS. High IL-10 production is also characteristic for liver MDC, as is shown by Goddard et al (20). Third, perfusate MDC were able to stimulate allogeneic T cell proliferation in contrast to freshly isolated blood MDC. Fourth, the MNC in perfusates had a low CD4/CD8 ratio and a high percentage of NK cells, which is indicative for liver-derived cells (26). Containing high numbers of MDC, perfusates are a rich source for the study of donor liver MDC.

Using perfusate, donor liver MDC were further immunophenotyped. The maturation state of hepatic MDC was compared with immature blood MDC and mature hepatic LN MDC (1, 21, 27). Perfusate MDC, like blood MDC, have a high expression of CD14 which indicates that these MDC are either derived from $CD14^+$ immature MDC in the blood, from $CD14^+$ bone-marrow precursors or from monocytes (28). The expression of DC-LAMP, a lysosomal protein that is up regulated in mature MDC (29), and HLA-DR is significantly lower on perfusate MDC than LN MDC. In addition, the co-stimulatory molecules CD80 and CD86 are expressed significantly lower on perfusate MDC than LN MDC. CCR7, normally expressed by mature MDC that have acquired the ability to home to secondary lymphoid tissue (30), is also expressed significantly lower on perfusate MDC than LN MDC. Together these results demonstrate that donor liver MDC have an immature phenotype.

Furthermore the majority of isolated perfusate MDC are vital and functional since they have the capacity to produce high amounts of IL-10, to stimulate allogeneic T cell proliferation, and to mature *in vitro* when exposed to the maturation stimulus LPS. These data show that perfusate MDC are functionally intact and can be used as a source for functional studies on donor liver MDC.

Another important implication of our study is that resident liver DC leave the liver graft via the vasculature during *ex vivo* perfusion. As the lymph vessels are transected during transplantation we suppose that DC also leave the transplanted graft after reperfusion via the vasculature and migrate into the blood circulation. However, this does not exclude another possible migration route namely via the transected lymph vessels into the peritoneum of the recipient and then via the lymphatics and blood circulation to the secondary lymphoid organs.

About 1 million MDC, that have the potential to become immunogenic, detach from the donor liver pretransplantation via vascular perfusion. We hypothesize that posttransplantation similar numbers of MDC, and probably even higher, will detach from the liver allograft, migrate into the recipient and subsequently induce acute rejection. Similar numbers of MDC are sufficient for induction of immunomodulatory processes; from a clini-

cal trial for treatment of advanced metastatic melanoma (stageVI), it is known that i.v. injection of as little as 0.9 x10^6 MDC can induce a tumor specific immune response (31). Thus the numbers of donor-derived MDC that detach during perfusion from donor livers are expected to be high enough to induce an allogeneic immune response in the recipient. Ischemia/reperfusion injury could elicit the maturation of donor liver DC *in vivo*. Upon arrival in the recipients LN or spleen these donor MDC are thought to present allogeneic MHC molecules to the recipient T cells which are recognized via the direct pathway causing acute rejection (32). From animal studies it is known that mature donor liver DC are indeed capable of inducing acute rejection and that on the contrary immature DC can induce tolerance (14, 16, 17, 33).

Since MDC in donor livers as well as in perfusates are immature they may still be sensitive to pharmacological targeting thereby preventing acute rejection (34). Pharmacological pre-treatment of the donor liver during the cold storage could be a promising new method to manipulate MDC to induce tolerance, and isolation of this cell fraction from perfusates would allow *in vitro* testing of such a model.

In conclusion, human donor livers contain exclusively immature MDC that detach in high numbers from the liver graft during perfusion before transplantation. These vital MDC have the capacity to stimulate allogeneic T cells and to mature upon activation, and could therefore play a major role in the induction of acute rejection.

ACKNOWLEDGEMENTS

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5

Migration of donor myeloid dendritic cells after human liver but not after kidney transplantation: implications for liver graft acceptance?

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submitted

ABSTRACT

The classical paradigm states that allogeneic T cell activation after organ transplantation results from transfer of donor-derived myeloid dendritic cells (MDC) from the graft into the recipient. Conversely, it has been postulated that the liver contains tolerogenic MDC, which may account for the tolerogenic properties of liver grafts. However, no formal proof that donor MDC migrate from human organ grafts into recipients has been provided. Here, we show that after human liver transplantation (LTx), but not after renal transplantation (RTx), donor-derived MDC circulate in the recipient. One day after LTx 4.2% of MDC were or donor origin, and 4 days later still 0.6%. MDC that detached from human liver grafts during vascular perfusion before transplantation were LFA-1^{high} and about 25% expressed CCR-7. Upon activation, about 44% acquired L-selectin, resulting in expression of all receptors required for homing into recipient secondary lymphoid tissues. Importantly, purified liver graft perfusate MDC stimulated with a physiological concentration of LPS were, in comparison with blood MDC, poor inducers of IFN-γ production in allogeneic T cells, although they were able to stimulate T cell proliferation. This was not due to LPS-resistance, since liver graft MDC produced higher amounts of cytokines after stimulation with LPS, and showed a higher expression of TLR-4 in comparison to blood MDC. In contrast to blood MDC, liver graft perfusate MDC produced high amounts of IL-10, which may account for their weak capacity to prime IFN-γ production in T cells.
In conclusion, human LTx, but not RTx, results in early transfer of donor-derived MDC into recipients. These hepatic MDC stimulate allogeneic T cell expansion but not IFN-γ production, probably due to their prominent production of IL-10. It is hypothesized that migration of donor-derived hepatic MDC after human LTx may contribute to LTx tolerance by early priming followed by activation-induced cell death of donor-reactive T cells.

INTRODUCTION

The classical paradigm states that organ transplantation results in transfer of donor-derived leukocytes from the graft into the recipient's. Pioneering experimental animal studies showed that these "passenger leukocytes" play an important role in the sensitization of the recipient's immune system to the allograft, by direct presentation of donor allo-antigens to recipient T cells (1-4). Donor-derived myeloid dendritic cells (MDC) are considered to be the most potent sensitizers of the recipient's immune system. In experimental animals, donor MDC migrating into the secondary lymphoid tissues of the recipient were found to prime recipient alloreactive T cells (4-6). Indeed, recipient's secondary lymphoid tissues are indispensable for the generation of effective rejection responses (7).

Liver grafts are less immunogenic than other organ grafts. Spontaneous acceptance of allogeneic liver grafts occurs in transplantations between several combinations of mouse and rat strains, and in outbreed species like pigs and primates (8). In the clinical setting, liver transplantation (LTx) does not require MHC-matching between donor and recipient, liver transplants can protect kidney transplants from the same donor from rejection (9), and immunosuppression can be more easily discontinued in LTx recipients than in patients transplanted with other solid organ grafts (10).

It has been postulated that the tolerogenic properties of liver grafts are related to their large numbers of passenger leukocytes (8, 11). Liver grafts contain larger numbers of passenger leukocytes compared to heart and kidney grafts (12), and after experimental LTx donor leukocytes migration into recipients is much more pronounced than after transplantation of other solid organ grafts (13-16). Although it has been shown that depletion of passenger leukocytes from liver grafts abrogates induction of spontaneous tolerance in several experimental animal models (17, 18), the passenger leukocyte subset responsible for induction of LTx tolerance has not been identified yet. Some studies have suggested that transfer of donor-derived T cells is important (19, 20), while others proposed that donor-derived myeloid dendritic cells (MDC) play a critical role in spontaneous tolerance to allogeneic liver grafts (21). Indeed, after experimental LTx donor-derived MDC have been detected in recipient spleen, lymph nodes and thymus (13, 22, 23). Thus, although donor-derived MDC are classically regarded as the most potent instigators of rejection, liver graft MDC have been proposed to contribute to liver graft acceptance. Supporting this concept, murine hepatic DC have been shown to possess tolerogenic properties (24). They are weak stimulators of allogeneic T cells (25, 26), and are able to prolong allograft survival when administered to recipients prior to transplantation (27). However, the current views on the immunological effects of donor MDC transfer in solid organ transplantation in general, and in liver transplant tolerance in particular, are based on evidence from experimental animal studies. No formal proof that donor MDC migrate after transplantation of human organ grafts into recipients has been provided. Recently, we observed

that donor-derived MDC easily detach from human liver grafts during vascular perfusion before transplantation (28). This observation prompted us to investigate whether MDC of donor-origin migrate into the circulation of recipients after human LTx, and to compare this with MDC chimerism after renal transplantation (RTx). In addition, we studied the immunological properties of hepatic MDC that detach from human liver grafts, in order to elucidate whether migrating human donor liver MDC may contribute to liver graft rejection or acceptance.

MATERIALS AND METHODS

Peripheral blood, liver graft perfusate, and donor spleen collection

For this study, 11 HLA-A2 negative LTx recipients, and 6 HLA-A2 negative renal transplant (RTx) recipients that were transplanted with a graft from an HLA-A2 positive donor, were selected. Immediately before transplantation, and at 1 day after LTx and RTx and 5 days after LTx, peripheral blood was collected. The immunosuppressive regime of both LTx and RTx recipients consisted of a combination of calcineurin inhibitors, IL-2 receptor blockers and steroids in the first week after transplantation. Peripheral blood mononuclear cells (PMBC) were prepared by Ficoll density gradient centrifugation. Ten of the LTx recipients were transplanted with a graft from a deceased donor, and one with a partial graft from a living donor. The RTx procedures were all living-related transplantations. Perfusates were collected from human liver grafts during the backtable procedure. Upon arrival in the hospital, the grafts were perfused through the portal vein with 1 to 2 L of University of Wisconson (UW)-solution to remove residual blood from the vasculature. Immediately before transplantation, the donor livers were perfused with 200 up to 500 ml of human albumin-solution. The latter perfusates were collected from the vena cava, and used to study hepatic MDC. Splenic tissue was obtained from the liver graft donors, and single cell suspensions were prepared by cutting splenic tissue into small pieces and passing splenic cells over a nylon mesh filter (200 µm pore diameter). Mononuclear cells (MNC) from liver graft perfusates and spleen were isolated within 12 hours by density gradient centrifugation. For immunophenotypic and functional studies of blood MDC, PBMC were isolated from healthy individuals. The Ethical Committee of the Erasmus MC approved the study protocol, and informed consent was obtained of each patient.

Monoclonal antibodies

The following mAbs were used: IgG1-FITC, IgG1-PE, IgG1-APC, IgG1-PerCP-cy5.5, IgG2a-FITC, IgG2b-FITC, anti-HLA-A2-FITC, CD4-PerCP, CD20 FITC, CD20-PerCP, CD20-APC, CD62L-APC and streptavidin-APC from BD Biosciences, San Jose, CA, USA; CD11a-FITC, CD45-FITC, CD3-PE, CD56-APC, from Beckman Coulter Immunotech,

Marseille, France; anti-BDCA1-FITC and anti-BDCA1-PE, CD19-microbeads and anti-PE-microbeads from Miltenyi Biotec, Bergisch Gladbach, Germany; CD8-APC and from Dako, Glostrup, Denmark; anti-CCR7-PE from R&D systems, Abingdon, United Kingdom; anti-TLR-2-bio and anti-TLR-4-bio from Biolegend, San Diego, USA.

Detection of donor-derived MDC, T cells and B cells

Donor-derived MDC, T cells and B cells were detected in the recipient circulation using a FITC-conjugated anti-HLA-A2 mAb, in combination with PE-conjugated anti-BDCA-1 and CD20-APC mAb to determine BDCA-1^{+}CD20^{-} MDC and CD20^{+} B cells, and CD3-PE plus CD56-APC to determine CD3^{+}CD56^{-} T cells. At least 1x10^{6} events were acquired on a FACS Calibur flowcytometer (BD Biosciences, San Jose, CA, USA). Dead cells were excluded from analysis by using 7-AAD (BD Biosciences Pharmingen, San Diego, USA), and binding of an appropriate isotype-matched FITC-conjugated control mAb was subtracted from binding of the anti-HLA-A2 mAb. Analysis was performed using CELLQuest Pro software (BD Biosciences, San Jose, CA).

Determination of lymphoid tissue homing receptors and TLR on MDC

Expression of lymphoid tissue homing receptors on liver graft- and blood MDC was compared by flowcytometry upon labeling of liver graft perfusate MNC and PMBC with CD11a-FITC, CCR7-FITC or L-selectin-APC mAb in combination with anti-BDCA-1-PE and CD20-APC mAb or CCR-7-PE mAb in combination with anti-BDCA-1-FITC and CD20-PerCP mAb. The effect of maturation of liver graft MDC on the expression of these homing receptors was established in overnight stimulations of liver graft perfusate MNC (3x10^{6} per 200 µl) at 37°C with 100 pg/ml LPS (Sigma Aldrich, Zwijndrecht, The Netherlands), or with synthetic double-stranded RNA (poly (I:C) 20µg/ml; Sigma Aldrich) in combination with recombinant human IFN-γ (1000 u/ml; Strathmann Biotech, Hannover, Germany) in RPMI-medium supplemented with 10% fetal calf serum (FCS; Hyclone, Logan, UT, USA), penicillin (100 U/ml) and streptomycin (100 µg/ml; both from Gibco BRL Life Technologies, Breda, The Netherlands). For analysis of TLR expression liver graft perfusate- and spleen MNC (from the same donor) and PBMC were labeled with biotinylated anti-TLR-2 or anti-TLR-4 mAb in combination with anti-BDCA-1-PE and CD20- FITC, followed by steptavidin-APC. Optimal dilutions of all mAb used were established in preliminary experiments, and analysis were performed using appropriate isotype-matched control mAb.

Isolation of MDC and T cells

BDCA-1^{+}CD20^{-} MDC were isolated from liver graft perfusate MNC and from PBMC by negative depletion of B cells using CD19-microbeads, followed by positive selection of MDC with anti-BDCA1-PE and anti-PE microbeads (Miltenyi Biotec, Bergisch Gladbach,

Germany) as described previously (28, 29). Purity and viability (as determined by flow-cytometry for 7-AAD) of magnetic bead-isolated liver perfusate MDC were 92±4% and 75±4% respectively, and of blood MDC 90±3% and 86±3% respectively. Alternatively, MDC were purified by labeling with PE-conjugated anti-BDCA-1 and CD20-APC mAb followed by sorting using a FACSAria flowcytometer (BD Biosciences). Purity and viability of magnetic bead isolated liver perfusate MDC were 93±3% and 86±5% respectively, and of blood MDC 98±1% and 93±2% respectively.
A batch of T cells was purified from buffy coat PBMC by incubation with CD14-PE, anti-BDCA1-PE and CD19 microbeads, and subsequently with anti-PE microbeads for 15 min at 4 °C. T cells were enriched by negative selection over a Large Seperation column using a MidiMACS separation device (Miltenyi Biotec) and contained 87% $CD3^+$ T cells and 11% $CD56^+$ cells.

Cytokine production by MDC

Magnetic bead isolated liver graft perfusate or blood MDC ($4x10^4$ per 200 µl) were stimulated for 24 hours at 37°C in flat-bottom plates with LPS (1 µg/ml), or with poly (I:C) (20 µg/ml) in combination with IFN-γ (1000 U/ml), or with $4x10^4$ CD154-transfected J558 plasmacytoma cells together with IFN-γ (1000 U/ml) in RPMI supplemented with 10% FCS, penicillin, streptomycin and recombinant GM-CSF (500 U/ml; Leucomax, Novartis Pharma, Arnhem, The Netherlands). In other experiments, MDC purified by flowcytometric sorting ($2x10^4$ per 200 µl) were stimulated with a low concentration of LPS (100 pg/ml) for 18 hours. Supernatants were harvested and concentrations of IL-6, IL-10, IL-12 p70 and TNFα in the supernatants were determined by standard enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions (eBioscience, San Diego, CA, USA).

Allogeneic T cell stimulatory capacity of MDC

MDC purified by flowsorting from liver graft perfusates or from blood were stimulated for 18 hours in two different concentrations (20 and 10 $x10^3$ cells/200 µl) in a flat bottom culture plate (Costar Cambridge, MA, USA) with 100pg/ml LPS in RPMI supplemented with 10% FCS, penicillin, streptomycin and recombinant GM-CSF (500 U/ml). Subsequently, MDC were washed and 1.5 $x10^5$ purified T cells purified from blood of a healthy individual were added. In all experiments, one batch of frozen T cells was used. All cultures were performed in triplicates. After 5 days, 50 µl culture supernatant was collected to determine cytokines. T cell proliferation was assessed by adding 0.5 µCi [^{3}H]-thymidine (Radiochemical Centre, Amersham, Little Chalfont, UK), harvesting the cells after 18 hours, and determination of [^{3}H]-thymidine incorporation. Phytohemagglutinin (PHA; 5 µg/ml, Murex, Paris, France) was added to T cells in the absence of MDC to assess their proliferative capacity. IL-2, IL-4, IL-10 and IFN–γ production in cocultures of MDC with

allogeneic T cells were determined by the cytometric bead array technology using human cytokine flex sets according to the manufacturer's instructions (BD Biosciences) . The data were analyzed on a FACSarray using CBA analysis software (BD Biosciences) .

Statistical analysis

All data are presented as means ± SEM. The Mann-Whitney test was used to analyze whether differences between unrelated groups were significant. For paired comparisons, the Wilcoxon Signed Ranks testa was used. A p-value <0.05 was considered as significant.

RESULTS

Donor MDC migrate into recipients after liver transplantation but not after kidney transplantation

To determine whether donor-derived MDC migrate into the recipient circulation after LTx, we selected HLA-A2 negative recipients who had been transplanted with a graft from an HLA-A2 positive donor. In these recipients circulating BDCA-1^{+}CD20^{-} MDC were HLA-2 negative before transplantation, while splenic MDC from their donors were HLA-A2 positive (Figure 1A). After LTx donor-derived HLA-A2 positive MDC were present in the circulation of 10 out of 11 selected recipients. One day after LTx 4.2% (range 0.0-18.1%) of circulating MDC were of donor origin. Five days after LTx still 0.6% (range 0.0-1.3%) of circulating MDC were donor-derived (Figure 1B). In addition, donor-derived B cells and T cells were detected in the recipient's circulation.

To establish whether leukocyte and MDC chimerism after organ transplantation is unique for LTx, we performed similar measurements in renal transplant (RTx) recipients. In contrast to LTx recipients, almost no donor-derived B cells, T cells and MDC could be detected in the circulation of RTx recipients (Figure 1C).

Ten out of 11 liver grafts in this study were from deceased donors, while all kidney grafts were from living donors. However, this does not account for the difference in leukocyte chimerism between LTx and RTx, since the recipient that was transplanted with a liver graft from a living donor showed high numbers of donor-derived leukocytes in the circulation (9.1% of circulating MDC, 0.7% of B cells, and 0.5% of T cells were donor-derived on day 1 after LTx).

Together, these data show that detectable leukocyte chimerism after human organ transplantation is unique for LTx, and that considerable numbers of donor-derived MDC detach from human liver grafts and migrate into the circulation of the recipient.

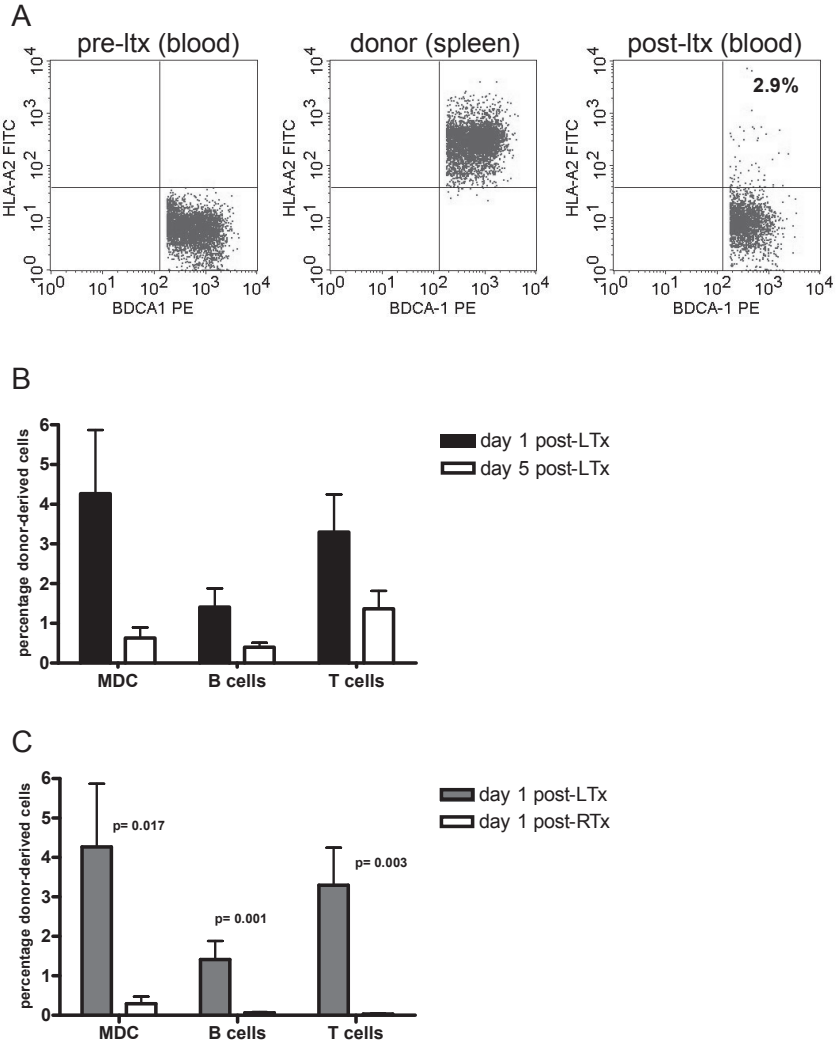


Figure 1. Donor MDC migration in recipients of liver and kidney grafts. To determine donor-derived leukocytes in the circulation after transplantation, presence of HLA-A2 positive MDC, B cells and T cells was analyzed in HLA-A2 negative recipients of liver or kidney grafts of HLA-A2 positive donors. Pre-transplant recipient PBMC, donor spleen MNC, and recipient PBMC collected 1 or 5 days after transplantation, were labeled with anti-BDCA-1-PE, CD20-APC and anti-HLA-A2-FITC mAb. (A) Representative dot plots of the expression of HLA-A2 on BDCA-1^{+}CD20^{-} MDC in pre-transplant recipient PMBC, donor spleen MNC and recipient PBMC collected 1 day after transplantation of an HLA-A2 positive liver graft into an HLA-A2 negative recipient. (B) Percentages of HLA-A2 positive (=donor-derived) MDC, B cells and T cells in the circulation of LTx recipients on day 1 (n=11) and day 5 (n=4) after transplantation. (C) Comparison of the percentages HLA-A2 positive (=donor-derived) MDC, B cells and T cells in the circulation of LTx recipients (n=11) and RTx recipients (n=6) one day after transplantation.

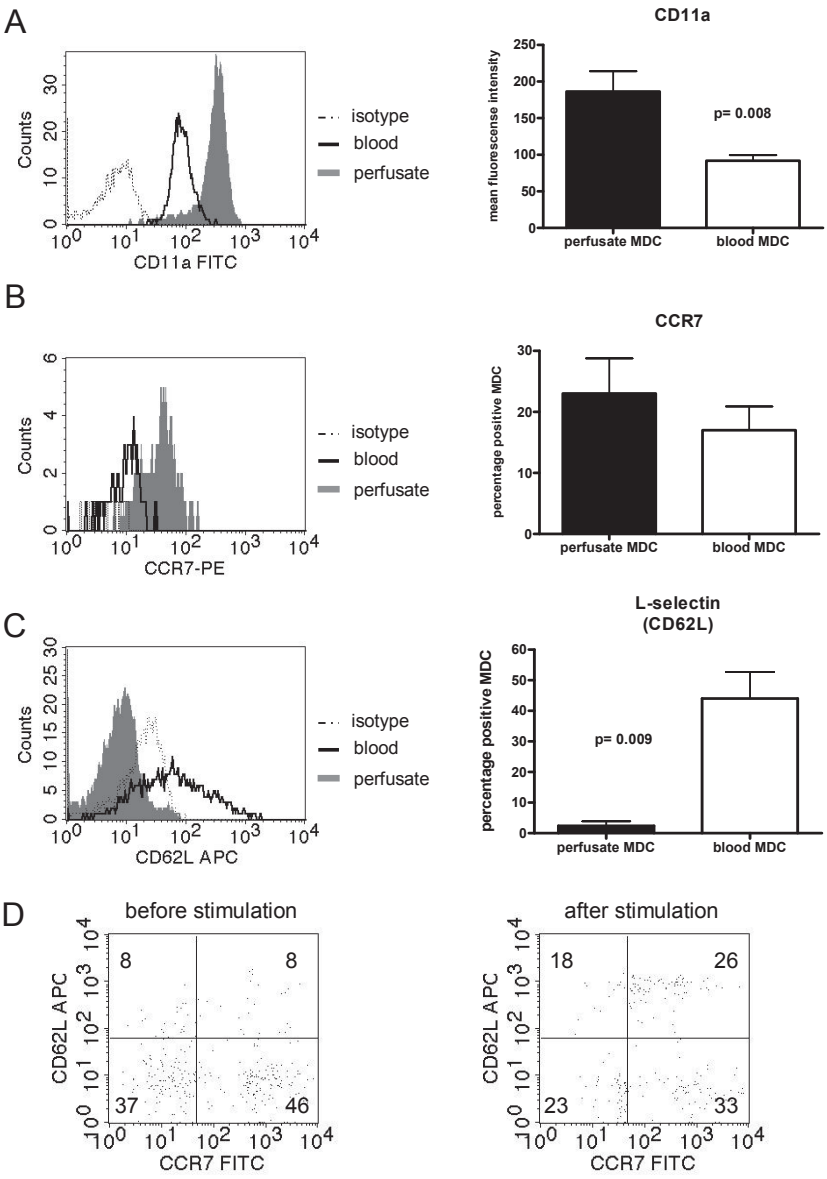
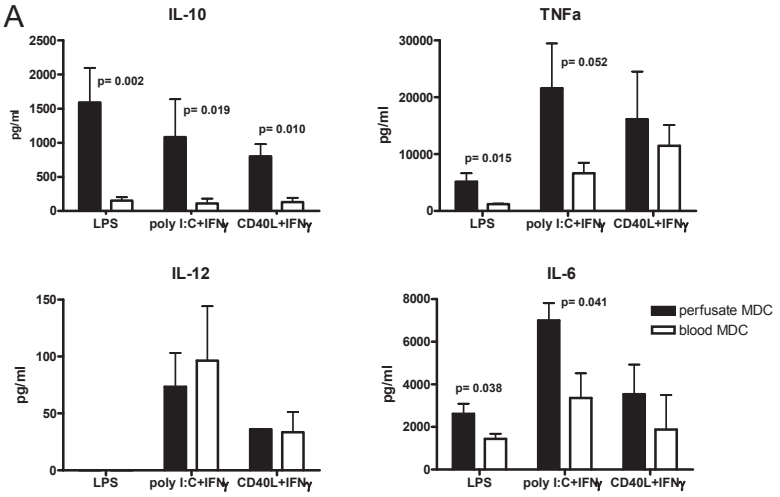


Figure 2. Expression of CD11a, CCR-7 and L-selectin on liver graft perfusate MDC. Comparison of CD11a (A), CCR-7 (B) and L-selectin (C) expression on BDCA-1^{+}CD20^{-} MDC in liver graft perfusate and peripheral blood. For each receptor a representative histogram of it's expression on liver perfusate and blood MDC is shown. In addition, bar plots depict mean fluorescence intensities (± SEM) for CD11a and percentages positive MDC (± SEM) for CCR-7 and L-selectin in 5 perfusates and 5 PBMC samples from different donors. (D) Perfusate MNC were stimulated with a physiological concentration of LPS (100 pg/ml) for 18 hours (n=2). Depicted are representative dot plots showing CCR-7 and L-selectin expression on BDCA-1^{+}CD20^{-} MDC before and after stimulation.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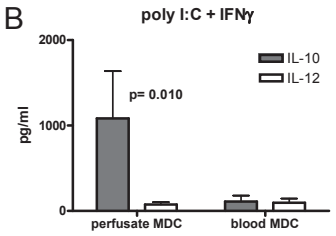
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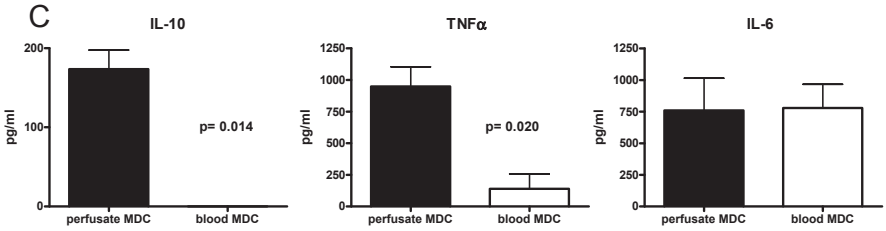
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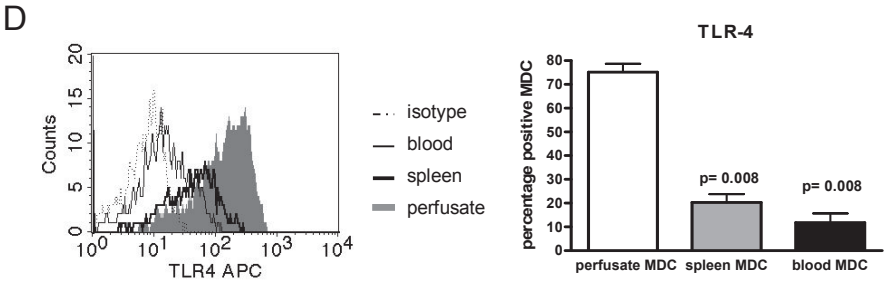
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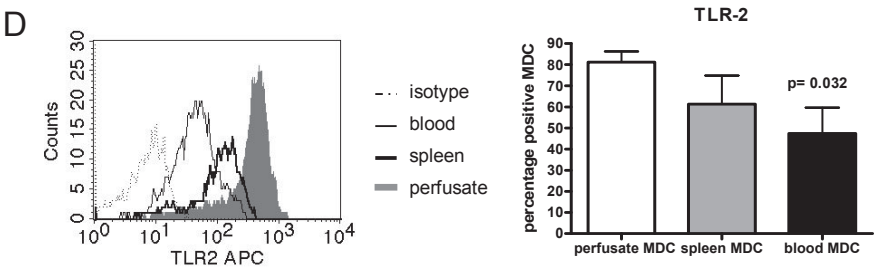

Figure 3. Cytokine production and TLR expression by liver graft and blood MDC. (A) MDC were purified from liver graft perfusates (n=7) and blood of healthy individuals (n=7) by immunomagnetic selection of BDCA-1$^+$CD19$^-$ cells. MDC (4x10^4 per well) were stimulated with LPS (1 μg/ml), poly (I:C) (20 μg/ml) and IFN-γ (1000 U/ml), or 4x10^4 CD40L-transfected J558 cells plus IFN-γ for 24 hours. Cytokine concentrations were determined in cell-free supernatants by ELISA. (B) Comparison of IL-10 and IL-12 production by liver graft MDC and blood MDC stimulated by poly (I:C) and IFN-γ. (C) MDC were purified from liver graft perfusates (n=4) and blood of healthy individuals (n=4) by flowcytometric sorting of BDCA-1$^+$CD20$^-$ cells, and 2x10^4 MDC were stimulated with a low concentration of LPS (100 pg/ml). After 18 hours cell-free supernatants were harvested and cytokine concentration determined by ELISA. (D) Expression of TLR-4 and TLR-2 on liver graft-, blood-, and splenic MDC. Representative histograms, and bar diagrams showing their expression on liver graft perfusate- (n=5), spleen- (n=5), and blood (n=5) BDCA-1$^+$CD20$^-$ MDC. P-values depicted are for comparison of perfusate MDC versus spleen or blood MDC.

MDC that detach from liver grafts upregulate homing receptors enabling migration into secondary lymphoid tissues upon activation

To characterize MDC that detach from liver grafts, we collected perfusates during vascular perfusion of liver grafts with albumin solution on the bench before transplantation. Previously, we have shown that these liver graft perfusates contain MDC that are immunophenotypically similar to hepatic MDC isolated from liver tissue, and differ from blood MDC in secreting high amounts of IL-10 upon LPS stimulation (28). To further establish their hepatic origin, we compared their expression of CD11a with blood MDC. CD11a is one of the components of the integrin LFA-1. It is highly expressed on liver lymphocytes, and mediates their adhesion to ICAM-1 on Kupffer cells and liver sinusoidal endothelial cells (30, 31). Figure 2A shows that CD11a expression was two-fold higher on BDCA-1$^+$CD20$^-$ MDC in liver graft perfusates compared to peripheral blood MDC, confirming their hepatic origin.

To study whether the donor-derived MDC that detach from liver grafts are able to home into T cell areas of recipient's lymphoid tissues, we analyzed their expression of CCR-7 and L-selectin. Both molecules are, together with LFA-1, essential for homing from the circulation into the T cell areas of lymph nodes (32, 33) and into splenic white pulp (34, 35).

CCR-7 was expressed on about 25% of perfusate MDC, similar to expression on blood MDC (Figure 2B). In contrast to blood MDC, perfusate MDC completely lack L-selectin expression (Figure 2C). However, upon stimulation *in vitro* with LPS in a concentration similar to that in the portal vein (100 pg/ml) (36.37); perfusate MDC upregulated L-selectin expression (Figure 2D). Comparable induction of L-selectin expression was observed upon stimulation of liver graft MDC with poly (I:C) and IFN-γ (data not shown). Together, these data demonstrate that MDC detaching from liver grafts become able to migrate into the T cell areas of recipient lymphoid tissues upon receiving a maturation signal. Therefore, to study the functional consequences of migrating liver graft MDC, we concentrated on stimulated liver graft perfusate MDC.

MDC that detach from liver grafts produce high amounts of pro- and anti-inflammatory cytokines and are highly responsive to LPS

Cytokine production profiles of purified perfusate MDC were compared with those of blood MDC. We used two types of pathogen-related stimuli to stimulate MDC (TLR-4 ligand LPS and TLR-3 agonist poly (I:C)), and one type of T cell stimulus (CD154- transfected cells). The latter two were combined with IFN-γ to provide optimal stimulation of IL-12 production (38). All three different stimuli resulted in much higher production of IL-10 by perfusate MDC compared to blood MDC (Figure 3A), demonstrating that this distinguishing property of hepatic MDC, that we and others reported in previous publications (28, 39), is independent of the mode of stimulation. In addition, liver perfusate MDC produced significantly more TNFα and IL-6 than blood MDC upon stimulation with LPS or poly (I:C) plus IFN- γ. Production of IL-12 was only observed in the presence of IFN-γ, and did not differ between hepatic and blood MDC. Interestingly, while blood MDC produced similar amounts of IL-10 and IL-12, liver graft MDC produced 20 times more IL-10 than IL-12 upon stimulation with poly (I:C) and IFN-γ (Figure 3B). The prominent capacity of liver graft MDC to produce IL-10 was not due to an effect of the storage of the liver graft in UW-preservation solution. When we incubated blood MDC for 8 hours at 4°C in UW-solution, and then stimulated them in culture medium at 37°C with LPS or poly (I:C) plus IFN-γ, or CD154 plus IFN-γ, they did not secrete higher amounts of IL-10 than untreated blood MDC (data not shown).

It has been reported that murine liver MDC exhibit a low expression of TLR-4, resulting in poor responsiveness to low amount of LPS (below 10 ng/ml) (26). In the experiments shown in Figure 3B, MDC were stimulated with a high concentration of LPS (1 μg/ml). To establish whether human liver MDC were also able to produce high amounts of cytokines upon stimulation with a lower and more physiological concentration of LPS, we compared cytokine production of liver perfusate MDC and blood MDC upon stimulation with 100 pg/ml LPS. Figure 3C shows that also in this condition liver graft MDC produced significantly higher amounts of IL-10 and TNFα than blood MDC, demonstrating that human hepatic MDC respond well to LPS in concentration similar to that present in portal blood. This result prompted us to

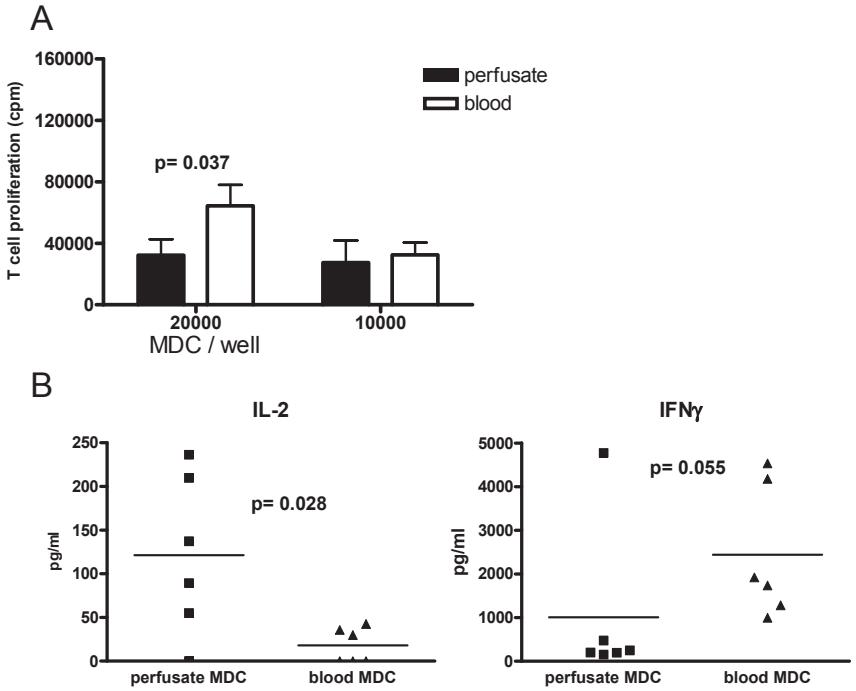

Figure 4. Allogeneic T cell stimulatory capacity of LPS activated liver graft and blood MDC. MDC were purified from liver graft perfusates (n=6) and blood of healthy individuals (n=6) by flowcytometric sorting, and $2x10^4$ or $1x10^4$ MDC per well were stimulated with a low concentration of LPS (100 pg/ml). After 18 hours MDC were washed, and $1.5x10^4$ allogeneic T cells were added. After 5 days 50 ul culture supernatant was harvested to quantify cytokine production, and [^{3}H]-thymidine incorporation was determined. (A) [^{3}H]-thymidine incorporation after 5 days of culture. (B) Production of IL-2, IL-4, IL-10 and IFN-γ was determined in cell-free supernatants harvested on day 5 from co-cultures of allogeneic T cells with $2x10^4$ LPS-stimulated MDC. IL-4 and IL-10 production was below detection limit (10 pg/ml).

compare the expression of TLR-4 on liver-, spleen, and blood MDC. As is shown in Figure 3D, human hepatic MDC express higher levels of TLR-4 than spleen- and blood MDC. TLR-2, another bacterial sensor expressed on the surface of MDC (40), also had higher expression on hepatic- than on spleen- and blood MDC. Collectively, these data show that MDC that detach from human liver grafts respond to various stimuli with the production of high amounts of IL-10, TNFα and IL-6, but low amounts of IL-12, and show no signs of LPS resistance.

MDC that detach from liver grafts are poor stimulators of Th1 cytokine production in allogeneic T cells

To determine the capacity of migrating liver graft MDC to activate allo-reactive T cells, we stimulated liver perfusate MDC with 100 pg/ml LPS and compared their allogeneic T cell stimulatory capacity with that of LPS stimulated blood MDC. Since the major immunological

barrier in organ transplantation is formed by allo-reactive memory T cells (41-43), we used unfractionated T cells in these experiments. Figure 4A shows that LPS stimulated liver graft MDC were well able to stimulate proliferation of allogeneic T cells, although in stimulations with $2x10^4$ MDC they were less potent compared to blood MDC. Most importantly, liver graft MDC from 5 out of 6 donors were very poor stimulators of IFN-γ production by allogeneic T cells compared to blood MDC (Figure 4B). IL-2 concentrations in cultures of T cells stimulated by liver graft MDC were higher than in cultures of T cells stimulated by blood MDC, probably reflecting higher consumption of IL-2 due to more proliferation of T cells in the co-cultures with blood MDC. No production of the T-helper 2 cytokine IL-4, neither of the immunoregulatory cytokine IL-10 was detected in both cultures of T cells with liver graft MDC and blood MDC.

DISCUSSION

The present study shows that leukocytes of donor origin, including T cells, B cells and MDC, migrate early after human LTx, but not after RTx, into the recipient's blood circulation. Secondly, we demonstrate that MDC that detach from human liver grafts acquire upon activation all receptors needed for homing from the circulation into T cell areas of recipient spleen and lymph nodes. Thirdly, MDC detaching from human liver grafts respond well to TLR-ligands, including LPS, and to T cell maturation signals, by production of high amounts of IL-6, IL-10 and TNF-α, but only minute amounts of IL-12. Finally, we show that activated liver graft MDC induce proliferation, but almost no IFN-γ production in allogeneic T cells.

According to the traditional paradigm, donor-derived MDC migrating from the organ grafts into the recipients are the prime stimulators of recipient T cells that recognize alloantigens via the direct pathway. On the other hand, in case of LTx, donor-derived MDC have been hypothesized to play a critical role in the development of spontaneous tolerance (21, 44). However, until now no formal proof of donor MDC migration into recipients has been provided in the clinical transplantation setting. A few research groups (45, 46), including ours (47), have shown that donor lymphocytes migrate into the recipient circulation after human LTx. The proportions of circulating T- and B cells that we observed to be of donor-origin early after LTx are comparable to those reported in these papers. However, none of these studied determined chimerism in circulating MDC after LTx. Moreover, to our best knowledge, no data have been published on donor leukocyte transfer after clinical transplantation of other organ grafts. Our observations show that transfer of donor leukocytes, including MDC, is much more prominent after clinical LTx than after RTx. The reasons for this difference are probably that the liver is a much larger organ than

the kidney, and is richer in immune cells (48-50). It has been calculated that murine livers contain 10-20 times more leukocytes than kidneys (12).

The MDC that detached from human liver grafts were partly CCR-7$^+$ but, in contrast to circulating MDC, invariable L-selectin negative. The same has been observed for CD8$^+$ T lymphocytes in human liver (31). Expression of CCR-7 on hepatic MDC may enable their migration to hepatic lymph nodes in the physiological situation. Sinusoidal endothelium and hepatic lymphatic endothelium secrete CCL-19 and CCL-21 (31, 51). Therefore, CCR-7 expression may enable hepatic MDC migration via the spaces of Disse to the portal tracts, and then via the hepatic lymphatics to the draining lymph nodes. This route has indeed been described as the physiological route of hepatic MDC migration (52, 53). However, after LTx, liver-draining lymphatics are disrupted, and MDC migration from the liver graft into the recipient is probably mainly via the blood circulation. For entry from the blood into secondary lymphoid tissues, like splenic white pulp and lymph nodes L-selectin expression is required (32-35). Our data show that a considerable proportion of liver graft MDC acquired L-selectin expression after activation with TLR-agonists or pro-inflammatory cytokine. Therefore, we propose that donor MDC from liver grafts enter recipient secondary lymphoid tissues, i.e. the tissues in which they can interact with recipient T cells, only after activation. Given the exposure of the liver graft to LPS from the commensal intestinal flora (36, 37), and the ischemia and reperfusion-related graft damage leading to the liberation of endogenous TLR-ligands and pro-inflammatory cytokines (54, 55), it is conceivable that liver graft MDC receive ample maturation signals after LTx.

Due to ethical constraints, it is not possible to study whether circulating donor MDC after human LTx indeed home into recipient secondary lymhoid tissues, but experimental animal studies have provided evidence that this occurs (13, 22, 23, 56).

Recently, we demonstrated that substantial numbers of donor-derived Foxp3$^+$ regulatory T cells migrate into recipients after human LTx (47). These donor-derived regulatory T cells were able to suppress allogeneic T cell responses, and may therefore contribute to induction of tolerance to the liver graft. Trying to elucidate how activated donor liver MDC may influence the recipient's immune reaction to the graft, we determined their capacity to stimulate allogeneic T cells. LPS activated liver graft MDC were able to stimulate allogeneic T cell proliferation (although slightly less effectively than blood MDC), but were poor stimulators of T cell IFN-γ production. Murine liver MDC exhibit a low expression of TLR-4 compared to splenic MDC, which was proposed to be due to continuous stimulation by gut-derived LPS, resulting in poor responsiveness to low amounts of LPS (26). In contrast, we found that human liver graft MDC showed a higher expression of TLR-4 compared to blood- and splenic MDC, and responded to LPS (both in high and low concentrations) by production of higher amounts of cytokines than blood MDC did. In addition, in a previous publication, we showed that a considerable proportion of liver graft perfusate MDC express the coreceptor for LPS, CD14, and strongly upregulate expression

of MHC class II and co-stimulatory molecules upon LPS stimulation (28). The weak ability of liver graft MDC to stimulate IFN-γ production by T cells can therefore not be attributed to LPS resistance.

The most striking difference in cytokine production between liver graft- and blood MDC was the high production of IL-10, which was observed with all types of stimuli used. Since IL-10 is known as a suppressor of IFN-γ production in T cells (57, 58), we hypothesize that the high amounts of IL-10 secreted by human hepatic MDC is the main factor contributing to their poor capacity to prime IFN-γ production in T cells. In the respect, human liver graft MDC show similarity to human Kupffer cells, which also produce, in addition to TNFα and IL-6, high amounts of IL-10 (59, 60).

It has been hypothesized (8) that spontaneous LTx tolerance in experimental animals is due to migration of large numbers of donor MDC from the graft into the recipient, resulting in profound early activation of allogeneic recipient T cells in recipient secondary lymphoid tissues (61), followed by their deletion due to activation-induced apoptotic death (15). Indeed, large numbers of apoptotic T cells have been observed in recipient lymphoid tissues and in infiltrates in liver grafts after experimental LTx, but not after RTx (62, 63). Our results are congruent with similar processes occurring after human LTx. Hepatic donor MDC migration occurring after human LTx but not after human RTx into recipient may stimulate expansion, but not effector function of recipient allogeneic T cells, ultimately leading to activation-induced apoptotic death in lymphoid tissues and/or in the graft. Indeed, early after human LTx, liver grafts often contain infiltrates of activated lymphocytes, even in the absence of any signs of graft damage (64, 65). These so-called "sub-clinical" rejection infiltrates may reflect a similar process of early immune activation as observed after LTx in experimental animals. Moreover, substantial numbers of apoptotic lymphocytes have been observed in post-transplant graft biopsies from human liver grafts, but not in human kidney grafts (66, 67). Apoptosis of human liver graft infiltrating lymphocytes is more profound during mild- than during severe acute rejections (68).

In conclusion, after human LTx, but not after RTx, donor-derived hepatic MDC migrate into the recipients. These MDC are able to stimulate allogeneic T cell proliferation, but not cytokine production, probably due to their prominent production of IL-10. We propose that early donor MDC migration after clinical LTx induces early expansion of allogeneic T cells followed by activation-induced cell death. This mechanism may account for the tolerogenic properties of human liver grafts.

ACKNOWLEDGEMENTS

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6

Impairment of circulating myeloid dendritic cells in immunosuppressed liver transplant recipients

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ABSTRACT

The aim of the present study was to elucidate the impact of liver transplantation (LTx) on myeloid dendritic cell (MDC) homeostasis. We observed a 3-fold reduction of circulating $CD1c^+$ MDC immediately after LTx ($n=16$; $p<0.01$), and normalization between 3 and 12 months after LTx. This decline was not due to recruitment of MDC into the liver graft, since numbers of MDC in post-LTx liver graft biopsies were not increased compared to pre-LTx biopsies ($n=7$). Moreover, no change in chemokine receptor expression on circulating MDC was observed, suggesting that their homing properties were not altered. Normalization of circulating MDC was associated with withdrawal of corticosteroid therapy, and not with changes in calcineurin inhibitor intake, indicating that corticosteroids are responsible for the observed changes in numbers of circulating MDC. During high-dose corticosteroid treatment early after LTx, circulating MDC showed a lowered maturation status with decreased expression of HLA-DR and CD86 compared to pre-LTx values ($p<0.01$). However, when MDC from blood of LTx recipients were matured *ex vivo*, they up-regulated HLA-DR and co-stimulatory molecules to a comparable extent as MDC from healthy individuals. In addition, *ex vivo* matured MDC from both groups had equal allogeneic T cell stimulatory capacity.

In conclusion, during the first months after LTx numbers and maturational status of circulating MDC are significantly impaired, probably due to a suppressive effect of corticosteroids on MDC. However, corticosteroid therapy does not imprint MDC with an intrinsic resistance to maturation stimuli.

INTRODUCTION

The earliest stage of the T cell response against allogeneic grafts consists of priming the recipient's T cells against donor antigens by dendritic cells (DC). In humans there are two main types of DC: myeloid DC (MDC) and plasmacytoid DC (PDC). Both donor- and host-derived MDC can activate allogeneic T cells after organ transplantation. Donor-derived MDC migrate from the graft via the blood circulation into the recipient's spleen and lymph nodes, and activate T cells of the recipient by direct presentation of donor allo-antigens (1-4). Host-derived MDC are recruited into the graft in response to ischemic injury (5), and subsequently migrate to the recipient's spleen and lymph nodes to prime recipient T cells by indirect presentation of donor-antigens (3). In contrast, PDC are in the transplantation setting probably involved in development of allograft tolerance (6, 7).

Whereas considerable knowledge is available on circulating donor-specific T cells after organ transplantation, only few studies have investigated changes in DC homeostasis. Longitudinal studies that have been performed, show that numbers of circulating MDC and PDC decrease immediately after cardiac- (8), kidney- (9, 10) and liver (LTx) (11) transplantation, but considerable differences in the duration of DC impairment have been reported. After renal- (9) and cardiac (8) transplantation, numbers of circulating MDC and PDC remained consistently lowered during the follow-up of 3 and 8 months. We reported that after LTx circulating PDC numbers decrease immediately and are only partially restored during the first year after transplantation (12). This was confirmed by cross-sectional studies which consistently showed that numbers of circulating PDC are reduced in patients late after organ transplantation compared to those in healthy controls (10, 13-15). Study results regarding the duration of MDC impairment after organ transplantation are, however, not uniform. Some studies reported reduced (10, 13), whereas others found normal (14, 15) numbers of circulating MDC in organ transplant recipients late after transplantation. Moreover, one study found that circulating MDC numbers were already restored to pre-transplant values 1 month after transplantation (11). Besides, it is unclear whether the maturational status and functional capacity of MDC are altered after organ transplantation.

We found that the decrease in circulating PDC numbers after LTx was due to induction of apoptosis in PDC by corticosteroids (12). Whether corticosteroid treatment is also responsible for the observed decline in circulating MDC numbers after organ transplantation is unknown. One cross-sectional study found that the decline in circulating MDC numbers after organ transplantation was associated with corticosteroid therapy (14), while two other studies did not (13, 15).

The aim of the present study was to establish the longitudinal course of numbers and maturational status of circulating MDC during the first year after LTx. In addition, we compared the functional capacity of blood MDC of LTx recipients with healthy indi-

viduals. Trying to explain our observations, we analyzed chemokine receptor expression on circulating MDC, quantified MDC in liver grafts, investigated associations between changes in circulating MDC and alterations in immunosuppressive therapy, and studied whether immunosuppressive drugs induce apoptosis of blood MDC.

MATERIALS AND METHODS

Patients and materials

Circulating MDC after LTx were analyzed in peripheral blood mononuclear cells (PBMC) obtained pre-, and at different time points posttransplantation from 16 LTx recipients transplanted between 1999 and 2001. Paraffin-embedded and frozen pre- and post-LTx liver graft biopsies were available from 7 patients. Pre-LTx biopsies had been collected at the end of the cold preservation of the graft. Post-LTx biopsies had been obtained as protocol biopsies or suspicion of graft dysfunction. The clinical characteristics of the included patients are summarized in Table I. Immunosuppressive therapy consisted of a calcineurin inhibitor (either Cyclosporin A or Tacrolimus) and corticosteroids. Before reperfusion the patients were given a single intravenous dose of 500 mg methylprednisolone (Solu-Medrol®, Pfizer, Capelle aan den IJssel, The Netherlands). During the first week after LTx intravenous prednisolone was given according to the following scheme: 100 mg on day 1 and 2, 75 mg on day 3 and 4, 50 mg on day 5 and 6 post-LTx. Thereafter treatment was changed to oral prednisone (15 mg per day) for the next 3 months. Subsequently, corticosteroid medication was slowly tapered over time, and was completely discontinued in 10/16 patients between 6 and 12 months (median: 10 months) after LTx. Ten patients were treated with CD25 antibody during the early post-transplant period.

For functional studies with purified blood MDC, fresh blood was collected from 8 LTx recipients at two weeks after transplantation, and from 11 healthy volunteers. These LTx recipients were transplanted for HBV cirrhosis (n=3), acute fulminant HBV infection (n=1), or HCV cirrhosis (n=4). Mean age was 47 (range: 23-61) years, and immunosuppressive therapy consisted of TAC or CsA supplemented with corticosteroids and CD25 antibody. The Medical Ethical Committee of the Erasmus Medical Center, Rotterdam approved the study protocol, and informed written consent was obtained from all patients.

Analysis of numbers and maturation status of circulating DC and monocytes

PBMC were isolated by Ficoll density centrifugation from heparinized venous blood, and stored in liquid nitrogen until analysis. After thawing, PBMC were incubated with combinations of the following monoclonal antibodies (mAb): CD1c-PE, CD14-PERCP, CD20-PERCP, CD80-FITC, CD86-APC, CCR7-FITC, CXCR4-FITC, anti-HLA-DR-FITC, or anti-HLA-DR-PERCP. MDC were defined as $CD1c^{+}CD14^{-}CD20^{-}$ cells. For determination

Table I. Characteristics of LTx recipients in which the longitudinal course of numbers and maturation status of circulating MDC were studied

	patients (n=16)
Age, yrs, mean (range)	48 (16 - 66)
Gender, male/female	7 / 9
Indication for liver transplantation	
Hepatitis B	3
Hepatitis C	3
PBC	3
PSC	3
Alcoholic liver cirrhosis	1
Insulinoma with liver metastasis	1
Auto-immune hepatitis	2
Immunosuppression	
CsA+prednisone	3
CsA+prednisone+αCD25	4
TAC+prednisone	3
TAC+prednisone+αCD25	6

of the relative numbers of monocytes and MDC, and for analysis of expression of HLA-DR and co-stimulatory molecules on monocytes, fifty thousand events were collected on a FACS Calibur flowcytometer. To analyze the expression of cell surface molecules on MDC, at least 200 $CD1c^+CD14^-CD20^-$ events were live-gated. Appropriate isotype control mAb were used to set gates for analysis. All mAb were obtained from BD Biosciences, Erembodegem, Belgium, except CD1c PE (Miltenyi Biotec, Bergisch Gladbach, Germany), CD80-FITC (Beckman Coulter Immunotech, Marseille, France), CCR7-FITC and CXCR4-FITC (R&D Systems, Abington, UK). Analysis was performed using CellQuest pro software.

Effect of immunosuppressive drugs on MDC survival *in vitro*

PBMC ($2x10^6$/ml) from healthy individuals were cultured for 18 hours in RPMI supplemented with penicillin (100 U/ml), streptomycin (100 µg/ml) (all from Gibco BRL Life Technologies, Breda, The Netherlands) and 10% fetal bovine serum (Hyclone, Logan, UT, USA), with or without CsA, TAC, prednisolone or methyl-prednisolone. After 18 hours expression of active caspase 3 was determined by flowcytometry after intracellular labeling with anti-active caspase 3-FITC mAb (BD Biosciences) using Intraprep permeabilization reagent (Beckman Coulter Immunotech). Before intracellular staining, surface antigens were labeled with CD1c-PE and CD20-APC mAb to determine MDC, CD14-PE mAb to determine monocytes, or anti-BDCA-4-PE (Miltenyi) and CD11c-APC (BD Biosciences) to determine $BDCA\text{-}4^+CD11c^-$ PDC.

Quantification of intragraft MDC and macrophages

Consecutive cryosections were stained with CD1c mAb (Miltenyi Biotec) and secondary peroxidase-conjugated goat anti-mouse Envision complex (Dako, Glostrup, Denmark) as previously described (4), or with DC-Lamp mAb (CD208; clone 104.G4) followed by rabbit anti-mouse antibodies and Alkaline-Phosphatase-Anti-Alkaline-Phosphatase complex (APAAP, Sertotec, UK) as previously described (16). Sections of paraffin-embedded biopsies were stained with CD68-Envision-peroxdiase (Dako, Glostrup, Denmark), after blocking for endogenous peroxidase by incubation with 0.2% sodium azide and 0.05% H_2O_2 in citric acid/phosphate-buffer (pH: 5.8), and antigen retrieval by Pronase (Sigma, Zwijndrecht, the Netherlands) incubation for 15 minutes and 37°C. CD1c and CD68 mAb were visualized using 3-amino-9-ethylcarbazole, and DC-Lamp mAb using Fast Blue/ASBI-phosphate substrate. Sections were only examined if stains with appropriate isotype-matched control mAb were negative. Positive cells were simultaneously counted by two observers. Numbers of positive cells in the parenchyma were counted in six randomly chosen microscopic fields of 400 times magnification. In addition, positive cells in portal fields were counted. If the difference in counts between the two observers exceeded 15%, recounting was done. For each biopsy the mean number of positive cells per portal field and per microscopic field of 400 times magnification of the parenchyma were calculated, and these numbers were used in the analysis.

Purification of blood MDC and determination of their functional capacity

MDC were isolated from blood of LTx recipients or healthy volunteers by immunomagnetic selection of $CD1c^+CD20^-$ cells, as described previously (17). The purity of MDC (defined as $CD1c^+$ $CD20^-$ cells) isolated from LTx recipients was 94±4% and from healthy individuals 96 ± 2%, and viability (determined by trypan blue exclusion) was 88 ± 4 and 89± 3%, respectively. For determination of their maturational capacity, MDC ($1x10^4$/200µl) were stimulated for 24 hours at 37°C with TNF-α (25 ng/ml) and IL-1β (50 ng/ml) (both from Strathmann Biotech, Hannover, Germany) in RPMI supplemented with penicillin, streptomycin, 10% fetal bovine serum, and GM-CSF (500U/ml; Leucomax, Novartis Pharma, Arnhem, The Netherlands). Before and after culture, MDC were labeled with CD1c-PE, CD20-PERCP, anti-HLA-DR-FITC, CD80-FITC, and CD86-APC, or appropriate isotype control mAb, and analyzed by flowcytometry.

To determine the capacity of MDC to acquire allogeneic T cell stimulatory capacity, graded numbers (5, 2.5, and 1.25 x 10^3 cells per well of a 96-well flat bottom plate) were stimulated to mature with TNF-α, IL-1β and GM-CSF for 24 hours. Thereafter, the culture medium was aspirated, MDC were washed two times to remove additives, and 1.5 x 10^5 allogeneic T cells were added. In all experiments the same batch of T cells was used, which were enriched by nylon wool filtration of PBMC from a buffy coat of a healthy blood donor and contained 83% $CD3^+$ cells. After 5 days, T cell proliferation was assessed

by determination of the incorporation of 0.5 µCi [^{3}H]thymidine (Radiochemical Centre, Amersham, Little Chalfont, UK) during 18 hours. Each condition was tested in triplicate from which means were calculated. These means were used in the analyses.

Statistical analysis

All data are expressed as mean ± SEM. Numbers of DC and macrophages in pre- and post-LTx biopsies were compared using the Wilcoxon test for paired data. All other differences were analyzed after log-transformation of data to obtain normal distribution, using either the independent samples t-test or the t-test for paired samples. Statistical analysis was performed using SPSS version11.0 software. A two-sided p-value of less than 0.05 was considered significant.

RESULTS

The longitudinal course of numbers of circulating MDC after LTx in relation to immunosuppressive medication

The relative numbers of circulating MDC and monocytes were determined in PBMC from 16 LTx recipients. To preclude effects of rejection treatment, only patients who had never been treated for acute rejection were included. The frequencies of CD1c$^+$ MDC in liver transplant recipients before transplantation were comparable to those in healthy individuals which we reported previously (17). Figure 1A shows that as early as 3 days after LTx the relative numbers of circulating MDC decreased 3-fold compared to pre-LTx values. This decline persisted up to 3 months post-LTx, and between 3 and 12 months after LTx the numbers of circulating MDC were restored to pre-transplant values.

To investigate the cause of the changes in numbers of circulating MDC, we compared longitudinal alterations in immunosuppressive therapy with MDC numbers (Figure 1C).

All LTx patients received prednisone in combination with either CsA or TAC. In the CsA treated group the intake of CsA was reduced between 1 and 3 months after LTx. This reduction did not coincide with recovery of circulating MDC. MDC numbers even tended to decrease in the period of CsA reduction (Figure 1B; p=0.43). During the period that MDC numbers were restored to pre-LTx values, i.e. between 3 and 12 months, CsA dosage was only slightly reduced. These data was confirmed in the TAC treated group, since between months 3 and 12 MDC numbers also increased (from 0.27±0.5 to 0.45±0.07; p=0.04) in this group, even though there were no alterations in the TAC dosage. Together, these observations support that the changes in circulating MDC numbers after LTx were not related to treatment with calcineurin inhibitors.

However, the recovery of MDC numbers between 3 and 12 months after LTx did coincide with tapering of prednisone treatment. In the subgroup of patients in which prednisone treatment was completely stopped in this time period (n=10), relative numbers of circulat-

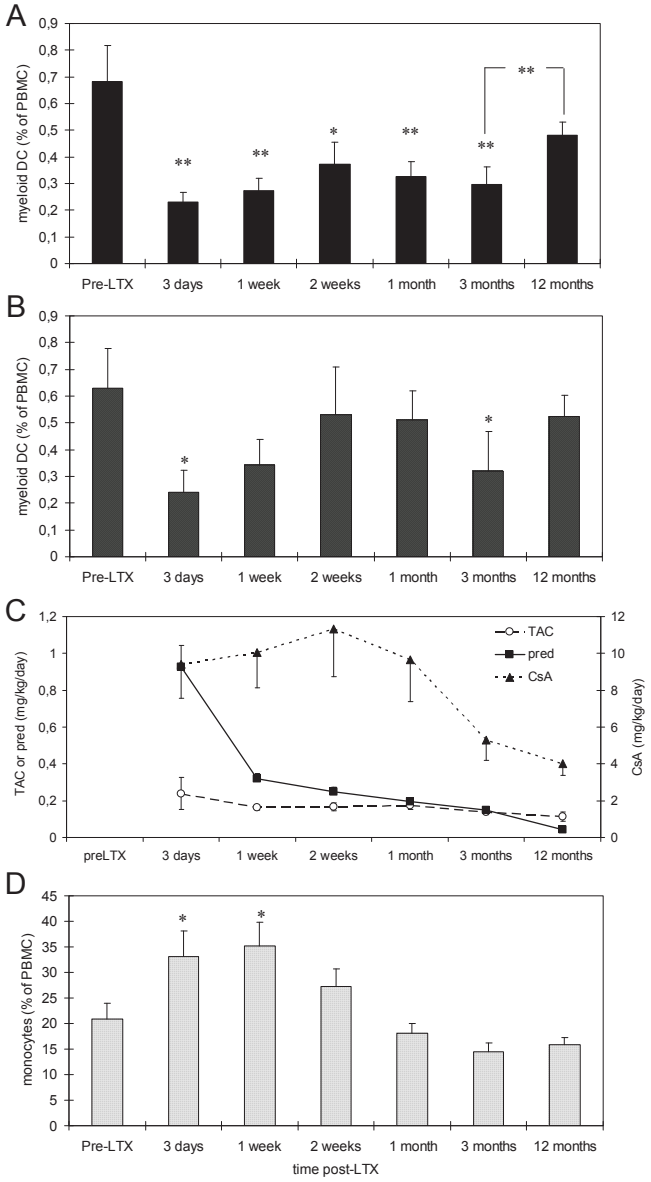

Figure 1. Longitudinal course of the numbers of circulating MDC and monocytes in LTx recipients. (A) Relative numbers of $CD1c^{+}CD14^{-}CD20^{-}$ MDC were determined in 16 LTx recipients at baseline, 3 days, 1 and 2 weeks, 1, 3 and 12 months after transplantation. (B) Idem for the LTx recipients that were treated with CsA (n=7). (C) Treatment doses of TAC, CsA and prednisolone/prednisone in the LTx recipients at the indicated time points. Left y-axis indicates daily TAC or prednisone doses, and right y-axis daily CsA dosis. (D) Relative numbers of $CD14^{+}$ monocytes in the 16 LTx recipients. ** $p<0.01$; * $p<0.05$ in comparison with pre-LTx values, or between indicated time-points.

ing MDC increased from 0.28 ± 0.10 % of PBMC at 3 months to 0.50 ± 0.05 % at 12 months after LTx (p=0.008). In the remaining 6 patients, prednisone intake was tapered (from 0.17 ± 0.03 mg/kg/day at 3 months to 0.11 ± 0.04 mg/kg/day at 12 months), which coincided with an increase in numbers of circulating MDC from 0.32 ± 0.08 % to 0.45 ± 0.12% of PBMC (p=0.04). This suggests that the alterations in numbers of circulating MDC after LTx may be related to corticosteroid treatment. The strong decline in MDC numbers immediately after LTx may be caused by high-dose prednisolone treatment during and shortly after transplantation.
In contrast to MDC, the relative numbers of circulating monocytes increased immediately after LTx (Figure 1D), and normalized again after 2 weeks, showing that the immunosuppressive therapy did not reduce all types of circulating APC.

Numbers of intragraft MDC and macrophages before and after LTx

To investigate whether the decline in circulating MDC after LTx resulted from MDC-recruitment into the liver graft, MDC and, for comparison, $CD68^+$ monocytes/macrophages were immunohistochemically quantified in liver graft biopsies taken before and after LTX. Paired pre- and post-LTx biopsies were available from 7 patients. The post-LTx biopsies had been taken on the average 12 (range: 6 – 24) days after transplantation, and did not show significant histological rejection activity (mean RAI-score 2; range: 0 - 5). CD1c mAb was used as a marker for both immature and mature liver MDC (4, 16, 18), and DC-Lamp as a marker for mature MDC (19). Figure 2A shows that $CD1c^{bright}$ MDC were predominantly located in portal fields, while $CD68^+$ macrophages/monocytes were observed both in portal fields and parenchyma. In the parenchyma these represent Kupffer cells. In post-LTx biopsies additional faint staining with CD1c mAb was observed in the parenchyma, in a pattern reminiscent of Kupffer cell staining. Therefore, only $CD1c^{bright}$ cells were considered as MDC. DC-$Lamp^+$ mature MDC were only sporadically observed both before and after LTx (not shown). Quantification of $CD1c^{bright}$ and DC-$Lamp^+$ MDC showed that their numbers were not significantly increased in post-LTx compared to pre-LTx liver graft biopsies (Figure 2B). In contrast, numbers of intragraft $CD68^+$ monocytes/macrophages increased after transplantation, the increase being significant in portal fields and borderline significant in the parenchyma. These data show that it is unlikely that the decline in circulating MDC after LTx is due to accumulation in the liver graft.

Expression of chemokine receptors on circulating MDC before and after LTx

To elucidate whether the migratory potential of circulating MDC changed after LTx, expression of CCR-7, the chemokine receptor responsible for homing to secondary lymphoid tissues (20), and of CXCR-4, the receptor for CXCL-12 which is constitutively expressed in lymphoid and non-lymphoid tissues (21, 22) including liver grafts (23), were analyzed. Before LTx, 56±9% of circulating MDC expressed CXCR-4 and 12±4% expressed CCR-7.

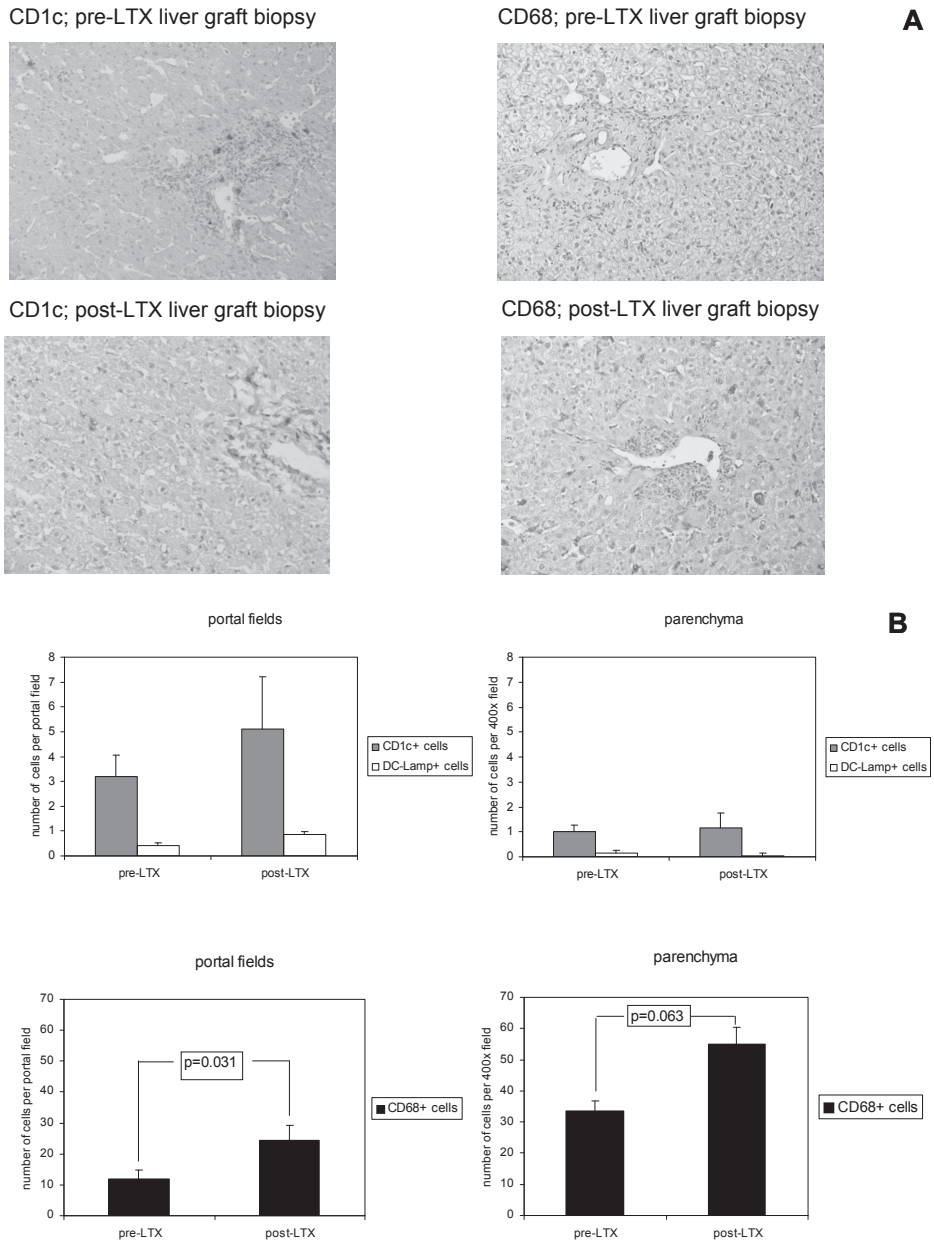

Figure 2. MDC and macrophages/monocytes in pre- and post-LTx liver graft biopsies. (A) Photomicrographs showing CD1c-stains of cryo-sections and of CD68-stains of paraffine-sections from pre- and post-LTx liver graft biopsies (200 times magnification). (B) Numbers of $CD1c^{bright}$ and $DC\text{-}Lamp^{+}$ MDC and $CD68^{+}$ monocytes/macrophages in paired pre- and post-LTx liver graft biopsies from 7 LTx-patients. Positive cells were quantified per portal field and per microscopic field of 400x magnification in the parenchyma as described in the Materials and Methods section.

Expression of these chemokine receptors measured at 3 days, and 1, 2 and 4 weeks after LTx, was not altered, suggesting that the decrease in relative numbers of circulating MDC is not due to enhanced homing capacity to secondary lymphoid organs (data not shown).

Effect of immunosuppressive drugs on MDC survival

Previously, we reported that PDC are very sensitive to apoptosis induction by corticosteroids (12). To study the pro-apoptotic effects of the immunosuppressive drugs on blood MDC, PBMC were cultured in the presence or absence of immunosuppressive drugs, and after 18 hours expression of the active form of caspase 3, the most downstream effector caspase, was determined in MDC, and for comparison also in monocytes and PDC. The effects of both corticosteroids used in the treatment of the LTx recipients (prednisolone and methyl-prednisolone) were studied. Different concentrations, corresponding with expected plasma concentrations in the patients were tested. Figure 3A shows that prednisolone or methyl-prednisolone did not induce expression of active caspase-3 in MDC or monocytes, but only in PDC (Figure 3A). In addition, relative numbers of MDC, monocytes and PDC were quantified after the cultures. Corticosteroids had almost no effects on the survival of MDC or monocytes *in vitro* (Figure 3B). In contrast, survival of PDC was clearly impaired, in accordance with our previous data (12). No effects of CsA or TAC on expression of active caspase 3 in MDC, monocytes or PDC, or on the survival of these cells, were observed (data not shown). Together, these data show that the reduction in circulating MDC after LTx, in contrast to the decline in PDC, is not due to impairment of MDC survival by immunosuppressive drugs.

Maturation status and functional capacity of circulating MDC after LTx in relation to immunosuppressive therapy

The expression of CD80 on circulating MDC was very low at baseline (0.6±0.4 % and positive MDC), and did not change after transplantation. In contrast, all circulating MDC expressed HLA-DR, and 71±4% of circulating MDC expressed CD86 before LTx. The expression of both molecules on circulating MDC was significantly reduced during the first 2 weeks after LTx (Figure 4A). A similar pattern was observed for the expression of HLA-DR and CD86 on circulating monocytes (Figure 4B). These data show that the maturation status of circulating MDC and monocytes was significantly reduced during the early post-transplant period. Comparison with the immunosuppressive drug regimen (Figure 1B) suggests that the depressed maturation status of MDC and monocytes may be related to high dose corticosteroid intake during the first few weeks after transplantation.

We next examined whether circulating MDC early after LTx were intrinsically impaired in their maturational capacity, or could still respond to maturation stimuli *ex vivo* in the absence of immunosuppressive drugs. For this purpose, MDC were purified from blood collected prospectively from eight LTx recipients at two weeks after transplantation, and from blood of 11 healthy individuals. MDC from both sources were stimulated *ex vivo* for

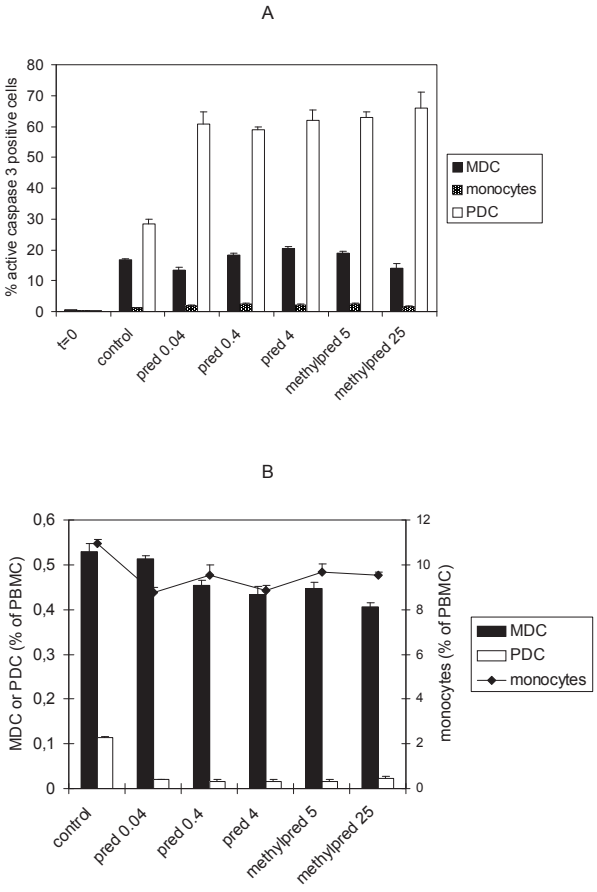

Figure 3. Effects of corticosteroids on survival of and active caspase 3 expression in MDC, monocytes and PDC. (A) After 18 hours incubation of PBMC from a healthy individual with different concentrations of corticosteroids, percentages of MDC, monocytes and PDC expressing active caspase 3 were determined by flow cytometry. Prednisolone was tested in a concentration of 0.4 µg/ml, which corresponds to the peak plasma level reached during daily treatment with 10 mg prednisone (37), at a ten-fold higher concentration because immediately after LTx the patients had been treated with daily doses of 100 mg, and at a ten-fold lower concentration. Methyl-prednisolone was tested at high concentrations which corresponded with the range of peak plasma concentrations reached after treatment with 500 mg, according to the manufacturer. Data are means with SEM of triplicate incubations from one experiment out of two independent experiments. t=0 means: expression before culture. Control = after incubation without corticosteroid; pred = after incubation with prednisolone; methylpred = after incubation with methylprednisolone. Concentrations (µg/ml) used are depicted in the numbers beneath x-axis. (B) Relative numbers of $CD1c^{+}CD20^{-}$ MDC, $CD14^{+}$ monocytes and $BDCA\text{-}4^{+}CD11c^{-}$ PDC were determined by flow cytometry after culture with different concentrations of corticosteroids. Data are means with SEM of tripicate incubations from one experiment out of three independent experiments. Left y-axis indicates percentages of MDC or PDC, and right y-axis percentages of monocytes within PBMC.

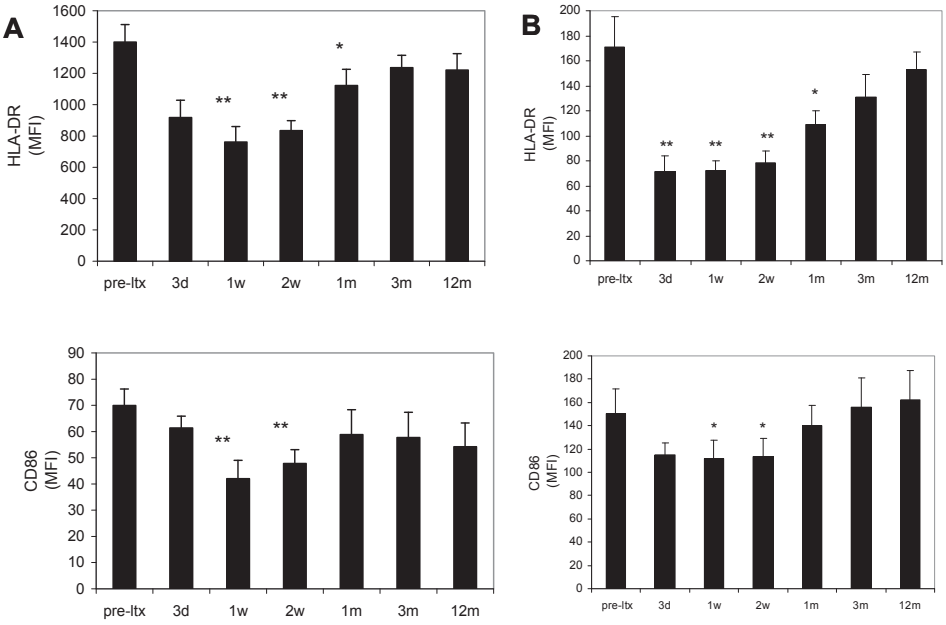

Figure 4. Maturation status of circulating MDC and monocytes after LTx. (A) Expression of HLA-DR and CD86 on circulating $CD1c^{+}CD14^{-}CD20^{-}$ MDC in 16 LTx recipients, depicted as mean fluorescence intensity (MFI). (B) Expression of HLA-DR and CD86 on circulating $CD14^{+}$ monocytes in 16 LTx recipients. ** p<0.01; *P<0.05 compared to pre-LTx values.

24 hours with proinflammatory cytokines (TNF-α and IL-1β). Figure 5A shows that MDC from LTx recipients and healthy individuals equally responded to the pro-inflammatory cytokines with up-regulation of HLA-DR and CD80. Only up-regulation of CD86 was slightly impaired in MDC from the LTx recipients. The capacity of the *ex vivo* matured MDC from LTx recipients and from healthy individuals to stimulate proliferation of allogeneic T cells was similar (Figure 5B). Together, these data show that circulating MDC of LTx recipients are not refractory to maturational stimuli, but functionally competent after they have been removed from the environment with immunosuppressive drugs.

DISCUSSION

The first key observation in this study is the reduction of the numbers and attenuation of the maturation status of circulating MDC after LTx. The maturation status was restored after 1 month, but the lower numbers of circulating MDC persisted until at least 3 months

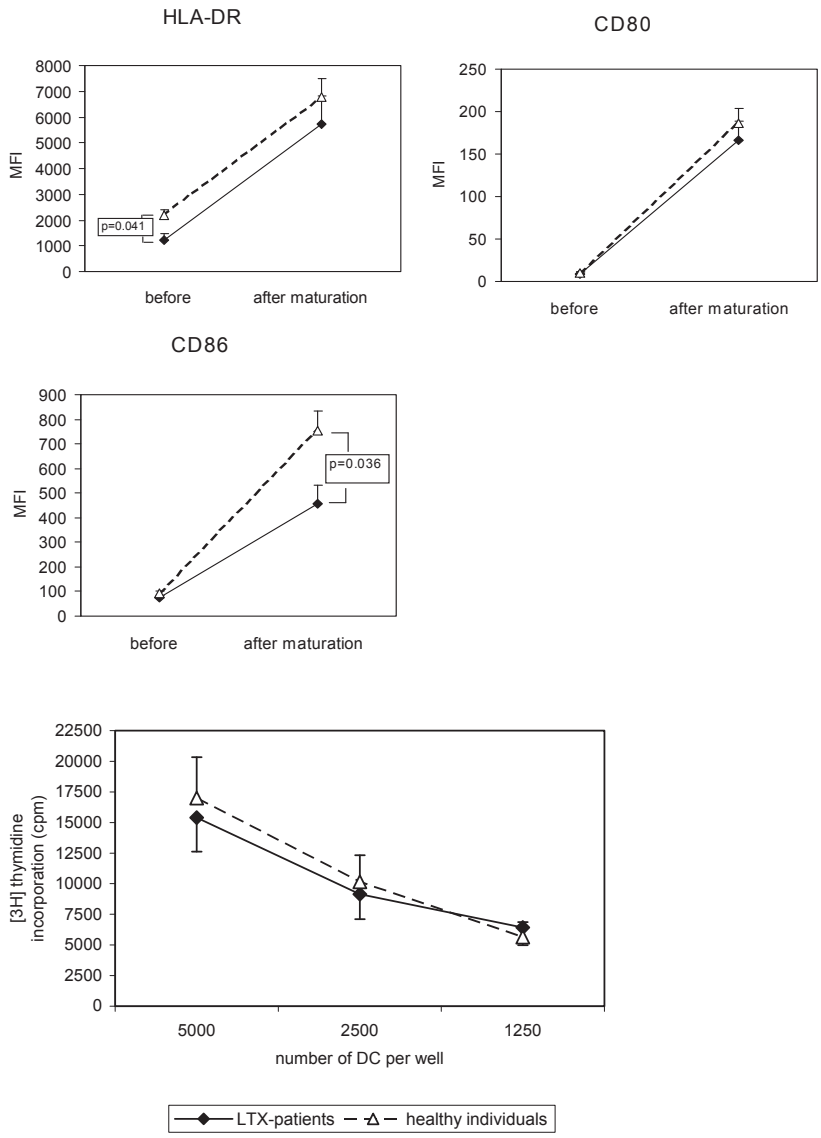

Figure 5. Comparison of maturational capacity and allogeneic T cell stimulatory capacity of purified blood MDC from LTx recipients and healthy individuals. MDC were immunomagnetically purified from blood collected 2 weeks after transplantation from 8 LTx recipients, and from blood collected from 11 healthy individuals. MDC were stimulated *ex vivo* with TNF-α and IL-1β for 24 hours. (A) Expression of HLA-DR, CD80, and CD86 before and after stimulation. Closed diamonds connected by solid lines depict expression on MDC from LTx recipients; open triangles with dotted lines depict expression on MDC from healthy individuals. (B) Allogeneic T cell stimulatory capacity after *ex vivo* maturation. Allogeneic T cells ($15x10^4$) were added to graded numbers of *ex vivo* matured MDC. After 5 days [^{3}H]-thymidine incorporation was determined.

after LTx. The second key observation is that the circulating MDC in LTx recipients nevertheless were functionally competent and responded well to maturation stimuli *ex vivo*.

Several factors might contribute to the decline in circulating MDC after LTx

First of all, the reduction could be due to recruitment of these cells into the liver graft. This possibility is contradicted, however, by our observation that MDC did not accumulate in the liver graft during the early post-transplant period, when circulating MDC numbers were most profoundly decreased.

Secondly, the reduction of circulating MDC might be caused by increased potential of these cells to home into lymphoid tissues. Immunosuppressive drugs can influence chemokine receptor expression (24) and migration of MDC (25). However, no change in the expression of CCR-7, the chemokine receptor responsible for homing to secondary lymphoid tissues (20), and of CXCR-4, the receptor for the homeostatic chemokine CXCL-12 which is constitutively expressed in many lymphoid and non-lymphoid tissues (21, 22), was observed upon transplantation. Although migration of circulating MDC into tissues might also be dependent on other factors, these results do not support the possibility that the decrease of circulating MDC after LTx is due to altered homing properties.

A third explanation for the reduced numbers of circulating MDC may be that immunosuppressive drug treatment negatively influence their survival. Though, neither corticosteroids nor CsA or TAC affected survival of blood MDC *in vitro*, nor induced expression of the active form of effector caspase 3. These observations are in agreement with data showing that these drugs do not induce apoptosis in MDC derived from monocytes (26) or $CD34^+$ hematopoietic progenitors *in vitro* (24, 27).

Fourthly, the decrease in circulating MDC numbers might be due to the surgical intervention. Indeed, numbers of circulating MDC are lowered immediately after abdominal surgery (9, 28), but this reduction is only temporary, and MDC counts normalize within 4 days (28).

Finally, immunosuppressive drugs may impair the generation of MDC from their precursors. The longitudinal association with corticosteroid intake strongly suggests that the impairment in circulating MDC is caused by corticosteroid treatment. *In vitro*, it has been clearly established that corticosteroids inhibit the differentiation of MDC from monocytes (29, 30) and from $CD34^+$ hematopoietic progenitors (27) with concomitant increased generation of macrophages (31). Moreover, *in vivo,* corticosteroid treatment resulted in reduced numbers of MDC and increased numbers of macrophages in the spleen in mice (32). In addition, corticosteroids inhibit maturation of MDC *in vitro*, suppressing the up-regulation of HLA- and co-stimulatory molecules (33, 34). Likewise, corticosteroids suppress maturation of monocytes *in vitro* (35). Thus, the observed decrease in HLA-DR- and CD86 expression on circulating MDC and monocytes may also be caused by corticosteroid treatment. Therefore, we hypothesize that the lowered numbers and maturation

status of circulating MDC after LTx is largely due to inhibition of their generation and maturation by corticosteroid treatment. This hypothesis in congruent with the observed increase in numbers of monocytes in the circulation and monocytes/macrophages in the graft after LTx, which may, at least partially, be due to suppression of their differentiation into MDC. Since all LTx recipients in our centre are treated with corticosteroids, we were unable to test this hypothesis formally.

Previous cross-sectional studies in LTx- and kidney transplant recipients (13-15) were discordant as to whether corticosteroid therapy influences the numbers of circulating MDC. These studies investigated the association between corticosteroids and circulating MDC several years after transplantation, when only low doses of corticosteroids are given. In contrast, by investigating the longitudinal course of circulating MDC early after transplantation, the present study gave the opportunity to observe a strongly suggestive association between corticosteroid therapy and circulating MDC.

Although circulating MDC during the first few weeks after LTx showed an impaired maturational status, these cells were functionally fully competent. Upon isolation they matured in a comparable way in response to pro-inflammatory cytokines to T cell stimulatory APC as MDC from non-treated individuals did. Apparently, corticosteroid therapy does not result in MDC that are refractory to maturation stimuli. The effect of corticosteroid treatment on MDC is thus quite different from the effect of chronic infection with e.g. HBV, which results in an intrinsic impairment, making MDC resistant to maturation stimuli *ex vivo* (18). This observation shows that circulating MDC in organ transplant recipients treated with corticosteroids differ from the "corticosteroid DC" that have been generated *in vitro* from monocytes in the presence of corticosteroids. The latter are refractory to maturation stimuli, and unable to acquire potent allogeneic T cell stimulatory capacity (30, 36). Indeed, "corticosteroid DC" are macrophage-like cells, with a high expression of CD14 and no expression of CD1a (29-31).

In conclusion, LTx recipients have during the first few weeks after transplantation reduced numbers of circulating MDC with an impaired maturational status. The paucity of circulating MDC persists up to at least three months after transplantation. Our results strongly suggest that the MDC impairment is caused by corticosteroid treatment. However, corticosteroid therapy does not result in the generation of maturation-resistant tolerogenic MDC. The unique role of corticosteroids in immunosuppressive protocols may be related at least partly to their inhibitory effects on MDC, thereby suppressing allogeneic T cell activation by the graft in it's earliest phase, namely by inhibition of donor antigen presentation. Therefore, in the design of corticosteroid-free immunosuppressive protocols it should be realized that omission of a drug that suppresses MDC requires compensational immunosuppressive drug treatment to avoid rejection.

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7

Dexamethasone transforms LPS-stimulated human blood myeloid dendritic cells (MDC) into MDC that prime IL-10 production in T cells

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Immunology, accepted

ABSTRACT

Myeloid dendritic cells (MDC) play an important role in antigen-specific immunity and tolerance. In transplantation setting donor-derived MDC are a promising tool to realize donor-specific tolerance. Current protocols enable generation of tolerogenic donor MDC from human monocytes during 1-week cultures. However, for clinical application in transplantation medicine, a rapidly available source of tolerogenic MDC is desired. In this study we investigated whether human blood MDC could be transformed into tolerogenic MDC using dexamethasone (dex) and lipopolysaccharide (LPS). Human blood MDC were cultured with dex and subsequently matured with LPS in the presence or absence of dex. Activation of MDC with LPS after pre treatment with dex did not prevent maturation into immunostimulatory MDC. In contrast, simultaneous treatment with dex and LPS yielded tolerogenic MDC, that had a reduced expression of CD86 and CD83, that poorly stimulated allogeneic T cell proliferation and production of Th1 cytokines, and primed production of the immunoregulatory cytokine IL-10 in T cells. *In vitro*, however, these tolerogenic MDC did not induce permanent donor-specific hyporeponsiveness in T cells. Importantly, tolerogenic MDC obtained by LPS stimulation in the presence of dex did not convert into immunostimulatory DC after subsequent activation with different maturation stimuli.

In conclusion, these findings demonstrate that combined treatment with dex and LPS transforms primary human blood MDC into tolerogenic MDC that are impaired to stimulate Th1 cytokines, but strongly prime the production of the immunoregulatory cytokine IL-10 in T cells, and are resistant to maturation stimuli. This strategy enables rapid generation of tolerogenic donor-derived MDC for immunotherapy in clinical transplantation.

INTRODUCTION

Induction of immunological tolerance, specific for transplant- or self-antigens while maintaining reactivity to other antigens, is an important goal in transplantation medicine and auto-immunity.

Myeloid dendritic cells (MDC) are the most potent antigen presenting cells and have the unique ability to initiate primary T cell responses (1). However, whereas mature MDC have a potent capacity to stimulate T cells, immature MDC poorly stimulate effector T cells which is associated with the induction tolerance (1).

This unique property of immature DC has been utilized in experimental animal transplantation models in which transfer of immature donor-derived MDC prolonged allograft survival (2, 3). However in order to use immature MDC as immunotherapy in humans it is pivotal that these MDC are refractory to maturation stimuli, since humans are constantly exposed to inflammatory stimuli from pathogens and other environmental danger signals that can trigger MDC maturation, leading to T cell activation instead of T cell tolerance (4).

Glucocorticoids are widely used as potent immunosuppressive and anti-inflammatory drugs to prevent allograft rejection and to treat autoimmune and allergic diseases (5, 6). Glucocorticoids affect growth, differentiation and function of many cell types, such as T cells, macrophages, monocytes and MDC (7, 8). Several studies investigated the feasibility of using glucocorticoids for developing tolerogenic MDC. It has been demonstrated that glucocorticoids are able to induce tolerogenic MDC when present during differentiation of human monocytes into MDC. The resulting monocyte-derived MDC (Mo-DC) remained immature upon exposure to maturation signals, as demonstrated by a low expression of the co-stimulatory molecules and a reduced T cell stimulatory capacity (8, 9). Importantly, these MDC were also able to induce hyporesponsiveness in alloreactive memory T cells by lack of costimulation and active suppression by B7-H1 and IL-10 (10).

In the transplantation setting, a major obstacle for induction of tolerance is the high frequency of pre-existing allo-reactive memory T cells that recognize donor HLA via the direct pathway, in other words presented on donor cells (11, 12). To induce hyporesponsiveness in these T cells, immunotherapy with donor-derived tolerogenic Mo-DC is an interesting option. However, in clinical transplantation practice, immunotherapy with glucocorticoid-induced tolerogenic Mo-DC will be limited to living donations, as culturing these MDC from monocytes will minimally take six days. Since most acute rejections occur within days, it is required to start immunotherapy directly after transplantation. A faster way of obtaining tolerogenic MDC could be the use of immature donor blood MDC that do not require 6 day long differentiation. However, to our knowledge, no studies have been done on the effects of glucocorticoids on primary human blood MDC.

Here, we studied whether pretreatment of freshly isolated primary immature human blood MDC with the synthetic glucocorticoid dexamethasone (dex), or combined treatment with dex and the Toll-like Receptor-4 agonist lipopolysaccharide (LPS), leads to the induction of stable tolerogenic MDC. Immunophenotype, cytokine production and T cell stimulatory capacity of dex-treated MDC were assessed. In addition, it was determined whether dex-treated human blood MDC were able to induce hyporesponsiveness in allogeneic T cells. To investigate whether glucocorticoid treatment had a sustained effect, dex-treated blood MDC were restimulated with different maturation stimuli.

MATERIALS AND METHODS

Antibodies

The following mAbs were used: IgG1-FITC, IgG1-PE, IgG1-APC, IgG1-PerCP-cy5.5, IgG2a-PerCP, CD4-PerCP, CD19-FITC, CD20-APC, CD40-APC, CD14-PE, HLA-DR-PerCP, and CD86-APC from Becton and Dickinson, Heidelberg, Germany; CD45-FITC, CD3-PE, CD56-APC, CD80-FITC from Beckman Coulter Immunotech, Marseille, France; CD8-APC from Dako, Glostrup, Denmark; CD83 FITC from Caltag Laboratories, Burlingame, California, USA; anti-BDCA-1 PE, CD19- and CD14 microbeads, anti-PE microbeads from Miltenyi Biotec, Bergisch Gradbach, Germany.

Isolation of human blood MDC and T cells

By means of Ficoll Isopaque gradient separation (Amersham Biosciences, Roosendaal, The Netherlands), peripheral blood mononuclear cells (PBMC) were isolated from buffycoats obtained from healthy blood donors (Sanquin Blood bank, The Netherlands). The study was approved by the Dutch blood transfusion organization Sanquin, and all donors gave written informed consent to use their buffy coat for research according to the ethical rules of blood donation in the Dutch blood donation law. MDC were isolated by positive selection of BDCA-1$^+$ cells after B cell depletion using magnetic microbeads, as previously described.(13) Purity of the isolated MDC was 96±4% (BDCA-1$^+$CD20$^-$ cells determined by flowcytometry) and viability was 96±2% determined by Trypan blue exclusion.
A batch of T cells was purified from buffy coat PBMC by incubation with CD14-PE, anti-BDCA1-PE and CD19 microbeads, and subsequently with anti-PE microbeads for 15 min at 4 °C. T cells were enriched by negative selection over a Large Seperation column using a MidiMACS separation device (Miltenyi Biotec) and contained 87% CD3$^+$ T cells and 11% CD56$^+$ cells.

Generation of control-, predex- and dex MDC

Isolated MDC (300.000 MDC/well in 200 µl) were cultured with or without 1 µM dexamethasone (dex) (Pharmacy, ErasmusMC, Rotterdam) for 18 hours in RPMI$^+$ with 10% FCS (Hyclone, Logan, UT, USA), penicillin (100U/ml), and streptomycin (100 ug/ml; Gibco BRL life technologies, Breda, The Netherlands) supplemented with 500 U/ml GM-CSF (Leucomax. Novartis Pharma, Arnhem, The Netherlands). Thereafter MDC were extensively washed and matured with 100 pg/ml lipopolysaccharide (LPS) (Sigma, Zwijndrecht, The Netherlands) for 24 hours. At this point MDC that had been cultured with dex were divided into two conditions; part of the MDC were matured in absence of dex (predex MDC) and part of the MDC were matured in presence of 1 µM dex (dex MDC). MDC cultured and matured in absence of dex are referred to as control MDC. After maturation MDC were immunophenotyped, and their cytokine production and allogeneic T cell stimulatory capacity were assessed. Recovery and viability were determined by trypan blue exclusion.

Second stimulation of control- and dex MDC

Control and dex MDC (300.000 MDC/well in 200 µl) were given a second maturation stimulus consisting of either 100 pg/ml LPS, 50 pg/ml TNF-α and 50 pg/ml IL-1β (both from Strathmann Biotech, Hannover, Germany) or CD40L-transfected J558 plasmacytoma cells(14) (5000 J558 cells) for 24 hours. After 24 hours of culture with the second maturation stimulus MDC were harvested and their immunophenotype, cytokine production and T cell stimulatory capacity were assessed.

Immunophenotyping of MDC

The following antibody-combinations were used to determine maturation of MDC: anti-BDCA1-PE in combination with CD80-FITC, anti-HLA-DR-PerCP, CD86-APC, CD83-FITC, CD40-APC. Non-viable MDC were excluded from analysis using 7-AAD (Becton and Dickinson, Heidelberg, Germany). Appropriate isotype-matched control antibodies were used. Optimal dilutions of all antibodies were established in preliminary experiments. The data were analyzed on a FACScalibur using Cellquest pro software (Becton Dickinson, CA, USA).

Allogeneic T cell stimulatory capacity of control-, predex- and dex MDC

Graded numbers of MDC (40, 20,10 and 5 x10^3 DC/200µl) were incubated with or without dex to generate control-, predex- and dex MDC. After 18 hours MDC were washed twice, after which 1.5 x10^5 purified allogeneic T cells were added to the MDC. Alternatively, to exclude an effect of MDC death on T cell stimulation, control-, predex- and dex MDC were recounted after they had been generated, and graded numbers (10, 5 and 2.5 x10^3 MDC/well) were cultured with 1.5 x10^5 allogeneic T cells. In addition, restimulated

control- and dex MDC (20 and 10 x10^3 MDC/well) were cultured with 1.5 x10^5 allogeneic T cells. After five days T cell proliferation was assessed by measuring the incorporation of [3H]-thymidine (Radiochemical Centre, Amersham, Little Chalfont, UK). 0.5 µCi was added per well and cultures were harvested 18 h later. T cells stimulated by phytohemagglutinin (5 µg/ml, Murex, Paris, France) served as a positive control. In addition, culture supernatants were collected at different time points of the MDC-T cell cultures for analysis of cytokine production.

Generation of mature monocyte-derived dendritic cells (Mo-DC)

Monocytes were isolated from buffy coat PBMC by positive selection using CD14 microbeads and a Large Separation column (both from Miltenyi Biotec) according to the manufacturer's protocol. The purity of monocytes determined by flowcytometry with CD14-PE was 99±0.2 %. Monocytes were cultured at a concentration of 1.5x10^6 cells per 2 ml in RPMI$^+$ supplemented with FCS, 50 U/ml GM-CSF and 125 U/ml IL-4 and cultured for 5 days at 37 ˚C, 5% CO_2. After 2 days medium was refreshed and after 5 days 100 ng/ml LPS was added together with fresh GM-CSF and IL-4, and cells were cultured for an additional 24h. At day 6 the cells were harvested, quantified and maturation of Mo-DC was assessed by flowcytometry with anti-BDCA-1 PE, HLA-DR-PerCP, CD40-APC, CD80-FITC and CD86-APC mAb together with appropriate isotype controls. Mo-DC were then frozen to be used as antigen presenting cells in restimulation experiments.

Restimulation of MDC-stimulated T cells

Control and dex MDC (2 x10^3 MDC) were cultured with 1.5 x10^5 purified allogeneic T cells for 7 days. On day 7 the culture medium was refreshed in order to rest the T cells. On day 10 T cells were harvested and quantified per condition. Mature Mo-DC, generated from the same donor as the MDC used in the primary stimulation, were thawed and used to re-stimulate the T cells. 7.5 x10^4 T cells were co-cultured with 7.5 x 10^3 mature Mo-DC for 4 days after which [^{3}H]-thymidine incorporation was determined. Supernatants from day 4 of the restimulation cultures were harvested and frozen to determine cytokine production.

Determination of cytokine production

Levels of IL-12, IL-10, IL-6 and TNFα in the supernatants of control-, predex- and dex MDC (300.000 MDC/ 200 µl), and of re-stimulated control- or dex MDC were determined by standard enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions (eBioscience, San Diego, CA, USA). IL-2, IL-4, IL-10 and IFN–γ production in co-cultures of MDC with allogeneic T cells were determined by the cytometric bead array technology using human cytokine flex sets according to the manufacturer's instructions

(Becton Dickinson). The data were analyzed on a FACSarray using CBA analysis software (Becton Dickinson).

Statistical analysis

Differences between groups of unpaired samples were statistically analyzed using the Mann Whitney test. The Wilcoxon Signed Ranks test was used to test whether differences between groups of paired samples. Analyses were performed using SPSS version 11.0 software. A p-value <0.05 was considered to be statistically significant. All data are presented as means ± standard error of the mean (SEM).

RESULTS

Dex partially blocks maturation of human blood MDC

To study whether pretreatment of freshly isolated human blood MDC with dex prevented maturation, MDC were cultured overnight in the presence dex. After 18 hours the MDC were washed and stimulated with LPS for another 24 hours. Non-treated MDC (control MDC) matured upon stimulation with LPS as demonstrated by an increased expression of CD86, CD80, CD83, CD40 and HLA-DR (Figure 1A and B). Pretreatment of MDC with dex (predex MDC) partially prevented maturation upon LPS activation as shown by a lower expression of expression of CD86 and CD83. HLA-DR and CD40 upregulation were not suppressed and CD80 expression was increased compared to control MDC (Figure 1B).

However when MDC were treated with dex before and during LPS activation (dex MDC) CD80 expression was not enhanced and upregulation of CD86 and CD83 were also partially inhibited (Figure 1A and B). Similar data for these markers were obtained when analyzing the percentages positive MDC (data not shown). The cytokine profiles of the control, predex and dex MDC were similar; during LPS stimulation they produced almost no detect IL-10, IL-12 and IL-6 (data not shown) and their TNFα production was not significantly different (978±102 pg/ml; 823±79 pg/ml; 657±156 pg/ml respectively).

Presence of dex during MDC activation primes IL-10 production and reduces Th1 cytokine production in T cells

Since both predex- and dex MDC had a lowered expression of co-stimulatory molecules needed for T cell activation, their allogeneic T cell stimulatory capacity was assessed. Pretreatment with dex did not suppress the capacity of LPS-stimulated MDC to induce allogeneic T cell proliferation. Presence of dex during MDC activation was required for suppression of their T cell stimulatory capacity (Figure 2A and B).

To exclude that the observed reduced T cell stimulatory capacity was a result of dex-induced MDC death, MDC recovery and viability after incubation with dex and matura-

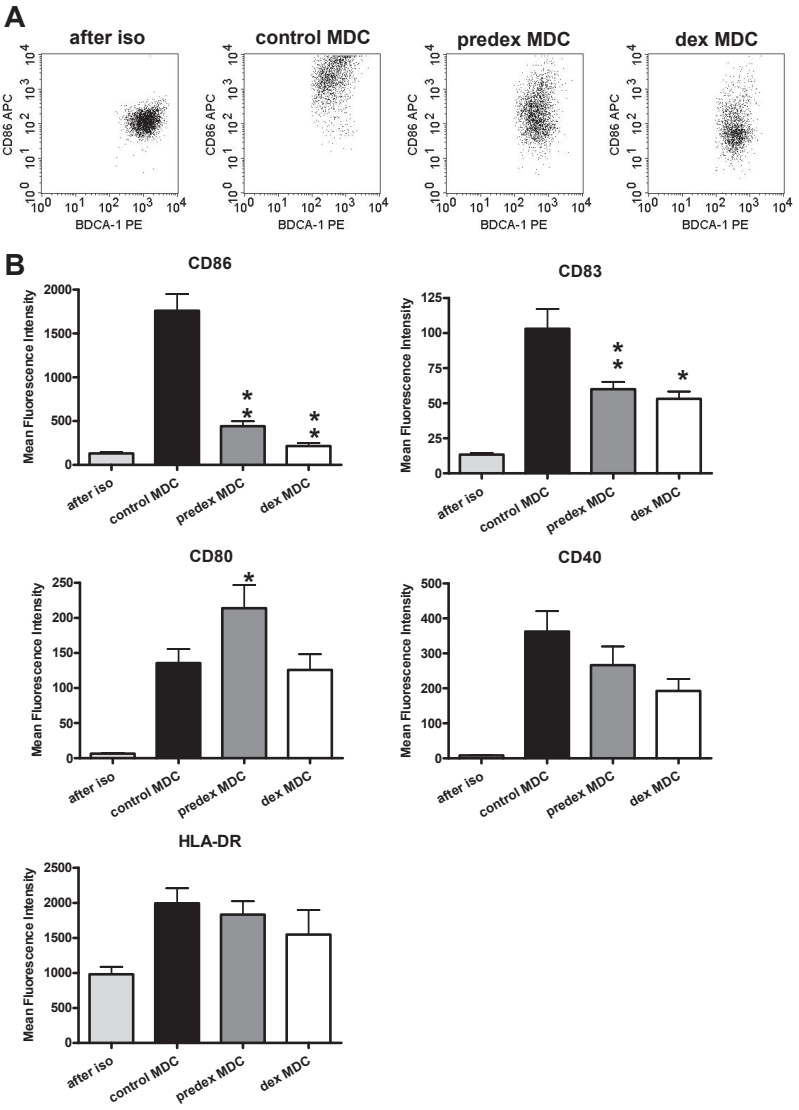

Figure 1. Effect of dex treatment on immunophenotype of human blood MDC. Control and predex MDC were generated by incubating isolated human blood MDC in the presence or absence of 1 μM dex for 18h and subsequently maturing them with 100 pg/ml LPS. Before addition of LPS the MDC were extensively washed to remove residual dex. Dex MDC were generated in the continuous presence 1 μM dex during both pre-incuation and LPS stimulation. (A) Representative dotplots of CD86 expression on freshly isolated (after iso), control-, predex- and dex MDC. (B) The immunophenotypic characteristics of freshly isolated (after iso), control-, predex- and dex MDC as analyzed by flow cytometry. Depicted is the mean fluorescence intensity of the various markers. Data represent the means with SEM of 10 separate experiments. * P<0.04, **P<0.02 for the comparison of predex or dex DC versus control MDC.

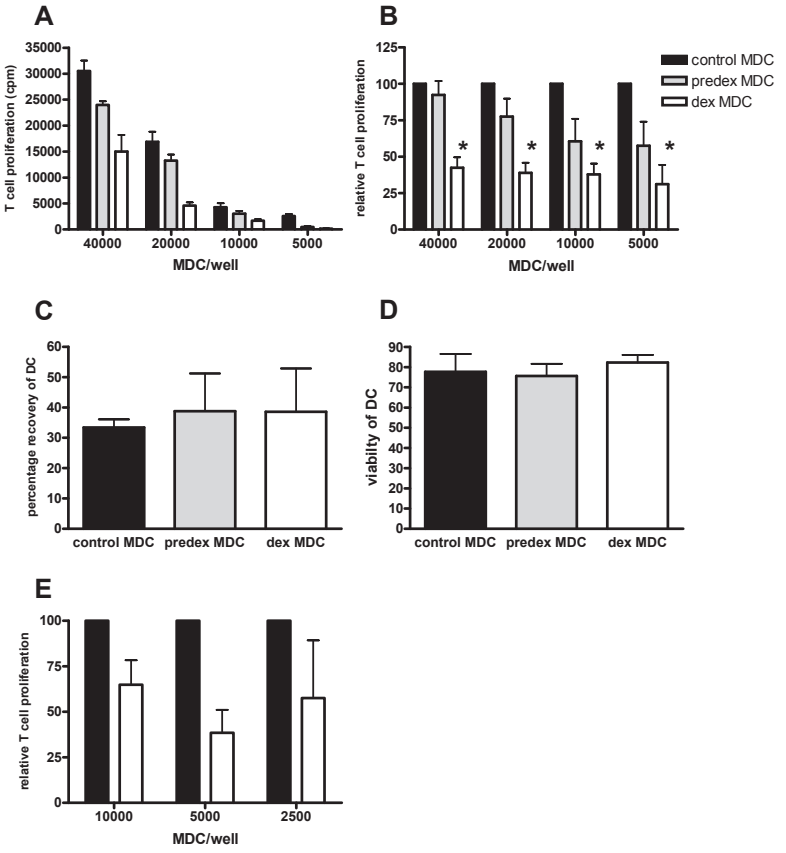

Figure 2. Continuous presence of dex during MDC maturation results in an impaired T cell stimulatory capacity of MDC. Control, predex and dex MDC were generated by incubating isolated human blood MDC in the presence or absence of 1 μM dex for 18h and subsequently maturing them with 100 pg/ml LPS in the presence or absence of 1 μM dex for 24h. Before addition of allogeneic T cells, MDC were extensively washed to remove dex from the cultures. Allogeneic T cell proliferation was assessed after 5 days of incubation with graded number of MDC by [^{3}H] thymidine incorporation. (A) A representative experiment showing T cell proliferation induced by control, predex and dex MDC from the same donor. (B) Relative T cell activation by predex and dex MDC compared to control MDC in 8 experiments. To exclude variations in absolute proliferation between experiments, [^{3}H]-thymidine incorporation in T cells stimulated with control MDC was normalized to 100% for each MDC number, thereby allowing easy pairwise comparison. Proliferation of T cells stimulated by predex and dex MDC was calculated by the following formula: Counts of T cells stimulated with predex or dex MDC divided by counts of T cells stimulated by control MDC times 100%. Data represent mean with SEM of 8 separate experiments. *$P<0.01$ for comparison of dex MDC versus control MDC. (C) Percentage recovery of control-, predex-, and dex MDC from wells after LPS activation. (D) Viability of MDC, determined by trypan blue exclusion, after LPS activation. (E) Relative T cell activation by recounted dex MDC compared to recounted control MDC. MDC were recounted after LPS stimulation and graded numbers of MDC were used to stimulate allogeneic T cells. Data represent mean with SEM of 5 separate experiments.

tion with LPS were analyzed. There were no significant differences in MDC recovery and viability, between control, predex and dex MDC (Figure 2C and D). Moreover, when we recounted MDC before adding T cells, dex MDC also exhibited a lower allogeneic T cell stimulatory capacity, indicating that dex only effects function and not survival of MDC (Figure 2E).

T cells activated with control MDC produced peak levels of IFN–γ on day 7 and of IL-2 on day 4 (data not shown). IFN–γ production was only significantly lowered in T cells stimulated by dex MDC, but not in T cells stimulated by predex MDC (Figure 3A), whereas IL-2 production was reduced in T cells activated with predex- and dex MDC (Figure 3B). T

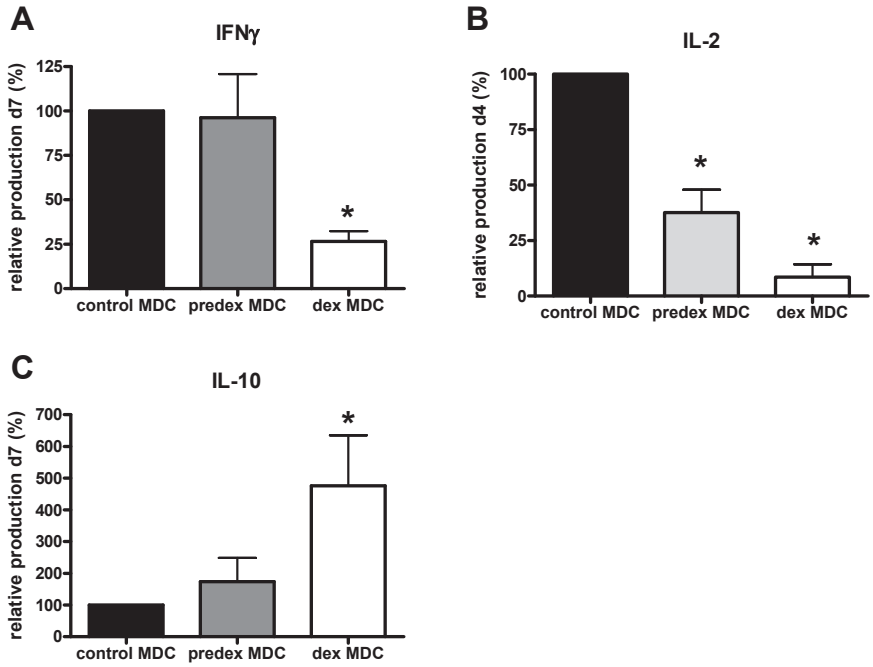

Figure 3. Dex MDC have reduced capacity to stimulate Th1 cytokine production but induce secretion of IL-10 by T cells. (A) Relative IFN–γ production by predex- and dex MDC compared to control-MDC on day 7. Supernatants of T cells stimulated by control MDC contained on average 456±131 pg/ml IFN-γ. To exclude variations in absolute IFN-γ production between experiments, IFN-γ concentration in culture media of T cells stimulated with control MDC was normalized to 100%, thereby allowing easy pairwise comparison. Data represent mean with SEM of 7 separate experiments. (B) Relative IL-2 production by control-, predex- and dex MDC on day 4. Supernatants of T cells stimulated by control MDC contained 260±100 pg/ml IL-2. (C) Relative IL-10 production by control-, predex- and dex MDC on day 7. Supernatants of T cells stimulated by control MDC contained 10±1.3 pg/ml IL-10. Data represent mean with SEM of 6 separate experiments. *P≤0.03 for comparison of dex MDC versus control MDC.

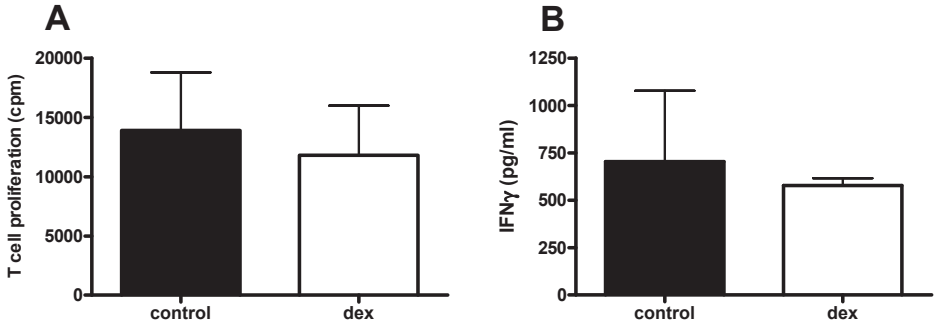

Figure 4. Upon restimulation dex MDC primed T cells proliferate and produce IFN–γ comparable to control MDC-primed T cells. 15 x 10^4 allogeneic T cells were primed with 20 x 10^3 control or dex MDC. At day 7 the T cells were rested for 2 days in refreshed medium. 75 x 10^3 T cells primed with control or dex MDC were restimulated with 75 x 10^2 mature mo-DC originating from the same donor as the control- and dex MDC. (A) T cell proliferation was assessed by [^{3}H] thymidine incorporation 4 days after restimulation of the control or dex MDC primed T cells. (B) IFN–γ production in the supernatant on day 4 of restimulation was determined by ELISA. Data represent mean with SEM of 3 separate experiments.

cells activated with dex MDC produced peak levels of IL-10 on day 7, whereas little IL-10 production was observed when T cells were stimulated with predex- or control MDC (figure 3C). IL-4 was not detected in the MDC-T cell cocultures (data not shown). These results show that continuous presence of dex during LPS stimulation inhibits maturation of primary human blood MDC to immunostimulatory MDC by reducing their capacity to stimulate proliferation and production of Th1 cytokines in T cells and by priming production of the immunoregulatory cytokine IL-10 in T cells. Therefore, the properties of these dex MDC were studied in more detail in the following experiments.

Dex MDC do not induce hyporesponsive T cells *in vitro*

To investigate whether dex MDC induced a permanent hyporesponsiveness in allogeneic T cells, T cells were recovered from the wells of the primary MDC-T cell cocultures and restimulated with mature Mo-DC generated from the same donor as from which the control- and dex MDC where isolated. T cells primed with either control or dex MDC displayed similar T cell proliferation upon restimulation with mature Mo-DC after 4 days of culture (Figure 4A). Additionally these T cells also produced similar levels of IFN–γ (Figure 4B) and IL-2 (data not shown) upon restimulation with mature Mo-DC. IL-10 and IL-4 production were not detectable in restimulations of T cells with Mo-DC. These data show that, although dex MDC have an impaired T cell stimulatory capacity, they do not induce permanent hyporesponsiveness in T cells *in vitro*.

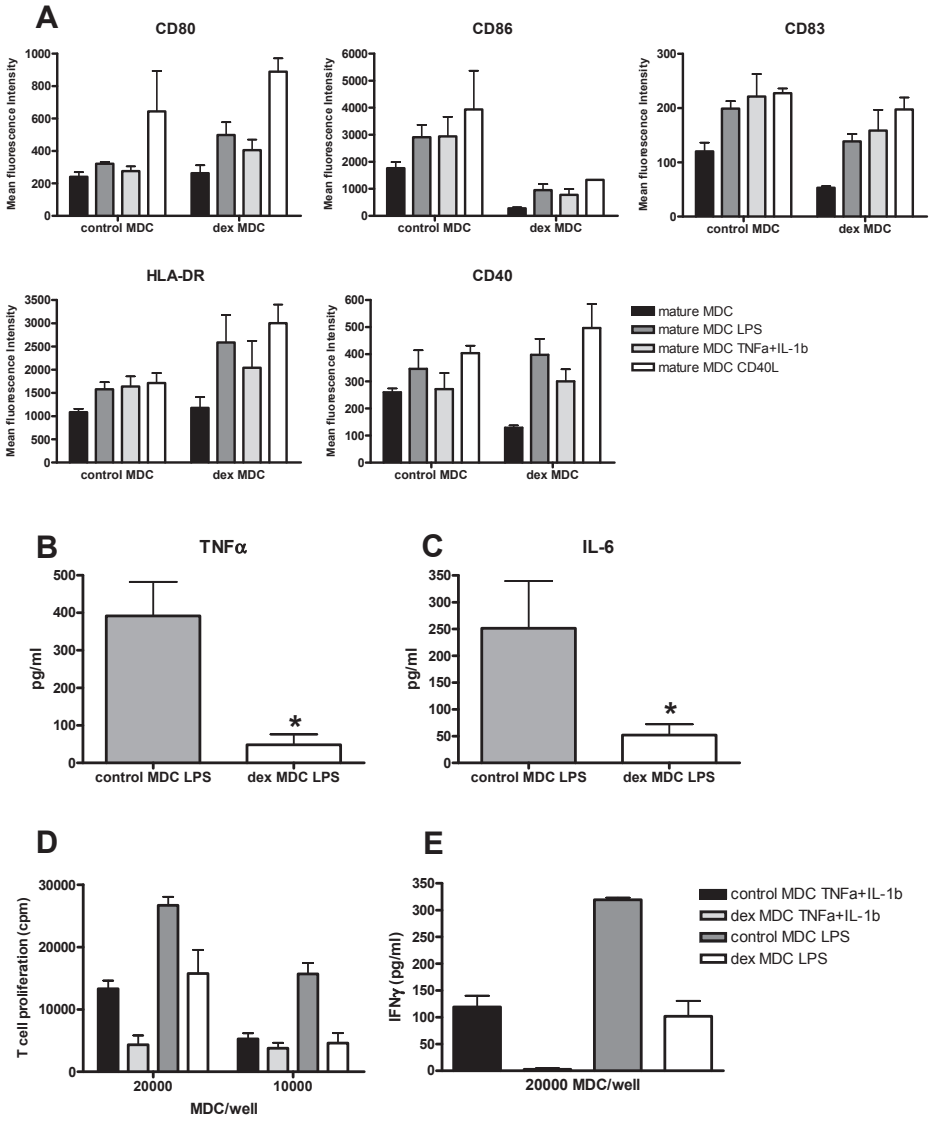

Figure 5. Dex MDC are functionally resistant to a second maturation stimulus in the absence of dex. Control- and dex MDC were exposed to a second maturation stimulus in the absence of dex consisting of either LPS (100pg/ml), TNFα + IL-1β (50 pg/ml) or CD40L (5 x 10^3 000 J558 cells) for 24h. (A) The immunophenotypic characteristics of these MDC were determined by flow cytometry. Depicted is the mean fluorescence intensity of the various markers on control and dex MDC before (mature MDC) and after the second stimulus (mature MDC LPS, mature MDC TNFα + IL-1β, mature MDC CD40L). Data represent mean with SEM of 3 separate experiments. (B) TNFα levels in the culture supernatants of control- and dex MDC 24 hours after restimulation with LPS. Data represent the mean with SEM of 6 separate experiments. * P=0.03 for the comparison of dex MDC

versus control MDC. (C) IL-6 levels in the culture supernatants of control- or dex MDC 24 hours after restimulation with LPS. Data represent the mean with SEM of 6 separate experiments. * P=0.03 for the comparison of dex MDC versus control DC. (D) Allogeneic T cell proliferation primed by control- or dex MDC from the same donor that had been re-stimulated with LPS or TNFα + IL-1β (one representative experiment out of three). (E) IFN–γ production in the supernatant on day 5 of the T cells stimulated by control or dex MDC that had been re-stimulated with LPS or TNFα + IL-1β. Data represent mean with SEM of 3 separate experiments.

Dex MDC are functionally resistant to a second maturation stimulus

To assess whether dex MDC are resistant to a second maturation stimulus, it was first studied whether dex MDC retain a relatively immature phenotype upon activation with a second stimulus consisting of either LPS, TNFα + IL-1β or CD40L. Figure 5A shows that both control MDC and dex MDC matured further upon stimulation with a second stimulus, but the expression of CD86 remained markedly lower on dex MDC compared to control MDC.

Functionally dex MDC produced very little TNFα and IL-6 when activated with a second stimulus consisting of LPS, in contrast to control MDC (figure 5B and C). Likewise, IL-6 production was significantly lowered compared to control MDC when dex MDC were stimulated with TNFα + IL-1β (data not shown). IL-10 and IL-12 production were not detectable in cultures of restimulated MDC. Furthermore, dex MDC activated with a second stimulus consisting of LPS or TNFα + IL-1β did display a reduced capacity to stimulate allogeneic T cell proliferation compared to control MDC (figure 5D). Also re-stimulated dex MDC hardly induced IFN-γ production by T cells (figure 5E). A similar difference in IL-2 production was detected when dex MDC and control MDC, upon restimulation with either LPS or TNFα + IL-1β, were co-cultured with allogeneic T cells (data not shown). IL-10 and IL-4 production were not detectable in the MDC-T cell co-cultures. Collectively, these data show that regulatory dex MDC are functionally resistant to re-stimulation with different maturation stimuli.

DISCUSSION

In this study we show that treatment of primary human blood MDC with the glucocorticoid dexamethasone (dex) in combination with the TLR-4 ligand LPS transforms human blood MDC into tolerogenic MDC. These dex MDC poorly stimulated T cell proliferation and production of Th1 cytokines (IFN–γ and IL-2), but primed production of the immunoregulatory cytokine IL-10 in T cells. Moreover, these dex MDC did not convert into immunogenic MDC after subsequent exposure to different maturation stimuli.

On the contrary, pretreatment of primary human blood MDC with dex did not prevent the generation of immunostimulatory MDC upon subsequent activation with LPS in the

absence of dex. Although dex pretreatment partially prevented immunophenotypic MDC-maturation upon stimulation with LPS, as shown by a reduced expression of CD83 and CD86, it did not prevent functional maturation to immunogenic MDC. This is in agreement with data showing that immature mo-DC treated with dex readily convert to immunogenic MDC after removal of glucocorticoids (15). In addition, we recently found that blood MDC from liver transplant recipients treated with glucocorticoids readily mature to immunostimulatory MDC when stimulated *ex vivo* in the absence of glucocorticoids (16). Apparently, corticosteroids alone do not imprint immature human blood MDC with a stable immunoregulatory function.

In contrast, treatment with dex and LPS simultaneously resulted in MDC which poorly stimulated allogeneic T cell proliferation. This is in agreement with the reported effects of continuous corticosteroid treatment during maturation of human mo-DC and murine MDC, that also inhibited immunophenotypic and functional maturation (15, 17-20). One reason that treatment with dex alone did not reduce the T cell stimulatory capacity of MDC, may be that it stimulated upregulation of CD80 upon LPS stimulation, while combined treatment with dex and LPS prevented this upregulation.

After restimulation, MDC that had been treated with the combination of dex and LPS hardly upregulated CD86. A durable effect of corticosteroids on CD86 expression on MDC was also observed *in vivo* in patients that were treated with high doses of glucocorticoids shortly after liver transplantation; when MDC from these patients were *ex vivo* stimulated with pro-inflammatory cytokines they showed impaired upregulation of CD86 expression (16). These data altogether show that the only durable effect of glucocorticoids on the immunophenotype primary human blood MDC is impairment of CD86 expression.

The secretion of DC derived immunoregulatory or immunostimulatory cytokines also plays a crucial role in the cascade of T cell priming. In contrast to human mo-DC(9, 15, 18, 19), primary human blood MDC did not produce detectable amounts of IL-6, IL-10 and IL-12 upon LPS-stimulation. TNFα production by human blood MDC stimulated by LPS was not suppressed by corticosteroid treatment. In this respect, primary human blood differ from human mo-DC or murine bone marrow derived MDC in which cytokine production is inhibited by dex (15, 18, 19, 21, 22). However, we did observe a late effect of dex on cytokine production by human blood MDC: upon restimulation, MDC that had been treated with dex and LPS hardly produced cytokines in contrast to untreated MDC.

The key observation of our study is that upon combined treatment with dex and LPS primary human MDC are impaired in their capacity to stimulate production of Th1 cytokines, but acquire the capacity to prime IL-10 production in T cells. Most likely, dex-MDC can prime type 1 regulatory T cells, which produce IL-10 (23, 24). However, T cells primed by dex MDC also produced IFN–γ in low amounts, but no Th2 cytokines. Since the ratio IL-10 and IFN–γ production by these T cells was 1:2, we propose that these T

cells represent a mixture of type 1 regulatory T cells and Th1 cells. MDC generated under the influence of LPS and dex from primary human blood MDC are reminiscent of murine MDC that have been stimulated with LPS in the presence of dex or stimulated with cytokines only. These so-called "alternatively activated", "semi-mature", or "modified" regulatory MDC similarly prime IL-10 production by T cells *in vitro* (20) and *in vivo* upon transfer into mice (21, 22, 25). Importantly, such tolerogenic murine MDC suppress Th1 responses *in vivo* and prevent allograft rejection (21, 22), Graft-versus-Host Disease(26), and protect against experimentally induced auto-immune diseases (25) upon transfer into mice.

Treatment of MDC with dex during LPS stimulation suppressed the upregulation of signal 2 (co-stimulatory molecules), but not of signal 1 (HLA-DR), suggesting that these MDC could induce T cell anergy. Unfortunately, our *in vitro* data showed that these tolerogenic dex MDC did not induce a permanent state of hyporesponsiveness in T cells. However this does not mean that hyporesponsiveness in T cells does not occur upon transfer of these MDC *in vivo*. Data from a murine study showed that alternatively activated MDC that did not induce hyporesponsiveness *in vitro* induced profound donor-specific hyporesponsiveness in T cells, and prolonged transplant survival when applied *in vivo* (22).

For use of MDC in immunotherapy to induce tolerance to allografts, it is essential that the MDC do not convert into immunogenic MDC *in vivo* upon encountering a host environment rich in pro-inflammatory stimuli, especially in transplant recipients which are highly susceptible for infections. The tolerogenic MDC obtained after simultaneous treatment with dex and LPS fulfill this requirement: After restimulation with different maturation stimuli they become unable to produce pro-inflammatory cytokines, and almost incapable to induce proliferation and cytokine production in T cells.

Donor blood is readily available at time of transplantation, and it is technically feasible to isolate immature blood MDC rapidly with the method described in the present study, using clinical grade equipment. Therefore, in clinical transplantation alternatively activated donor blood MDC are an attractive option for cell-based immunotherapy to induce donor-specific tolerance. For this purpose the culture period must be shortened. We anticipate that the pre-incubation period with dex can be reduced considerably. In case of liver transplantation an additional source of immature donor MDC is readily available, namely immature donor liver MDC obtained during the backtable perfusion of a liver graft (13). In addition, alternatively activated recipient blood MDC may be suitable for induction of tolerance in recipient T cells that recognize donor alloantigens via the indirect pathway of antigen presentation. However, for this purpose effective means of loading MDC with donor HLA have to be developed.

In conclusion human blood MDC treated with dex and LPS are transformed in tolerogenic MDC that are strongly impaired in their capacity to stimulate allogeneic T cell proliferation and Th1 secretion, but that prime T cells to produce the immunoregulatory cytokine

IL-10. Moreover, these tolerogenic MDC were functionally unresponsive to maturation stimuli. These findings indicate alternatively activated primary blood MDC may be a promising option for cell-based immune modulatory therapy to induce donor-specific tolerance in transplant recipients.

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8

Summary and discussion

Samenvatting en discussie

Dankwoord

Curriculum Vitae

SUMMARY AND DISCUSSION

The tightly regulated immune responses against food antigens from the gut and the commensal intestinal flora and the low incidence of chronic rejection after liver transplantation compared to other solid organs, support the paradigm that the liver is an immunotolerant organ. The underlying mechanisms explaining the tolerogenic properties of the liver are poorly understood. The general theory is that the liver contains specific immune cells with tolerogenic capacities, resulting in induction of tolerance rather than immunity. However, the cell types responsible for this phenomenon have not been identified yet. Most likely, these tolerogenic hepatic immune cells act through production of certain immunoregulatory cytokines and chemokines, or alternatively by inducing deletion or anergy of antigen-specific effector T cells and/or by activating or inducing regulatory T cells that can actively suppress an immune response.

Unraveling the mechanisms responsible for immunological tolerance in the liver may allow the design of rational therapies for liver diseases that result from poor hepatic immune responses, such as persistent infections with hepatitis viruses. Furthermore, insight into the mechanisms responsible for tolerance induction after liver transplantation (LTx) may enable devising strategies to facilitate acceptance of other organ transplants. Finally, insight in hepatic immunological tolerance will contribute to understand mechanisms of peripheral immunological tolerance in general. Since myeloid dendritic cells (MDC) are regarded as the main instigators and regulators of T cell responses, these cells are the main focus in this thesis.

Chapter two describes the functional and phenotypical differences between MDC derived from skin/muscle draining lymph nodes (LN) and MDC from liver draining LN. Studying MDC in draining LN provided the opportunity to clarify whether MDC of the liver undergo a differential maturation process *in vivo* compared to MDC of the skin/muscle. The comparison between these two types of MDC was made because the skin is the major barrier to the external environment, and disturbance of this barrier results in a strong immune response, whereas immune responses to external stimuli in the liver are tightly controlled and in general more tolerogenic. We observed that hepatic LN MDC had a two-fold reduced T cell stimulatory capacity compared to skin/muscle draining LN MDC. This lower T cell stimulatory capacity confirms that liver-derived MDC have weaker immunogenic properties that skin/muscle-derived MDC. The explanation for this observation was not the maturation state of the MDC since, surprisingly, hepatic LN MDC even had a more mature immunophenotype than the skin/muscle draining LN MDC. Conversely hepatic LN MDC almost completely lacked the ability to produce cytokines. This deficiency most likely explains their weak ability to activate T cells. We hypothesize that this highly mature exhausted status of MDC in hepatic LN is due to a continuous stimulation with gut-derived components.

The liver is an lymphoid organ with 10-20 times more leukocytes than kidneys. In experimental animal models it has been described that after LTx these graft derived immune cells migrate into the recipient where they influence the recipient's immune response against the allograft. Therefore it has been postulated that the tolerogenic properties of liver grafts are related to their large numbers of passenger leukocytes. However, the passenger leukocyte subset responsible for induction of LTx tolerance has not been identified yet. **Chapter three** describes the presence of regulatory T cells (Treg) in liver graft perfusates obtained prior to transplantation and the subsequent presence of donor-derived Treg in the circulation of the patient after transplantation. An increased proportion of $CD25^+FoxP3^+$ and $CD25^+CTLA\text{-}4^+$ cells was found within the population of $CD4^+$ T cells in the perfusates compared to the proportion in peripheral blood of healthy controls. These donor liver-derived Treg were able to suppress the direct pathway of allorecognition as shown by the inhibition of proliferation and cytokine production of recipient-responder T cells upon stimulation with donor cells. In addition, we demonstrated the presence of substantial numbers of donor-derived $CD4^+CD25^+CTLA\text{-}4^+$ T cells in the circulation of recipients after liver transplantation. The chimerism of donor Treg may contribute to suppression of the direct pathway of the alloresponse, which is the dominant antigen presentation pathway responsible for acute rejection early after transplantation. However, since MDC are the initiators of an immune response, the presence, phenotype, migration and function of liver graft-derived MDC were established in **chapters four and five**. **Chapter four** revealed that MDC were present in the portal fields of human donor livers and that resident donor liver MDC had an immature phenotype as demonstrated by a low expression of CD80 and CD83. During vascular perfusion of the donor liver before transplantation, on average one million MDC detached from graft. These liver-derived MDC had an immature phenotype intermediate between circulating blood MDC and hepatic LN MDC. In contrast to blood MDC, freshly isolated perfusate MDC were able to stimulate allogeneic T cell proliferation. Moreover, MDC from liver graft perfusates were responsive to stimulation with lipopolysaccharide (LPS). In **chapter five** these perfusate MDC were studied in more detail and the presence of donor MDC in the recipient after LTx was determined and compared with kidney transplantation (RTx). This study showed that leukocytes of donor origin, including MDC, migrate early after human LTx, but not after RTx, into the recipient's blood circulation. Even on day five after LTx donor MDC could be traced in the circulation of the recipient. LPS activated liver graft MDC were able to stimulate allogeneic T cell proliferation (although slightly less effective than blood MDC), but were poor stimulators of T cell IFN-γ production. The most striking difference in cytokine production between liver graft- and blood MDC was the high production of IL-10. This difference was observed with all types of stimuli used. Since IL-10 is known as a suppressor of IFN-γ production in T cells, we hypothesized that the high amounts of IL-10 secreted by human hepatic MDC is the main factor contributing to their poor capacity to prime IFN-γ production in T

cells. We propose that early donor MDC migration after clinical LTx may either stimulate expansion, but not effector function of recipient allogeneic T cells, ultimately leading to activation-induced apoptotic death in lymphoid tissues and/or in the graft. This mechanism may account for the tolerogenic properties of human liver grafts.

Besides the type of donor MDC that enter the patient after transplantation and directly effect the immune response against the graft, the effect of the immunosuppressive regimen on MDC from both recipient and donor origin also has to be taken into account when investigating the immune response after organ transplantation. Recipient MDC play an important role in the regulation of an anti-donor immune response since they can take up donor allo-antigens and present these indirectly to the T cells, thereby instigating (chronic) rejection. In **chapter six** we demonstrated that there is a threefold decline in circulating MDC directly after LTx and that MDC numbers are normalized between 3 and 12 months after LTx. This decline was not due to recruitment of MDC into the graft, and also no change in chemokine receptor expression on circulating MDC was observed, indicating that their homing properties were not altered. A strong association with the use of corticosteroids suggests that the decline in MDC numbers was due to corticosteroid treatment. Indeed, normalization of MDC numbers was associated with withdrawal of corticosteroid therapy and not with changes in calcineurin intake. Shortly after transplantation circulating MDC exhibited a reduced expression of HLA-DR and CD86 compared to pre-LTx values, but *ex vivo* maturation of the MDC resulted in an upregulation of HLA-DR and co-stimulatory molecules to the same extent as MDC from healthy controls. *Ex vivo* matured MDC from both groups also had an equal allogeneic T cell stimulatory capacity. This altogether indicates that corticosteroids probably have an immunosuppressive effect on MDC *in vivo*, but that this therapy does not imprint MDC with an intrinsic resistance to maturation stimuli. The unique role of corticosteroids as immunosuppressive medication may be partly related to their inhibitory effects on MDC. By inhibiting donor antigen presentation by MDC, corticosteroids suppress allogeneic T cell activation against the graft in its earliest phase. Since corticosteroids are known to induce tolerogenic MDC when present during the generation of MDC from monocytes or bone marrow precursors *in vitro* and can also modulate MDC *in vivo* after LTx (**chapter six**), the effect of corticosteroids on human blood MDC was studied in **chapter seven**. The ultimate goal was to create tolerogenic MDC by *ex vivo* treatment with corticosteroids, to be used for immunotherapy to promote graft acceptance. Successful outcomes in rodent models have indicated that DC based therapy may provide a novel approach to induce transplant tolerance. The feasibility and safety of DC based therapy in humans is supported by promising results for treatment of advanced melanoma. In **chapter seven** we report that treatment of primary human blood MDC with the glucocorticoid dexamethasone (dex) alone did not result in tolerogenic MDC. Although it partially prevented the immunophenotypic maturation of MDC, it did not prevent functional maturation to immunogenic MDC upon exposure to

maturation stimuli. This in agreement with the data of **chapter six**, showing that blood MDC from liver transplant recipients treated with glucocorticoids readily mature when *ex vivo* stimulated in the absence of glucocorticoids. However, treatment of primary human blood MDC with dex in combination with LPS transformed human blood MDC into tolerogenic MDC. These dex MDC poorly stimulated T cell proliferation and production of the Th1 cytokines (IFN–γ and IL-2), but primed the production of the immunoregulatory cytokine IL-10 in T cells. Moreover these dex MDC did not convert into immunogenic MDC after subsequent exposure to different maturation stimuli in the absence of dex. This is essential for the use of MDC in immunotherapy to induce tolerance to an allograft, since for this application tolerogenic MDC should not convert into immunogenic MDC *in vivo* upon encountering a host environment rich in inflammatory stimuli, especially in transplant recipients which are highly susceptible for infections. Unfortunately, blood MDC treated with dex plus LPS did not induce hyporesponsiveness in allogeneic T cells *in vitro*, casting doubt upon their potential to prevent graft rejection *in vivo*. The source of donor MDC that could be used in clinical practice for generating tolerogenic MDC could be donor blood MDC, which are readily available at time of transplantation, but in case of LTx there is an additional source of MDC, namely immature perfusate MDC (**chapter four and five**) obtained during the backtable perfusion of the liver graft.

Overall, liver grafts are unique compared to other solid organs since they contain high numbers of passenger leucocytes, including Treg and MDC, with immunomodulatory properties. Liver-derived Treg can suppress the direct pathway of allorecognition and this might contribute to the suppression of the direct pathway of alloresponse early after LTx. Liver graft MDC produce high levels of the immunoregulatory cytokine IL-10 and most likely in that way modulate the anti-donor T cell response. Additionally, corticosteroid therapy after LTx probably has an immunosuppressive effect on MDC thereby contributing to the prevention of rejection. Donor blood and liver graft perfusates are sources of donor MDC that may be used for MDC based immunotherapy to induce tolerance. In future, corticosteroid in combination with LPS treated donor MDC might even be used as MDC-based immunotherapy for the induction of tolerance provided that they induce donor-specific hyporesponsiveness *in vivo* and regulatory properties in allogeneic T cells.

Samenvatting en discussie

SAMENVATTING EN DISCUSSIE

De strak gereguleerde immuunrespons tegen voedsel antigenen afkomstig uit de darm en tegen de aanwezige darmflora in combinatie met de lage incidentie van chronische afstoting na levertransplantatie vergeleken met andere solide organen, ondersteunen het paradigma dat de lever een immuuntolerant orgaan is. De onderliggende mechanismen die de tolerogene eigenschappen van de lever kunnen verklaren worden nog slecht begrepen. De algemene theorie is dat de lever specifieke immuuncellen met tolerogene eigenschappen bevat, hetgeen resulteert in de inductie van tolerantie in plaats van immuniteit. Echter, de celtypen verantwoordelijk voor dit fenomeen zijn nog niet geïdentificeerd. Waarschijnlijk functioneren deze tolerogene lever immuuncellen via de productie van immuunregulerende cytokinen en chemokinen. Het is ook mogelijk dat de tolerogene lever immuuncellen een regulerend effect hebben op T cellen, bijvoorbeeld depletie van antigeen specifieke effector T cellen en/of activatie van regulatoire T cellen die actief de immuunrespons kunnen onderdrukken.

Het begrijpen van de mechanismen die verantwoordelijk zijn voor de immunologische tolerantie van de lever kan bijdragen aan de ontwikkeling van specifieke therapieën voor leverziekten die het gevolg zijn van de matige immuunrespons in de lever, zoals persistente infecties met hepatitis virussen. Verder zal inzicht in de mechanismen die verantwoordelijk zijn voor tolerantie inductie na levertransplantatie (LTx) het mogelijk maken om strategieën te ontwikkelen om acceptatie van donororganen te bewerkstelligen. Tot slot zal inzicht in de immunologische tolerantie bijdragen aan het begrijpen van de mechanismen van perifere tolerantie in het algemeen. Aangezien myeloïde dendritische cellen (MDC) beschouwd worden als de belangrijkste cellen om een T cel respons te initiëren en te reguleren, ligt de nadruk in dit proefschrift op dit celtype.

Hoofdstuk twee beschrijft de functionele en fenotypische verschillen tussen MDC afkomstig uit huid/spier drainerende lymfeklieren (LN) en MDC uit lever LN. Het vergelijken van MDC in de huid/spier drainerende LN met MDC uit lever LN gaf de mogelijkheid om te onderzoeken of MDC uit de lever *in vivo* een ander maturatieproces ondergaan dan MDC uit de huid/spieren. De vergelijking tussen deze twee typen MDC werd gemaakt omdat de huid de grootste barrière is met de buitenwereld en verstoring van deze barrière leidt tot een sterke immuunrespons, terwijl imuunresponsen tegen externe stimuli in de lever strak gecontroleerd en in het algemeen ook meer tolerogeen zijn. We hebben gezien dat lever LN MDC een twee keer zo lage T cel stimulatoire capaciteit hebben in vergelijking met huid/spier drainerende LN MDC. Deze lagere T cel stimulerende capaciteit bevestigt dat leverafkomstige MDC minder immunogene eigenschappen hebben dan huid/spierafkomstige MDC. De verklaring voor deze waarneming was niet de maturatiestatus van de MDC aangezien, tot onze verbazing, lever LN MDC een meer matuur fenotype hadden dan huid/spier drainerende LN MDC. Lever LN MDC waren echter bijna niet in

staat om cytokinen te produceren. Dit gebrek verklaart waarschijnlijk hun zwakke T cel stimulerende capaciteit. Wij veronderstellen dat de zeer mature en uitgeputte status van de MDC uit de lever LN wordt veroorzaakt door de continue stimulatie met darmafkomstige componenten.

De lever is een lymfoïd orgaan dat 10-20 keer meer leukocyten bevat dan de nier. In experimentele diermodellen is beschreven dat na LTx immuuncellen afkomstig uit het donororgaan de ontvanger in migreren waar zij de immuunrespons van de ontvanger tegen het donororgaan beïnvloeden. Dit heeft geleid tot de aanname dat de tolerogene eigenschappen van donorlevers een gevolg kunnen zijn van de grote aantallen migrerende leukocyten. De migrerende leukocytpopulatie die verantwoordelijk is voor de inductie van tolerantie na LTx is echter nog niet geïdentificeerd. **Hoofdstuk drie** beschrijft ten eerste de aanwezigheid van regulatoire T cellen (Treg) in de donor lever perfusaten die verkregen zijn voor transplantatie en bovendien de aanwezigheid van donorafkomstige Treg in de circulatie van de patiënt na transplantatie. Binnen de $CD4^+$ T cel populatie van de perfusaten werd een verhoogd percentage van $CD25^+CTLA\text{-}4^+$ en $CD25^+Foxp3^+$ T cellen aangetroffen in vergelijking met de percentages in bloed van gezonde controles. Deze donorleverafkomstige Treg waren in staat om de antigeen presentatie te remmen, hetgeen blijkt uit de inhibitie van proliferatie en cytokine productie door ontvanger T cellen na stimulatie met donor cellen. Verder hebben wij de aanwezigheid van substantiële donorafkomstige $CD4^+CD25^+CTLA\text{-}4^+$ T cellen in de circulatie van de ontvanger aangetoond. Chimerisme van donor Treg draagt mogelijk bij aan de suppressie van de directe allogene respons, de dominante weg van antigeen presentatie die verantwoordelijk is voor acute afstoting vroeg na transplantatie. Aangezien MDC echter de aanstichters zijn van een immuunrespons zijn de aanwezigheid, fenotype, migratie en functie van leverafkomstige MDC onderzocht in **hoofdstuk vier en vijf**. **Hoofdstuk vier** laat zien dat MDC aanwezig zijn in de portale velden van humane donor levers en dat MDC in de donor lever een immatuur fenotype hebben gekenmerkt door een lage expressie van CD80 en CD83. Tijdens vasculaire perfusie van de donorlever voor transplantatie laten gemiddeld één miljoen MDC los uit het donororgaan. Deze leverafkomstige MDC hadden een immatuur fenotype intermediair tussen circulerende bloed MDC (=immatuur) en lever LN MDC (=matuur). In tegenstelling tot bloed MDC, waren deze vers geïsoleerde perfusaat MDC in staat om allogene T cel proliferatie te induceren. Tevens waren perfusaat MDC gevoelig voor stimulatie met de bacteriële stimulus lipopolysaccharide (LPS). In **hoofdstuk vijf** zijn deze perfusaat MDC in meer detail bestudeerd en werden bovendien de aanwezigheid van donor MDC in de ontvanger na LTx bepaald en vergeleken met de aanwezigheid van donor MDC na niertransplantatie (NTx). Deze studie toonde aan dat donor MDC vroeg na LTx, maar niet na NTx, aanwezig waren in de bloedcirculatie van de ontvangers. Zelfs op dag vijf na LTx zijn donor MDC nog aanwezig in de circulatie van de ontvanger. LPS geactiveerde donorlever MDC waren in staat om proliferatie van allogene T cellen te stimuleren (iets minder

goed dan bloed MDC), maar zij waren slechte stimulatoren van T cel IFN-γ productie. Het meest opvallende verschil in cytokine productie tussen donorlever- en bloed MDC was de hoge productie van IL-10, geproduceerd na stimulatie met verschillende stimuli. Aangezien bekend is dat IL-10 de productie van IFN-γ door T cellen onderdrukt, denken wij dat de secretie van relatief veel IL-10 door de humane lever MDC de voornaamste factor is die bijdraagt aan de lage productie van IFN-γ door de T cellen. Wij veronderstellen dat vroege migratie van donor MDC na levertransplantatie mogelijk wel de expansie van ontvanger T cellen stimuleert, maar niet de effector functie, wat leidt tot activatie geïnduceerde celdood van de T cellen in lymfoide weefsels en/of het donororgaan. Dit mechanisme draagt mogelijk bij aan de tolerogene eigenschappen van humane donor levers.

Naast het type donor MDC dat de patiënt in migreert na transplantatie en op deze manier direct een effect heeft op het immuunrespons tegen het donororgaan, is ook het effect van het immuunsuppressieve regime op zowel donor als ontvanger afkomstige MDC van belang bij de immuunrespons na transplantatie. Ontvanger MDC spelen ook een belangrijke rol in de regulatie van de antidonor immuunrespons omdat zij donor antigenen kunnen opnemen en op een indirecte manier kunnen presenteren aan de T cellen waardoor (chronische) afstoting kan optreden. In **hoofdstuk zes** laten wij zien dat er een drievoudige afname is van circulerende MDC direct na LTx en dat MDC aantallen normaliseren tussen 3 en 12 maanden na LTx. Deze afname was niet het gevolg van rekrutering van MDC in de donorlever en er was ook geen verandering in de chemokine receptor expressie op de circulerende MDC. Dit wijst erop dat hun eigenschappen om naar de lymfoide organen te kunnen migreren niet waren veranderd. Een sterke associatie tussen het gebruik van corticosteroïden en de afname van MDC suggereert dat de behandeling met corticosteroïden hiervoor verantwoordelijk is. Normalisatie van MD was inderdaad geassocieerd met het staken van de therapie met corticosteroïden en niet met verandering in het gebruik van calcineurine inhibitors. Direct na transplantatie hebben circulerende MDC een verlaagde expressie van HLA-DR en CD86 in vergelijking met preLTx waarden, maar *ex vivo* maturatie van deze MDC resulteerde in een verhoging van expressie van HLA-DR en de co-stimulatoire moleculen tot dezelfde hoogte als MDC van gezonde controles. *Ex vivo* gematureerde MDC van beide groepen hadden ook eenzelfde capaciteit om T cellen te stimuleren. Dit tezamen wijst erop dat corticosteroïden waarschijnlijk een immuunonderdrukkend effect hebben op MDC *in vivo*, maar dat deze therapie MDC niet inprent met een intrinsieke resistentie tegen maturatie stimuli. De unieke rol van corticosteroïden als immuunonderdrukkende medicatie is waarschijnlijk gedeeltelijk gerelateerd aan hun remmende effect op MDC. Door het onderdrukken van de presentatie van donor antigenen door MDC zouden corticosteroïden de activatie van T cellen tegen het donororgaan kunnen remmen vanaf het allereerste begin. Aangezien van corticosteroïden ook bekend is dat zij tolerogene MDC kunnen induceren tijdens de generatie van MDC uit monocyten of uit voorlopercellen afkomstig uit beenmerg en zij bovendien MDC *in vivo* kunnen

moduleren na LTx (**hoofdstuk zes**),werd het effect van corticosteroïden op verse humane bloed MDC onderzocht in **hoofdstuk zeven**. Het ultieme doel was het creëren van tolerogene MDC door *ex vivo* behandeling met corticosteroïden die gebruikt kunnen worden voor immuuntherapie om acceptatie van het donororgaan te stimuleren. Succesvolle resultaten in diermodellen laten zien dat therapie met DC een mogelijkheid is om tolerantie na transplantatie te induceren. De haalbaarheid en veiligheid van deze aanpak in mensen wordt ondersteund door de veelbelovende resultaten van studies waarin DC therapie wordt toegepast bij uitgezaaide melanomen. In **hoofdstuk zeven** laten wij zien dat behandeling van primaire humane bloed MDC met alleen het glucocorticoïd dexamethason (dex) niet resulteerde in tolerogene MDC. Hoewel de fenotypische maturatie gedeeltelijk voorkomen werd, voorkwam het niet de functionele maturatie in immunogene MDC na blootstelling aan een maturatie stimulus. Dit komt overeen met de data uit **hoofdstuk zes**, waarin aangetoond werd dat bloed MDC afkomstig van patiënten na levertransplantatie onder behandeling van glucocorticoïden snel matureren wanneer ze *ex vivo* werden gestimuleerd in afwezigheid van glucocorticoïden. Echter behandeling van primaire humane bloed MDC met dex in combinatie met LPS veranderde humane bloed MDC in meer tolerogene MDC. Deze dex MDC hadden een verlaagde T cel stimulerende capaciteit en produceerden weinig Th1 cytokinen (IFN–γ en IL-2), maar induceerden wel de productie van het immuunregulerende cytokine IL-10 in T cellen. Bovendien veranderde deze dex MDC niet in immunogene MDC na blootstelling aan verschillende maturatie stimuli in de afwezigheid van dex. Dit is essentieel voor het gebruik van MDC als immuuntherapie om tolerantie te induceren, omdat voor deze toepassing tolerogene MDC *in vivo* niet mogen veranderen in immunogene MDC wanneer zij worden blootgesteld aan een omgeving die rijk is aan maturatie stimuli, zoals transplantatiepatiënten die zeer gevoelig zijn voor infecties. Helaas induceerden de bloed MDC die behandeld waren met dex en LPS *in vitro* geen hyporesponsiviteit in de allogene T cellen, hetgeen het twijfelachtig maakt of zij *in vivo* bruikbaar zijn om tolerantie te induceren. In de praktijk kunnen donor bloed MDC die voorradig zijn ten tijde van de transplantatie gebruikt worden als bron van donor MDC om tolerogene MDC te genereren, maar in het geval van LTx is er nog een andere bron namelijk de immature perfusaat MDC (**hoofdstuk vier en vijf**) die verkregen worden tijdens de perfusie van de donorlever.

Donorlevers zijn uniek in vergelijking met andere solide organen omdat zij een groot aantal migrerende leukocyten bevatten, waaronder Treg en MDC, met immuunmodulatoire eigenschappen die na transplantatie de patiënt in migreren. Deze donorleverafkomstige Treg waren in staat om de directe weg van antigenpresentatie te remmen en dragen mogelijk op deze manier bij aan het onderdrukken van de allogene respons vroeg na LTx. Donorlever MDC produceren veel IL-10 en dragen op deze manier waarschijnlijk bij aan de modulatie van de antidonor T cel respons. Verder heeft therapie met corticosteroïden na LTx waarschijnlijk een immuunonderdrukkend effect op MDC en draagt op deze manier

bij aan het voorkomen van afstoting. Donor bloed en perfusaten van donorlevers kunnen worden benut als bron van donor MDC en kunnen gebruikt worden voor immuuntherapie met MDC. In de toekomst zouden donor MDC behandeld met corticosteroïden en LPS mogelijk gebruikt kunnen worden voor immuuntherapie met MDC om tolerantie te bewerkstelligen, mits zij *in vivo* donorspecifieke hyporesponsiviteit en regulerende eigenschappen in allogene T cellen induceren.

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CURRICULUM VITAE

Brenda Margret Bosma werd op 13 maart 1978 geboren in Apeldoorn. Zij behaalde in 1997 haar Gymnasium diploma aan het Stedelijk Gymnasium in Leiden. In datzelfde jaar startte zij met haar studie (Medische) Biologie aan de Universiteit Leiden. Tijdens haar studie deed zij een onderzoeksstage van 12 maanden bij de onderzoeksgroep chirurgische oncologie, onderdeel van de afdeling Heelkunde, in het LUMC. Onder beleidng van Dr. P. Kuppen en drs. R.I.J.M. Aalbers deed zij onderzoek naar een onbekend antigen op ratten T cellen die bij activatie zeer snelle celdood induceerde. In 2003 legde zij met succes haar doctoraalexamen af. Aansluitend startte zij met haar promotieonderzoek op de afdeling Maag-, Darm- en Leverziekten van het ErasmusMC in Rotterdam op het gebied van immuunmodulatie na levertransplantatie. Hierbij werd de rol die donor-afkomstige immuun cellen kunnen spelen bij het afstotingsproces na transplantatie onderzocht en werd ook het effect van corticosteroïden op dendritische cellen bepaald. Onder begeleiding van Prof. dr. E.J. Kuipers (promotor), Prof.dr. H.J. Metselaar (copromotor) en Dr. J. Kwekkeboom (copromotor) werd het onderzoek verricht dat in dit proefschrift beschreven is. Sinds november 2007 is zij werkzaam bij Wyeth Pharmaceuticals als Medical Scientific Liaison in de transplantatie geneeskunde.