Regulation of Immunity by Dendritic Cells in the Setting of Liver Transplantation

Patrick P.C. Boor

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Regulation of Immunity by Dendritic Cells in the Setting of Liver Transplantation

Regulatie van immuniteit door dendritische cellen in de context van levertransplantatie

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CONTENTS

Chapter 1	General introduction and outline of the thesis	9
Part I.	Myeloid dendritic cells: characterization in liver tissue and hepatic lymph nodes	
Chapter 2	Immunomagnetic selection of functional dendritic cells from human lymph nodes. *Immunol Lett. 2005 Jul 15;99(2):162-8*	27
Chapter 3	Characterization of human liver dendritic cells in liver grafts and perfusates *Liver Transpl. 2006 Mar;12(3):384-93*	39
Chapter 4	Characterization of dendritic cell subtypes in human hepatic lymph nodes	57
Part II.	Plasmacytoid dendritic cells: Induction of regulatory T cells and effects of immuno-suppressive drugs	
Chapter 5	Human plasmacytoid dendritic cells induce $CD8^+LAG3^+$ $FoxP3^+CTLA4^+$ regulatory T cells that suppress allo-reactive memory T cells *Eur J Immunol. 2011 Jun;41(6):1663-74*	77
Chapter 6	Prednisone suppresses the function and promotes apoptosis of plasmacytoid dendritic cells *Am J Transplant. 2006 Oct;6(10):2332-41*	97
Chapter 7	Corticosteroids abrogate inhibition of hepatitis C infection by plasmacytoid dendritic cells	113
Chapter 8	Rapamycin has suppressive and stimulatory effects on human plasmacytoid dendritic cell functions *Revision submitted*	123
Chapter 9	Summary and discussion	139
	Nederlandse samenvatting	149
	Dankwoord	157
	Curriculum Vitae	159
	Portfolio	161
	List of publications	165

chapter 1

General introduction and outline of the thesis

The immune system

The immune system is the defense mechanism of the human body for protection against infections and other unwanted biological invasions. There are two types of immune responses: the innate immune response and the adaptive immune response.
Innate immune recognition relies on a limited number of germline-encoded innate immune receptors that recognize conserved products of microbial metabolism produced by pathogens, but not by the host. Recognition of these molecules allows the immune system to distinguish infectious nonself from noninfectious self (1). Antigen presenting cells (APC) form the bridge between the innate and the adaptive immune system, which has more variability and specificity. B en T cells are the mediators of the adaptive immune system. B-cells are the precursors of antibody secreting cells and can directly recognize antigens via their B-cell receptors, however T cells need the antigen to be processed and presented to them by an APC. The T-cell receptor (TCR) recognizes fragments of antigen in combination with molecules of the major histocompatibility complex (MHC). Two types of MHC molecules exist: MHC class I that is expressed by all nucleated cells in the human body and MHC class II that is expressed exclusively by APC, which present antigenic peptides to cytotoxic T cells and Thelper cells, respectively.

Dendritic cells (DC) are a specialized type of APC that can stimulate T cells very efficiently, being able to stimulate naïve T cells. Myeloid DC (MDC) arise in the bone marrow from hematopoetic stem cells and migrate via the blood circulation to all tissues in the body. In their immature state MDC can take up pathogens or antigens using their abundantly expressed innate immune receptors. The antigens enter the endocytic pathway of the DC and are digested to small peptides that are loaded in MHC molecules. After capturing antigens, DC are activated and travel to the T cell areas of lymphoid tissues like regional lymph nodes or spleen. They up regulate MHC-peptide complexes, co-stimulatory molecules such as CD80 and CD86, and secrete cytokines such as IL-12 and IL-6, to initiate T cell activation. The activated T cells begin to proliferate, produce cytokines, become cytotoxic and migrate from the lymphoid tissues to the site of inflammation (2-4).

Liver transplantation and rejection

The liver is the largest glandular organ of the body. Its average weigh is 1.5 kg and it is divided into four lobes of unequal size and shape. The liver lies on the right side of the abdominal cavity beneath the diaphragm. Liver tissue is composed of thousands of lobules, and each lobule consists of plates of hepatocytes, the basic cells of the liver. These hepatocytes play an essential role in metabolism. They synthesize plasma proteins, bile, cholesterol and hormones such as angiotensinogen, thrombopoietin, somatomedin C and Hepcidin. Furthermore they are involved in the detoxification of e.g. ammonia, drugs and steroids (5). The hepatic artery and the portal vein supply the liver with blood. The heptic artery carries oxygen-rich blood from the aorta. The portal vein carries blood containing digested nutrients from the small intestine. These blood vessels subdivide in the liver repeatedly and merge into very small capillaries, named sinusoids, between the hepatocyte plates.

In case of end-stage liver disease, liver transplantation is the only available life-saving therapy. Liver transplantation is the surgical process wherein a diseased liver is remo-

ved and replaced with a healthy one from another human being. The first successful human liver transplantation was performed by Thomas Starzl in 1967 (6) and since then surgical techniques improved to a great extent. After a liver transplantation the immune system will respond to the foreign antigens of the donor graft in order to eliminate the graft by causing rejection. Two types of allo-recognition that lead to graft rejection have been described: the direct and the indirect pathway (7). In the indirect pathway recipient T cells with the appropriate T-cell receptor are activated by recipient APCs that present peptides from donor origin in their major MHC-molecules. The most immunogenic peptides are those derived from donor MHC-molecules that are not expressed by the recipient (mismatched MHC), because MHC-molecules are highly polymorphic and for that reason there is a lot of variance between different individuals. The activated T cells start to proliferate and migrate to the graft where they cause organ damage. This is actually the normal mechanism for generating a T-cell response. In the direct pathway donor APCs migrate from the donor graft to the lymphoid organs of the recipient where they activate naïve but especially memory T cells from the recipient. These T cells express T-cell receptors that can respond to intact foreign/donor MHC-molecules. Recognition of intact foreign MHC can either be dependent or independent of the peptides that are presented in these MHC-molecules. On average 3 to 8% of the recipient T-cell repertoire is able to respond to intact foreign (allogeneic) MHC molecules, dependent on the number of MHC mismatches between the donor and the recipient (8). Most of these allogeneic T cells have a memory phenotype. They are self-MHC restricted and are directed to microbial-derived peptides (mostly of viral origin) but cross-react with allogeneic MHC. Since humans are exposed to a large repertoire of pathogens during their lifes, it is obvious that any given individual harbors a considerable number of pathogen-specific T cells that are potentially alloreactive (9-12).

The direct and the indirect pathway can both mediate acute and chronic rejection of the liver graft, therefore liver transplant recipients are in need of life-long immunosuppressive drug treatment.

Immunosuppressive drugs

In the early days of transplantation only corticosteroids were available as immunosuppressive drug. Corticosteroids are non-specific anti-inflammatory agents and their primary action is inhibition of cytokine gene transcription. They diffuse through the cell membrane, and upon binding to the corticosteroid receptor the newly formed complex translocates to the nucleus where it can bind to glucocorticoid response elements in the promoter regions of cytokine genes (13). Corticosteroids inhibit the function of both APC and T-cells at the level of proliferation and cytokine production (14). The use of steroids is associated with multiple side effects. In common with all potent immunosuppressive drugs, sepsis and infections are frequently encountered. In addition, corticosteroids can cause hypertension, Cushingoid appearance, personality changes, development of cataracts, weight gain, dyslipidaemia, osteoporosis, hyperglycaemia and diabetes (15).

In the late 70s the immunosuppressive effects of cyclosporine were discovered and applied with immense success in transplant patients (16-17). This gave an enormous boost to the transplantation field and changed liver transplantation from an experimental procedure into a standard clinical treatment. Cyclosporine belongs together with tacrolimus to the group of calcineurin inhibitors (CNI). Both agents depend on inhibition

of the activation of calcineurin, but differ in that cyclosporine binds to cyclophilins while tacrolimus binds to FK506 binding protein (18). Inhibition of calcineurin prevents the activation of the transcription factor NFAT and thereby impairing interleukin-2 (IL-2) production in T-cells (19). This has a profound effect on the process of rejection since IL-2 is a key cytokine for the activation and proliferation of T cells and various other immune cells. The two CNI have a different side-effect profile, which is due to the slight difference in mechanisms of action. Both agents can result in nephrotoxicity and haemolytic uremic syndrome, but cyclosporine has a higher risk of inducing hyperlipidemia, hypertension and cosmetic problems (growth of facial en body hair), while tacrolimus is more toxic for the central nervous system and the islets of Langerhans (20).

Mycophenolate mofetil (MMF) is a prodrug that releases mycophenolic acid and acts as a lymphocyte proliferation inhibitor. For the replication of DNA lymphocytes depend on the production of guanosine monophosphate nucleotides by an enzyme named iosine monophosphate dehydrogenase (IMPDH). This enzyme is blocked by MFF and thereby the proliferation of T en B cells (21). The drug is easy to use without the need for monitoring. The side-effects are gastrointestinal symptoms (mainly diarrhea), anemia and leukopenia (20).

Rapamycin is a relatively new immunosuppressive drug and until now in liver transplantation it is used only in experimental therapeutic regimens. Rapamycin is structurally related to tacrolimus and forms a complex with FK506 binding protein but does not inhibit calcineurin, but the mammalian target of rapamycin (mTOR) instead (20). mTOR is an evolutionary conserved serine-threonine kinase that senses the environmental en cellular nutrition status to control metabolism and cell growth. In T cells binding of rapamycin to mTOR interrupts the signal from the IL-2 receptor and the receptors for other cytokines and growth factors, which are required for the progression of cytokine-stimulated T cells from the G1 to the S-phase, thus suppressing the interleukin-driven T cell proliferation (22-24). The adverse effects of rapamycin include hyperlipidemia, thrombocytopenia and impaired wound healing. Mouth ulcers, skin lesions and pneumonitis are also reported (20).

Current immunosuppressive treatment consists of 3 phases: induction, maintenance en treatment of acute rejection (25). The induction phase comprises the first 30 days after liver transplantation when alloreactivity is at its peak. The most common induction therapy is a high dose of corticosteroids combined with a calcineurin inhibitor (CNI), like cyclosporine or tacrolimus, sometimes supplemented with mycophenolic acid (MMF). Clinical outcome is better with tacrolimus than with cyclosporine during the first year of liver transplantation (26). Maintenance therapy starts about 30 days after liver transplantation and lasts indefinitely. In this phase the corticosteroid dose is slowly lowered and ultimately discontinued and the CNI dose is reduced. After the first year patients are usually receiving monotherapy with just a CNI. Incidence of acute rejection depends on the underlying liver disease. Young patients with autoimmune disease have the highest incidence of acute rejection while older patients with alcoholic liver disease have the lowest incidence (27). Due to the intense immunosuppressive medication, currently the majority of liver transplant recipients do not experience acute rejection, and most acute rejection episodes are mild and are successfully treated with a high intravenous dose of methylprednisolone (3 times 500-1000mg). If the acute rejection is steroid-resistant patients are treated with anti-lymphocyte-depleting antibodies (28-29).

The liver as a tolerant organ

Currently used immunosuppressive drugs have a number of disadvantages. They have to be taken lifelong and have serious side-effects. The general shortcoming of contemporary immunosuppressive drugs is that they not only suppress the immunity against the graft but immunity in general, making the recipients much more susceptible for bacterial, viral and fungal infections and the development of malignancies. Therefore a major aim of transplantation research is to target the activated T cells directed against donor antigens to make them unresponsive or tolerant for the graft while leaving all other T cells untouched. Multiple therapeutic strategies are capable of inducing allograft tolerance in experimental animal models. The most successful strategies combined inactivation of alloreactive T cells via induction of anergy (e.g. by co-stimulatory blockade) or depletion (with anti-lymphocyte antibodies) and induction or transfer of regulatory T cells (30). However, in primates and humans all these attempts have proven unsuccessful, probably due to the large repertoire of donor reactive memory T cells that are relatively resistant to co-stimulation blockade and lymphocyte depletion (31-33).

Interestingly, there is a large amount of evidence that livers itself possesses tolerogenic properties, far more than other solid organs. Human liver allografts have a lower susceptibility to rejection than other organs and chronic rejection is extremely rare (34). Liver transplants are matched for ABO blood groups but no MHC matching of donor and recipient is required (35-36), and liver transplants protect kidney transplants from rejection after a combined liver-kidney transplantation (37-38). Moreover, in contrast to recipients of heart and kidney transplants it is possible to completely withdraw immunosuppression in about 20% of liver transplant recipients with stable liver graft function (39-41). The down site of this phenomenon is that chronic viral and parasitic infections as well as metastasis of malignancies develop relatively easy in the liver (42).

About 80% of the liver's blood supply is from the portal vein, that carries digested nutrients from the small intestine along with antigens and microbial products that originate form the bacteria present in the small and large intestines. Food antigens are harmless non-self molecules that do not pose any treat, and must therefore be ignored by the immune system. Indeed, the immune system responds to antigens administered orally by tolerance. Oral tolerance depends on the connection of the blood flow between intestine and liver (43). The important role of the liver in oral tolerance has been demonstrated by the infusion of donor lymphoid cells into the portal vein which resulted in extension of survival time of skin grafts (44), cardiac grafts (45), kidney grafts (45) and small intestinal transplants (46). One of the bacterial products entering the liver via the portal vein is an endotoxin named lipopolysaccharide (LPS), which is a product from the cell-wall of gram negative bacteria and a very potent activator of the immune system (47). Under normal circumstances, human blood contains no LPS, because the cells lining the liver sinusoids (i.e. sinusoidal endothelial cells and Kupffer cells) express the LPS receptors, CD14 and TLR4 and effectively remove most of the LPS out of the circulation (48-49).

However, immune cells in the liver are continuously exposed to LPS and non-self food antigens in the absence of an infectious treat. Therefore the local immune system of the liver responds differently to these agents compared to other tissues. The tolerogenic properties of the liver are currently explained by 3 different mechanisms:

1. Interleukin-10 production by Kupffer cells. Kupffer cells are a large population of sinusoidal macrophages that express CD14 and TLR4. When exposed to LPS, they produce the anti-inflammatory cytokine interleukin-10 (50) and when they are depleted or inactivated the induction of portal vein tolerance is prevented (51). Likewise, liver BDCA1$^+$ MDC and hepatic stellate cells also produce IL-10 upon LPS binding (52-53).
2. Clonal deletion or unresponsiveness of CD8$^+$ T cells in the liver. This theory states that CD8$^+$ T cells can be activated in the liver itself, and not only in secondary lymphoid. However, while CD8$^+$ T-cell activation in lymphoid organs is productive and causes inflammation (54-55), activation within the liver does not induce effector functions (56-57). Hepatocytes and especially liver sinusoidal endothelial cells (LSEC) play a role in this. Hepatocytes express low amounts of MHC class I molecules that can prime CD8$^+$ T cells. These T cells initially undergo clonal expansion, but are soon eliminated by apoptosis probably due to lack of costimulation (58-59). LSEC express high amounts of MHC class I molecules and are able to cross present soluble antigens to CD8$^+$ T cells. An LSEC primed CD8$^+$ T cells lack effector function. They do not acquire cytotoxic capability and fail to produce IFN-γ and IL-2 upon restimulation. The coinhibitory molecule PDL-1 (CD274) plays an important role in this. LSEC not only prime naïve CD8$^+$ T cells, but can also inhibit activation and proliferation of existing effector CD8$^+$ T cells in the liver. However, viral infection of the LSEC promotes differentiation into effector cells again (60-63).
3. Regulatory T cells (Treg). Treg are a subpopulation of T cells that suppress the immune system to prevent pathological self-reactivity. The majority of Treg are derived from the thymus as CD4$^+$CD25$^+$ T cells that express the transcription factor Foxp3 and are constitutively immunosuppressive (64-65). A small group of Treg originate in the periphery and are known as induced Treg (iTreg) (66). In addition to CD4$^+$CD25$^+$Foxp3$^+$ Treg, several different types of iTreg have been described and may be involved in graft acceptance. CD4 and CD8 double-negative T cells that have been associated with graft acceptance (67-68). CD3/CD46-induced regulatory CD4$^+$ T cells that can lyse activated effector T cells by using granzymes and perforin (69). CD3$^+$γδTCR$^+$ T cells (Vδ1-type) that produce high amounts of IL-10 and were found to be elevated in peripheral blood of tolerant liver transplant recipients (70-71). Invariant natural killer T cells, which lyse autologous T and B cells in a perforin-dependent manner may also play a role in transplant tolerance (72-73). IL-10 producing Tr1 cells that downmodulate MHC class II and co-stimulatory molecules on APCs (66, 74), and TGF-β producing Th3 cells (75-76). Besides CD4$^+$ Tregs, CD8$^+$ Tregs have been described. Phenotypically, the following types of CD8$^+$ Treg have been reported in the literature: CD8$^+$CD103$^+$ (77), CD8$^+$CD94$^+$NKG2A$^+$ (78), CD8$^+$CD25$^+$Foxp3$^+$ (79), CD8$^+$CD28$^-$ (80) and CD8$^+$LAG-3$^+$ which inhibit via CCL4 (81). There is only indirect evidence for a role of Treg in liver tolerance. In autoimmune liver disease and in primairy biliary cirrhosis the number of circulating Treg is reduced compared to controls and even more reduced during active disease (82-83). In liver transplant recipients with stable graft function, CD4$^+$ T cells were hypo responsive to allo-stimulation, but depletion of Tregs restored the allo-immune response partially (84). However direct evidence that prove the mechanistic function of Treg in liver immunology is lacking (42).
Many aspects of T-cell responses and the induction of tolerance in the liver are very well studied. However, in general T-cell responses are not initiated in parenchymal organs, but in lymph nodes, and up till now the knowledge about the role of liver draining lymph nodes in liver tolerance is very limited.

The chance of success to induce graft tolerance after transplantation is probably higher

in liver transplantation compared to other solid organ transplantation due to the tolerogenic properties of the liver. In experimental animal models adoptive transfer of *in vitro* expended $CD4^+CD25^+$ Treg can overcome rejection of allografts (85-87). However regulatory T-cell therapy has never been tested on solid organ transplantation in the clinic, although progress is being made. It is now possible to isolate en expand human $CD4^+CD25^+Foxp3^+$ regulatory T cells *ex vivo*, and in 2011 three clinical trials have been started to evaluate the safety and efficacy of Treg therapy in graft-versus-host-disease, showing promising safety and potentially efficacy profiles (88-91). Unfortunately, alloreactive T cells in humans contain many memory T cells (9-11), which are resistant to suppression by $CD4^+CD25^+Foxp3^+$ Tregs (92-93), Therefore it is a matter of debate whether transfer of this type of Treg will induce transplant tolerance in humans. For this reason, it is highly important to identify clinically applicable approaches that can inhibit allogeneic memory T-cell responses in humans.

Plasmacytoid dendritic cells

Plasmacytoid dendritic cells (PDC) are a rare (~0.2% of the human peripheral blood) distinct immune cell type that are specialized in direct virus recognition and in producing high amounts op interferon-alpha (IFN-α). PDC arise in the bone marrow and are thought to develop in the same pathway as myeloid dendritic cells (MDC). They share a progenitor termed the common DC progenitor that depends on the cytokine Flt3-ligand (Flt3L) for its development (94-95). Flt3L induces STAT3 phosphorylation what leads to expression of class I basic helix-loop-helix transcription factor E2-2 (96-97). E2-2 is essential for the development of PDC. Knock down or overexpression of E2-2 in human progenitors reduces or increases PDC development. The induced E2-2 enhances other key transcription factors, like IRF8 and SpiB, leading to PDC specific gene expression (98-99). Mature PDC also depend on expression of E2-2. Without E2-2 a mature PDC will spontaneously differentiate towards a cell with MDC properties (100). GM-CSF induces STAT5 phosphorylation and subsequent expression of the transcription factor Id2 (96). Id2 promotes MDC generation but inhibits the development of PDC (101-102).

Human PDC have high expression of the IL-3 receptor (CD123) and are characterized by the surface molecules BDCA2 (CD303) and BDCA4 (CD304) that are almost exclusively expressed on PDC (103). Antibodies against BDCA2 block the IFN-α production of the PDC, but antibodies against BDCA4 have no substantial effect on PDC function and can therefore be used for the isolation of PDC (104).

When PDC are fully developed they are released into the blood stream, and then migrate directly to the T-cell-rich areas of lymphoid organs via the high endothelial venules (HEV) (105-106). The interactions of CD62L on PDC that bind to peripheral node addressin (PNAd) on the HEV and CCR7 with CCL19 en CCL21 on the stromal cells appear to play a prominent role in the migration from blood in to lymph nodes (107-108).

PDC can take up antigen-antibody complexes via CD32 and present them to T cells (109), But it remains controversial whether PDC can phagocytose and process antigens independently from Fc receptors (110). PDC present peptides less efficiently to $CD4^+$ T cells than MDC. Compared to MDC, PDC accumulate MHC-II-peptide complexes less abundantly on their surface membrane and their turnover is higher, due to continuous production and degradation of MHC-II molecules (111-112). In contrast to mouse PDC,

human PDC are capable to cross-present viral antigens in MHC class I molecules, although the mechanism is not fully clear yet (113-115).

PDC sense viruses via toll like receptors (TLR). They express TLR-7, which recognizes single stranded RNA viruses like influenza, and TLR-9 which binds DNA viruses like herpes simplex virus. Both receptors are expressed in endosomal compartments (116-119). The binding of viral nucleotides to TLR-7 or TLR-9 leads to the recruitment of the adaptor molecule MyD88 and activates a pathway that ultimately leads to the phosphorylation of the key transcription factor IRF7. IRF7 translocates to the nucleus and initiates the transcription of IFN-α (120-121). In contrast to MDC, PDC express IRF7 constitutively and therefore they can produce very rapidly high amounts of IFN-α (121). The TLR-MyD88 pathway also activates NF-κB, which leads to the production of the pro-inflammatory cytokines TNF-α and IL-6. In addition, MHC and co-stimulatory molecules are up-regulated, giving PDC the ability to stimulate naïve and memory T cells (122).

Besides anti-viral responses PDC are also involved in the induction of immunological tolerance. Human thymic PDC can drive natural development of Treg from $CD4^+CD8^+$ and $CD4^+CD8^-CD25^-$ thymocytes. These natural Treg are more efficient in producing IL-10 than TGF-β (123-124), indicating that thymic PDC play a role in the selection of Treg that preferentially secrete IL-10 in response to auto-antigens in the periphery. In mice it was shown that peripheral immature PDC can transport peripheral antigens to the thymus and subsequent delete antigen-reactive thymocytes (125). Human PDC have an intrinsic capacity to induce hypo-responsiveness in naïve allogeneic Thelper cells, and to stimulate the differentiation of IL-10 producing Treg from naïve Thelper cells in vitro (126-130). In addition, it has been demonstrated that human PDC can induce the generation of Treg from naïve cytotoxic T cells in vitro (131). These $CD8^+$ Treg inhibit in an IL-10 dependent way. The mechanism by which PDC induce Treg is not fully established. Some studies suggest that kynurenine, the breakdown product of degradation of tryptophan by the enzyme indoleamine 2,3 dioxygenase, is mechanistically responsible for the generation of Treg (129-130), while others show that Treg generation is dependent on the interaction of ICOS-ligand on the PDC with ICOS on T cells (128). In mice, the binding of the notch ligand Delta-like-4 on the PDC to the notch receptor on the T-cells is also described as a mechanism for il-10 induction (132). Immature PDC can also directly suppress T-cell proliferation. Upon exposure to IL-3 or IL-10 they produce granzyme B, but not perforin, which results in inhibition of T cell expansion (133).

In experimental animals, PDC have been shown to be involved in several forms of immunological tolerance. Antigen presentation by PDC was observed to protect against experimental autoimmune encephalomyelitis (134). PDC also play a role in the induction of oral tolerance. Systemic depletion of PDC in mice prevented oral tolerance (135). These PDC deleted a large fraction of antigen specific $CD8^+$ T cells in the liver and also triggered the suppressive function of $CD4^+Foxp3^+$ Treg in secondary lymphoid organs (136). In a mucosal tolerance model, wherein a PDC depleted mouse developed asthma-like symptoms upon inhalation of inert antigens, adoptive transfer of antigen presenting PDC prior to sensitization abrogates this effect (137). The tolerogenic properties of PDC may be dependent on their anatomical location. It was found that unlike splenic PDC, PDC from peyer's patches are unable to produce IFN-α after TLR stimulation (138), and in a transplantation model the generation of Treg and prolonged graft sur-

vival was abrogated when the homing of PDC to lymph nodes was prevented (139).

Aim and outline of the thesis

The first part of this thesis describes studies on the possible involvement of myeloid dendritic cells in liver lymph nodes in the maintenance of liver tolerance. The second part shows that plasmacyoid dendritic cells can induce the generation of a peculiar subset of $CD8^+$ regulatory T cells that are able to inhibit alloreactive memory T-cell responses in a donor-specific fashion. This knowledge could in the future be utilized to develop a novel immunotherapic regimen aiming to induce donor-specific Treg in liver transplant recipients, or to treat liver transplant recipients with autologous Treg that are expanded *ex vivo* using donor-derived PDC. Moreover, we describe how immunosuppressive drugs affect PDC survival and functions, including the generation of $CD8^+$ Treg.

Part I. Myeloid dendritic cells: characterization in liver tissue and hepatic lymph nodes

In **chapter two** a technique to enrich MDC and PDC from cell suspension of inguinal lymph nodes was developed.
In **chapter three** 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 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 four** it was investigated whether hepatic MDC undergo in vivo an alternative maturation program leading to the presence tolerogenic effector MDC in the hepatic lymph nodes. Immunophenotypical en functional characteristics of human hepatic lymph node MDC were compared with those of skin/muscle draining inguinal lymph node MDC, spleenic and liver perfusate MDC.

Part II. Plasmacytoid dendritic cells: Induction of regulatory T cells and effects of immunosuppressive drugs

In **chapter five** it was investigated whether allogeneic PDC can induce regulatory T cells from unfractionated $CD3^+$ T cells, whether these Treg can inhibit effector T cells in a donor specific fashion, and whether these Treg can inhibit alloreactive memory T cells. The mechanism of Treg induction by the PDC and the mechanisms of suppression by the Treg was also determined.
In **chapter six** the effects of corticosteroids and CNI on the survival and functions of PDC were determined.
In **Chapter seven** these studies were extended by establishing the effect of corticosteroids on the anti-viral activity of PDC on HCV in an in vitro HCV-replicon model.
In **chapter eight** the effects of the immunosuppressive drug rapamycin on different functions of PDC, including the generation of Treg, were studied.

Finally an integrated view on the studies described in this thesis is provided, discussed and summarized in **chapter nine**.

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chapter 2

Immunomagnetic Selection of Functional Dendritic Cells from Human Lymph Nodes

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ABSTRACT

It was investigated whether positive immunomagnetic selection with two novel DC-specific mAb allowed purification of functional myeloid dendritic cells (MDC) and plasmacytoid dendritic cells (PDC) from human lymph nodes (LN). The results were compared with enrichment of DC by low-density Nycodenz gradient centrifugation followed by immunomagnetic depletion of residual B- and T-cells (Nycodenz method). MDC were selected from inguinal LN cell suspensions using CD1c mAb, and PDC using anti-BDCA-4 mAb. Immunomagnetic selection with anti-CD1c mAb yielded highly pure MDC-preparations (90%±3% MDC; n=7), provided that B-cells were thoroughly depleted by using CD19 magnetic beads and Large Depletion columns prior to selection of MDC. The purified MDC comprised both mature and immature cells, and were functional, secreting large amounts of cytokines upon stimulation, and strongly stimulating allogeneic T-cell proliferation. Immunomagnetic selection with anti-BDCA-4 mAb enriched PDC 70-fold to a purity of 59%±26% (n=8). The contamination consisted mainly of BDCA-4$^+$ T-cells and NK-cells. The previously used Nycodenz method yielded mixtures of MDC and PDC, not allowing functional studies of MDC and PDC separately.
In conclusion, positive immunomagnetic selection with CD1c mAb from human LN cell suspensions yields almost pure MDC-preparations, which are, in contrast to those obtained by the Nycodenz method, not contaminated with PDC. Moreover, these MDC are functionally intact. Selection with anti-BDCA-4 mAb does enrich PDC from human LN, but the resulting preparations are contaminated with T-cells and NK-cells.

INTRODUCTION

Two distinct types of dendritic cells (DC) have been identified in humans: myeloid DC (MDC), and plasmacytoid DC (PDC). MDC are present in non-lymphoid tissues as surveillance cells, specialized in recognition and capture of pathogens and processing of their antigens. After migration to regional lymph nodes (LN) and maturation, MDC present the antigenic peptides to T-cells and thereby stimulate T-cell responses. PDC on the other hand, migrate from the blood circulation into lymphoid tissues, and are the principal producers of Interferon-α (IFN-α), thereby being of major importance in the defence against viral infections (1).
Most studies on human MDC have been conducted with DC differentiated in vitro in the presence of GM-CSF and IL-4 from monocytes. Although these monocyte-derived DC share many characteristics with MDC present in vivo, they also differ from them in several aspects (2). In addition, there is considerable variation in phenotype and function of DC present at different anatomical localizations (3). Therefore, to get insight into DC function in vivo, it is preferable to isolate them directly from blood or tissues.
Until recently, purification of DC was difficult because of the lack of specific surface markers. Enrichment techniques involved low density gradient separation, using media supplemented with BSA, metrizamide, or Nycodenz, followed by flow-cytometric or immunomagnetic depletion of the residual contaminating cells (4-8).
To study the specific characteristics of DC in human lymph nodes (LN) at different anatomical locations, we initially isolated DC using Nycodenz density gradient centrifugation, followed by depletion of residual lymphocytes by immunomagnetic selection (9). However, this technique yielded mixtures of MDC and PDC, and was time-consuming. After it had been shown that MDC (10) and PDC (11) could be separately purified from blood by positive selection with the novel monoclonal antibodies (mAb) CD1c and anti-BDCA-4, respectively, we investigated whether these mAb were also suited for DC-purification from human LN. Here we report that immunomagnetic selection with CD1c mAb yields pure preparations of functional MDC from human LN, but that the anti-BDCA-4 mAb is not specific enough for purification of PDC from human LN. The DC-preparations obtained by selection with these mAb are compared with those enriched previously with the Nycodenz method.

MATERIALS AND METHODS

Patients and Lymph Nodes
Inguinal LN were obtained from kidney graft recipients during transplantation, and hepatic LN were obtained from donor livers during liver transplantation. LN were immediately transported in University of Wisconsin solution to the laboratory. The Medical Ethical Committee of the Erasmus Medical Center approved the study protocol, and informed consent was obtained from all patients.

Antibodies
CD11c-FITC, CD3-FITC, CD16-FITC, CD19-FITC, CD20-FITC, and CD80-FITC were from Beckman Coulter Immunotech, Marseille, France; CD11c-APC, CD14-FITC, CD20-FITC, CD56-FITC, CD86-APC, CD123-PE, CD123-Biotin, HLA-DR-PERCP, and Streptavidin-PerCP from BD Biosciences, Heidelberg, Germany; CD1c-PE (anti-BDCA-1), anti-BDCA 4-PE, CD3-microbeads, CD19-microbeads, Goat-anti-mouse Ig microbeads and anti-PE-microbeads were from Miltenyi Biotec, Bergisch Gladbach, Germany, CD83-PE from Caltach,

Burlingame, CA, USA and CD19 from Dako, Glostrup, Denmark.

Immunomagnetic selection of DC from lymph node cell suspensions
LN were cut into small pieces, and tissue-fragments were removed by filtration over nylon mesh. Mononuclear cells (MNC) were isolated by Ficoll density gradient centrifugation (Pharmacia Biotec, Uppsala, Sweden). If at least $40x10^6$ MNC were available, they were divided into two equal fractions, and one fraction was used for MDC-isolation and the second for PDC-isolation. If less than $40x10^6$ MNC were obtained, only one DC-type was isolated. MNC were suspended in 80 µl PBS supplemented with 2mM EDTA, 5 mg/ml BSA and 2.5 mg/ml human immunoglobulins (Octagam, Octapharma, Lachen, Switzerland), and incubated for 5 minutes to prevent subsequent FcR-mediated mAb binding. For isolation of MDC, 20 µl CD1c-PE mAb and 100 µl CD19 MACS-beads were added, and cells were incubated for 15 minutes at 4°C. Hereafter, B-cells were depleted by separation over a Large Separation (LS) or a Large Depletion (LD) column using a MidiMACS separation device (Miltenyi Biotec). The non-adherent cells were incubated for 15 minutes at 4°C with 50 µl anti-PE mAb-conjugated MACS-beads 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. For isolation of PDC, MNC were resuspended in 180 µl PBS supplemented with 2mM EDTA, 5 mg/ml BSA and 2.5 mg/ml human immunoglobulins, and incubated with 20 µl anti-BDCA4-PE mAb. Subsequently the cells were incubated with 50 µl anti-PE mAb-conjugated MACS-beads, and PDC were isolated by two rounds of separation over MS columns. Viability of cells was determined by trypan blue exclusion.

Isolation of DC from human lymph nodes by the nycodenz method
Enrichment of DC from LN MNC was done as described in (9). Briefly, monocytes and macrophages were depleted by adherence to culture plastic, and low-density cells were isolated by centrifugation over a gradient of 12.5% (w/v) Nycodenz (Nycomed Pharma, Oslo, Norway; density = 1.069 g/cm^3). Residual lymphocytes were removed by separation over a MS-column after sequential labelling with CD19 and CD20 mAb, CD3-conjugated MACS-beads, and Goat-anti-Mouse antibody-conjugated MACS-beads.

Flow cytometry
Purity of MDC isolated by immunomagnetic selection with CD1c-PE mAb was determined by labelling with a lineage-FITC cocktail (containing CD3, CD14, CD16, CD19, and CD56 mAb) and CD11c-APC. MDC were defined as $CD1c^+lin^-$ or $CD1c^+D11c^+$ cells. The maturation stage of MDC was determined by labelling with HLA-DR-PERCP, CD80-FITC and CD86-APC. Purity of PDC isolated by immunomagnetic selection with anti-BDCA-4 PE mAb was determined by labeling with lineage-FITC mAb and CD123-biotin followed by incubation with streptavidin-PerCP. PDC were defined as $BDCA\text{-}4^+CD123^+lin^-$ cells. The composition of the DC-enriched cell preparations isolated by the Nycodenz method was determined by labelling with CD11c-FITC, CD123-PE, and HLA-DR-PerCP. MDC were defined as $HLA\text{-}DR^+CD11c^+CD123^{low}$ cells and PDC as $HLA\text{-}DR^+CD11c^-CD123^{high}$ cells. In some isolations with the Nycodenz method, MDC were identified by labelling with CD83-PE and CD20-FITC, and defined as $CD83^+CD20^-$ cells. Optimal dilutions of all antibodies were established in preliminary experiments. Appropriate isotype-matched control antibodies were included for each analysis, and flow-cytometry was performed on a FACSCalibur flow cytometer using Cellquest software (Becton Dickinson).

Cytokine production

Purified MDC were cultured at a concentration of $4x10^4$ cells/200 µl in 96-wells flat bottom Costar plate (Costar Cambridge, MA) at 37°C in complete medium supplemented with GM-CSF (Leucomax; 500 U/ml; Novartis Pharma, Arnhem, The Netherlands), and stimulated with either polyriboinosinic-polyribocyticylic acid (poly (I:C), 20 µg/ml; Sigma-Aldrich, St. Louis, MA) and recombinant human IFN-γ (1000 U/ml; Strathmann Biotech, Hannover, Germany), or with Staphylococcus aureus Cowan strain I (SAC; 75 µg/ml, Calbiochem, San Diego, CA). Purified PDC ($4x10^4$ cells/200 µl) were cultured in the presence of IL-3 (10 ng/ml), and stimulated with SAC. After 24 hours supernatants were harvested and the levels of TNF-α, IL-6, IFN-α, and IL-10 were determined by specific sandwich ELISA, using pairs of mAb and recombinant cytokine standards from Biosource International, Camarillo, CA, USA.

Allogeneic T-cell stimulatory capacity of purified DC

Purified MDC or PDC were co-cultured at different concentrations (10, 5, 2.5, and 1.25 $x10^3$ cells/200 µl) in flat-bottom culture plates with 1.5 x 10^5 nylon-wool purified T-cells from blood of a healthy volunteer. After 5 days, cell proliferation was assessed by determination of the incorporation of [^{3}H] thymidine (Radiochemical Centre, Amersham, Little Chalfont, UK). 0.5 µCi was added per well, and cultures were harvested 18 hours later. Phytohemagglutinin (5 µg/ml, Murex, Paris, France) was added to T-cells as a positive control.

RESULTS

Composition of MDC- and PDC-preparations obtained by positive immunomagnetic selection

To obtain pure MDC suspensions by immunomagnetic selection with CD1c mAb, it was found to be essential that all B-cells (part of which express the CD1c antigen) were thoroughly depleted prior to selection of MDC. In a first set of isolations we used Large Separation (LS)-columns for B-cell depletion, and obtained cell preparations containing only 34%±27% MDC (mean ± SD; n=6) after selection with CD1c mAb. On the average 65% of the contaminating cells in these preparations were B cells. Thereafter, we started using Large Depletion (LD)-columns, which are especially designed for cell depleti-

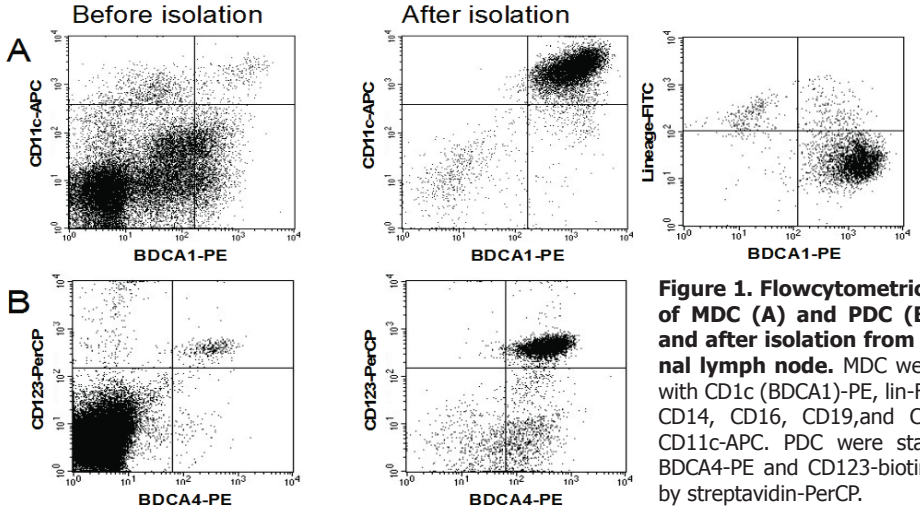

Figure 1. Flowcytometric analysis of MDC (A) and PDC (B) before and after isolation from an inguinal lymph node. MDC were stained with CD1c (BDCA1)-PE, lin-FITC (CD3, CD14, CD16, CD19,and CD56) and CD11c-APC. PDC were stained with BDCA4-PE and CD123-biotin followed by streptavidin-PerCP.

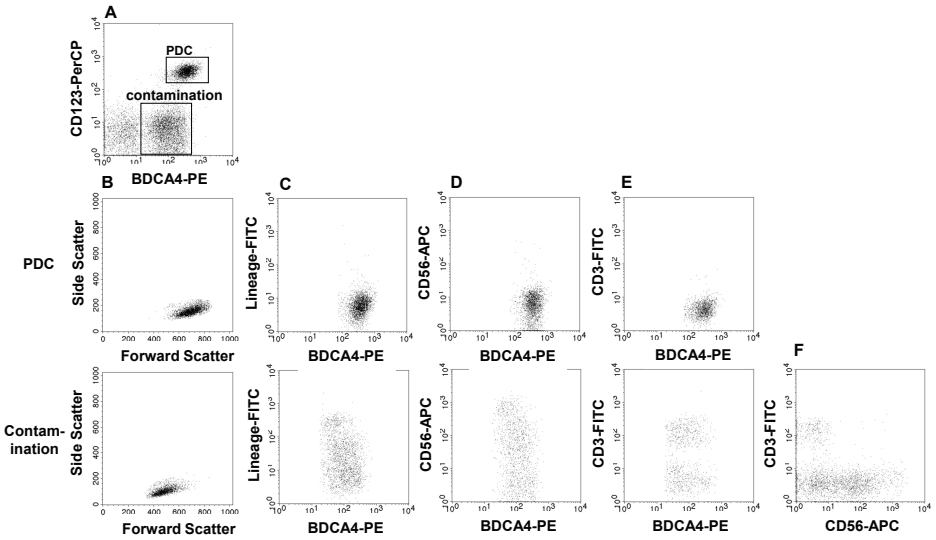

Figure 2. Immunophenotyping of contaminating cells in PDC-preparations isolated by positive selection with anti-BDCA-4 mAb from human inguinal LN. The major contamination consisted of BDCA^{-}4^{+}CD123^{-} cells (A). Depicted are the forward/side scatter properties (B), expression of lineage markers (CD3, CD14, CD16, CD19, and CD56) (C), CD56 (D), CD3 (E), and CD56 plus CD3 (F) on the BDCA-4^{+}CD123^{-} cells.

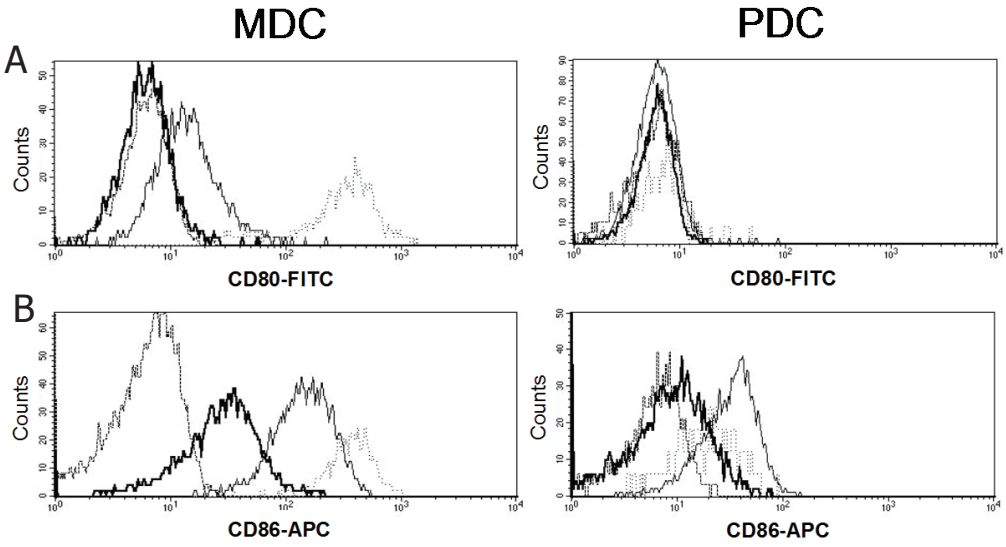

Figure 3. Comparison of expression levels of CD80 (A) and CD86 (B) on MDC (left panels) and PDC (right panels) isolated from different tissues. The bold solid line depicts blood, the thin solid line inguinal LN, the dotted line liver LN, and the dashed line the binding of isotype control mAb on blood MDC.

on, to remove B-cells. After depletion of B-cells with these columns, immunomagnetic selection of $CD1c^+$ cells from inguinal LN MNC which contained 0.88%±0.18% MDC, resulted in highly pure MDC-preparations (Figure 1A) containing 90%±3% $CD1c^+lin^-$ cells, i.e. a 100-fold enrichment (n=7). Comparable figures were found when MDC were defined as $CD1c^+CD11c^+$ cells. Immunomagnetic selection with anti-BDCA-4 mAb from LN MNC enriched PDC 70-fold (Figure 1B): from 0.86%±0.39% to 59%±26% $BDCA\text{-}4^+CD123^+lin^-$ PDC (n=8). The resulting PDC-preparations were mainly contaminated with $BDCA\text{-}4^+CD123^-$ cells (25%±13%; Figure 2A). These cells had forward/side scatter properties similar to lymphocytes (Figure 2B), and were partially positive for a cocktail of lineage markers (Figure 2C). Further immunophenotyping indicated that part of these cells expressed CD56 and another part CD3 (Figure 2D,E), but none of these cells were CD3CD56-double positive (Figure 2F). This suggests that the contaminating cells were partly T-cells and partly NK-cells.

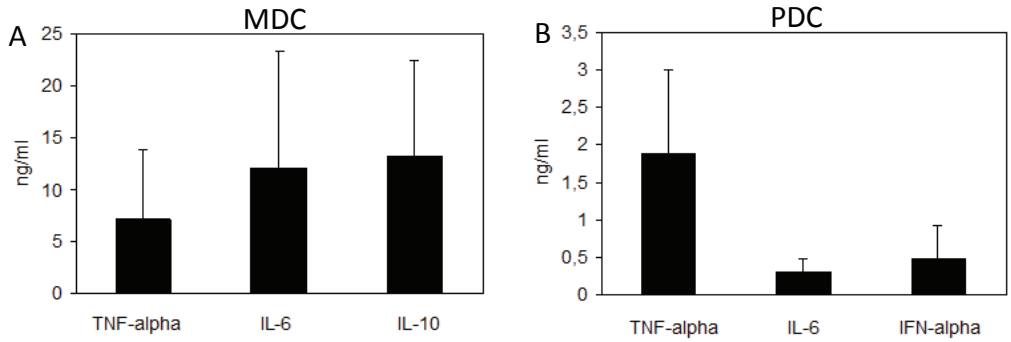

Figure 4. Production of cytokines by MDC and PDC purified by positive immunomagnetic selection from human inguinal LN. (A) TNF-α secreted by $4x10^4$ MDC into 200 µl culture medium was determined after 24-hour stimulations with poly (I:C) and IFN-γ. The production of IL-6 and IL-10 was determined after 24-hour stimulation with SAC. (B) TNF-α, IL-6 and IFN-α secreted by $4x10^4$ PDC into 200 µl culture medium were determined after 24-hour stimulation with SAC. Data are depicted as mean concentrations ± SD in culture medium derived from four different experiments.

Maturation stage, viability and functional capacity of DC

The MDC population isolated from inguinal LN using the CD1c mAb contained both mature and immature cells: 37%±32% expressed CD86, and 16%±10% CD80 (n=5). MDC isolated from blood by the same technique for another study (12) expressed no CD80 (Figure 3A) and CD86-expresssion was lower compared to MDC from inguinal LN (Figure 3B), while MDC isolated from liver LN were more mature compared to inguinal LN. As shown in Figure 3, MDC from liver LN contained almost no $CD80^-$ cells, which indicates that MDC preparations from LN were not contaminated with blood MDC. PDC isolated by positive selection with anti-BDCA-4 mAb were predominantly immature: only 5±5% expressed CD80 (n=6; Figure 3).

The viability of the DC isolated by the immunomagnetic method, as assessed by trypan blue exclusion, was moderate: 61%±8% were trypan blue-negative, and 72%±18% of PDC. Nevertheless, MDC were able to secrete substantial amounts of pro-inflammatory (IL-6 and TNF-α) and anti-inflammatory (IL-10) cytokines (Figure 4A). PDC secreted TNF-α and IFN-α, but relatively small amounts of IL-6 (Figure 4B). In addition, as shown in Figure 5, MDC were strong stimulators of allogeneic T-cell proliferation. As few as 5000 DC induced T-cell proliferation similar to that induced by PHA. PDC were less effective in stimulating allogeneic T-cell proliferation. This may have been due to a

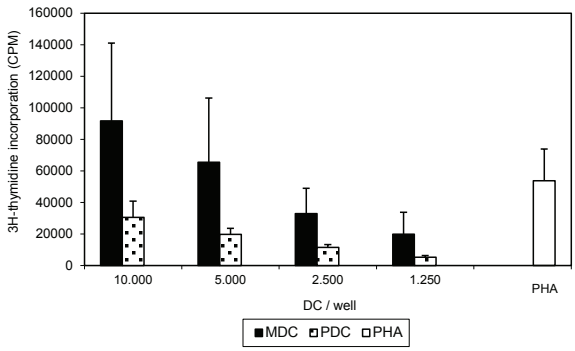

Figure 5. Allogeneic T-cell stimulatory capacity of MDC and PDC purified by positive immunomagnetic selection from inguinal LN. Different numbers of DC were co-cultured with $1.5x10^5$ allogeneic T-cells isolated from blood of a healthy volunteer. [^{3}H]-thymidine incorporation was determined after 5 days. As a positive control T-cells were stimulated with PHA. Data are depicted as means±SD derived from stimulations with MDC isolated from 6 human LN and with PDC from 5 human LN.

much lower expression of HLA-DR on isolated PDC as compared to isolated MDC (Mean Fluoresescence Intensities 371±121, and 2399±920; n=6), respectively.

Composition of DC-preparations enriched by the nycodenz method
The technique to enrich DC that we used previously, i.e. Nycodenz gradient centriguation followed by immunomagnetic depletion of residual T- and B-cells (Nycodenz method), yielded mixtures of MDC and PDC. During the time period in which we used this technique the CD1c mAb was not available. Therefore, in a first set of experiments, we defined MDC as HLA-DR$^+$CD11c$^+$CD123^{low} cells, and PDC as HLA-DR$^+$CD11c$^-$CD123^{high}. The DC-enriched preparations obtained contained 26%±12% MDC and 16%±13% PDC (n=5). In another set of experiments, in which we defined MDC as CD83$^+$CD20$^-$ cells, the mixture of DC obtained with this enrichment technique contained 40%±22% MDC and 11%±10% PDC (n=4).

DISCUSSION

This is the first report showing that positive immunomagnetic selection with the novel CD1c mAb is suitable for the isolation of pure and functional MDC from human LN. Selection with anti-BDCA-4 mAb from LN cell suspensions resulted in considerable enrichment of PDC, but the preparations obtained were contaminated with lymphocytes.
To obtain pure MDC, it appeared essential to deplete B-cells, part of which express the CD1c antigen, thoroughly by using a LD column instead of a LS column. MDC isolated from inguinal LN contained different maturation stages. Previously, all available MDC-specific mAb, like CD1a or CD83, recognized antigens which are restricted in their expression to specific type or maturation stage of MDC, and are therefore not suited for positive selection of the complete MDC-population. The latter is especially relevant as, in contrast to what has been believed for a long time, LN do not only contain mature MDC, but also immature differentiation stages (13). However, it should be realized that by positive selection with CD1c mAb some MDC subsets which are CD1c-negative, e.g. BDCA$^-$3$^+$ DC (10), may be missed.
Using anti-BDCA-4 mAb for positive selection, PDC-preparations with purities of more than 95% have been isolated from peripheral blood (11,14,15). However, upon selection with this mAb from LN cell suspensions, BDCA-4$^+$ lymphocytes were co-purified. Part of these cells were CD3-positive. According to Dzionek et al, these BDCA-4 expressing T-cells may be follicular B helper T-cells (16). In addition, we found that another part of BDCA-4$^+$ lymphocytes isolated by this technique from human LN expressed CD56,

suggesting that these cells are of NK-cell origin. Since several different BDCA-4$^+$ cell populations together with PDC are selected by the anti-BDCA-4 mAb, it will be difficult to optimize this technique for the isolation of pure PDC from human LN. The use of anti-BDCA-2 mAb for purification of PDC from LN might be a better alternative, because BDCA-2 expression in human LN is restricted to PDC (16). However, it has the disadvantage that binding of anti-BDCA-2 mAb to the BDCA-2 antigen suppresses one of the main functi ons of PDC, IFN-α production (17).

Although their viabilities, as assessed by the trypan blue dye exclusion test, were moderate, the MDC and PDC isolated by positive immunomagnetic selection were functional. Firstly, they were able to produce substantial amounts of pro- and anti-inflammatory cytokines, comparable to the amounts produced by highly viable MDC and PDC which we isolated from peripheral blood using the same technique (12). Secondly, the isolated MDC, although they were relatively immature, were strong stimulators of allogeneic T-cell proliferation. Apparently, MDC and PDC isolated from human LN by this technique can be used for functional studies.

Previously, we isolated DC from human LN by a technique in which first monocytes and macrophages were depleted by adherence to culture plastic, subsequently DC were enriched by a low-density Nycodenz gradient centrifugation step, and finally residual B- and T-cells were removed by immunomagnetic selection with CD3, CD19 and CD20 mAb and MACS-beads. Nycodenz gradient separation has been used by several research groups to enrich MDC from blood and lymphoid organs (7,8), but was not developed for enrichment of PDC. Our results show however, that it also enriches PDC from human LN. However, since this enrichment technique did yield mixtures of MDC and PDC, it is not suited for the study of functional properties of MDC and PDC separately.

In conclusion, positive immunomagnetic selection with anti-CD1c mAb from human LN yields pure and functional MDC. In addition, PDC can be enriched from human LN by positive immunomagnetic selection with anti-BDCA-4 mAb, but the resulting preparations are contaminated with BDCA-4$^+$ lymphocytes.

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chapter 3

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-BDCA1 monoclonal antibody (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 (MNC) contained 1.5% (range, 0.3-6.6%) MDC with a viability of 97±2%. Perfusates were a rich source of hepatic MDC since $0.9x10^6$ (range, $0.11\text{-}4.5x10^6$) 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), CD86 (P=0.003), and CCR7 (P=0.014) than mature hepatic lymph node (LN) MDC, and similar CD86 (P=0.140) and CCR7 (P=0.262) as and more DC-LAMP (P=0.007) and CD80 (P=0.002) than immature blood MDC. Perfusate MDC differed from blood MDC in producing significantly higher amounts of interleukin (IL)-10 in response to lipopolysaccharide (LPS), 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 toward 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 dendritic cells (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 (7) 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 major histocompatibility complex barriers and induce donor-specific tolerance without anti-rejection 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 major histocompatibility complex but few co-stimulatory molecules (11-13). In addition, DC generated *in vitro* from murine liver leukocytes 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 hematopoietic 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 that 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 major histocompatibility complex classes II and CD86, but a relatively 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 than 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 suggest that they facilitate organ graft survival (24), we have focused on the classical MDC. MDC were visualized by immunohistochemistry on liver cryosections using the anti-BDCA1 monoclonal antibody (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 flow cytometry. 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 10 liver grafts and used for isolation of liver leukocytes (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 Center during the back-table procedure were perfused through the portal vein with 1 to 2 L of University of Wisconsin 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 LN (n=12) were obtained from the hepatoduodenal ligament of donor livers during preparation of the liver pretransplantation. Blood samples were collected from 3 of the multiorgan donors and from 12 healthy volunteers. Nine hepatic LN were obtained from the same donors as perfusates; 5 of 10 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 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, IgG1-PerCP-Cy5.5, IgG2a-FITC, CD4-PerCP, CD19-FITC, CD20-PerCP, CD14-FITC, HLA-DR-FITC, CD86-APC, and streptavidin-APC from BD Bioscience, Erembodegem, Belgium; CD45-FITC, CD3-PE, CD56-APC, CD80-FITC, and anti-DC-LAMP-PE from Beckman Coulter Immunotech, Marseille, France; anti-BDCA1 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, CA; anti-CCR7-PE from R&D systems, Abingdon, UK.

Immunohistochemistry
To identify the location of MDC in donor livers pretransplantation, 5-µm 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 minutes), after which endogenous peroxidase 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 minutes, 20°C). The slides were washed with Tris-buffered saline (pH 7.4) twice, after which anti-BDCA1 or IgG2a isotype-matched controls were applied in optimal concentrations in Tris-buffered saline (pH 7.4) supplemented with 0.01% Normal Human Plasma for 18 hours at 4°C. Binding of anti-BDCA1 mAb was detected by incubation with conjugated goat anti-mouse Envision-PO (Envision™, DAKO, Glostrup, Denmark) for 30 minutes at room temperature and visualized

using 3-amino-9-ethylcarbazole. The sections were counterstained with Mayers hematoxylin (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-conjugated rabbit anti-FITC immunoglobulins (DAKO). Than slides were incubated overnight at 4°C with anti-BDCA1, and binding was detected with PO-Envision. Visualization of alkaline phosphatase was performed by incubation in Fast Blue salt/naphtol AS-BI phosphate solution supplemented with levamisole, giving a blue precipitate. Revelation of peroxidase was performed with 3-amino-9-ethylcarbazole, giving a red precipitate.
Six portal fields and 6 microscopic fields of 400x magnification in the parenchyma were analyzed, immunohistochemically positive cells were counted, and means with standard deviation (SD) were calculated.

Isolation of MNC
Cells were harvested from the perfusion fluid by centrifugation and resuspended in 30 ml of 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 that 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% fetal calf serum (Hyclone, Logan, UT), pencillin (100 U/mL), and streptomycin (100 µg/mL; Gibco BRL Life Technologies, Breda, The Netherlands) for 24 hours at 37°C in the presence of 100 ng/mL lipopolysaccharide (LPS) (Sigma, Zwijndrecht, the Netherlands) in 24-well plates using $1x10^6$ MNC per well. After 24 hours the cells were harvested and expression of maturation antigens and co-stimulatory molecules on MDC was determined using flow cytometric analysis.

Isolation of MDC from Blood and Perfusate MNC
For isolation of MDC 80 µl of PBS supplemented with 2 mmol/l ethylenediamine tetraacetic acid and 5 mg/ml bovine serum albumin (BSA), 100 µl of CD19 microbeads, and 20 µl of anti-BDCA1-PE were added per $100x10^6$ MNC, and the cells were incubated for 15 minutes at 4°C. Hereafter, B-cells were depleted by separation over a large depletion column using a Midi-MACS separation device (Miltenyi Biotec). The nonadherent cells were incubated for 15 minutes at 4°C with 50 µl of anti-PE microbeads and separated over a mini-separation 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 flow cytometry, 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%.

Flow Cytometry
Flow cytometric analysis was used to immunophenotype the MNC. MNC were resuspended in PBS with human immunoglobulin G (IgG) (1.25 µg/ml Octagam; Octapharma, Langenfeld, Germany) to prevent aspecific binding of antibodies to Fc-receptors on DC. Per labeling $1x10^6$ 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 $BDCA1^+$ and $CD20^-$ cells and PDC as $BDCA2^+$ cells. To determine the phenotype of MDC, MNC were labeled with the following antibody combinations: anti-BDCA1-PE and CD20-PerCP in combination with anti-HLA-DR-FITC, CD83-APC, CD80-FITC, and 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, then permeabilized and fixed with IntraPrep Permeabilization Reagent according to the manufacturer's protocol (Beckman Coulter Immunotech, Marseille, France) before adding the anti-DC-LAMP-PE. Cell death of the DC was determined using 7-amino-actinomycin-diacetate staining (BD Biosciences Pharmingen, San Diego, CA). 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, and $1.25x10^3$ cells/200 µl) in a flat-bottom Costar culture plate (Costar, Cambridge, MA) with $1.5x10^5$ purified T-cells from the blood of a healthy volunteer. After 5 days, cell proliferation was assessed by measuring the incorporation of [^{3}H]-thymidine (Radiochemical Center, Amersham, Little Chalfont, UK). 0.5 µCi was added per well and cultures were harvested 18 hours 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 minutes at 4°C. T-cells were enriched by negative selection over a large separation 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 $4x10^4$ cells/200 µL in a 96-well flat-bottom culture plate in $RPMI^+$ supplemented with 10% fetal calf serum (Hyclone, Logan, UT), pencillin (100 U/mL), streptomycin (100 µg/mL; Gibco BRL Life Technologies, Breda, the Netherlands), and granulocyte-macrophage colony- stimulating factor (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 hours, supernatants were harvested and the level of IL-10 was determined by specific sandwich enzyme linked immunosorbent assay, using a mAb and recombinant cytokine standard from Biosource International (Camarillo, CA).

Statistical Analysis

The Kruskal-Wallis and Mann-Whitney tests 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 of <0.05 was considered significant. All data are presented as means±SD.

RESULTS

MDC in Human Donor Livers

MDC were visualized in wedge biopsies of donor livers (n=7) by immunohistochemistry with anti-BDCA1 mAb. BDCA1$^+$ 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 BDCA1 is also expressed on a subpopulation of B-cells (25), additional double stains with anti-BDCA1 and CD19 mAb were performed (Figure 1C and D). Portal fields contained on average 4.9±6.0 BDCA1$^+$CD19$^-$ MDC per portal field, which is comparable to the number of BDCA1$^+$ cells observed in the single stains with anti-BDCA1 mAb (P=0.351). Both in portal tracts and parenchyma only few BDCA1/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%) BDCA1$^+$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).

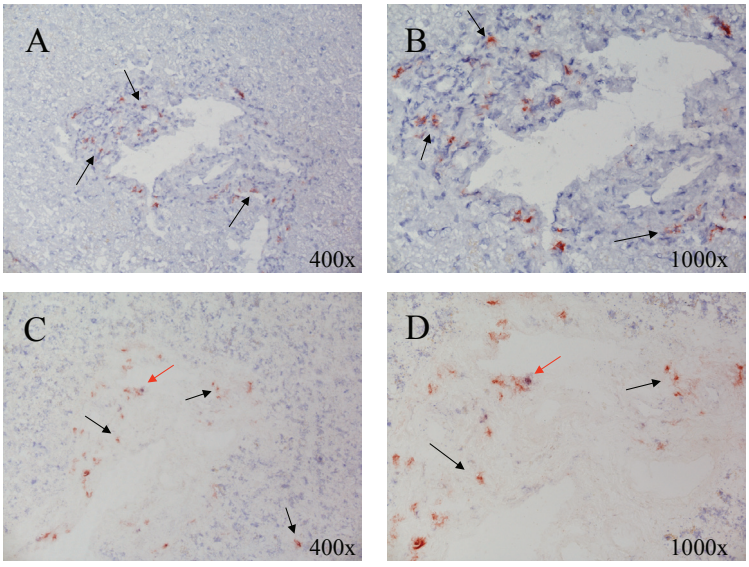

Figure 1. Localization of MDC in human donor livers. (A,B) MDC detected in a donor 5-µm liver cryosection by immuno- histochemistry with anti-BDCA1 mAb (red). The tissue was counterstained with hematoxylin (blue). (C,D) MDC detected in a donor 5-µm liver cryosection by immunohistochemistry with anti-BDCA1 mAb (red) and CD19 (blue). The black arrows indicate BDCA1$^+$CD19$^-$ MDC. The red arrow indicates a BDCA1$^+$CD19$^+$ B-cell.

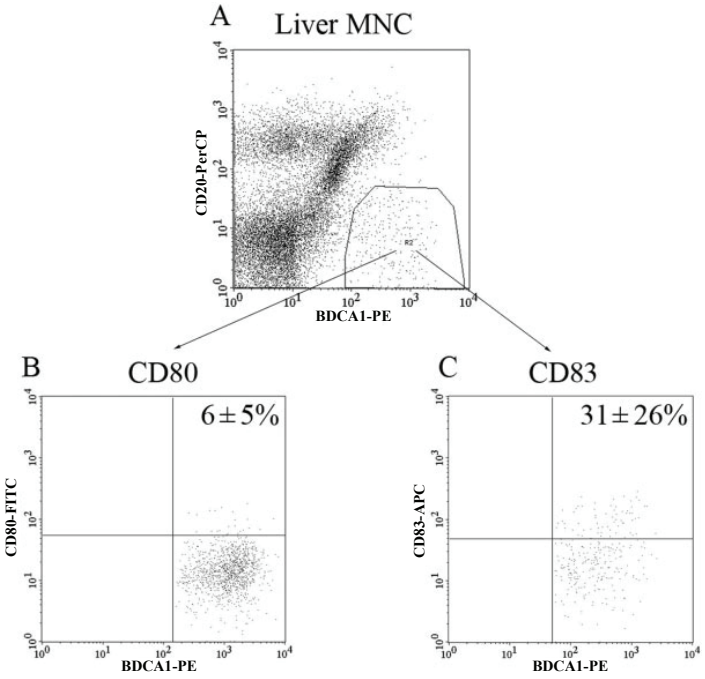

Figure 2. Characterization of MDC in human donor livers. (A) Liver MNC isolated from a donor liver wedge biopsy with MDC gated in R2. MDC were defined as BDCA1$^+$CD20$^-$ cells. A representative example of the expression of (B) CD80 and (C) CD83 on MDC in donor liver MNC. Data of MDC from 10 donor livers 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. The MNC present in the perfusates (99±2%) 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 (P=0.014) MNC differed significantly from blood (3.6±1.9) (Figure 3). Also, the natural killer 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 natural killer cells between liver and perfusate and the significantly distinct ratios of these cells in blood strongly suggest that leukocytes present in perfusates are predominantly liver tissue derived.

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 0.9x10^6 (range, 0.11-4.5x10^6). 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

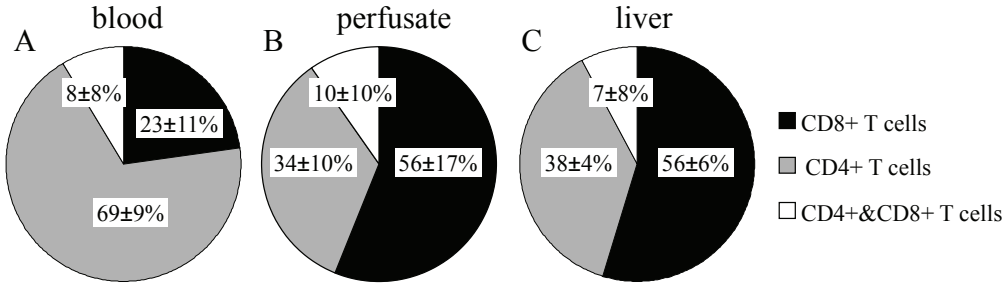

Figure 3. Composition of T-cells in perfusate compared with blood and liver MNC. MNC were isolated from (A) blood (n=8), (B) perfusate (n=17), and (C) donor liver biopsies (n=10). MNC were incubated with CD45-FITC, CD3-PE, CD4-PerCP, and CD8-APC mAbs, and T-cell subsets were analyzed by flow cytometry.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

0.3-0.7%; P=0.10). The total number of PDC that detached from donor livers during vascular perfusion pretransplantation was $0.5x10^6$ (range, $0.1\text{-}1.3x10^6$). The variation in volumes and numbers of MNC did not correlate with differences in cold ischemic times (Bosma, B.M., data not shown). Altogether, these data indicate that a high number of DC detach during perfusion from donor livers pretransplantation.

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 pairwise 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 obtained from the same multiorgan donors. 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 and that these MDC can be used to study the properties of hepatic 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

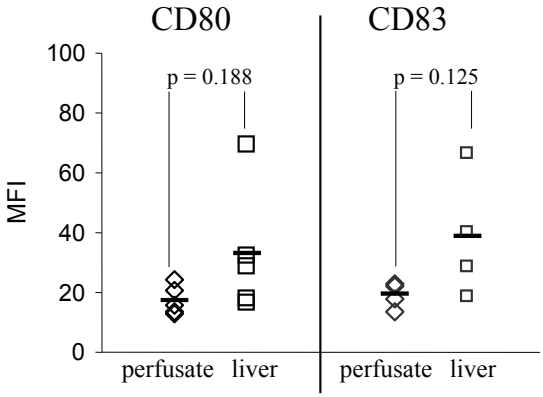

Figure 4. Expression of CD80 and CD83 on MDC from perfusate and liver MNC obtained from the same donor. The expression of the molecules on MDC was determined by flow cytometric analysis of MNC. MDC were defined as $BDCA\text{-}1^{+}$ and $CD20^{-}$ cells. Expression of CD80 (n=5) and CD83 (n=4) was similar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

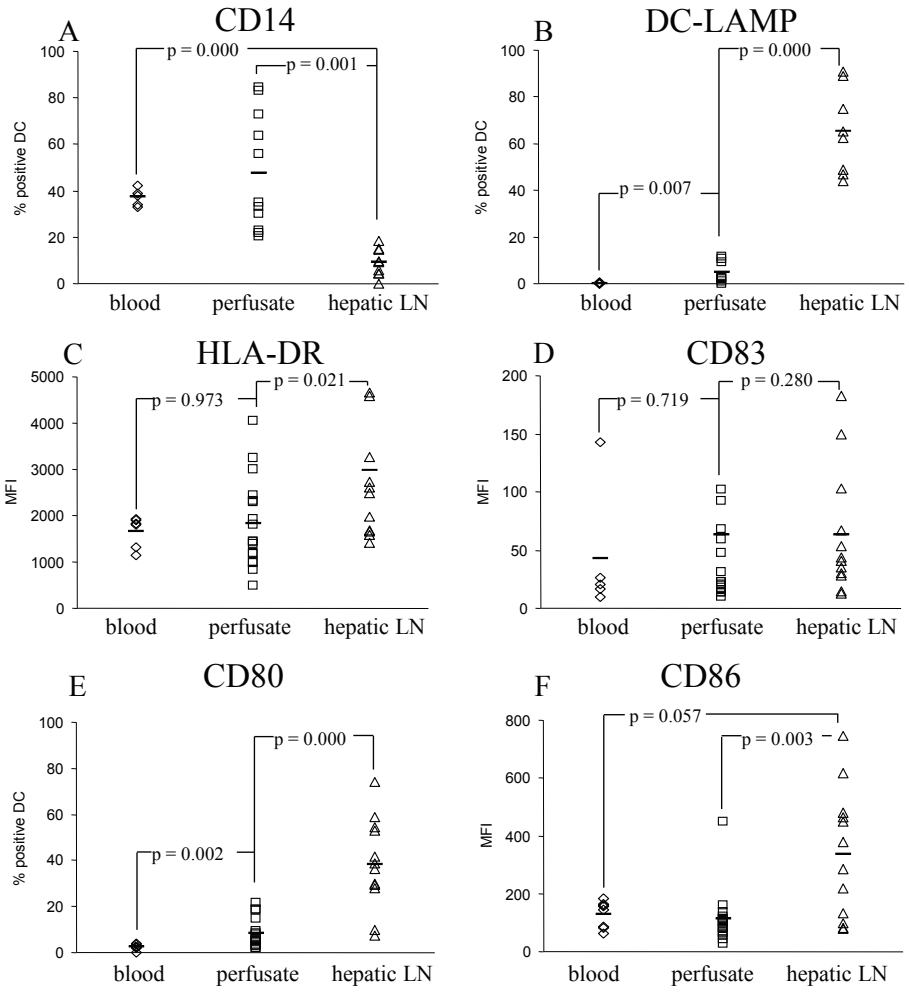

Figure 5. Maturation state of MDC derived from liver perfusate compared with blood and hepatic LN. The expression of maturation molecules on MDC was determined by flow cytometric analysis of MNC. MDC were defined as BDCA1$^+$ and CD20$^-$ cells. (A) Expression of CD14 was significantly higher on perfusate (n=11) and blood (n=5) MDC than on 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 significantly 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).

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

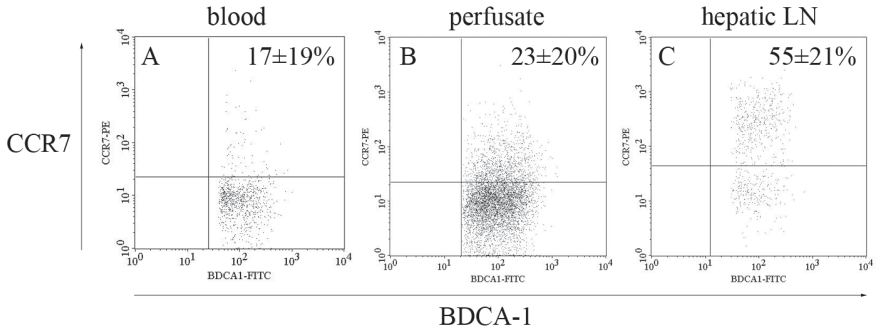

Figure 6. CCR7 expression on MDC from perfusate compared with blood and LN. CCR7 expression on MDC was determined by flow cytometric analysis of MNC. MDC were defined as BDCA1$^+$ and CD20$^-$ cells. (A) Blood MDC (n=4) and (B) perfusate MDC (n=12) had a similar expression of CCR7, but this was significantly higher on (C) LN MDC (n=10) (P<0.014). Data are expressed as mean±SD.

confirmed in paired comparisons of MDC from the same multiorgan donor (n=9): CD80 (P=0.012), CD86 (P=0.004), CD14 (P=0.016), and DC-LAMP (P=0.063). MDC in blood and perfusate expressed significantly lower levels of CCR7 than 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.

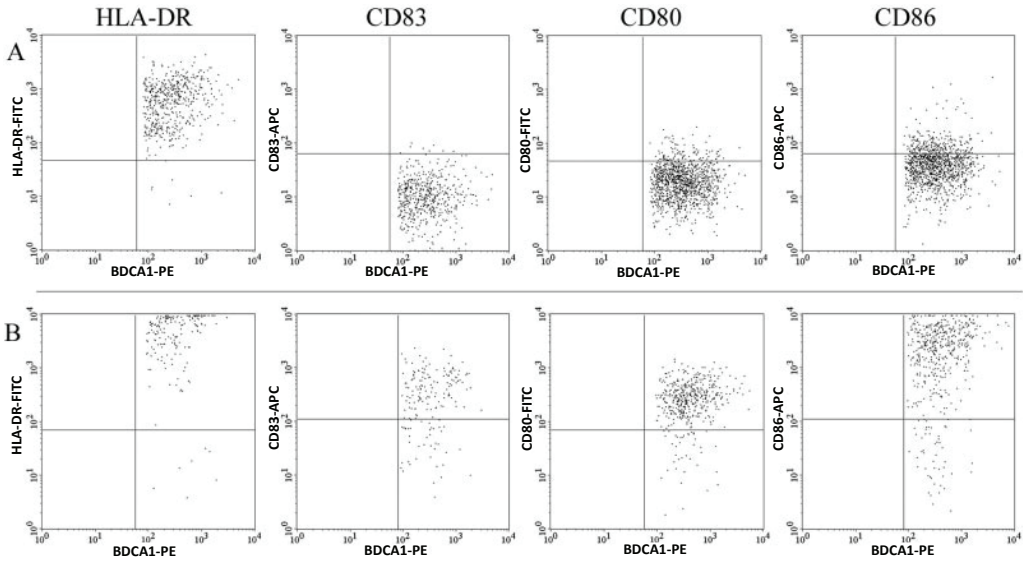

Figure 7. Phenotype of perfusate MDC after 24 hours maturation with LPS. Perfusate MNC were incubated with 100 ng/ml LPS at 37°C for 24 hours. Flow cytometric analysis was used to determine the maturation state of perfusate MDC at t=0 and t=24 hours. MDC were defined as BDCA1$^+$ and CD20$^-$ cells. (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=24 hours. Data shown are from 1 representative sample (n=3).

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 production 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).

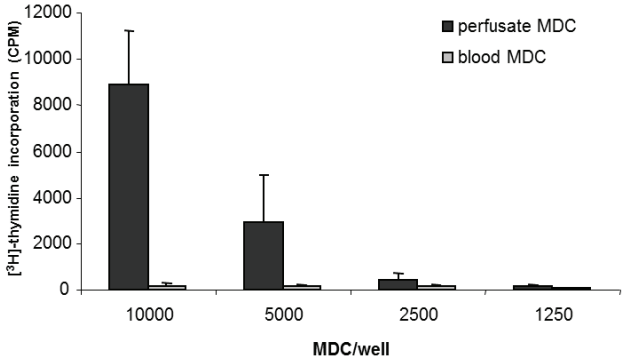

Figure 8. Allostimulatory capacity of perfusate MDC in comparison with blood MDC. Graded numbers of freshly isolated MDC were cultured with $1.5x10^5$ allogeneic T-cells. After 5 days the cells were pulsed for another 18 hours with [^{3}H]-thymidine. Perfusate MDC (n=3) stimulated T-cell proliferation, 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 flow cytometric analysis. The obtained data indicate that MDC from human donor livers have a relatively low expression of the maturation marker CD83 and costimulatory 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 major histocompatibility complex class II, but few co-stimulatory molecules (11, 13). The immunochemical stains with anti-BDCA1 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, natural killer 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 $0.9x10^6$ MDC and $0.5x10^6$ PDC. Since PDC are less efficient in antigen presentation and first data on their role in transplantation suggest 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 as resident liver MDC. Furthermore, CD80 expression was slightly higher on perfusate MDC than on blood MDC. Second, perfusate MDC produced significantly higher amounts of IL-10 than blood MDC upon stimulation with LPS. High IL-10 production is also characteristic for liver MDC, as

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 natural killer 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 derived either 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 one million MDC, which 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 clinical trial for treatment of advanced metastatic melanoma (stage VI), it is known that intravenous injection of as little as $0.9x10^6$ 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 recipient's LN or spleen these donor MDC are thought to present allogeneic major histocompatibility complex 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 pretreatment 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.

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chapter 4

Characterization of dendritic cell subsets in human hepatic lymph nodes

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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. In general T cell responses are not initiated in parenchymal organs, but in secondary lymphoid organs like spleen and LN. Therefore we compared numbers, immunophenotypical and functional characteristics of plasmacytoid dendritic cells (PDC), myeloid dendritic cell (MDC) subtypes, and macrophages (MF) in hepatic lymph nodes (LN) with those in skin- and muscle-draining inguinal- and iliacal LN, spleen and liver tissue.

Hepatic LN, iliacal LN, spleen and liver perfusate were obtained from multiorgan donors, and inguinal LN were optained from kidney transplant recipients. DC and MF were immunophenotyped by flowcytometry. BDCA1^{+}BDCA3^{+} MDC and PDC were isolated by positive immunomagnetic selection and tested for their capacity to produce cytokines and stimulate allogeneic T cells.

Hepatic LN contain significantly lower numbers of BDCA1^{+}BDCA3^{+} MDC compared to inguinal LN and spleen. Compared to spleen, numbers of BDCA3^{high} MDC were low in both types of LN. Both BDCA1^{+}BDCA3^{+} MDC and BDCA3^{high} MDC showed a more mature immunophenotype (higher HLA-DR, CD40, CD86 and PDL-1 expression) than those in inguinal LN, spleen and liver tissue. Despite their higher maturation status, BDCA1^{+}BDCA3^{+} MDC isolated from hepatic LN have a lower T-cell stimulatory capacity and produce less cytokines (IL-10, IL-12, TNFα and IL-6) than BDCA1^{+}BDCA3^{+} MDC from inguinal LN. The number of PDC in hepatic LN is comparable to the number in spleen and liver, but ~7 times lower than in inguinal LN. CD40 and HLA-DR expression on PDC did not differ between the tissues. Hepatic LN contain 2 times less CD14^{+}CD163^{+}DC-SIGN^{+} MF than inguinal LN. In the hepatic LN these CD14^{+} cells have a lower CD86 and HLA-DR expression than CD14^{+} cells in inguinal LN.

In conclusion, hepatic LN contain a low numbers of three types of antigen presenting cells (i.e lower numbers of MDC, PDC and CD14^{+} MF). In addition, BDCA1^{+}BDCA3^{+} MDC in hepatic LN are functionally impaired. Both phenomena may result in a reduced chance of T-cell activation in liver LN, thereby contributing to the relatively low immunogenicity of the liver environment.

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 important. However, as a consequence the liver is prone to chronic viral and parasitic infections, and metastases of malignant diseases develop relatively easy 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 malaria 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 (4). 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 20 percent of liver transplant recipients (7-10).

It has been 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). Two main subtypes of DC can be distinguished: Myeloid DC (MDC) and plasmacytoid DC (PDC). Immature 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 LN, MDC complete their maturation and acquire T-cell stimulatory capacity (12-14). The characterization of human MDC subtypes has for a long time been thwarted by their rarity, lack of distinctive markers, and limited access to human tissues. MDC are classified as antigen-presenting leucocytes that lack leucocyte lineage markers (CD3, 14, 15, 19, 20 and 56) and express high levels of MHC class II molecules (12). They express CD11c and lack CD123 and can be subdivided in CD1c (BDCA1)-expressing MDC and CD141 (BDCA3)-expressing MDC (15). Both subtypes appear to originate out of a shared precursor, but have unique gene expression profiles, which predicts that they have different functions (15-19). Recently, computational genome-wide expression profiling clustered human $CD141^+$ MDC and $CD1c^+$ MDC with the mouse $CD8\alpha^+$ and $CD8\alpha^-$ MDC (20). Indeed, human $CD141^+$ MDC and mouse $CD8\alpha^+$ MDC share a number of similarities. They both express Toll like receptor-3 and the C-type lectin CLEC9a and both can cross-present antigens in MHC class I molecules (21-24). As with all non-lymphoid tissue MDC (25), freshly isolated human and murine liver MDC are predominantly immature, expressing surface MHC class II molecules but low amounts of co-stimulatory molecules (11, 26-30). However liver MDC have some unique characteristics. We and others showed that human liver MDC produce higher amounts of the anti-inflammatory cytokine IL-10 compared to blood or skin MDC (28, 31). Liver MDC are also less efficient in stimulating allogeneic T-cell proliferation compared to spleen or skin MDC (28, 30-33). MDC propagated from murine liver progenitors even have the capacity to induce donor-specific hyporesponsiveness and prolong graft

survival when injected prior to allogeneic organ or tissue transplantation (32, 34-35). However, human liver MDC are not invariably tolerogenic, but can upon activation via Toll-like Receptors also produce pro-inflammatory cytokines and acquire T-cell stimulatory capacity (36).
PDC are unique in rapidly producing massive amounts of type I interferon upon recognition of viral nucleotides or self-DNA-protein complexes by their Toll-Like receptors (TLR). Upon maturation, PDC can also prime productive $CD4^+$ and $CD8^+$ T cell responses (37). On the other hand they possess the capacity to generate $CD4^+$ and $CD8^+$ regulatory T cells (Treg) from naïve $CD4^+$ or $CD8^+$ T cells, respectively (38).
In general T-cell responses are not initiated in parenchymal organs, but in secondary lymphoid organs like spleen and LN. Major liver lymph vessels leave the liver at the hilus, pass the hepatoduodenal ligament, and liver-derived lymph drains through LN situated at the hilus and along the hepatic artery and portal vein, in which MDC migrating from the liver are trapped (39-40). Indeed, T cells specific for viral antigens encoded in the liver are preferentially located in the hepatic LN and not in the liver itself (41). In addition, immunological tolerance to antigens delivered into the portal vein coincides with induction of hyperresponsiveness in T cells in the hepatic LN in mice (42).
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 human liver-draining LN. We hypothesized that, in order to prevent immune reactivity to non-hazardous gut-derived antigens, subtype composition and function of DC in liver draining LN may differ from that in LN located at sites of the body where immunogenic responses are required. The aim of this study was to compare immunophenotypical and functional characteristics of PDC and MDC subtypes in hepatic LN with DC in skin- and muscle-draining LN, spleen and liver tissue.

MATERIALS AND METHODS

Collection of lymph nodes, spleen and liver perfusate
Hepatic LN were obtained from the hilus and along the hepatic artery and portal vein from multi-organ donors. Iliacal LN and spleen tissue were also obtained from multi-organ donors. Liver leukocytes were collected during routine perfusion of liver grafts immediately before transplantation, as we described previously (28). Inguinal LN were obtained from kidney transplant recipients and are skin/muscle draining LN. The Ethics Committee of the Erasmus MC approved the study protocol and informed consent of each patient was obtained.

Reagents
The following mAbs and reagents were used: IgG1-FITC, IgG1-PerCP-Cy5.5, IgG2a-APC, IgG1-PB, CD14-PE, CD45-HorizonV500, streptavidin-PerCP, 7AAD and Lineage cocktail-FITC (CD3, 16, 19, 20, 14, 56) from BD Biosciences, Erembodegem, Belgium; CD80-FITC from Beckman Coulter, Woerden, the Netherlands; anti-BDCA1-PE, anti-BDCA2-FITC, anti-BDCA2-biotin, anti-BDCA4-PE, anti-BDCA3-APC, anti-Clec9a-VioBlue, CD19 microbeads and anti-PE-microbeads from Miltenyi Biotec, Bergisch Gladbach, Germany; CD1a-PB, CD209 (DC-SIGN)-PECy7, CD206-eFluor450, CD40-APC and CD274 (PDL1)-PECy7 from eBioscience, Vienna, Austria; CD86-APC, CD86-PB, CD163-PerCP-Cy5.5, CD40-PerCP-Cy5.5, HLA-DR-APC-Cy7 and CD209 (DC-SIGN)-APC from Biolegend, London, United Kingdom.

Isolation of mononuclear cells (MNC)
Lymph nodes and spleen were cut into small pieces and treated with 500 µg/ml collagenase and 200 µg/ml DNAse I (both from Sigma Aldrich, Zwijndrecht, the Netherlands) at 37°C. After 20 minutes the collagenase reaction was stopped by adding fetal bovine serum and the tissue pieces were passed over a nylon mesh filter (100 µm pore diameter) to obtain a single cell suspension. To determine possible effects of collagenase on surface molecule expression, a few LN and spleens were divided into two parts, and one part was treated with collagenase and the other part not. MNC were obtained from single cell suspensions of tissue by Ficoll Paque (GE Healthcare Biosciences AB, Uppsala, Sweden) density centrifugation. Cell yield and viability was determined by counting with trypan blue.

Flowcytometry
Flowcytometric analysis was used to immunophenotype splenic, lymph node, and liver DC. MNC were labeled with the following antibody combinations: (1) lineage-FITC, BDCA1-PE, 7AAD, BDCA3-APC, CD45-HorizonV500 and CD1a-PB; (2) lineage-FITC, BDCA1-PE, 7AAD, BDCA3-APC, CD45-HorizonV500 and Clec9a-Vioblue; (3) BDCA2-FITC, BDCA4-PE, 7AAD, CD40-APC, HLA DR-APC-Cy7 and CD45-HorizonV500; (4) CD80-FITC, CD14-PE, CD86-APC, CD163-PerCP-Cy5.5, CD209 (DC-SIGN)-PECy7, HLA DR-APC-Cy7 and CD45-HorizonV500; (5) Lineage-FITC, BDCA1-PE, CD40-PerCP-Cy5.5, BDCA3-APC, CD274 (PDL-1)-PECy7, HLA DR-APC-Cy7, CD45-HorizonV500 and CD86-PB. Appropriate isotype matched control antibodies were used. DC subsets were determined within a MNC gate after exclusion of $CD45^-$ non-hematopoietic cells and $7AAD^+$ dead cells. MDC subsets were distinguished on basis of BDCA1 and BDCA3 expression. PDC were defined as $BDCA2^+BDCA4^+$, and monocytes/macrophages as $CD14^+$. The data were analyzed on a FACSCanto II using FACSDiva 6.1 software.

Isolation of MDC and PDC
MDC were isolated by labeling LN MNC with anti-BDCA1-PE and CD19 microbeads and depletion of 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 a 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-BDCA4-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%.

Cytokine production and T-cell stimulatory capacity of DC
Purified MDC were cultured at a concentration of $4x10^4$ cells/200ul in flat bottom culture plates (greiner Bio-one, Alphen a/d Rijn, the Netherlands) in RPMI1640 supplemented with 10% FCS (Hyclone, Logan, UT, USA), penicillin (100 U/ml), streptomycin (100 U/ml) and GM-CSF (500 U/ml; Leucomax, Novartis Pharma, Arnhem, the Netherlands) and stimulated with either 20 µg/ml synthetic double-stranded RNA (polyriboinosinic-polyribocytidylic acid; poly I:C; Sigma-Aldrich, St. Louis, MO) and 1000 U/ml IFN-γ or CD40L-transfected J558 plasmacytoma cells ($4x10^4$ cells/well) and 1000 U/ml IFN-γ for 24 hours and 37°C. After 24 hours, supernatants were harvested and levels of IL-12,

IL-10, IL-6 and TNF-α were determined by standard enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions (eBioscience, Vienna, Austria). The T-cell stimulatory capacity was assessed by co-culturing MDC and PDC at different concentrations (10, 5, 2.5, $1.25x10^3$ cells/200 ul) in flat bottom culture plates in RPMI1640 supplemented with 10% FCS, penicillin and streptomycin with $1.5x10^5$ T cells isolated from blood of a healthy volunteer. In all experiments T cells from the same individual were used. After 5 days, T-cell proliferation was assessed by measuring the incorporation of [^{3}H]-thymidine (Radiochemical Centre, Amersham, Little Chalfont, UK). 0.5 µCi was added per well and cultures were harvested 18 hours later. Phytohemagglutinin (PHA; 5 µg/ml, Murex, Paris, France) was added to the T cells as a positive control for the proliferative potential.

Statistical analysis
The unpaired t-test was used to test whether differences between hepatic and inguinal LN were statistically significant. The paired t-test was used for differences between hepatic lymph nodes, iliacal LN, spleen and liver. A $p<0.05$ was considered significant. Microsoft office excel 2003 software was used to perform the statistical tests. All data are presented as means±SEM.

RESULTS

Myeloid dendritic cell subtypes in lymphoid organs and liver
To study MDC subsets, MNC freshly isolated from hepatic LN, inguinal LN, spleen, and liver graft perfusates were analyzed for expression of BDCA1, BDCA3, and lineage markers (CD3, CD16, CD19, CD20, CD14 and CD56). Since leukcoytes in liver graft perfusates accurately represent leukocytes present in liver tissue (28, 36, 43-45) we will refer to liver perfusate DC as liver DC. $CD45^+Lineage^-BDCA1^+$ and $CD45^+Lineage^-BDCA3^+$ cells were gated, and both gates were combined to determine MDC subsets using BDCA1 and BDCA3 antibodies (figure 1A). Three different populations were observed: $BDCA1^-BDCA3^+$, $BDCA1^+BDCA3^+$, and $BDCA1^+BDCA3^-$ cells. Within $BDCA1^-BDCA3^+$ cells, $BDCA3^{bright}$ and $BDCA3^{dim}$ subsets could be distinguished. All $BDCA3^{bright}BDCA1^-$ cells expressed CD11c (Figure 1B). In liver the $BDCA3^{bright}BDCA1^-$ population also expressed Clec9A (figure 1C), identifying them as the recently discovered human equivalent of mouse $CD8α^+$ MDC, which have a superior ability to cross-present antigens compared to other MDC subsets (18). However, Clec9a expression was reduced on splenic $BDCA3^{bright}$ MDC, and very low on $BDCA3^{bright}$ MDC in LN (figure 1C). Clec9a is a C-type lectine receptor expressed on immature $BDCA1^-BDCA3^{bright}$ MDC and cell surface expression is lost after MDC maturation (46). As shown in Figure 2B, LN $BDCA1^-BDCA3^{bright}$ MDC show a higher maturation status compared to splenic and liver MDC, explaining the absence of Clec9a expression in LN. In none of the tissues $BDCA1^-BDCA3^{bright}$ MDC expressed CD1a, CD206, or DC-SIGN, indicating that both types of LN, as well as spleen and liver, contain a homogeneous population of $BDCA1^-BDCA3^{bright}$ MDC that correspond to blood $BDCA1^-BDCA3^{bright}$ MDC (18-19). All $BDCA1^+BDCA3^+$ cells in the examined tissues expressed CD11c (Figure 1B), identifying them as bona fide MDC, confirming that BDCA1 and BDCA3 are co-expressed on the major MDC subset in LN, as was previously reported (47). In all examined tissues 10%-20% of $BDCA1^+BDCA3^+$ MDC expressed CD1a and a small proportion expressed CD206, but they were negative for DC-SIGN (data not shown). Together, these data show that a minority of $BDCA1^+BDCA3^+$ MDC (the ones that express CD1a) represent migratory MDC derived from skin or other epithelia,

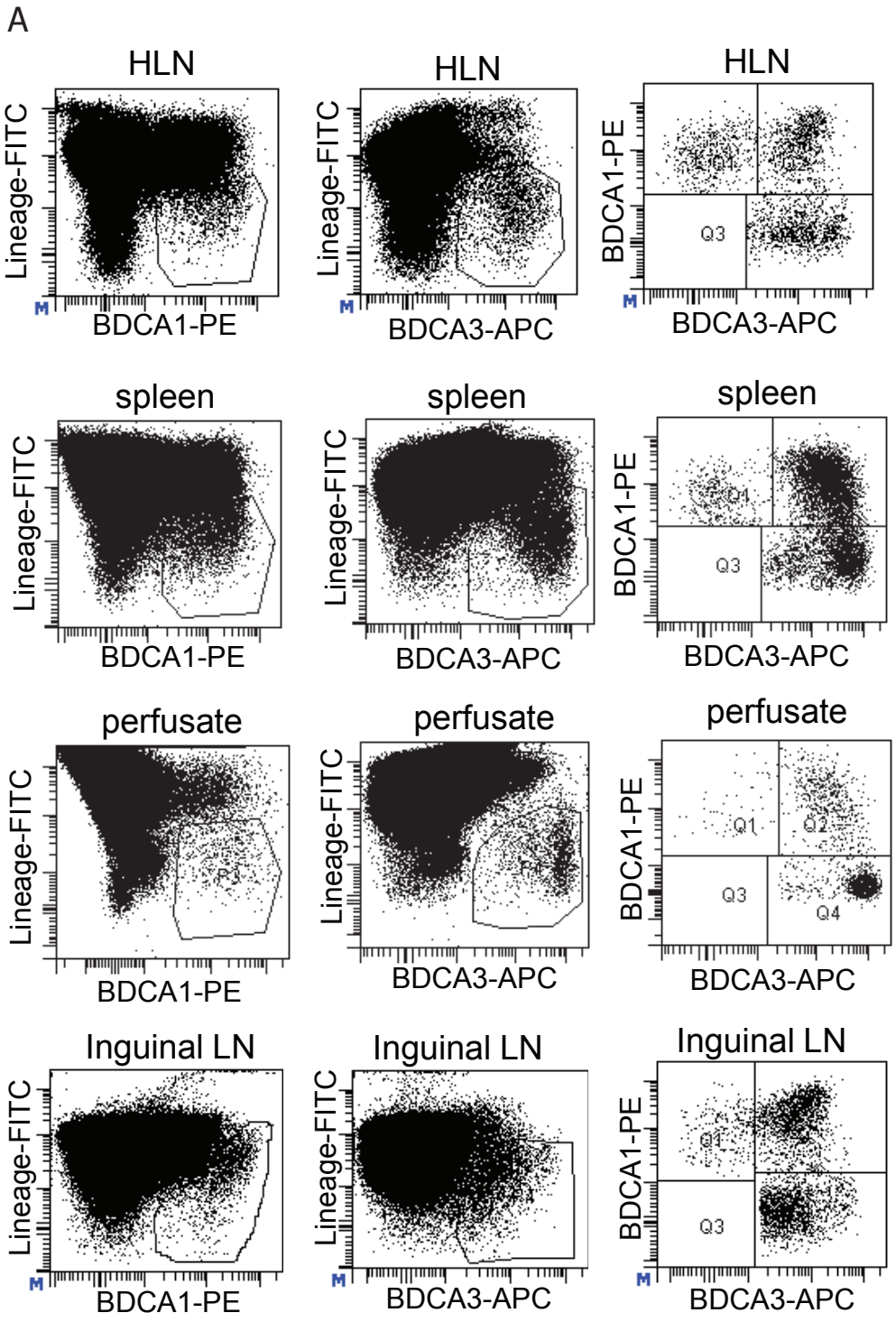
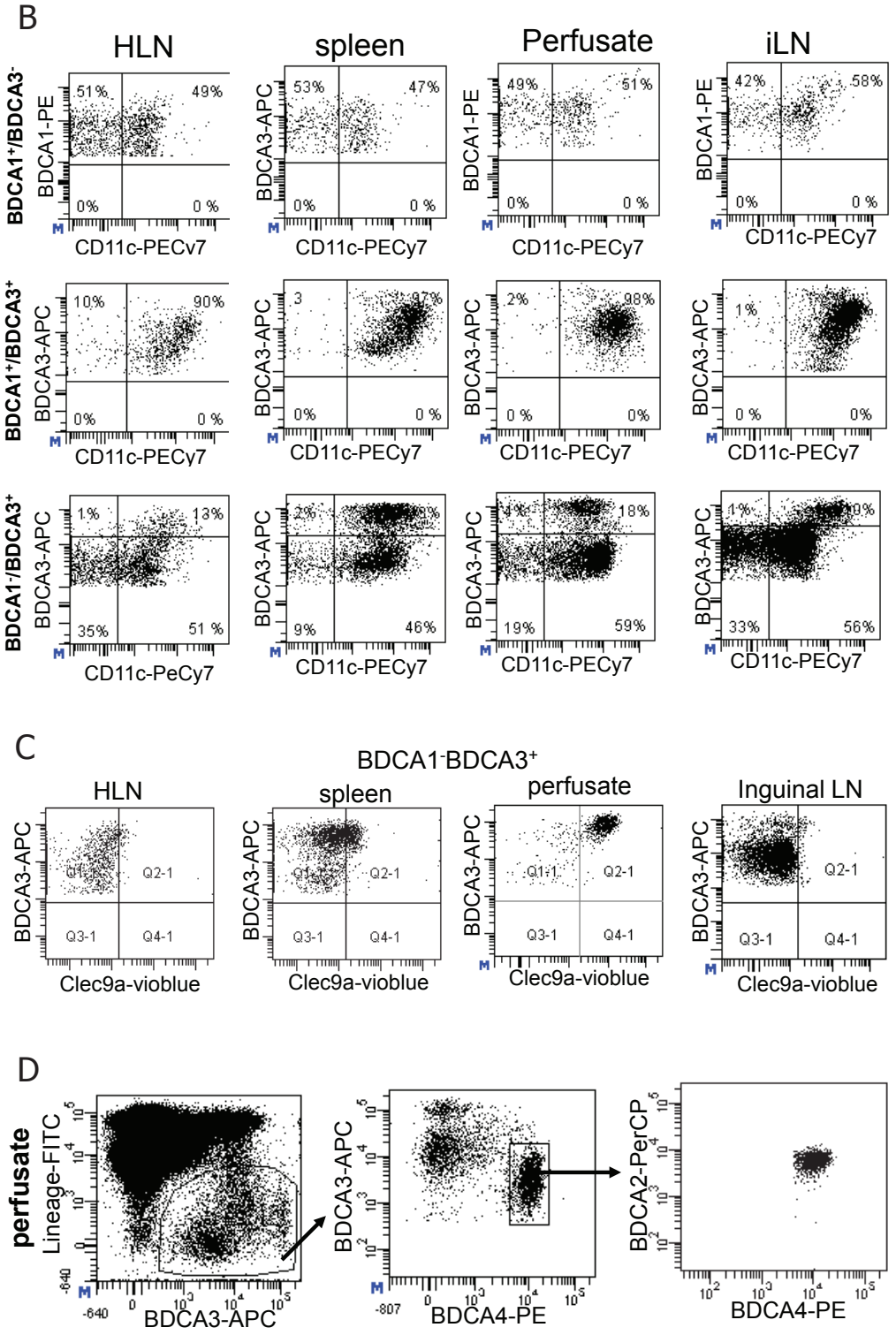


Figure 1. Identification of MDC subsets in hepatic and inguinal lymph nodes, spleen, and liver. (A) $CD45^+Lineage^-BDCA1^+$ and $CD45^+Lineage^-BDCA3^+$ cells were gated in MNC isolated from hepatic and inguinal lymph nodes, spleen and liver perfusate, and both gates were combined to determine MDC subsets.


B
HLN
spleen
Perfusate
iLN
BDCA1+/BDCA3-
BDCA1+/BDCA3+
BDCA1-/BDCA3+
BDCA1-PE
BDCA3-APC
CD11c-PECy7
CD11c-PECy7
CD11c-PECy7
CD11c-PECy7
CD11c-PeCy7
51%
49%
0%
0 %
53%
47%
49%
51%
42%
58%
10%
90%
3
97%
2%
98%
1%
1%
13%
2%
18%
1%
35%
51 %
9%
46%
19%
59%
33%
56%
C
BDCA1-BDCA3+
HLN
spleen
perfusate
Inguinal LN
BDCA3-APC
Clec9a-vioblue
Q1-1
Q2-1
Q3-1
Q4-1
D
perfusate
Lineage-FITC
BDCA3-APC
BDCA4-PE
BDCA2-PerCP
BDCA4-PE

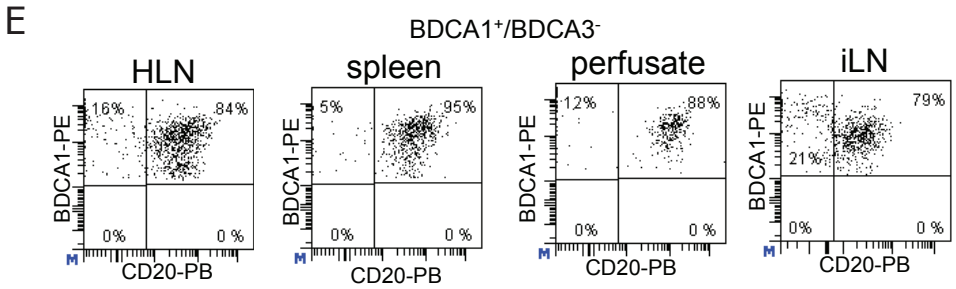


Figure 1. Identification of MDC subsets in hepatic and inguinal lymph nodes, spleen, and liver. (B) BDCA1$^-$BDCA3$^+$, BDCA1$^+$BDCA3$^+$, and BDCA1$^+$BDCA3$^-$ cells were gated as indicated in (A) and analyzed for CD11c expression. (C) In addition, BDCA1$^-$BDCA3$^+$ cells were analyzed for Clec9A expression. (D) BDCA2 and BDCA4 expression showed that the BDCA3^{low} population consists largely of BDCA2$^+$BDCA4$^+$ PDC. (E) BDCA1$^+$BDCA3$^-$ cells were gated using a lineage cocktail containing the B-cell antibody CD19-FITC but not CD20-FITC, and CD20-pacific blue was added. Expression of CD20 shows that the majority of BDCA1$^+$BDCA3$^-$ cells are B-cells.

e.g. the Glisson's capsule of the liver, while the majority of BDCA1$^+$BDCA3$^+$ MDC in the examined tissues are similar to the main subset of MDC in blood.

The BDCA1$^-$BDCA3^{dim} subset was partially CD11c$^-$ (Figure 1B) and the majority of these cells expressed both BDCA2 and BDCA4, identifying them as PDC and not MDC (Figure 1D), which is in agreement with a previous report (17). The numbers of BDCA1$^+$BDCA3$^-$ MDC were small, especially in spleen en liver (<0.05% of MNC), and they were partially CD11c$^-$ (Figure 1B) When stainings were performed with CD19-FITC, but not CD20-FITC in the lineage cocktail, while CD20-Pacific blue was added, we observed that the majority of BDCA1$^+$BDCA3$^-$ cells actually were B-cells (Figure 1E) and not MDC.

Like in blood (18-19), LN and liver contain few BDCA1$^-$BDCA3^{bright} MDC, while these cells are much more prominent in spleen (Figure 2A). In LN BDCA1$^+$BDCA3$^+$ MDC are the more prominent subset, but hepatic LN contain 2.2 times less BDCA1$^+$BDCA3$^+$ MDC compared to inguinal LN and 2.9 times less BDCA1$^+$BDCA3$^+$ MDC compared to spleen.

The expression of HLA DR, the co-stimulatory molecules CD40 and CD86, and the co-inhibitory molecule PDL-1, was analyzed on both MDC subsets in all tissues. Expression of HLA DR on both subsets was higher in LN compared to spleen and liver, but did not significantly differ between hepatic and inguinal LN (figure 2B). Intestingly, both BDCA1$^+$BDCA3$^+$ MDC and BDCA1$^-$BDCA3^{bright} MDC in hepatic LN showed significantly higher expression of CD40, CD86 and PDL-1 compared to inguinal LN, spleen and liver, indicating that both MDC subsets in hepatic LN are highly activated. To exclude possible differences between multi-organ donors and kidney transplant recipients as a confounder, paired comparisons of expression of HLA-DR, CD40, CD80, CD83, CD86 and PDL-1 on BDCA1$^+$BDCA3$^+$ MDC in hepatic and iliacal LN derived from the same multi-organ donors were performed. The data derived from these measurements confirm that MDC in hepatic LN have a more mature immunophenotype compared to their counterparts in non liver-draining LN (Figure 2C).

BDCA1$^+$BDCA3$^+$ MDC in hepatic lymph nodes are poor cytokine producers and poor T-cell stimulators

We compared the functional properties of the most prominent MDC-subset present in LN, i.e. the BDCA1$^+$BDCA3$^+$ cells. For this purpose, we purified BDCA3$^+$BDCA1$^+$ MDC

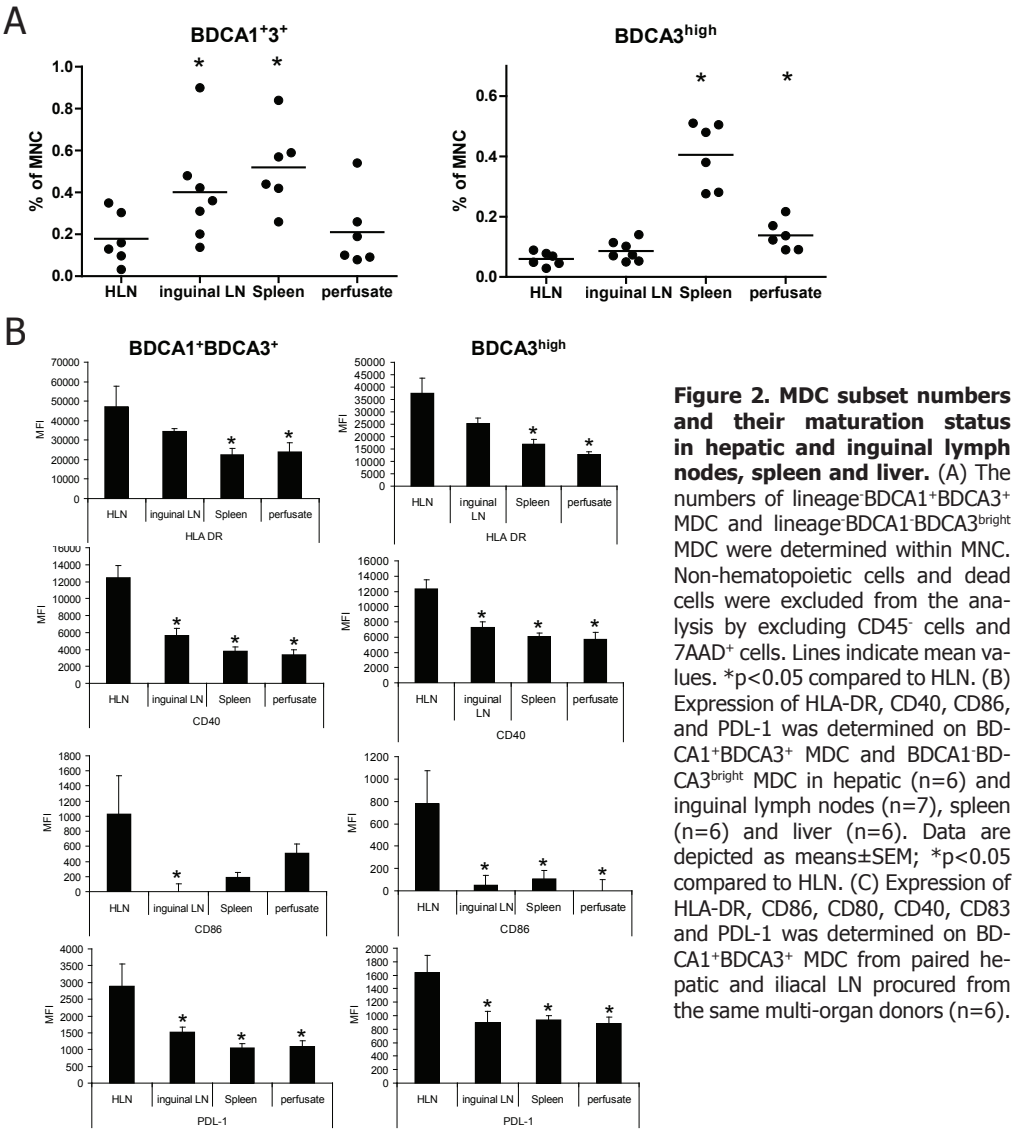


Figure 2. MDC subset numbers and their maturation status in hepatic and inguinal lymph nodes, spleen and liver. (A) The numbers of lineage$^-$BDCA1$^+$BDCA3$^+$ MDC and lineage$^-$BDCA1$^-$BDCA3^{bright} MDC were determined within MNC. Non-hematopoietic cells and dead cells were excluded from the analysis by excluding CD45$^-$ cells and 7AAD$^+$ cells. Lines indicate mean values. *p<0.05 compared to HLN. (B) Expression of HLA-DR, CD40, CD86, and PDL-1 was determined on BDCA1$^+$BDCA3$^+$ MDC and BDCA1$^-$BDCA3^{bright} MDC in hepatic (n=6) and inguinal lymph nodes (n=7), spleen (n=6) and liver (n=6). Data are depicted as means±SEM; *p<0.05 compared to HLN. (C) Expression of HLA-DR, CD86, CD80, CD40, CD83 and PDL-1 was determined on BDCA1$^+$BDCA3$^+$ MDC from paired hepatic and iliacal LN procured from the same multi-organ donors (n=6).

from hepatic and inguinal lymph node MNC, and analyzed their cytokine production upon *ex vivo* stimulation with the TLR3-agonist poly I:C or CD154-tranfected fibroblasts in the presence of IFN-γ. Skin/muscle draining inguinal LN BDCA1$^+$BDCA3$^+$ MDC secreted the pro-inflammatory cytokines IL-12, TNF-α and IL-6 as well as the immune-regulatory cytokine IL-10. Hepatic LN BDCA1$^+$BDCA3$^+$ MDC only produced biologically relevant levels of TNF-α, although in significantly lower amounts than skin/muscle LN BDCA1$^+$BDCA3$^+$ MDC did (figure 3A).

In addition, we compared their T-cell stimulatory capacity. BDCA3$^+$BDCA1$^+$ MDC from liver LN had a significantly lower allogeneic T-cell stimulatory capacity compared to BDCA3$^+$BDCA1$^+$ MDC isolated from inguinal LN (Figure 3B). Blocking of PDL-1 and PDL-2 on hepatic LN BDCA3$^+$BDCA1$^+$ MDC during co-culture with allogeneic T cells did not enhance their T-cell stimulatory capacity (data not shown), indicating that their weak T-cell stimulatory capacity is not caused by ligation of PD1 on T cells.

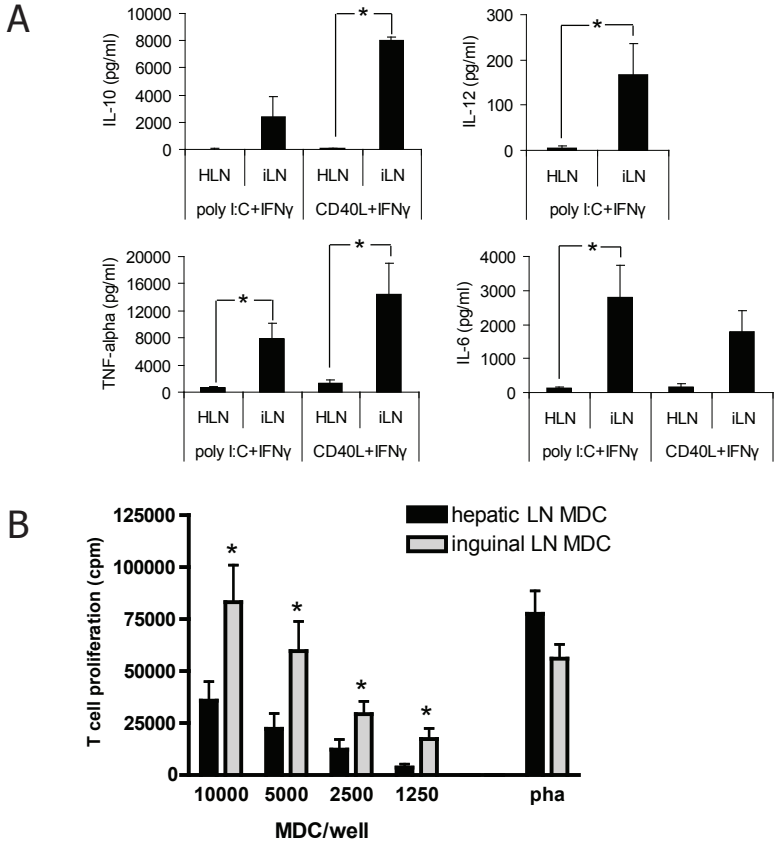


Figure 3. Hepatic LN BDCA1$^+$BDCA3$^+$ MDC produce almost no cytokines compared to skin/muscle draining LN BDCA1$^+$BDCA3$^+$ MDC and have a lower stimulatory capacity. (A) BDCA1$^+$BDCA3$^+$ MDC isolated from LN MNC were cultured at a concentration of 4x10^4 cells/200 µl and stimulated with either 20 µg/ml poly IC and 1000 U/ml IFN-γ or with CD40L-transfected plasmacytoma cells (4x10^4 J558 cells) and 1000 U/ml IFN-γ for 24 hours at 37°C. After 24 hours, supernatants were harvested and levels of IL-10, IL-12, TNF-α and IL-6 were determined. Data represent the mean±SEM; *p<0.03 for comparison of hepatic LN BDCA1$^+$BDCA3$^+$ MDC (n=8) with skin/muscle draining LN BDCA1$^+$BDCA3$^+$ MDC (n=5). (B) Graded numbers of hepatic and inguinal LN BDCA1$^+$BDCA3$^+$ MDC were co-cultured with allogeneic T cells (from one batch) and T-cell proliferation was assessed after 5 days by [^{3}H]-thymidine incorporation. Data represent the mean±SEM. Stimulation with PHA was used as a positive control for T-cell proliferation. *p<0.05 for comparison of hepatic LN BDCA1$^+$BDCA3$^+$ MDC (n=8) with skin/muscle draining LN BDCA1$^+$BDCA3$^+$ MDC (n=8).

Plasmacytoid dendritic cells in lymphoid organs and liver

PDC numbers were determined by staining with BDCA2 and BDCA4 antibodies (figure 4A). Hepatic LN contain on average 0.13% PDC, which is comparable to the numbers of PDC in spleen and liver (0.19%), but ~7 times lower than PDC numbers in inguinal lymph nodes (0.95%; figure 4B). The maturation status of the PDC was assessed by determination of the expression of HLA-DR, CD40, CD86, and PDL-1. In none of the examined tissues CD86 expression was detected on PDC, and PDL-1 expression was very low (data not shown). CD40 and HLA-DR expression on PDC did not differ between the tissues (figure 4C). In addition, PDC isolated from liver LN and inguinal LN showed comparable T-cell stimulatory capacity (Figure 4D).

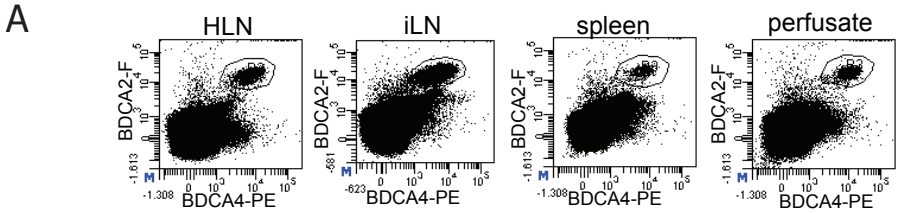


Figure 4. PDC numbers and maturation status in hepatic and inguinal lymph nodes, spleen and liver. (A) Stainings with BDCA2 and BDCA4 antibodies on hepatic and inguinal lymph node, spleen, and liver MNC (B) The numbers of $BDCA2^{+}BDCA4^{+}$PDC were determined within MNC. Non-hematopoietic cells and dead cells were excluded from the analysis by CD45-expression and 7AAD. Lines indicate mean values. *$p<0.05$ compared to HLN. (C) Expression of HLA-DR and CD40 on PDC in hepatic (n=5) and inguinal lymph nodes (n=7), spleen (n=5) and liver (n=5). Data are depicted as means± SEM; *$p<0.05$ compared to HLN. (D) Graded numbers of hepatic (n=6) and inguinal LN PDC (n=5) were co-cultured with allogeneic T-cells (from one batch) and T-cell proliferation was assessed after 5 days by [^{3}H]-thymidine incorporation. Data represent the mean±SEM

CD14⁺ cells in lymphoid organs and liver

In addition to the two MDC-subsets and PDC, we detected abundant numbers of CD14⁺ cells in spleen and liver (5.0%±0.5% and 11.3%±2.8% of total MNC respectively). In contrast, these cells were rare in LN, and hepatic LN contained two-fold lower numbers compared to inguinal LN (0.23±0.06% and 0.53±0.03%of total MNC, respectively; Figure 5A). The CD14⁺ cells in hepatic and inguinal LN expressed high levels of DC-SIGN (figures 5B and C), while those in spleen and liver expressed low levels. In experiments in which we compared MNC isolations from LN or spleen with or without collegenase treatment, we observed that DC-SIGN expression was affected by collagenase digestion

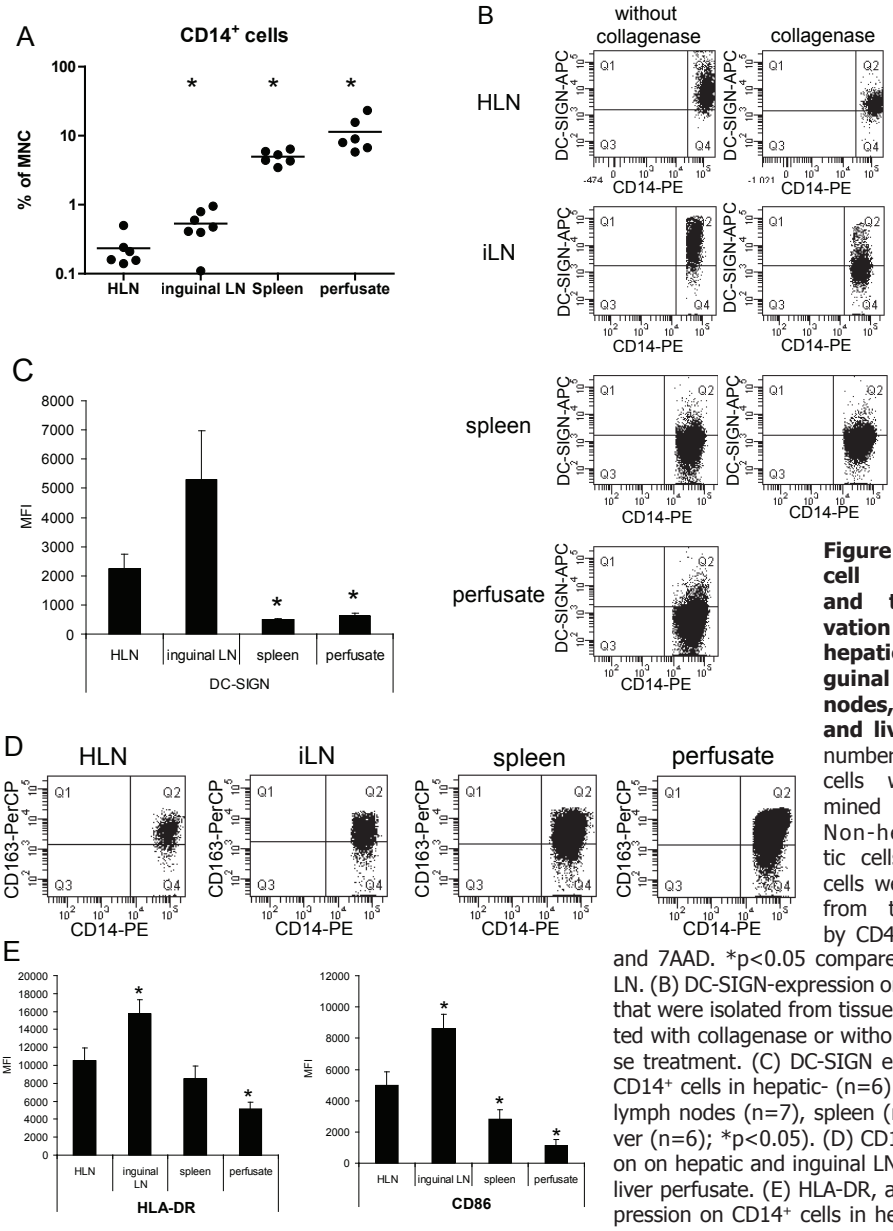


Figure 5. CD14⁺ cell numbers and their activation status in hepatic and inguinal lymph nodes, spleen, and liver. (A) The numbers of CD14⁺ cells were determined within MNC. Non-hematopoietic cells and dead cells were excluded from the analysis by CD45-expression and 7AAD. *p<0.05 compared to hepatic LN. (B) DC-SIGN-expression on CD14⁺ cells that were isolated from tissues either treated with collagenase or without collagenase treatment. (C) DC-SIGN expression on CD14⁺ cells in hepatic- (n=6) and inguinal lymph nodes (n=7), spleen (n=6), and liver (n=6); *p<0.05). (D) CD163 expression on hepatic and inguinal LN, spleen and liver perfusate. (E) HLA-DR, and CD86 expression on CD14⁺ cells in hepatic- (n=6) and inguinal lymph nodes (n=7), spleen (n=6), and liver (n=6); *p<0.05).

of the tissue (figure 5B). Therefore, the expression levels of DC-SIGN on cells isolated after collagenase digestion depicted in Figure 5C are probably under-estimations, except for liver cells which were isolated without collagenase treatment. In all examined tissues $CD14^+$ cells expressed CD163 (figure 5D), but not CD206, suggesting that these cells represent macrophages (47-48). Liver $CD14^+$ cells represent most probably Kupffer cells, which have been shown to express low levels of DC-SIGN (49). The activation status of the $CD14^+$ cells was determined by the expression of HLA-DR, CD80 and CD86. None of $CD14^+$ cells in the examined tissues expressed CD80. Surprisingly, compared to $CD14^+$ cells in inguinal LN, $CD14^+$ cells in hepatic LN showed a higher expression of HLA-DR and CD86, indicating a more activated status (figure 5E).

DISCUSSION

DC play an important role in maintaining a fine balance between immune responsiveness and unresponsiveness. DC take up antigens in non-lymphoid tissues and migrate via the lymph to regional LN where they present antigen to T cells in the paracortex. The sentinel LN of the liver are located in the hilus and along the hepatic artery and portal vein (39-40). Therefore, we hypothesized that DC subsets in hepatic LN may show peculiar properties that contribute to the immunological tolerogenicity of the liver environment, and compared them with DC subsets from skin/muscle-draining LN. The skin is a major barrier against the external environment and disturbances of this barrier cause strong immune responses. In addition, we compared DC in liver LN with those the spleen, which harbours DC derived from blood, and with those in the liver itself, which are thought to contain the predecessors of hepatic LN MDC.
The present study shows that liver LN contain relatively low numbers of $BDCA1^+BDCA3^+$ MDC compared to skin/muscle-draining inguinal LN and spleen, while $BDCA1^-BDCA3^{bright}$ MDC are rare in both hepatic and inguinal LN. Spleen contains ~6 times higher numbers of $BDCA1^-BDCA3^{bright}$ MDC compared to LN. The lower numbers of $BDCA1^+BDCA3^+$ may result in a reduced chance of T-cell activation in liver LN, thereby contributing to the relatively low immunogenicity of the liver environment.
However both $BDCA1^+BDCA3^+$ and $BDCA3^{bright}$ MDC displayed a more mature phenotype in hepatic LN compared to inguinal LN, iliacal LN, spleen and liver, with higher expression of co-stimulatory molecules. Interestingly, rat liver lymph has also been shown to contain highly mature MDC (50). Nevertheless, $BDCA1^+BDCA3^+$ MDC isolated from hepatic LN showed a weaker T-cell stimulatory capacity compared to those isolated from inguinal LN, which may further contribute to the tolerogenicity of the liver environment. Hepatic lymph node MDC (both $BDCA1^+BDCA3^+$ and $BDCA3^{bright}$ MDC) also have higher expression of PDL-1 compared to inguinal LN, iliacal LN, spleen and liver. PDL-1 belongs to the B7 family and has been shown to downregulate T cell activation and proliferation (51). Therefore, we postulated that PDL-1 on MDC in hepatic LN may reduce their T-cell stimulatory capacity, but this appeared not to be the case.
Upon stimulation via TLR3 or CD40, $BDCA1^+BDCA3^+$ MDC from hepatic LN hardly produced any cytokines, while inguinal LN MDC produced IL10, IL12, IL6, and TNF-α. Whether the deficiency in cytokine production explains the reduced T-cell stimulatory potential of $BDCA1^+BDCA3^+$ MDC isolated from liver LN remains to be established.

Hepatic LN contained 7 times less PDC than skin/muscle draining inguinal LN, which is in agreement with our previous finding (52). PDC from both types of LN showed similar expression of HLA-DR, CD40 and CD86, and equivalent T-cell stimulatory capacity. PDC

possess both activating and tolerogenic properties (38, 53), but the circumstances in which the balance tips towards activation or tolerance are not well understood, and probably depend on the microenviromental conditions in the tissue in which they reside. PDC interact with MDC and this interplay synergizes the induction of immune responses (54). This interaction is bi-directional. PDC provide help to MDC to induce an optimal CTL response in LN during viral infection (55), and MDC promote PDC survival and enhance their T-cell stimulatory capacity (56). These interactions depend on both soluble factors and cell-cell contact (57). The low numbers of MDC and PDC in hepatic LN compared to skin/muscle draining inguinal LN may lead to fewer interactions between PDC and MDC and thereby prevent the induction of optimal immune responses.

The number of $CD14^+$ monocytes/macrophages in hepatic LN is two times lower than in skin/muscle draining inguinal LN. This shows again that an antigen presenting cell subset is rare in hepatic LN. In contrast to MDC, $CD14^+$ cells in hepatic LN have a lower expression of CD86 and HLA-DR compared to $CD14^+$ cells in inguinal LN. These cells correspond to the $CD14^+$DC-$SIGN^+CD206^-$ cells located in the subcapsular area and along the trabeculae radiating into LN as observed by Angel CE et al (58), and identified by Segura et al (47) as macrophages. The precise function of these cells is still open to speculation.

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 (2) and could cause the higher maturation status of MDC in hepatic LN. Interestingly, MDC present in the human liver itself are fully capable to produce pro- and anti-inflammatory cytokines upon stimulation via TLR or CD40 (36). Therefore, we would like to speculate that the deficiency of $BDCA1^+BDCA3^+$ MDC in hepatic LN to produce cytokines is induced by the continuous presence of maturation stimuli in the liver environment, which may drive functional exhaustion of liver-derived MDC during their migration to the hepatic LN (59).
PDC migrate directly from blood to the LN via the high endothelial venules (60-61). Therefore, they are probably less exposed to maturation stimuli present in the liver environment, which may explain the similar maturation status and T-cell stimulatory capacity of PDC in hepatic and inguinal LN. More recently it was shown in mice that PDC can also enter the LN via the lymphatic route (62). When this is also true in the human situation, PDC might also enter hepatic LN via the lympatics. This would mean that part of the hepatic LN PDC are probably exposed to endotoxins present in the liver, but because PDC lack toll-like receptor 4 (TLR-4) (63) they can not respond to these stimuli.

$CD14^+$ cells in hepatic LN are less mature compared with inguinal LN. This was unexpected, since $CD14^+$ cells, including Kupffer cells, express CD14/TLR-4, and therefore can respond to endotoxins in the liver. The origin of $CD14^+CD163^+$DC-$SIGN^+$ in liver LN is currently unknown. One possibility is that (a subset of) Kupffer cells migrate via lymph to hepatic LN, but in rat it has been shown that liver lymph does not contain Kupffer cells (39). Since $CD14^+CD163^+$DC-$SIGN^+$ cells were also found in inguinal LN, even in higher numbers, we favour the possibility that these cells represent macrophages derived from circulating monocytes. This hypothesis also explains the relatively low activation status of these cells in liver LN, because they have, like PDC, not been exposed to endotoxins present in the liver.

In summary, we observed that hepatic LN low numbers of BDCA1^{+}BDCA3^{+} MDC, which cannot produce cytokines and have a weaker T-cell stimulatory capacity compared to the same subset in skin/muscle-draining LN. In addition, hepatic LN contain lower numbers of PDC and macrophages compared to skin/muscle-draining LN. The paucity of several types of Antigen-Presenting Cells together with the functional deficiency of BDCA1^{+}BDCA3^{+} MDC in liver LN may contribute to the tolerogenicity of the liver environment against foreign compound and pathogens. Whether PDC, macrophages or BDCA1^{-}BDCA3^{bright} MDC in liver LN also show functional deficits has still to be investigated.

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chapter 5

Human plasmacytoid dendritic cells induce CD8$^+$LAG-3$^+$Foxp3$^+$CTLA-4$^+$ regulatory T cells that suppress allo-reactive memory T cells

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ABSTRACT

Allo-reactive memory T cells are a major barrier for induction of immunological tolerance to allografts in humans. Here we report that stimulation of unfractionated human T cells with TLR-stimulated allogeneic plasmacytoid dendritic cells (PDC) induces $CD8^+$ regulatory T cells (Treg) that inhibit T-cell allo-responses, including those of memory T cells.

$CD3^+$ T cells were primed for 7 days with allogeneic PDC which had been pre-stimulated with TLR-7- or TLR-9 ligands. While the T cells proliferated and produced cytokines during the priming culture, they were profoundly hypo-responsive to re-stimulation with the same allo-antigen in a second culture. Moreover, T cells primed by PDC exerted donor-specific suppression on allo-responses of both unfractionated and memory $CD3^+$ T cells. The regulatory capacity of PDC-primed T cells was confined to $CD8^+LAG\text{-}3^+Foxp3^+CTLA\text{-}4^+$ T cells, which suppressed allogeneic T-cell responses through a CTLA-4-dependent mechanism. Induction of $CD8^+$ Treg by PDC could be partially prevented by 1-methyl tryptophan, an inhibitor of indoleamine 2,3-dioxygenase.

In conclusion, stimulation of human T cells by TLR-stimulated allogeneic PDC induces $CD8^+$ Treg, that inhibit allogeneic T-cell responses, including memory T cells. Donor-derived PDC may be considered as an immunotherapeutic tool to prevent activation of the recipient allo-reactive (memory) T-cell repertoire after allogeneic transplantation.

INTRODUCTION

To prevent rejection of their graft, the majority of organ transplant recipients need life-long treatment with immunosuppressive drugs. These agents have specific adverse effects, such as nephrotoxicity and hypertension, and side effects due to non-specific immunosuppression, such as enhanced susceptibility to infections and cancer. Induction of specific transplant tolerance is, therefore, a major goal in transplantation research.
For this purpose, extensive effort is being devoted to the development of immunotherapeutic protocols using regulatory T cells (Treg) (1). Long-term allograft tolerance is thought to be dependent on the presence of Treg which suppress alloreactive T-cells (2). Several subsets of Treg have been described, including $CD4^+CD25^+Foxp3^+$ Treg (2), TCR-$\alpha\beta^+$ CD4 and CD8 double negative Treg (3), interleukin-10 (IL-10) secreting $CD4^+$ T cells (4), and $CD8^+$ Treg (5-11). Although naturally occurring $CD4^+CD25^+Foxp3^+$ Treg have shown an ability to suppress transplant rejection by co-transferred effector T cells in immunodeficient experimental animals (12-13), evidence is accumulating that these Treg are not able to control the large repertoire of alloreactive T cells in immunocompetent animals (14-16).
At least 50% of alloreactive T cells in humans belong to the memory subset (17-18), which has a lower threshold for activation (19-21) and is relatively resistant to suppression by $CD4^+CD25^+Foxp3^+$ Treg (22-23). Memory alloreactive T cells have compromised attempts to induce transplant tolerance in humans by co-stimulatory blockade or T-cell depletion (21, 24-27). For this reason, it is highly important to identify approaches that inhibit allogeneic memory T-cell responses (28).
Plasmacytoid dendritic cells (PDC) have been found to be involved in tolerance to allografts in experimental animal models (29), and treatment of recipient mice with donor-derived PDC prolonged allogeneic cardiac graft (30) and skin graft survival (31), and prevented acute Graft-versus-Host Disease after allogeneic bone marrow transplantation (32). In addition, involvement of PDC in natural tolerance to oral antigens (33-34) and protection against experimental autoimmune encephalomyelitis (35) has been demonstrated. In several of these models, PDC mediate tolerance induction by generating Treg (11, 29, 34, 36). Human PDC have an intrinsic capacity to induce hypo-responsiveness in naïve T cells, and to stimulate the differentiation of $CD4^+$ and $CD8^+$ Treg from naïve T cells *in vitro* (37-42). However, it is unknown whether human PDC induce hypo-responsiveness in alloreactive memory T cells, and whether Treg generated by PDC are able to inhibit alloreactive memory T cells, questions which are much more relevant for their potential application in allogeneic transplantation in humans.
In this study, we determined whether human PDC meet these criteria, which may advocate their application in immunotherapeutic protocols aiming to induce durable allograft acceptance in humans. We investigated whether human PDC in vitro can: (i) render unfractionated allogeneic T cells (containing both naïve and memory T cells) hypo-responsive to re-stimulation with allo-antigen; (ii) induce donor-specific Treg. In addition, we elucidated the immunophenotypic and functional characteristics of PDC-induced Treg.

MATERIALS AND METHODS

Reagents
Interleukin-2 (IL-2), Interleukin-3 (IL-3), anti-BDCA2-FITC, anti-BDCA4-PE, anti-BDCA1-PE mAb, CD15-microbeads, CD235-microbeads, CD14-microbeads, anti-PE-microbeads, MS and LD-columns were obtained from Miltenyi Biotec, Bergisch Gladbach,

Germany. Anti-IL-10 receptor (clone 3F9, neutralizing), IgG1-APC, IgG1-PE, anti-perforin-PE, CD3-PerCP, CD4-PerCP, CD4-APC-H7, CD14-PE, CD25-PE and CD56-PE mAb were obtained from BD Biosciences, Erembodegem, Belgium, and IgG1-FITC, anti-granzyme A-PE and anti-granzyme B-PE from Sanquin, Amsterdam, the Netherlands. IgG2a-PE, CD152 (clone BNI3, neutralizing), CD152-PE, CD4-FITC, CD19-PE, CD28-FITC, CD38-FITC, CD45-FITC, CD94-PE and CD123-PE mAb were purchased from Beckman Coulter, Immunotech, Marseille, France, and IL-4, CD45RA-PE, functional grade irrelevant control IgG1 and IgG2a, CD8-APC, anti-Foxp3-APC and Foxp3 staining buffer from eBiosciences, San Diego, CA. IL-2, IL-6, IL-10 and TNF-α ELISA-kits were also obtained from eBiosciences, and IFNγ and IL-4 ELISA's from Invitrogen, Breda, the Netherlands. CFSE was also obtained from Invitrogen. CpG A ODN2336 and loxoribin were purchased from InVivogen, San Diego, USA. Anti-CXCR3-PE, anti-CXCR4-PE and anti-CCR4 (clone 24006, neutralizing) mAb and recombinant human latency-associated peptide were purchased from R&D systems, Abingdon, UK. Anti-LAG-3 mAb (clone 17B4, neutralizing) was obtained from Enzo Life Sciences, Plymouth Meeting, USA), and caspase-3 inhibitor (Z-DEVD-FMK) and granzyme B inhibitor (Z-AAD-FMK) from EMD4Biosciences, Gibbstown, USA. GM-CSF was a kind gift of Schering-Plough, Kenilworth, USA. 1-methyl-DL-tryptophan was from Sigma Aldrich, Zwijndrecht, the Netherlands, goat-anti-mouse-PE from DAKO, Heverlee, Belgium, CD38-pacific blue from Exbio, Vestec, Czech Republic, fix&perm cell permeabilization kit from An der Grub Bio Research, Austria, and PHA was obtained from Murex, Paris, France.

Purification of PDC, T cells and monocytes from human blood

PBMC were isolated from buffy coats of healthy blood-bank donors by Ficoll density centrifugation. For isolation of PDC, PBMC were incubated with anti-BDCA4-PE mAb. Subsequently, the cells were washed and incubated with anti-PE-microbeads. After a second wash, PDC were isolated in two rounds of separation over MS columns. Purity of isolated PDC, as assessed by staining with anti-BDCA2-FITC and flowcytometry, was > 94%. T cells were purified from PBMC by negative selection. PBMC were labeled with PE-conjugated antibodies against BDCA1, CD14, CD19, CD56, CD123 as well as CD15- and CD235-microbeads. After 15 minutes of incubation, the cells were washed and incubated with anti-PE microbeads. T cells were isolated over an LD column. In some experiments memory T cells were isolated by adding CD45RA-PE mAb to the antibody cocktail. The purity of the T cells was analyzed after labeling with CD3-PerCP and CD45-FITC, and was >97%. Monocytes were isolated with CD14 Microbeads over an MS column. Purity was >98%. For the generation of monocyte-derived DC (MoDC) monocytes ($1x10^6$/ml) were cultured in RPMI supplemented with 10% FCS, 50 ng/ml GM-CSF and 200 U/ml IL-4 for 7 days. On day 6, 500 ng/ml LPS was added to stimulate MoDC maturation.

Stimulation of T cells with allogeneic PDC

Purified PDC ($2x10^4$/200 µl RPMI supplemented with 10% FCS) were cultured in round-bottom wells (Greiner Bio-one, the Netherlands) in the presence of 10 ng/ml IL-3 with or without 5 µg/ml CpG A ODN2336 or 400 µM loxoribin. After 18 hours, PDC were washed and unfractioned allogeneic T cells were added ($1x10^5$/200 µl RPMI supplemented with 10% FCS). In some cases CFSE-labeled allogeneic T cells were added. The cells were cultured at 37ºC with 5% CO2. After 7 days cell-free supernatant was collected, and interleukin-10, interleukin-6, TNF-α and IFN-γ concentrations were determined by ELISA according to the manufacturer's instructions. T cell proliferation was analyzed by

determination of the incorporation of 0.5 µCi/well [^{3}H]thymidine (Radiochemical Centre, Amersham, Little Chalfont, UK) during the last 18 hours or the culture. T cells stimulated with PHA (5 µg/ml) served as a positive control. Alternatively, cells were collected from the wells and labeled for immunophenotypical analysis with different combinations the following antibodies: CD4-PerCP, CD4-APC-H7, CD8-APC, CD38-FITC, CD25-PE, CD4-PerCP, anti-CXCR3, anti-CXCR4-PE, anti-Foxp3-APC, anti-CLTA-4, and anti-LAG-3. Intracellular Foxp3 expression was determined according to the manufacturer's instructions. Anti-LAG-3 was combined with goat-anti-mouse-PE. Intracellular CTLA-4 staining was performed using the fix&perm cell permeabilization kit according to the manufacturer's instructions. Cells were analyzed on a Canto II flowcytometer using Diva 6.0 software (Becton Dickinson) or a Calibur flowcytometer with CellQuest Pro 5.2 software. Isotype-matched irrelevant mAb labeling was used to analyze expression of these molecules appropriately. When indicated, PDC-primed T cells were separated by flowcytometric sorting using a FacsAria Cell Sorter (Becton Dickinson) into CD38$^-$, CD4$^+$CD38$^+$ and CD8$^+$CD38$^+$ T cells, or CD8$^+$CD38$^+$LAG-3$^-$ and CD8$^+$CD38$^+$LAG-3$^+$ T cells, and the resulting subpopulations were tested separately in re-stimulation and suppression assays.

Re-stimulation of PDC-primed T cells

To assess responsiveness to secondary allogeneic stimulation, PDC-stimulated T cells ($1x10^5$/200 µl) were re-stimulated in round bottom wells with irradiated (3000 rad) mature MoDC ($1.5x10^4$) from the same source as the PDC. After 5 days T cell proliferation was determined by pulsing the cells for 18 hours with 0.5 µCi/well [^{3}H]-thymidine, and IL-2 was measured in culture medium by ELISA. Where indicated, IL-2 was added to the re-stimulation cultures.

Suppression assay

To assess their suppressive capacity, PDC-stimulated T cells were added in graded doses to cultures of unfractionated CD3$^+$ T cells or, when indicated, CD45RO$^+$ memory T cells ($1x10^5$/200 µl), that were stimulated with allogeneic irradiated (3000rad) donor-specific MoDC or third-party MoDC ($1.5x10^4$) in round bottom wells. After 5 days, proliferation was assessed by determination of [^{3}H]thymidine incorporation for 18 hours. Where indicated, neutralizing antibodies against IL10-receptor, CCR-4, or CTLA-4 (all: 15 µg/ml) were added to the cultures. In other experiments functional inhibitors of caspase-3 or granzyme B were added.

Determination of MoDC survival in the presence of PDC-stimulated CD8+CD38+ T-cells

CD38$^+$CD8$^+$ PDC-stimulated T-cells were co-cultured with allogeneic MoDC derived from the same individual as PDC, in a ratio of 1.5 : 1. After 18 hours incubation at 37°C the cells were washed and stained with CD3 mAb. 7AAD was added to exclude dead cells. To quantify the absolute number of MoDC, a fixed amount of beads (CaliBRITE unlabeled beads, BD Biosciences, San Jose, CA) was added to each sample and measured by flowcytometry. For each well, the absolute number of living MoDC (=CD3-negative cells) was calculated as a proportion to the number of beads, as described previously (64).

Statistical analysis

All experiments were performed n times, as indicated in the figure legends, with cells from different individuals, and mean values±SEM were calculated. Significance of differences between paired observations was tested in the paired T test using SPSS v15.0

software. A p-value of less than 0.05 was considered significant.

RESULTS

TLR-stimulated PDC induce profound hypo-responsiveness in allogeneic T cells
Freshly purified human blood PDC were cultured for 18 hours in the presence of IL-3 as survival factor, with or without CpG-A or LOX to stimulate their maturation. After extensive washing to remove TLR-agonists, unfractionated allogeneic CD3$^+$ T cells were added, and after 7 days T cell proliferation and cytokine production was measured. Figure 1A shows that non-stimulated PDC were weaker stimulators of allogeneic T cell proliferation compared to LPS-stimulated monocyte-derived DC (moDC). After induction of PDC-maturation by CpG-A or LOX, the allogeneic T-cell stimulatory capacity of PDC was enhanced, but still lower than that of mature moDC. Figure 1B shows that CpG-A and

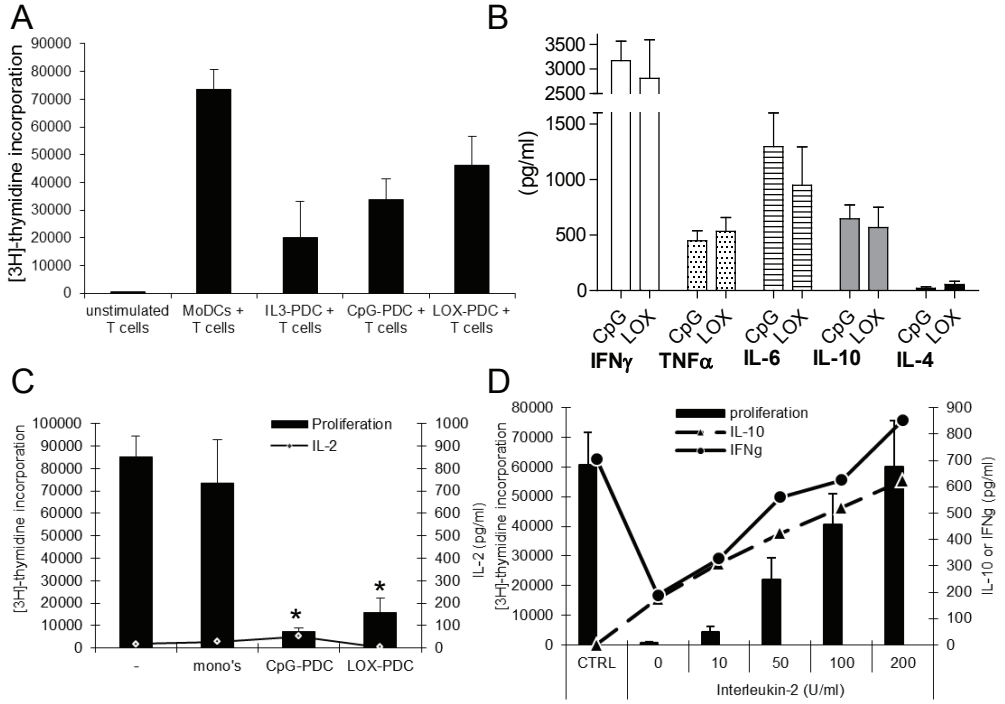


Figure 1: TLR-stimulated PDC induce hypo-responsiveness in allogeneic T cells. (A) PDC were cultured for 18 hours, without or with CpG-A or LOX to stimulate their maturation and in the presence of IL-3 as survival factor. MoDC were stimulated for 18 hours with LPS to stimulate maturation. CD3$^+$ T cells were added to allogeneic PDC or MoDC, and after 7 days T cell proliferation was determined by [^{3}H]-thymidine incorporation (mean±sem, n=7). Cultures of T cells alone served as negative control (unstimulated T cells). (B) Production of INF-γ, TNF-α, IL-6, IL-10, and IL-4 was determined in supernatants of co-cultures of T cells with allogeneic TLR-stimulated PDC harvested at day 7 (mean±sem, n=7). Concentrations of cytokines in supernatants of 7-day cultures of TLR-stimulated PDC alone were below detection limit (30 pg/ml). (C) Allogeneic CD3$^+$ T cells primed by CpG-A- or LOX-stimulated PDC, or by LPS-stimulated monocytes, were harvested at day 7 and re-stimulated with LPS-activated MoDC derived from the same donor as the PDC. After 5 days T cell proliferation was determined by [^{3}H]-thymidine incorporation (mean±sem, n=6). Primary T cell stimulation by LPS-MoDC was included as a control. IL-2 production was determined in supernatants of the re-stimulation cultures harvested at day 5 (*p≤0.001 using the paired T-test). (D) Allogeneic CD3$^+$ T cells primed by CpG-A-stimulated PDC were re-stimulated with LPS-stimulated MoDC in the presence of increasing concentrations of IL-2. After 5 days T-cell proliferation, IL-10 and IFN-γ production was determined. Primary T cell stimulation by LPS-MoDC was taken as a control (mean±sem, n=4).

LOX-stimulated PDC induced similar cytokine production in allogeneic T cells. PDC-primed T cells produced the Th1 cytokines IFN-α, IL-6 and TNF-α, the immunoregulatory cytokine IL-10, but hardly any IL-4 (Fig. 1B).
Interestingly, T cells primed by mature PDC were profoundly hypo-responsive upon re-stimulation with the same alloantigen (Fig. 1C). In contrast, no hypo-responsiveness was induced in T cells upon priming with LPS-stimulated monocytes. While re-stimulation with allogeneic MoDC induced substantial proliferation of control (non-primed) T cells or T cells primed by monocytes, this was not accompanied by higher concentrations of IL-2 in the culture medium compared to conditions in which PDC-primed T cells were re-stimulated (Fig. 1C). Probably, this was due to consumption of IL-2 in conditions of a strong proliferative response. Nevertheless, hypo-responsiveness of PDC-primed T cells was fully reversed by the addition of IL-2 to the re-stimulation cultures. Not only proliferation, but also cytokine production of PDC-primed T cells was restored upon addition of IL-2 to re-stimulation cultures (Fig 1D). In contrast, T-cell hypo-responsiveness induced by PDC could not be elevated by addition of a neutralizing antibody against IL-10 receptor to the re-stimulation cultures (data not shown),

TLR-stimulated PDC induce alloantigen-specific regulatory T cells which are able to inhibit memory T cell responses

To analyze whether PDC induce regulatory capacity in allogeneic T cells, we added PDC-primed T cells in graded numbers to co-cultures of autologous CD3+ T cells which were stimulated with allogeneic LPS-MoDC derived from the same donor as the PDC.

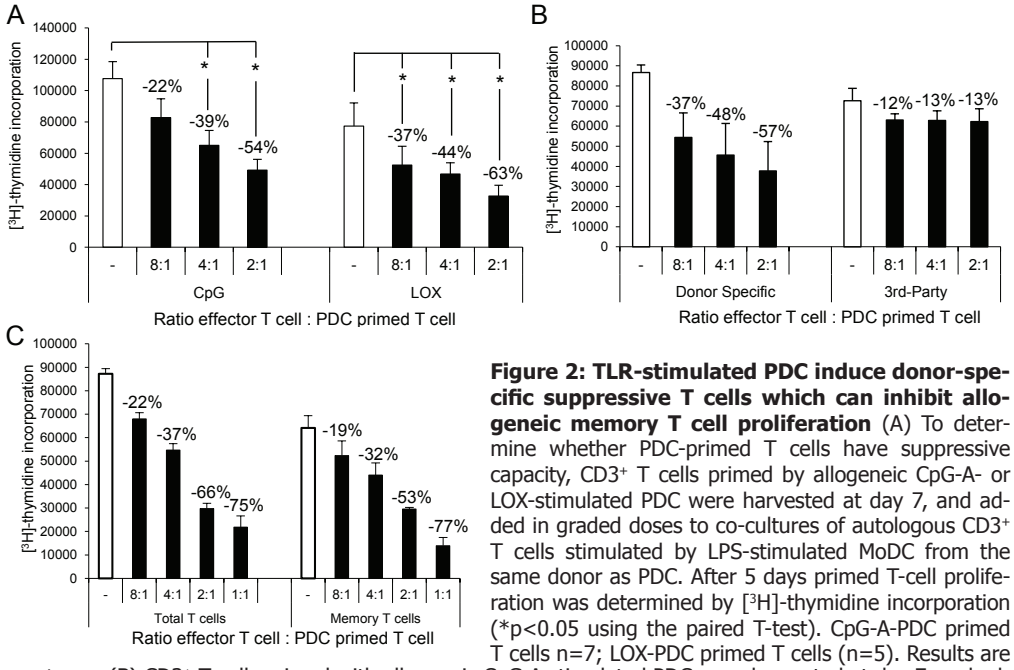
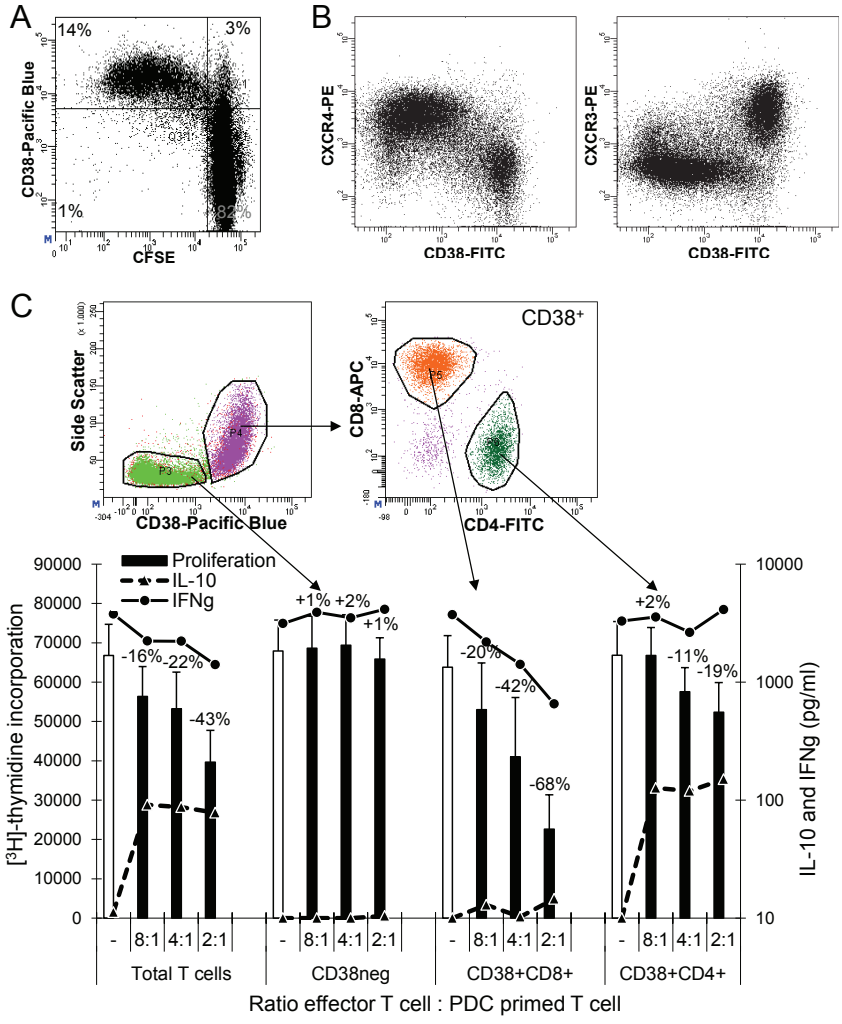


Figure 2: TLR-stimulated PDC induce donor-specific suppressive T cells which can inhibit allogeneic memory T cell proliferation (A) To determine whether PDC-primed T cells have suppressive capacity, CD3+ T cells primed by allogeneic CpG-A- or LOX-stimulated PDC were harvested at day 7, and added in graded doses to co-cultures of autologous CD3+ T cells stimulated by LPS-stimulated MoDC from the same donor as PDC. After 5 days primed T-cell proliferation was determined by [3H]-thymidine incorporation (*p<0.05 using the paired T-test). CpG-A-PDC primed T cells n=7; LOX-PDC primed T cells (n=5). Results are mean±sem. (B) CD3+ T cells primed with allogeneic CpG-A stimulated PDC were harvested at day 7, and added in graded doses to CD3+ T cells stimulated with allogeneic LPS-stimulated MoDC. The MoDC were either from the same source as the PDC (donor-specific) or from an individual fully mismatched for HLA-A, HLA-B and HLA-DR loci with the individuals from which PDC were isolated (3rd-party). After 5 days T cell proliferation was determined by [3H]-thymidine incorporation (mean±sem, n=4). (C) CD3+ T cells primed with allogeneic CpG-PDC were added in graded doses to autologous CD3+ T cells or purified CD3+CD45RO+ memory T cells stimulated with LPS-MoDC which were derived from the same individual as PDC (mean±sem, n=5).

PDC-primed T-cells inhibited the proliferation of responder T cells in a dose-dependent manner (Fig. 2A). CpG-A and LOX-stimulated PDC induced comparable suppressive capacity in $CD3^+$ T cells. Notably, priming with allogeneic LPS-stimulated monocytes did not induce suppressive capacity in T-cells (data not shown). To investigate whether the suppression exerted by PDC-induced regulatory T cells is antigen-specific, we compared their capacity to suppress proliferation of autologous $CD3^+$ T cells that were stimulated with either MoDC derived from the same individual as the PDC, or with third-party MoDC fully mismatched on HLA-A, B and DR loci with the donor from which the PDC had been isolated. PDC-primed T cells did not inhibit proliferation of T cells stimulated by fully MHC-mismatched third-party MoDC (Fig. 2B), indicating that suppression exerted by PDC-primed T cells is alloantigen-specific.

Many attempts to induce transplant tolerance have failed due to the presence of allo-reactive memory T cells. To investigate whether T cells primed by mature PDC are able to suppress allo-reactive memory T cells, we added PDC-primed T cells to purified autologous $CD3^+CD45RO^+$ memory T cells that were stimulated with allogeneic MoDC from the same priming donor. Figure 2C shows that PDC-primed T cells suppress memory T cell proliferation with similar potency as they inhibit proliferation of unfractioned T cells.



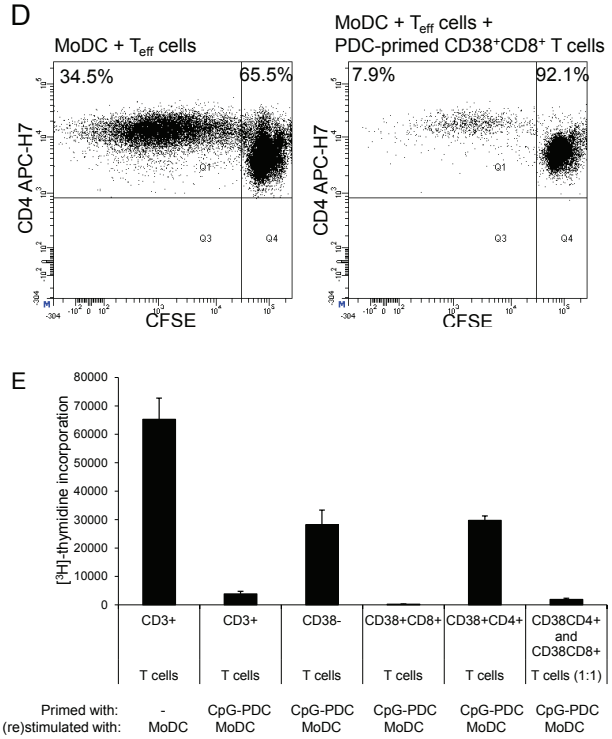


Figure 3: TLR-stimulated PDC induce CD8+ regulatory T-cells. (A) CFSE-labeled CD3+ T cells were primed with allogeneic CpG-A stimulated PDC, and after 7 days CFSE-dilution and CD38-expression were determined. (B) CD3+ T cells primed by CpG-A stimulated PDC for 7 days were examined for CD38, CXCR4 and CXCR3 expression. (C) CD3+ T cells primed with allogeneic CpG-A stimulated PDC for 7 days were separated into CD38-, CD38+CD4+ and CD38+CD8+ subpopulations by flowcytometric cell sorting, which were added in graded numbers to autologous CD3+ responder T cells stimulated with allogeneic LPS-MoDC (from the same donor as PDC were derived from) to test their suppressive capacity on the T-cell proliferation (mean±sem, n=5). IFN-γ and IL-10 production were determined in supernatants of these cultures by ELISA. (D) CD8+CD38+ T-cells were sorted from CD3+ T cells primed by allogeneic CpG-A stimulated PDC for 7 days, and added at a 1:2 ratio to autologous CFSE-stained CD3+ T cells stimulated with allogeneic LPS-MoDC derived from the same donor as PDC. After 5 days CFSE-dilution was determined by flowcytometry. To avoid interference of the CD8+CD38+ T cells in the analysis, CFSE-dilution of CD4+ T cells was analyzed. (E) Upon priming of CD3+ T-cells with allogeneic CpG-A stimulated PDC, total PDC-primed CD3+ T cells, or CD38-, CD4+CD38+ or CD8+CD38+ subpopulations obtained by sorting, or a 1:1 mix of CD4+CD38+ and CD8+CD38+ T-cells were re-stimulated with allogeneic LPS-MoDC derived from the same individual as PDC. Proliferation was determined by [3H]-thymidine incorporation at day 6. Fresh T-cells were used as a control (mean±sem, n=5).

TLR-stimulated PDC induce CD8+ regulatory T cells

Depletion of CD25+ T cells from CD3+ T cells prior to PDC-priming did not diminish the induction of suppressive capacity in T cells by allogeneic PDC (data not shown), indicating that naturally occurring CD4+CD25+ Treg are not involved in the induction of Treg by PDC. Therefore, we hypothesized that PDC induce suppressive capacity in another T-cell subset. To enable separation of T cells that respond to stimulation with allogeneic PDC from those that do not respond, we stimulated CFSE-labelled CD3+ T cells with allogeneic PDC and tested which activation marker selectively identified divided T cells. It appeared that CD38 reliably marked T cells that divided upon stimulation by allogeneic PDC (Fig. 3A). The use of CD38 for identification of activated T cells is further supported by differential expression of the chemokine receptors CXCR3 and CXCR4 on PDC-stimulated T cells (Fig. 3B). CD3+CD38- T cells expressed CXCR4 which is down-regulated on activated CD38+ T cells, while CXCR3 is up-regulated on CD38+ T cells, accordant with published data (43) (Fig. 3B).

To examine which T-cell subpopulation mediates suppressive capacity, allogeneic CD3+ T cells primed by mature PDC were separated into CD38-, CD4+CD38+ and CD8+CD38+ cells by flowcytometric cell sorting, and the suppressive capacity of each fraction was separately tested. Figure 3C shows that the suppressive capacity almost completely resided in CD8+CD38+ T cells, while CD4+CD38+ suppressed weakly and CD3+CD38- T cells did not suppress responder T-cell proliferation and IFN-γ production. Interestingly,

IL-10 was produced by $CD4^+CD38^+$ T cells, but not by $CD8^+CD38^+$ T cells, showing that the suppressive capacity was not related to IL-10 secretion. The potent suppressive capacity of PDC-primed $CD38^+CD8^+$ T cells was confirmed by measuring CFSE-dilution of $CD4^+$ responder T-cells (Fig. 3D). The proportion of $CFSE^{low}$ dividing $CD4^+$ T cells was reduced by 77% (from 34.5 to 7.9%) when $CD38^+CD8^+$ PDC primed T cells were added at a ratio of $CD38^+CD8^+$ PDC primed T cell : $CD4^+$ responder T cell of 1:2.
$CD38^+CD8^+$ T cells appeared to be completely non-responsive to re-stimulation with donor-specific MoDC, while $CD38^-$ T cells and $CD4^+CD38^+$ T cells were only partially hypo-responsive (Fig 3E). The non-responsiveness of $CD38^+CD8^+$ T cells to re-stimulation was not due to the lack of $CD4^+$ T-cell help, because a mixture of $CD38^+CD4^+$ and $CD38^+CD8^+$ T cells (1:1) did also not respond to re-stimulation. Notably, the mixing experiment shows that $CD38^+CD8^+$ T cells suppressed proliferation of $CD38^+CD4^+$ T cells upon re-stimulation with alloantigen. Together, these data show that mature PDC induce suppressive $CD8^+$ T cells.

Induction of $CD8^+$ regulatory T cells is partially mediated by indoleamine-2,3 dioxygenase in PDC

We investigated whether IL-10 or indoleamine-2,3 dioxygenase (IDO) are involved in the generation of $CD8^+$ Treg by PDC. $CD3^+$ T cells were primed by allogeneic CpG-A stimulated PDC in the presence of either a neutralizing anti-IL-10 receptor antibody or 1-metyl-DL-tryptophan (1-MT). After seven days $CD8^+$ $CD38^+$ T cells were sorted from PDC-primed T cells and tested for their suppressive capacity. Blocking IL-10 during the induction of Treg had no effect on the suppressive capacity of $CD8^+CD38^+$ T cells (data not shown), but inhibition of IDO by 1-MT partially reduced the suppressive capacity of the $CD8^+$ Treg induced by PDC (Fig. 4). Higher concentrations (up to 1mM) of 1-MT did not reduce suppression function further.

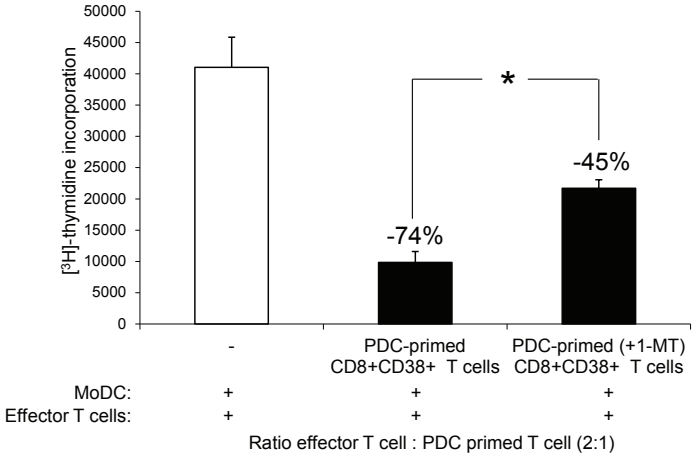


Figure 4: Induction of $CD8^+$ regulatory T cells by PDC is partially mediated by indoleamine-2,3 dioxygenase. $CD3^+$ T cells were primed by allogeneic CpG-A stimulated PDC in the presence or absence of 1-MT (250 μM). After 7 days the $CD8^+CD38^+$T cells were purified from PDC-primed T cells by flow-cytometric sorting, and added to autologous responder T cells stimulated with allogeneic MoDC at a Treg : Tresponder ratio of 1:2. After 6 days proliferation was determined (mean±sem, n=6; *p=0.001 using paired T-test).

PDC-induced $CD8^+$ Treg express LAG-3, Foxp3 and CTLA-4

Phenotypically, several types of $CD8^+$ Treg have been observed in humans: $CD8^+CD103^+$, $CD8^+CD94^+NKG2A^+$, $CD8^+CD25^+Foxp3^+$, $CD8^+CD28^-$, and $CD8^+LAG\text{-}3^+$ Treg (7-8, 44-47). PDC-primed $CD38^+CD8^+$ T cells were $CD28^+$ and did not express CD94 or CD103 (data not shown). Interestingly, the cells with the highest CD38-expression were $Foxp3^+$ and $CLTA\text{-}4^+$, and expressed lymphocyte activation gene-3 (LAG-3; CD223) on their surface (Fig 5A). $CD8^+CD38^+LAG\text{-}3^+$ and $CD8^+CD38^+LAG\text{-}3^-$ populations were sorted, and their ability to suppress effector T-cells was tested individually. Figure 5B shows

that the CD8+CD38+LAG-3+ population suppressed responder T cells ~3 times more efficiently than the CD8+CD38+LAG-3- cells. Thus, CD8+ Treg induced by allogeneic PDC are characterized by surface expression of LAG-3 and intracellular expression of Foxp3 and CLTA-4. Importantly, T cells with the same immunophenotype are present at a very low frequency in PBMC of healthy individuals (0.22 ± 0.08% of CD8+ T cells; n=5). After stimulation with TLR-activated PDC, 37.6 ± 8.9% of CD8+ T cells co-expressed these markers. Interestingly, the induction of CD8+ Treg was not dependent on TLR-stimulation of PDC. PDC pre-cultured with IL-3 alone also induced suppressive CD38+CD8+ T cells, but with three times lower yield (data not shown).

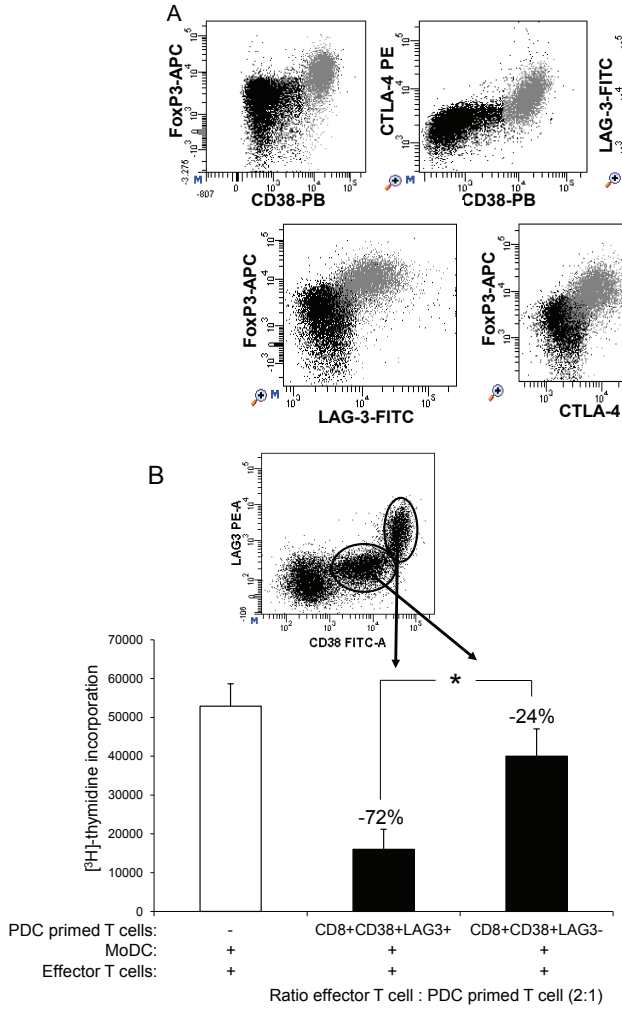


Figure 5: PDC-primed CD8+ Treg express Foxp3, CTLA-4 and LAG-3 (A) Allogeneic CD3+ T cells were stimulated by CpG-PDC, and after 7 days surface-stained for CD8, CD38 and LAG-3 and intracellularly stained for Foxp3 and CTLA-4. Dot plots show are gated on CD8+ T cells. (B) PDC-primed CD8+ T cells were divided into CD38+LAG-3+ and CD38+LAG-3- cells by flowcytometric sorting, which were separately tested for their suppressive capability (mean±sem, n=6). *p=0.001 using paired T-test.

Suppression PDC-primed CD8+ Treg is mediated by CTLA-4

One possible explanation for the mechanism of action of PDC-primed CD8+ T reg is allo-specific cytotoxicity to stimulator cells (MoDC) in re-stimulation assays. Figure 6A shows that PDC-primed CD38+CD8+ T cells express both granzyme A and granzyme B intracellularly, but no perforin. It is generally thought that both granzyme A and granzyme B use perforin to enter a target cell (48-49), but for granzyme B a perforin-independent way of target cell killing has been described (50-51). To test if suppression by PDC-induced CD8+ Treg is caused by cytotoxic killing of allogeneic stimulator cells, we

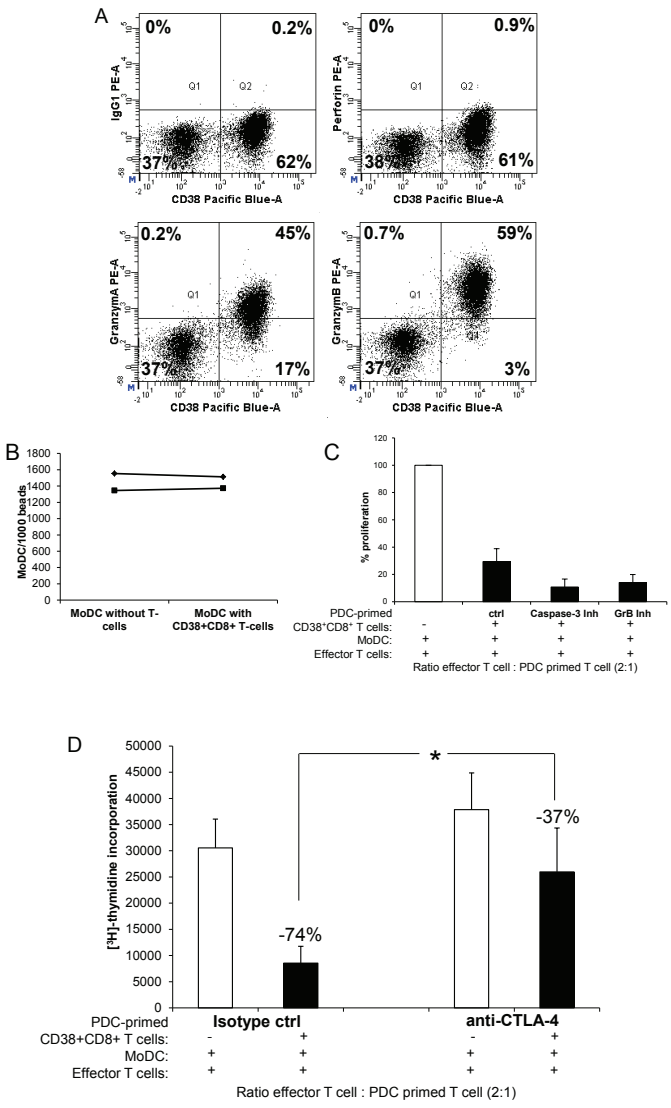


Figure 6: Suppression PDC-primed CD8$^+$ Treg is not mediated by cytotoxicity but partly dependent on CTLA-4. (A) CD3$^+$ T cells were stimulated by allogeneic CpG-PDC and after 7 days stained for CD8, CD38 and either anti-perforin, anti-granzyme A, anti-granzyme B, or isotype-matched irrelevant control mAb. In the dotplots gated CD8$^+$ T-cells are shown. (B) MoDC were cultured alone or together with PDC-stimulated allogeneic CD38$^+$CD8$^+$ T-cells for 18 hours at a ratio of 1.5 : 1. After co-culture, absolute MoDC numbers were determined by flowcytometry with normalization by adding known numbers of beads. Data from two independent experiments are shown. (C) The CD8$^+$CD38$^+$ cell fraction was purified from PDC-primed CD3$^+$ allogeneic T cells by flowcytometric sorting, and tested in suppression assays to which either a caspase-3 inhibitor (Z-DEVD-FMK; 20 μM) or a granzyme B inhibitor (Z-AAD-FMK; 20 μM) were added. At day 3 the inhibitors were refreshed and at day 6 proliferation of responder T cells was determined (mean±sem, n=3). Addition of the solvent DMSO alone did not affect the suppressive capacity of PDC-primed CD8$^+$CD38$^+$ T cells (data not shown). (D) CD8$^+$CD38$^+$ T cells were purified from PDC-primed CD3$^+$ allogeneic T cells, and tested in a suppression assay to which a neutralizing antibody against CTLA-4 or an isotype-matched control antibody (both at 15ug/ml) were added (mean±sem, n=5; *p=0.034 using paired T-test).

compared numbers of moDC after co-culture with PDC-primed CD8$^+$CD38$^+$ Treg with those after culture of moDC alone. Figure 6B shows that PDC-primed CD8$^+$CD38$^+$ Treg did not kill allogeneic moDC. In addition, we added a caspase-3 inhibitor (Z-DEVD-FMK) or a granzyme B inhibitor (Z-AAD-FMK) to suppression assays. Figure 6C shows that neither inhibition of caspase-3 nor neutralization of granzyme B activity abrogated suppression of allo-antigen-driven responder T-cell proliferation by PDC-primed CD8$^+$ Treg, indicating that suppression is not mediated by cytotoxic killing of stimulator cells. Since suppression could not be prevented by a caspase-3 inhibitor, the involvement of an apoptosis inducing ligand, like FAS-ligand or TRAIL was also ruled out.

To investigate whether suppression was exerted by soluble factors secreted by PDC-induced CD8$^+$ Treg, we harvested conditioned media at day 5 from cultures in which PDC-primed CD38$^+$CD8$^+$ T-cells were re-stimulated with allogeneic MoDC. The conditioned media did not suppress proliferation of CD3$^+$ responder T cells stimulated by allogeneic MoDC (data not shown). In addition, we investigated whether PDC-induced CD8$^+$

T cells exerted their suppression via CCL-4 (8), IL-10 (37) or TGF-β. However, adding neutralizing antibodies to CCL4 or IL-10 receptor, or addition of human latency-associated peptide to neutralize TGF-β, had no effect on their capacity to suppress allogeneic T-cell proliferation (data not shown). In addition, also neutralizing antibodies against LAG-3 had no effect. However, addition of a neutralizing antibody against CTLA-4 in the suppression assay significantly prevented inhibition of allogeneic T-cell proliferation by PDC-primed $CD38^+CD8^+$ T cells (figure 6D). Thus, PDC-induced $CD8^+$ Treg suppress effector T cells via a CTLA-4-dependent mechanism.

DISCUSSION

Attempts to induce tolerance to allogeneic organ transplants in primates or humans have been compromised by their large repertoire of allo-reactive memory T cells (21, 24-27). Therefore, clinically applicable tools to silence allo-reactive memory T cells are highly relevant for transplantation medicine.

In this study we show that human PDC induce profound hypo-responsiveness in unfractionated allogeneic $CD3^+$ T cells that contain both naïve cells and memory cells. Hypo-responsiveness is mediated by $CD8^+$ Treg that exert donor-specific suppression on allo-reactive T cells, including allo-reactive memory T-cells. These Treg are characterized by surface expression of LAG-3 and intracellular expression of Foxp3 and CLTA-4, and exert their suppressive capacity at least partially via CTLA-4.

Previous studies have shown that human PDC stimulated by a virus, cell-bound CD154, or CpG DNA induce the generation of $CD4^+$ Treg from naïve $CD4^+$ T cells (38-42), or $CD8^+$ Treg from naïve $CD8^+$ T cells (37). More relevant to the allogeneic transplant situation in humans, we found that $CD8^+$ Treg are induced in unfractionated allogeneic $CD3^+$ T cells upon co-culture with allogeneic human PDC. Although we observed up-regulation of CD25 and Foxp3 in $CD4^+$ T cells upon co-culture of $CD3^+$ T cells with TLR-stimulated allogeneic PDC (data not shown), $CD4^+$ T cells purified from PDC-T cell co-cultures had only weak suppressive capacity. Probably memory $CD4^+$ T cells and/or $CD8^+$ T cells present within unfractionated $CD3^+$ T cells prevent either the acquisition of suppressive capacity by $CD4^+Foxp3^+$ Treg during the co-culture with allogeneic PDC, or suppress their suppressive function in suppression assays. Interestingly, treatment of rat with CD40Ig induces $CD8^+CD45C^{low}$ Treg, but not $CD4^+$ Treg *in vivo*. These $CD8^+CD45C^{low}$ Treg mediated tolerance to allogeneic cardiac transplants. The dynamics of co-localization of $CD8^+CD45C^{low}$ Treg and PDC in these animals, first in the allograft and subsequently in the spleen, suggest that PDC play an important role in the induction of $CD8^+$ Treg *in vivo* (11). These data corroborate our observation that in the presence of all subsets of T cells PDC preferentially induce $CD8^+$ Treg and not $CD4^+$ Treg. However, $CD8^+$ Treg induced by allogeneic PDC in the current study differ in their mechanism of suppression from those induced by CD40Ig-treatment in the rat model used by the group of Anegon et al, since these exert their suppressive capacity via IFN-γ, fibroleukin and IDO, but not via CTLA-4 (10-11).

$CD8^+$ Treg induced by PDC in unfractionated allogeneic $CD3^+$ T cells differ also from those induced by PDC in naïve $CD8^+$ T cells, since the latter exerted their suppressive capacity through secretion of IL-10 (37). In addition, they are different from $CD8^+CD28^-$ Treg described by the group of Suciu-Foca (52) because they express CD28 but not CD103. They also differ from $CD8^+CD103^+$ Treg described by the group of ten Berge et al, because they do not express CD103, and inhibit allogeneic T cells in a donor-specific manner, while $CD8^+CD103^+$ Treg suppress allogeneic T cells non-specifically (7). The $CD8^+$

Treg described in the present study resemble immunophenotypically those found in blood of individuals infected with Mycobacterium tuberculosis or vaccinated with bacillus Calmette-Guérin (BCG) (8). Both express LAG-3, CTLA-4 and Foxp3. However, whereas $CD8^+LAG\text{-}3^+$ Treg from BCG-vaccinated individuals inhibit T-cell activation via secretion of CC chemokine ligand-4, $CD8^+$ Treg induced by allogeneic PDC exert their suppressive capacity not via soluble factors. Although highly expressed on PDC-induced $CD8^+$ Treg, LAG-3 is not involved in their suppressive activity. Accordingly, although LAG-3 has been shown to play a role in the suppressive activity of mouse $CD4^+CD25^+Foxp3^+$ Treg (53-54), a role in the suppressive function of human $CD4^+$ Treg has never been observed (8, 55). Like naturally occurring $CD4^+CD25^+Foxp3^+$ Treg (56-57), PDC-induced $CD8^+$ Treg suppress allogeneic T-cell proliferation via a CTLA-4-dependent mechanism. Therefore, PDC-induced $CD8^+$ Treg are probably equivalent to $CD8^+CD25^+Foxp3^+$ Treg present in the human thymus (6, 44), which also exert their suppressive function via CTLA-4. We hypothesize that the minor $CD8^+LAG\text{-}3^+CTLA\text{-}4^+$ population that we detected in blood of healthy individuals are emigrants of a more extensive population of this type of Treg in the thymus. Suppression of T-cell responses by CTLA-4 may be exerted by competition for co-stimulatory molecule binding with CD28, by down-regulation of CD80 and CD86 expression on Antigen-Presenting Cells, or by upregulation of IDO-activity in APC (58). Whether Foxp3 is critical for development or suppressive function of $CD8^+$ Treg described in this study is unclear. However, the subpopulation of activated $CD38^+CD8^+$ T cells that expressed Foxp3 together with CLTA-4 and LAG-3 exerted a much stronger suppression on allogeneic T cell responses compared to the $LAG\text{-}3^-CLTA\text{-}4^-Foxp3^-CD38^+CD8^+$ subset. This observation suggests that Foxp3 is associated with the regulatory properties of the $CD8^+$ Treg induced by allogeneic PDC.

Human PDC express IDO, which is further up-regulated by TLR-signaling, and use the IDO pathway to drive the differentiation of $CD4^+CD25^+Foxp3^+$ Treg from naïve $CD4^+$ T cells (41-42). The present study shows that PDC use the same mechanism to induce $CD8^+$ Treg from unfractionated $CD3^+$ T cells. Since $CD8^+LAG\text{-}3^+CTLA\text{-}4^+$ are present, although as a minor $CD8^+$ subset, in blood of healthy individuals, allogeneic PDC may selectively stimulate expansion of pre-existing $CD8^+$ Treg. Alternatively, PDC may induce their de novo generation from either naïve or memory $CD8^+$ T cells. Whatever the mechanism of $CD8^+$ Treg induction may be, we observed a more than 150-fold increase in the numbers of $CD8^+LAG\text{-}3^+CTLA\text{-}4^+$ Treg during 7-day co-cultures with allogeneic TLR-stimulated PDC. Notably, non-activated PDC also induce $CD8^+$ Treg, but with three times lower yield. This is consistent with the observation that PDC constitutively express IDO (41-42).

We conclude that TLR-stimulated PDC induce $CD8^+LAG\text{-}3^+Foxp3^+CTLA\text{-}4^+$ Treg from unfractionated allogeneic $CD3^+$ T cells *in vitro*, and that these Treg exert a dominant donor-specific suppressive effect on unfractionated allo-reactive T cells and render unfractionated allogeneic T cells hypo-responsive to re-stimulation with allo-antigen. These findings are highly relevant to clinical transplantation medicine, and support the use of donor-derived PDC as a means to prevent activation of recipient allo-reactive (memory) T cells. Whereas experimental animal studies have shown that PDC *in vivo* can either induce immunity or tolerance to protein antigens, the majority of studies investigating the effects of PDC on allogeneic immune responses show that PDC promote tolerance to allo-antigens *in vivo* (29-32). Only one study found that allogeneic PDC primed an allogeneic T cell response, but only in an inflammatory environment created by host irradiation (59). Importantly, we observed that TLR-stimulated PDC indeed stimulate allogeneic T cells to proliferate (although less efficient compared to moDC; figure 1a)

and to produce cytokines (figure 1b). However, during the same stimulation, $CD8^+$ Treg are induced that render these T cells hypo-responsive to re-stimulation with the same allo-antigen. Notably, Treg were selectively induced in activated $CD8^+CD38^+$ T cells. These data indicate that T cell activation precedes and is required for the induction of $CD8^+$ Treg by PDC.

In current clinical practice, PDC are almost eliminated from organ transplant recipients by the usual corticosteroid medication (60). Therefore, we advocate avoidance of corticosteroids as much as possible. In addition, an immunotherapeutic protocol can be envisaged in which PDC are purified from organ donor blood using clinical grade immuno-magnetic equipment, stimulated *ex vivo* by a clinical grade TLR-ligand, and administered to the transplant recipient. Alternatively, PDC can be used for *ex-vivo* generation of donor-specific $CD8^+$ Treg from recipient T cells, which subsequently are administered to the recipient. Both approaches have potential advantages and disadvantages. *Ex-vivo* matured donor-PDC can be administered within 1 day after transplantation, while *ex vivo* generation of PDC-primed Treg takes some time, precluding their use to prevent early rejection episodes. Conversely, administration of donor-derived PDC has the danger of short-term stimulation of the patient's immune response to donor allo-antigens, because PDC stimulate proliferation and pro-inflammatory cytokine production in allogeneic T cells before rendering them hypo-responsive (Figure 1 A and B). It may however be envisaged that the effects of the pro-inflammatory cytokines produced by PDC-primed T-cells are counterbalanced by the immune-regulatory cytokine IL-10 that they also secrete. In addition, TLR-stimulated PDC may stimulate the recipient immune system by secreting type I Interferons. To prevent type I Interferon production by TLR-stimulated PDC, it may be necessary to add a compound that inhibits Interferon-production, like CD303 antibodies (61), to the *ex vivo* stimulation protocol. Interestingly, a recent study showed that administration of large numbers of donor-derived activated macrophages to renal transplant recipients was safe (62-63), and has paved the way to clinical testing of donor-derived cellular therapeutics in organ transplant recipients.

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chapter 6

Prednisolone suppresses the function and promotes apoptosis of plasmacytoid dendritic cells

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ABSTRACT

Organ transplant recipients are highly susceptible to viral infections early after transplantation. Plasmacytoid dendritic cells (PDC) play a major role in anti-viral immunity. Therefore, we determined the numbers of circulating PDC after liver transplantation (LTX) and established the effects of immunosuppressive drugs on PDC-survival and function.

PDC were determined longitudinally in 13 LTX recipients treated with prednisone and cyclosporin or tacrolimus. Purified PDC were cultured with or without clinically relevant concentrations of cyclosporin, tacrolimus or prednisolone. Apoptosis induction was monitored by determination of active caspase-3, nuclear condensation and annexin-V/7AAD staining.

After LTX a 4-fold reduction in the number of circulating PDC was observed ($p<0.01$), which recovered partially after discontinuation of prednisone treatment. *In vitro*, prednisolone induced apoptosis in PDC, while cyclosporin and tacrolimus did not. Higher doses of prednisolone were needed to induce apoptosis in Toll-like receptor (TLR)-stimulated PDC. However, non-apoptosis inducing concentrations of prednisolone suppressed interferon-alpha production, upregulation of co-stimulatory molecules and allo-stimulatory capacity of TLR-stimulated PDC.

In conclusion, prednisolone induces apoptosis in PDC, which explains the decline in circulating PDC numbers after transplantation. Moreover, prednisolone suppresses the functions of TLR-stimulated PDC. Therefore, corticosteroid-free immunosuppressive therapy may reduce the number and severity of viral infections after transplantation.

INTRODUCTION

During the early post-transplantation period organ transplant recipients are highly susceptible to viral infections, such as the Herpesviridae, Epstein-Barr virus (EBV), and Varicella-Zoster virus. Primary infection with Cytomegalovirus (CMV), which can cause prolonged episodes of fever and pneumonia, and EBV, which is a cofactor in the development of post-transplantation lymphoproliferative disease, both can be life-threatening (1). After liver transplantation (LTX), recurrence of HCV-infection is universal. Progression to cirrhosis is much more rapid as compared with non-transplanted individuals, and is a major cause of graft loss (2).
It is generally accepted that the high incidence and severity of viral infections after organ transplantation is due to suppression of T-cell function by immunosuppressive drug treatment. Besides the adaptive immune response, innate anti-viral responses are important to counteract viral infections. A major component of the innate anti-viral immune response is the plasmacytoid dendritic cell (PDC). Upon viral infection, PDC are the principal producers of type I interferons (3), which are potent suppressors of viral replication. The importance of type I interferons in combatting viral infections is exemplified by the fact that Interferon-alpha (IFN-α)-treatment is the most effective drug for eradication of chronic HCV-infection (4).
PDC arise from hematopoietic stem cells in the bone marrow, and migrate via the blood circulation to lymph nodes, where they are mainly present in the T-cell rich areas around the high endothelial venules. They sense viruses via Toll-like receptors (TLR), including TLR-7 and TLR-9 which recognize single-stranded RNA viruses and unmethylated CpG-rich regions in the genome of DNA-viruses, respectively (3). Upon stimulation PDC maturate to Antigen-Presenting Cells (APC) which can prime T cell responses (3). PDC highly express the receptor for interleukin-3 (CD123), and IL-3 is essential for their survival and differentiation.
It has been observed that the numbers of circulating PDC decline after heart and kidney transplantation (5-8). This has been attributed to immunosuppressive therapy. However, the mechanism of the post-transplant reduction has not been fully elucidated. One possible explanation might be induction of apoptotic cell death in PDC by immunosuppressive drugs. In this respect, corticosteroids are of special interest, as Shodell et al (9) observed a reduction of circulating PDC after prednisone-treatment in healthy individuals. Corticosteroids are part of immunosuppressive induction regimens used in nearly all kinds of allogeneic transplantation. They inhibit activation of T-cells (10), and induce apoptosis in thymocytes (11), eosinophils and basophils (12). In addition, corticosteroids down-regulate multiple aspects of macrophage function and inhibit the differentiation of monocytes into macrophages. However, the effects of corticosteroids on PDC function and survival have never been investigated.
The aim of this study was to determine the kinetics of circulating PDC after LTX *in vivo*, and to establish the effects of immunosuppressive drugs on PDC survival and functions *in vitro*.

MATERIALS AND METHODS

Patients
Retrospectively, 13 liver graft recipients, transplanted in 1999 and 2000 from which archival peripheral blood mononuclear cells (PBMC) were available, were included in this study. None of the patients were treated with corticosteroids before transplantation.

To exclude effects of acute rejection and/or rejection treatment, only patients without clinically evident acute rejection were included in this study. Table I shows the clinical characteristics of the included patients. The dosage schedules of immunosuppressive treatment were extracted from the patient files. In the anhepatic phase of surgery the patients were given a single intravenous dose of 500 mg methylprednisolone. During the first week after transplantation intravenous prednisolone was given, after which the treatment was changed to oral prednisone. After 3 months prednisone was tapered over time and was discontinued in 11/13 patients between 6 and 12 months (median: 10 months post-LTX). Two patients were still treated with low doses of prednisone 12 months post-LTX.

Table I. Patient characteristics

	Patients (n=13)
Age, yrs, mean (range)	49 (34 - 59)
Gender, male/female	7 / 6
Indication for liver transplantation	
Hepatitis B	3
Hepatitis C	3
PBC	4
PSC	1
Alcoholic liver cirrhosis	1
Insulinoma with liver metastasis	1
Immunosuppression	
CsA+prednisone+azathioprine	2
CsA+prednisone+αCD25	3
TAC+prednisone	2
TAC+prednisone+αCD25	6

Reagents and materials

Anti-BDCA2-FITC mAb, anti-BDCA4-PE mAb, anti-PE-microbeads and MS-columns were obtained from Miltenyi Biotec, Bergisch Gladbach, Germany; anti-active caspase-3-FITC mAb, 7-amino-actinomycin D (7-AAD), CD86-APC, CD40-APC, CD123-biotin mAb, streptavidin-PerCP and annexin V-APC were obtained from BD PharMingen, Erembodegem, Belgium. IgG1-APC mAb was obtained from BD Biosciences, Erembodegem, Belgium, and IgG1-FITC mAb was from Sanquin, Amsterdam, the Netherlands. Vectashield supplemented with DAPI was obtained from Vector Laboratories, Burlingame, USA. CD80-FITC mAb and Intraprep permeabilization reagent were obtained from Beckman Coulter Immunotech, Marseille, France, and anti-CCR7-FITC mAb from R&D systems, Abingdon, United Kingdom. Prednisolone was obtained from Sigma, Zwijndrecht, the Netherlands. CpG A ODN2336 and loxoribin were obtained by InvivoGen, San Diego, USA, and caspase inhibitors Z-IETDS-FMK and Z-LEDH-FMK were from Calbiochem, Amsterdam, the Netherlands. Tacrolimus (TAC) was a kind gift from Fujisawa Pharma BV, Houten, the Netherlands, and Cyclosporin (CsA) was a kind gift from Novartis Pharma AG, Basle, Switzerland. 96-wells culture plates were obtained from Greiner Bio-one, Alphen a/d Rijn, The Netherlands Finally, phytohemagglutinin (PHA) was obtained from Murex, Paris, France.

Determination of the relative numbers of circulating PDC and monocytes
Venous blood was obtained in sodium-heparinized tubes before LTX, and 3 days, 1 week, 2 weeks, 1 month, 3 months and 12 months after transplantation. The Medical Ethical Committee of the Erasmus Medical Center, Rotterdam approved the study protocol, and informed consent was obtained from all patients. PBMC were isolated by Ficoll density centrifugation and stored in liquid nitrogen until analysis. Before analysis, PBMC were thawed and incubated with CD14-PE or anti-BDCA4-PE and biotinylated CD123 mAb followed by streptavidin-PerCP for 15 minutes in the dark at room temperature. Cells were washed and analyzed on a FACScalibur flow cytometer using CellQuest pro software (BD Biosciences). PDC were identified as CD123 and BDCA4 double-positive cells.

Purification of blood PDC
PBMC were isolated from buffy coats of healthy blood bank donors by Ficoll density centrifugation, and labeled with anti-BDCA4-PE mAb. Subsequently, the cells were washed and incubated with anti-PE-microbeads. After a second wash, PDC were isolated by two rounds of separation over MS columns. Purity of isolated PDC as assessed by staining with anti-BDCA2-FITC was typically more than 90%.

Determination of apoptosis
Isolated PDC ($2{,}5x10^4$/200 µl) were incubated in RPMI1640 medium containing 10% heat-inactivated fetal calf serum (Perbio Science HyClone, Logan, UT, USA) with or without CsA, TAC or prednisolone in the presence of IL-3. Prednisolone is the biologically active form of prednisone, and therefore prednisolone was used *in vitro*. After 4 and 18 hours the number of active caspase-3 expressing cells was determined by intracellular labeling with anti-active caspase-3-FITC mAb using Intraprep permeabilization reagens according to the manufacturer's instructions, after membrane labeling with anti-BDCA4-PE. To determine apoptotic cell death, cytospin preparations of PDC were stained with DAPI, and the appearance of their nuclei was visualized using fluorescence microscopy. Apoptotic cell death was confirmed by annexin V-APC/7-AAD staining. For this purpose, PDC that were cultured with or without prednisolone were suspended in 10 mM Hepes, 140 mM NaCl and 0.25 mM $CaCl_2$ (pH 7.4), incubated with annexin V-APC and 7-AAD for 15 minutes in the dark, and analyzed by flow cytometry.

Effects of prednisolone on cytokine production and immunophenotype
Purified PDC ($3x10^4$/200 µl) were stimulated with 5 µg/ml CpG A ODN2336 or 400 µM loxoribin with or without prednisolone in the presence of IL-3. After 20 hours cell-free supernatant was collected, and IFN-α (Bender MedSystems, Vienna, Austria) and interleukin-6 (IL-6; Bioscource International, Nivelles, Belgium) concentrations were determined by standard enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions. PDC were harvested from the wells and labeled for immunophenotypical analysis with the following antibodies: anti-BDCA4-PE, CD40-APC and anti-CCR7-FITC, or anti-BDCA4-PE, CD80-FITC and CD86-APC. Expression of these molecules was analyzed on vital PDC by exclusion of 7AAD-positive cells. Isotype-matched irrelevant mAb labeling was used to analyze expression of these molecules appropriately.

Analysis of allogeneic T cell stimulatory capacity of PDC
Purified PDC ($1x10^4$ and $5x10^3$ cells/200 µl) were stimulated with 5 µg/ml CpG A

ODN2336 or 400 µM loxoribin with or without prednisolone in the presence of IL-3. After 24 hours the culture supernatant was aspirated and the cells were washed twice with PBS. Allogeneic T cells, purified by negative selection (depletion of CD19, CD14, CD123 and BDCA1 positive cells) using magnetic cell separation (MACS) from PBMC of a healthy volunteer. $1.5x10^5$ T-cells/well were added to the PDC. After 5 days cell proliferation was assessed by determination of [^{3}H]thymidine (Radiochemical Centre, Amersham, Little Chalfont, UK) incorporation. 0.5 µCi/well was added and cultures were harvested 18 hours later. PHA (5 µg/ml) was added to T cells as a positive control.

Statistical analysis
Significance of differences between paired observations was tested by the Wilcoxon Signed Rank test using SPSS v11.0 software. A p-value of less than 0.05 was considered significant. All values are expressed as mean±SEM

RESULTS

Numbers of circulating PDC decrease after liver transplantation
The kinetics of circulating PDC after LTX were determined in 13 liver transplant recipients who were never treated for acute rejection. As is shown in Figure 1A, immediately after LTX the relative numbers of circulating PDC decreased 4-fold compared to the pre-LTX value. This decline persisted up to 3 months post-LTX. Between 3 and 12 months post-LTX PDC number started to rise, but still did not reach pre-LTX values at 12 months post-LTX. Since prednisone treatment was discontinued in 11 out of 13 patients between 3 and 12 months after transplantation, while the dosages of CNI (either CsA or TAC) did not change in this period (Figure 1B), it is likely that the increase in PDC-numbers was due to discontinuation of prednisone. Supporting this conclusion, no change in PDC-numbers was observed between 1 and 3 months after transplantation, when CsA-dosages were reduced. Moreover, in one of the two patients that were still on prednisone therapy on month 12 post-LTX, prednisone dose was tapered from 5

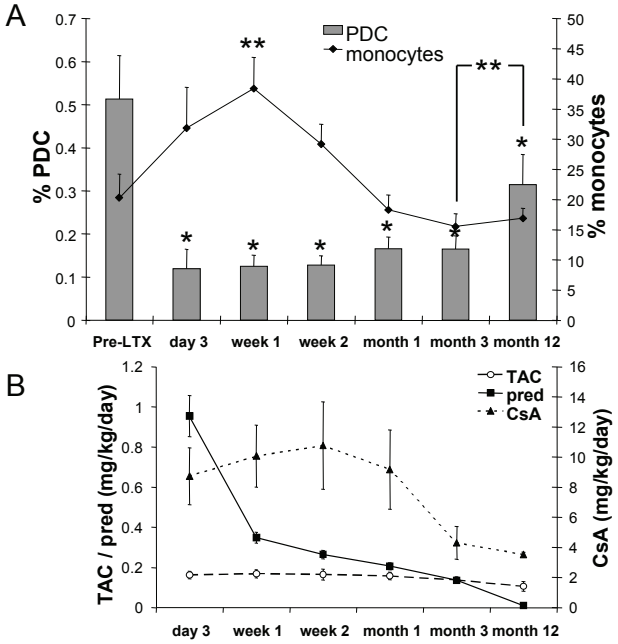

Figure 1. Longitudinal course of circulating PDC and monocytes after liver transplantation. (A) PDC (bars) and monocyte (lines) numbers were determined at baseline, 3 days, 1 and 2 weeks, 1 month, and 3 and 12 months after LTX; n=13, *P<0.02 compared to baseline, **P<0.05. (B) Treatment doses of TAC, CsA and prednisolone/prednisone in the LTX-patients at the indicated time-points.

mg on month 3 to 2.5 mg per day on month 12 post-LTX. This patient also showed an increase in circulating PDC-numbers between month 3 and 12 (from 0.27% to 0.46%). In the other patient the prednisone dose was not changed between 3 and 12 months (5 mg per day), and PDC numbers remained low over this time period (0.09% at month 3; 0.04% at month 12). In contrast to PDC, the relative numbers of monocytes increased after LTX, reaching its peak 1 week post-LTX, and decreased to normal levels at 1 month. This shows that not all types of APC decrease after LTX.

Prednisolone induces apoptosis in PDC, while CsA and TAC do not

To determine whether the decline in circulating PDC numbers after transplantation might be caused by apoptosis induced by immunosuppressive drugs, PDC were isolated from buffy coats of healthy blood bank donors, and incubated with CsA, TAC, or prednisolone. The concentration of CsA (600 ng/ml) used in these *in vitro* experiments was similar to C2-levels found in blood of LTX-recipients in our center (13). The concentration of

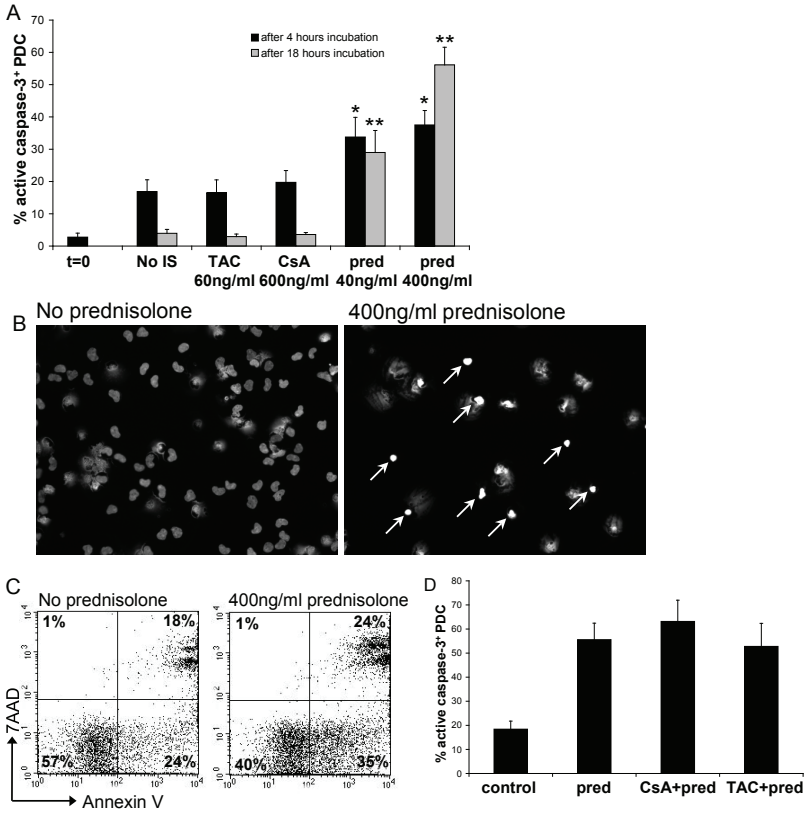

Figure 2. Prednisolone induces apoptosis in PDC. (A) PDC were cultured with or without CsA (600 ng/ml, TAC (60 mg/ml), or prednisolone (40 ng/ml or 400 ng/ml) in the presence of IL-3, and after 4 and 18 hours the numbers of active caspase-3$^+$ cells were determined by flow cytometry. Data are means±SEM of seven (4 hours incubation) or nine (18 hours incubation) independent experiments; *P<0.02, **P<0.01 compared to control cultures without immunosuppressive drug. (B) DAPI staining of prednisolone-treated and untreated PDC. PDC were cultured with or without 400 ng/ml prednisolone in the presence of IL3, and after 18 hours the cells were spinned on a slide and stained with DAPI. The arrows indicate PDC with condensed nuclei, undergoing apoptotic cell death. (C) Annexin-V/7AAD staining on PDC that were cultured for 4 hours with or without 400 ng/ml prednisolone in the presence of IL-3. (D) Addition of CsA or TAC has no effect on prednisolone-induced apoptosis of PDC. PDC were cultured with prednisolone (400 ng/ml) alone, 600 ng/ml CsA and 400 ng/ml prednisolone, or 60 ng/ml TAC and 400 ng/ml prednisolone in the presence of IL-3. After 4 hours the numbers of active caspase-3 positive PDC were determined (n=4).

TAC (60 ng/ml) was based on extrapolation of TAC trough levels in the patients included in the present study to C_{max} levels. TAC trough levels in our patients varied between 10 and 15 ng/ml. According to Kimikawa et al (14) and min et al (15) accompanying C_{max} levels of TAC in kidney transplant- and LTX-recipients vary between 20 – 60 ng/ml. The highest prednisolone concentration (400 ng/ml) used was comparable with peak plasma levels reached during daily treatment of 10 mg prednisolone (16). In these experiments IL-3 was added as a survival factor, since in the absence of IL-3, PDC undergo rapidly spontaneous apoptosis (17). After 4 and 18 hours the numbers of PDC expressing the active form of the effector caspase-3 were determined. As shown in Figure 2A, after 4 hours of culture in the presence of IL-3 about 15% of PDC were positive for active caspase-3. This is in agreement with previous observations that spontaneous apoptotic cell death of PDC *in vitro* is not completely prevented by IL-3 (18, 19). After 18 hours almost no expression of active caspase-3 was observed in control cells, which suggests that after 4 hours of culture no additional PDC entered the apoptotic pathway spontaneously, and that the pro-apoptotic PDC that were present at 4 hours had died at 18 hours. As shown in Figures 2A, addition of 600 ng/ml CsA or 60 ng/ml TAC did not affect the numbers of active caspase-3 positive PDC. However, during the first month after transplantation average CsA trough levels in the patients included in the present study varied between 180 and 260 ng/ml, which is considerably higher than those late after transplantation (13). According to Cantarovich et al (20), these trough levels result in C2-levels of about 1000 ng/ml. Moreover, Hesselink et al (21) showed that C_{max} levels of CsA in LTX-recipients are about 200 ng/ml higher than C2-levels. Consequently, it is conceivable that during the first month after transplantation, CsA peak levels of about 1200 ng/ml may have been reached in our study cohort. Therefore, we additionally investigated whether concentrations of CsA up to 1200 ng/ml could induce active caspase-3 expression in PDC, but we found no induction of active caspase-3 (data not shown).
In contrast, after 4 hours of culture with 400 ng/ml prednisolone, the number of active caspase-3 positive PDC increased more than 100% in comparison with control incubations. Between 4 and 18 hours of culture the numbers of pro-apoptotic PDC increased further, indicating that in the presence of prednisolone PDC continuously enter the apoptotic pathway. A ten-fold lower concentration of prednisolone induced apoptosis at similar rates after 4 hours and at halve of the rate after 18 hours compared to 400 ng/ml prednisolone.
To determine whether active caspase-3 positive PDC really undergo apoptotic cell death, PDC cultured with and without 400 ng/ml prednisolone were stained with DAPI, or with annexin-V and 7AAD. As is shown in Figure 2B, many condensed nuclei, which is one of the main features of apoptotic cell death, were visible after 18 hours in the prednisolone treated PDC, whereas untreated PDC show no condensed nuclei. In addition, after 4 hours of culture in the presence of prednisolone an increase in both single annexin-V^+ and Annexin-V/$7AAD^+$ PDC was observed in comparison to PDC cultured in the absence of prednisolone (Figure 2C).
All LTX-recipients included in the present study, had been treated with prednisone in combination of either CsA or TAC. To investigate whether CsA or TAC influence prednisolone-induced apoptosis, PDC were co-cultured with prednisolone in combination with CsA or TAC. As is shown in Figure 2D, the addition of CsA or TAC has no effect on the prednisolone-induced expression of active caspase-3.

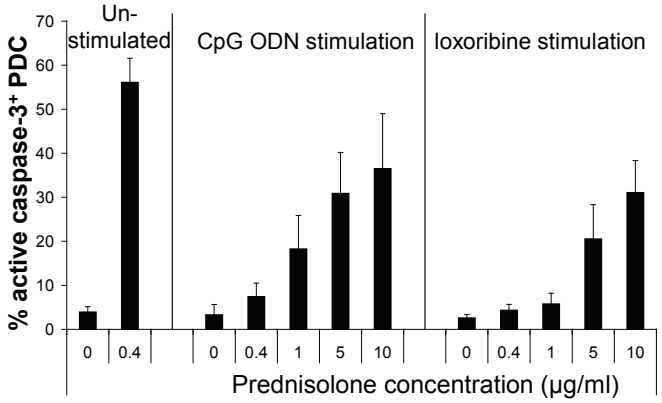

Figure 3. Higher doses of prednisolone induce apoptosis in TLR-stimulated PDC. PDC were stimulated with CpG ODN2336 or loxoribin and exposed to increasing concentrations of prednisolone in the presence of IL-3. For comparison, also non-stimulated PDC were cultured with prednisolone. After 18 hours the percentages of active caspase-3$^+$ PDC were determined (n=3).

Stimulated PDC are less sensitive to prednisolone-induced apoptosis

To investigate the effect of viral stimulation of PDC on their sensitivity to prednisolone-induced apoptosis, we stimulated PDC via TLR-9 with CpG A ODN2336, or via TLR-7 with loxoribin, and added increasing concentrations of prednisolone. Figure 3 shows that higher concentrations of prednisolone were required to induce expression of active caspase-3 in TLR-stimulated PDC compared to unstimulated PDC. A concentration of 400 ng/ml prednisolone, which rapidly induced apoptosis in resting PDC, did not induce active caspase-3 expression in TLR-stimulated PDC. Induction of active caspase-3 expression required concentrations above 1 µg/ml prednisolone.

Prednisolone reduces TLR-stimulated PDC function

Stimulated PDC are less sensitive to apoptosis-induction by lower doses of prednisolone, but at these concentrations prednisolone might still affect PDC function. To investigate the effect of prednisolone on IFN-α and IL-6 production, PDC were stimulated with CpG A ODN or loxoribin and prednisolone was added in concentrations that did not induce apoptotic death of TLR-stimulated PDC. Figure 4A shows that after CpG A stimulation, prednisolone at concentrations of 400 and 40 ng/ml reduced IFN-α production by 34% and 22% respectively. The inhibitory effect of prednisolone on IFN-α production after TLR-7 stimulation was stronger, 81% and 62% suppression, respectively. Prednisolone inhibited the IL-6 production by 57% and 55% respectively in case of CpG A ODN stimulation and by 82% and 74% respectively in case of loxoribin stimulation.

In addition, the effects of prednisolone on the upregulation of the co-stimulatory molecules CD40, CD80, CD86 and of the lymph node homing receptor CCR7 after TLR-stimulation were determined (Figure 4B). Prednisolone significantly inhibited the upregulation of these molecules, both at 400 ng/ml and at 40 ng/ml. To establish whether prednisolone affected the allogeneic T-cell stimulatory capacity of PDC, PDC were stimulated with CpG A ODN or loxoribin with or without 400 ng/ml prednisolone. After 24 hours the PDC were thoroughly washed and allogeneic T cells were added. After 5 days ^{3}H-thymidine incorporation was determined. As shown in Figure 4C prednisolone inhibited the allogeneic T cell stimulatory capacity of TLR-stimulated PDC. The inhibition was most pronounced upon stimulation with loxoribin. To exclude a direct effect of remaining prednisolone on T-cell proliferation, wells without PDC were incubated with 400 ng/ml prednisolone, washed in the same way as wells filled with PDC, and PHA-stimulated T-cell proliferation was compared in prednisolone-treated and non-treated wells. No differences were observed (data not shown), indicating that measured differences were mediated by the effect of prednisolone on PDC and not by a direct effect on T-cells.

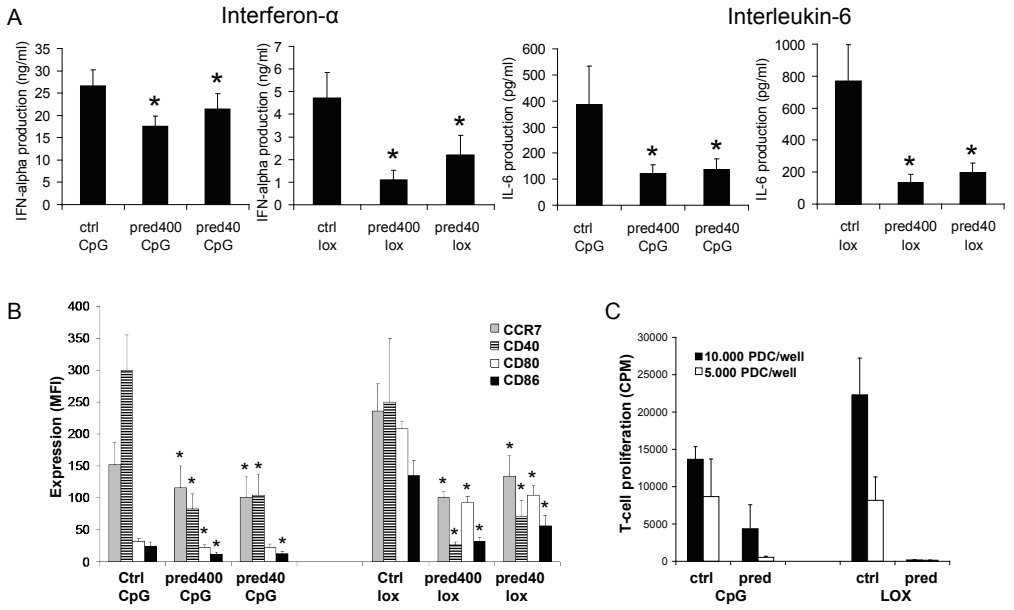

Figure 4. Prednisolone suppresses the functions of TLR-stimulated PDC. PDC were stimulated with CpG A ODN2336 or loxoribin with or without 400 and 40 ng/ml prednisolone in the presence of IL-3. (A) After 20 hours the production of IFN-α and IL-6 was determined in the supernatant; n=6, *p<0.03. (B) PDC from these cultures were harvested and expression of CD40, CD80, CD86 and CCR7 were determined by flowcytometry. As a measure for expression the geometric mean of fluorescence intensity was analysed; n=6; *P<0.03. (C) Effect of prednisolone on the allogeneic T-cell stimulatory capacity of TLR-stimulated PDC. PDC were stimulated with CpG A ODN or loxoribin in the presence of IL-3 with or without 400 ng/ml Prednisolone. After 24 hours the supernatant was thoroughly washed away and allogeneic T-cells were added. After 5 days [^{3}H]-thymidine incorporation was determined. One representative experiment out of three is shown.

Together, these results show that concentrations of prednisolone which are similar to plasma concentrations in stable liver transplant recipients more than two weeks after LTX, and which do not induce apoptosis in TLR-stimulated PDC, significantly suppress their function.

Prednisolone does not interfere in the signaling of IL-3 and induces apoptosis in PDC via the extrinsic pathway

A possible mechanism by which prednisolone may induce apoptosis of PDC is by interfering with the signaling pathway of IL-3. To investigate this item, PDC were cultured in the absence of IL-3 with or without prednisolone. Figure 5A shows that in the absence of IL-3 higher numbers of PDC became pro-apoptotic in comparison to cultures in the presence of IL-3. However, addition of prednisolone to cultures without IL-3 further increased the numbers of pro-apoptotic PDC, suggesting that prednisolone does not exert its pro-apoptotic effect by interfering with the signaling of IL-3.

Apoptosis can be induced via an extrinsic or an intrinsic signaling pathway. The extrinsic pathway depends on activation of caspase-8, which either directly activates caspase-3, or induces mitochondrial disruption and cytochrome c release, which leads to consecutive activation of caspases-9 and 3. The intrinsic pathway does not involve caspase-8, but is provoked by stress factors that result in permeablization of the mitochondrial outer membrane and subsequent cyctochrome c release, caspase-9 and caspase-3 activation (22). To investigate via which pathway prednisolone induces PDC-apoptosis,

the effects of caspase-8 inhibition and caspase-9 inhibition on prednisolone-induced expression of active caspase-3 were determined. Figure 5B shows that addition of a caspase-9 inhibitor reduced the expression of active caspase-3 in PDC considerably, but not completely. In contrast, addition of a caspase-8 inhibitor blocked the activation of caspase-3 completely, indicating that prednisolone induces apoptosis in PDC via the extrinsic pathway.

DISCUSSION

The present study shows for the first time that human plasmacytoid dendritic cells undergo apoptotic cell death when exposed to prednisolone, even in the presence of survival factor IL-3. In contrast, another type of frequently used immunosuppressive drugs, the calcineurin inhibitors cyclosporin and tacrolimus do not induce apoptosis in PDC. Induction of apoptosis by prednisolone, the active metabolite of prednisone explains the strong decline in the numbers of circulating PDC observed shortly after LTX (this study), heart and kidney transplantation (5-8). This explanation is supported by the partial, but significant, restoration of circulating PDC numbers in the LTX-patients 1 year after LTX, since prednisone treatment was discontinued or further tapered between 3 and 12 months post-LTX, while the calcineurin inhibitor treatment was not changed during this time period. Our data confirm the association between corticosteroid therapy and low numbers of circulating PDC numbers that has been found both in patients and healthy volunteers treated with corticosteroids only (9, 23, 24).
The concentrations of prednisolone which induced apoptosis in non-stimulated PDC (400 and 40 ng/ml), are comparable to or lower than peak plasma levels found in transplant recipients during daily treatment of 10 mg prednisolone (16). TLR-stimulated PDC were less sensitive to prednisolone-induced apoptosis than non-stimulated PDC, and needed higher concentrations (above 1 µg/ml) of prednisolone to undergo apoptosis. However, intra-operatively and early after transplantation, LTX-recipients are treated with much

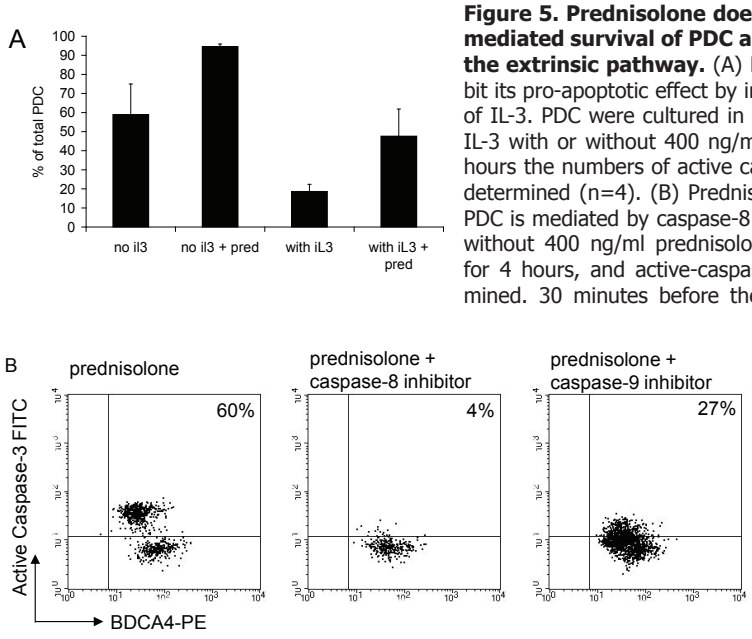

Figure 5. Prednisolone does not interfere with IL-3 mediated survival of PDC and induces apoptosis via the extrinsic pathway. (A) Prednisolone does not exhibit its pro-apoptotic effect by interfering with the signaling of IL-3. PDC were cultured in the absence or presence of IL-3 with or without 400 ng/ml prednisolone, and after 4 hours the numbers of active caspase-3 positive cells were determined (n=4). (B) Prednisolone-induced apoptosis of PDC is mediated by caspase-8. PDC were cultured with or without 400 ng/ml prednisolone in the presence of IL-3 for 4 hours, and active-caspase-3 expression was determined. 30 minutes before the prednisolone was added, PDC were treated with 10 µM Z-IETD-FMK (caspase-8 inhibitor) or 10 µM Z-LEHD-FMK (caspase-9 inhibitor). Representative flow cytometric dot plots of one out of four experiments are shown.

higher doses of prednisolone (500 – 25 mg/daily). It is therefore conceivable that early after LTX not only unstimulated, but also virus-stimulated PDC will undergo apoptosis.
The second major observation in our study is that concentrations of prednisolone that did not induce apoptosis in TLR-stimulated PDC, were able to suppress their functional capacity. Low non-apoptosis inducing concentrations of prednisolone inhibited IFN-α and IL-6 production, up regulation of CCR7, CD40, CD80 and CD86, and T cell stimulatory capacity of TLR-stimulated PDC. Therefore, it can be envisaged that the doses of prednisone used to treat LTX-recipients later than 3 months after LTX suppress the major functions of PDC upon encounter of a virus, i.e. production of the anti-viral cytokine IFN-α and T-cell activation.
The inhibition of PDC functions by prednisolone was more pronounced after TLR-7 stimulation than after TLR-9 stimulation with CpG A ODN. This observation confirms recent data of Hackstein et al. who showed a stronger reduction of IFN-α production in blood of prednisone-treated kidney transplant recipients compared to blood of healthy individuals after Resiquimod (ligand for TLR-7) stimulation than after CpG A ODN stimulation (8). The differences in sensitivity of IFN-α production to prednisolone between TLR-7 and TLR-9 stimulation are probably related to differences in signaling pathways in PDC that are elicited by CpG A ODN and TLR-7 ligands. Unmethylated CpG A ODN have an extraordinary capacity to stimulate IFN-α synthesis in PDC compared to CpG type B ODN (25). We observed that CpG A ODN stimulated about five-fold higher production of IFN-α compared to loxoribin. CpG A ODN have been reported to stimulate two distinct regulatory pathways of IFN-α synthesis in human PDC. One pathway is dependent on the IFN receptor-mediated positive feedback loop and one pathway is independent of that loop (25). Probably, prednisolone inhibits the induction of IFN-α synthesis via the signaling pathway that is independent from the IFN receptor, but not the positive feedback loop. Therefore, the extent of suppression of IFN-α synthesis by PDC by prednisone treatment *in vivo*, may depend on the type of virus infection: during infections with viruses that stimulate PDC via TLR-7 or via TLR-9 in a CpG B ODN-like manner, the suppressive effect of prednisone on IFN-α production will be larger compared to viruses that stimulate like CpG A ODN do.
While this study was in progress, a report was published showing that dexamethasone enhances apoptosis of mouse PDC. However no effect of dexamethasone treatment on *ex vivo* PDC function was observed (26). Probably, the inhibition of PDC functions by corticosteroids is reversible, and continuous presence of corticosteroid is needed to observe functional suppression of PDC.
By virtue of their high capacity to secrete IFN-α, PDC play an important role in the innate immune response against viruses (3). Therefore, the decline in PDC numbers and suppression of their IFN-α production by prednisolone may substantially contribute to the increased susceptibility of organ transplant recipients for viral infections. HCV-infection is a main indication for LTX. Recurrent HCV infection progresses more rapidly to cirrhosis than before transplantation and leads to graft loss. It is generally accepted that the rapid progression to cirrhosis after LTX is due to immunosuppressive treatment. Whether corticosteroids accelerate or delay histological HCV-recurrence is at present unclear. Many studies with conflicting results have been published on this item during the last few years (27-30). However, the increased HCV replication after LTX as compared to before LTX (2) has been definitely associated with corticosteroid treatment (31, 32). IFN-α is the most effective cytokine suppressing HCV-replication (4) and Horsmans et al (33) demonstrated that treatment of patients with chronic HCV-infection with a TLR-7 agonist resulted in rapid reduction of HCV-RNA coinciding with increased IFN-α

plasma levels. Therefore, we hypothesize that the increased HCV-replication in corticosteroid-treated LTX-recipients is due to induction of PDC-apoptosis and suppression of IFN-α production by PDC.

PDC are important sensors of viruses circulating in the blood stream, and can present viral antigens to T-cells. Therefore, the suppression of their TLR-induced maturation and acquisition of T-cell stimulatory capacity by prednisolone implies that during corticosteroid treatment PDC are also impaired in priming adaptive T-cell responses against circulating viruses. This may further contribute to the high incidence and severity of viral infections in organ transplant recipients.

Increased ratio's of circulating PDC and myeloid DC have been associated with successful weaning of immunosuppressive drugs in LTX-recipients (34, 35). Moreover, infusion of PDC in mice prolonged allogeneic heart survival (36, 37). Therefore, PDC might play a role in the development of tolerance against the graft, and PDC-apoptosis induced by the corticosteroid therapy after organ transplantation may counteract the induction of tolerance against the transplanted organ.

The intracellular signaling pathways that lead to apoptosis involve sequential activation of pro-caspases to active enzymes. We demonstrate that prednisolone-induced apoptosis in PDC is mediated by caspase-8, similar to corticosteroid-induced apoptosis in thymocytes (11). Caspase-8 is involved in the extrinsic signaling pathway of apoptosis induction that is normally activated by ligation of membrane receptors, like the TNF-receptor or CD95. Instead of binding to a membrane receptor prednisone acts by binding to the intracellular glucocorticoid receptor (GR). However caspase-8 can also be activated without triggering membrane receptors (38, 39). We found that inhibition of caspase-9, a mediator of the intrinsic pathway, reduced the activation of caspase-3, but did not completely block its expression in prednisolone treated PDC. This partial effect of prednisolone can be explained, as active caspase-8 induces activation of caspase-3 by two pathways: either directly or indirectly via mitochondrial disruption and caspase-9. As a consequence, by inhibiting caspase-9, the amplifying effect of the mitochondrial route is blocked, while the direct activation of caspase-8 remains intact (40, 41).

In conclusion, prednisolone induces apoptosis in PDC, which may explain the rapid decline of circulating PDC after organ transplantation. TLR-stimulated PDC are less sensitive to prednisolone-induced apoptosis, but prednisolone suppresses their production of IFN-α and reduces their maturation to APC. On basis of these data corticosteroid-free immunosuppressive therapy can be expected to reduce the number and severity of viral infections after organ transplantation, and should be persued especially in HCV-positive LTX recipients. Recently, several clinical studies show that corticosteroid-free therapy is feasible without additional risk of rejection (28, 29, 31, 42).

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chapter 7

Corticosteroids abrogate the antiviral activity of plasmacytoid dendritic cells on hepatitis C virus

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ABSTRACT

Chronic hepatitis C virus (HCV) infection is one of the leading indications for liver transplantation (LTX), but outcomes are compromised by re-infection of the graft. Several studies have indicated that glucocorticosteroid-based immunosuppression is a risk factor for accelerated HCV recurrence after LTX. However, the mechanism responsible for the glucocorticosteroid-mediated effect on HCV recurrence is unknown. The aim of this study was to investigate the effects of glucocorticosteroids on the antiviral activity of IFN-α and on inhibition of HCV replication by the primary IFN-α-producing cells, plasmacytoid dendritic cells (PDC). HCV replication and IFN-α signal transduction were studied in Huh7 cells stably expressing luciferase reporter genes linked to HCV IRES (Huh7-ET) or to an interferon response element (Huh7-ISRE-luc). Cells were treated with IFN-α in the presence of different doses of the glucocorticosteroids prednisolone or dexamethasone. Human PDC, stimulated with a Toll-Like Receptor (TLR)-7 ligand, or by co-culture with Huh7-ET, in the presence or absence of glucocorticosteroids, were tested for their effect on HCV replication. Glucocorticosteroids did not directly inhibit HCV replication or IFN-α signalling in Huh7 cells. Conditioned media of TLR-7 stimulated PDC potently suppressed HCV replication. This antiviral capacity of PDC was abrogated by glucocorticosteroids and by pre-treating Huh7 cells with an IFN-α/β receptor blocking antibody. In addition, HCV-replication in Huh7-ET was suppressed by co-culture with PDC, and this effect was also abrogated by glucocorticosteroids. In conclusion, these data indicate that the inhibition of PDC function by glucocorticosteroids may contribute to the observed accelerated HCV recurrence after LTX, and support the notion to carefully consider the use of glucocorticosteroids in HCV infected liver transplant recipients.

INTRODUCTION

End-stage liver disease caused by chronic Hepatitis C (HCV) infection is one of the leading indications for liver transplantation (LTX) in the world (1). A major complication after LTX is the universal recurrence of HCV infection with accelerated levels of HCV replication (2), liver fibrosis and development of cirrhosis (3). HCV recurrence has a severe impact on patient and graft survival (4). Usually, these patients have a much poorer response to standard therapy with pegylated interferon-alpha and ribavirin compared to non-transplanted patients (5).

Immunosuppressive drugs are one of the major factors that are associated with the more aggravated course of HCV infection after LTX. In particular, glucocorticosteroid pulses given for acute cellular rejection are associated with more severe HCV recurrence (6). Prednisolone (PRED) and its close analogue dexamethasone (DEX) are potent suppressors of the immune system and are used in a broad range of autoimmune and inflammatory diseases. LTX-patients are treated with glucocorticosteroids as part of induction protocols and as maintenance immunosuppression in combination with other drugs to prevent acute rejection. Glucocorticosteroid boluses are used to treat acute rejection episodes. However, the use of glucocorticosteroid boluses is associated with increased HCV replication and progression of fibrosis (6-7). The effect of glucocorticosteroid maintenance therapy on HCV related liver graft injury is still a matter of debate. Some clinical studies found a beneficial effect of maintenance corticosteroids, while others are in favor of a steroid-free regimen (7).

The reason why standard therapy with pegylated interferon-alpha and ribavirin is less effective after LTX is still unknown.

The mechanism of the glucocorticosteroid-mediated effects on HCV replication is not fully elucidated. An earlier study by our group showed that glucocorticosteroids do not directly stimulate replication of HCV in an *in vitro* replicon model (8), which was confirmed in a later study with an infectious HCV model (9). However, Ciesek et al. did show that glucocorticosteroids increase HCV cell entry through upregulation of the cell entry factors occludin and scavenger receptor class B type 1 (9). It is unlikely that this effect alone counts for the negative impact of glucocorticosteroids on HCV recurrence, because of the pleiotropic nature of steroids. Apart from direct effects on viral entry receptors on hepatocytes, effects of glucocorticosteroids on immune cells may indirectly act on HCV infection by reducing immune control of the virus.

Plasmacytoid dendritic cells (PDC) are the principal producers of interferon-alpha (IFN-α), and play a major role in anti-viral immunity (10-11). PDC are abundant in HCV infected livers (12), and HCV-infected cells trigger IFN-α secretion by PDC via a mechanism that requires active viral replication, direct cell-cell contact, CD81/CD9-associated membrane microdomains, and Toll-like receptor 7 (TLR-7) signalling (13-14). Previously, we have shown that glucocorticosteroids suppress IFN-α production by PDC (15). However, the effect of glucocorticosteroids on inhibition of HCV replication by PDC has not been investigated so far. In addition, whether glucocorticosteroids interfere with IFN-α-mediated suppression of HCV infection or with IFN-receptor signalling is unknown.

The aim of this study is to investigate the effect of glucocorticosteroids on the antiviral activity of IFN-α and IFN-α regulated gene expression, and the impact of glucocorticosteroids on suppression of HCV replication by PDC. We report that glucocorticosteroids did not directly inhibit IFN-α signalling in hepatocytes but abrogated the antiviral capacity of PDC. Clinical implications of these findings will be discussed.

MATERIALS AND METHODS

Reagents
Interferon-α2a was provided by Roche Ltd (Basel, Switzerland). Prednisolone and dexamethasone were obtained from Sigma (Sigma-Aldrich Chemie B.V. Zwijndrecht, the Netherlands). Beetle luciferin potassium salt was obtained from Promega (Promega Benelux BV, Leiden, The Netherlands). Loxoribin was from Invivogen, San Diego, CA. Interleukin-3, anti-BDCA2-FITC, anti-BDCA4-PE, anti-PE-microbeads and LS-columns were obtained from Miltenyi Biotec, Bergisch Gladbach, Germany. Mouse anti-human neutralizing interferon alpha/beta receptor chain 2 monoclonal antibody was from Millipore (Millipore BV, Amsterdam, the Netherlands).

Cell lines
The human hepatoma cell line Huh7-ET-luc, stably tranduced with the HCV bicistronic replicon (I389/NS3-3V/LucUbiNeo-ET) containing the non-structural coding sequences of HCV and the luciferase gene was used as a model for HCV replication (16). The cells were cultured in DMEM (Lonza Benelux B.V., Breda, the Netherlands), with 10% fetal calf serum (Sigma-Aldrich Chemie B.V. Zwijndrecht, the Netherlands), 2 mM L-glutamine, 100 U/ml penicillin, 100 U/ml streptomycin and 500 µg/ml G418 (Life Technologies Europe BV, Bleiswijk, Netherlands). To measure interferon stimulated gene expression we used the Huh7 cell line Huh7-ISRE-luc, stably transduced with a luciferase reporter gene controlled by the ISRE promoter element (17), cultured in DMEM (10% FCS, glutamine, pen/strep). Huh7 cells, stably transduced with a lentiviral vector constitutively expressing luciferase controlled by a PGK promoter (Huh7-PGK-luc) (17), were used as control cells to measure the effects of compounds on the expression of luciferase (cultured in DMEM, 10% FCS, glutamine, pen/strep).

Purification of PDC from human blood
PBMC were isolated from buffy coats of healthy blood-bank donors by Ficoll density centrifugation. For isolation of PDC, PBMC were incubated with anti-BDCA4-PE mAb, washed, and incubated with anti-PE-microbeads. After a second wash, PDC were enriched over an LS-column, followed by flowcytometric sorting on a FacsAria II cellsorter. Purity of isolated PDC, as determined by staining with anti-BDCA2-FITC and flowcytometry, was ≥ 99%. Purified PDC were cultured in a 96-well plate ($2x10^4$ cells/200 µl) in the presence of the survival factor interleukin-3 (20 ng/ml), and stimulated with 400 µM loxoribin in the presence or absence of PRED or DEX. After 24 hours of culture, conditioned media (PDC-CM) were collected and stored at -20°C.

Luciferase measurement in living cells
Cell lines were seeded in white walled 96-well plates at 70-80% confluency, and cultured overnight. The next day cells were treated with IFN-α, PRED, DEX or a combination, or with 25% PDC-CM. After 24 hours, 100 mM luciferin potassium salt was added to the cells, and they were incubated for 30 minutes at 37°C. Luciferase activity was measured in a LumiStar Optima luminescence counter (BMG LabTech, Offenburg, Germany). All measurements were performed in triplicate.
For co-culture experiments, Huh7-ETluc cells were seeded in a 96-well plate and allowed to attach. Purified PDC were added ($2x10^4$ cells/well) in the presence of the survival factor interleukin-3 (20 ng/ml), and in the presence or absence of PRED or DEX. After 24 hours of culture luciferase was measured.

To neutralize the IFN–α receptor on the Huh7-ET cells, the cells were seeded in a 96-well plate and treated with 10 µg/ml anti-human interferon alpha/beta receptor chain 2 monoclonal antibody for 15 minutes at 37°C, after which PDC-CM was added. Luciferase activity was measured after 18 hours.

RESULTS

Inhibition of HCV replication by interferon-α is not affected by glucocorticosteroids

The effects of increasing doses of PRED and DEX on the replication of HCV were measured in Huh7-ET-luc cells, where the expression of luciferase is a direct measure for hepatitis C replication. In agreement with previous studies, glucocorticosteroids had no direct effect on HCV replication. HCV replication was inhibited by 5 IU/ml IFN-α to more than 80% of baseline levels. The addition of PRED and DEX had no significant effect on the IFN-mediated inhibition of HCV replication (Figure 1A).

To investigate potential non-specific effects of glucocorticosteroids and IFN-α on Huh7 cells or luciferase activity, Huh7-PGK-luc cells were tested, which express the luciferase reporter gene under control of the constitutive PGK promoter element. Both PRED and DEX enhanced the luciferase signal by 1.5 fold, but this was independent of the dose. IFN-α slightly decreased luciferase activity, while dexamethasone restored it to baseline levels (Figure 1B). These data are in agreement with a previous study in which we showed that glucocorticosteroids increase the cellular protein content of Huh7 cells (8). Together, these data demonstrate that glucocorticosteroids do not interfere with the antiviral activity of IFN-α.

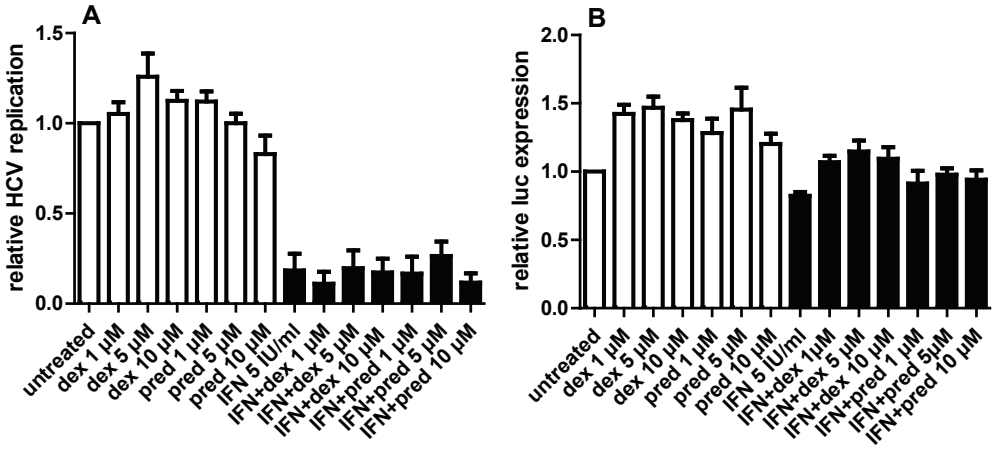

Figure 1. A. Dexamethasone and prednisolone do not affect HCV replication in the presence or absence of IFN-α. (A) Huh7-ET cells were cultured for 24h with increasing concentrations of PRED or DEX, in the absence or presence of 5 IU/ml IFN-α. The luciferase activity in these cells was used as a direct measure of HCV replication. Results are means±SEM of at least 4 independent experiments. (B) DEX and PRED increase luciferase activity in Huh7-PGK-luc cells, both in the presence or absence of IFN-α. Results are means±SEM of 3 independent experiments.

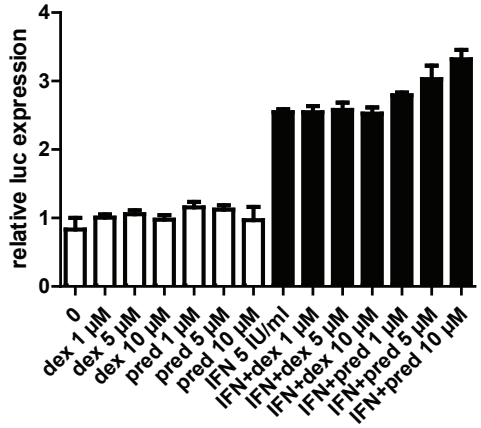

Figure 2. Dexamethasone and prednisolone do not inhibit IFN-α mediated gene expression. IFN-regulated gene expression was measured with increasing concentrations of DEX and PRED, in the presence or absence of 5 IU/ml IFN-α. ISRE-regulated luciferase expression was increased 2.5 fold by IFN-α. DEX and PRED did not inhibit IFN regulated gene expression, either in the absence or presence of IFN-α. Results are the means±SEM of 6 observations from 2 independent experiments.

Effect of glucocorticosteroids on interferon-α mediated gene expression

To assess the effect of PRED and DEX on IFN-α mediated gene expression we used Huh7-ISRE-luc cells that contain a luciferase gene under the control of an interferon-stimulated response element. The cells were incubated for 24 hrs with increasing doses of PRED and DEX, in the presence or absence of 5 IU/ml IFN-α. The expression of luciferase was stimulated 2.5-fold with 5 IU/ml IFN-α. DEX had no significant effect on IFN regulated luciferase expression, either in the absence or presence of IFN-α. Increasing concentrations of PRED stimulated IFN regulated gene expression by maximal 30% (Figure 2). These data show that glucocorticosteroids do not inhibit IFN-α mediated gene expression.

Glucocorticosteroids suppress the antiviral activity of plasmacytoid dendritic cells

HCV-infected cells stimulate IFN-α production by PDC via TLR-7 (13-14). Therefore, to determine the effects of glucocorticosteroids on the antiviral activity of PDC, we stimu-

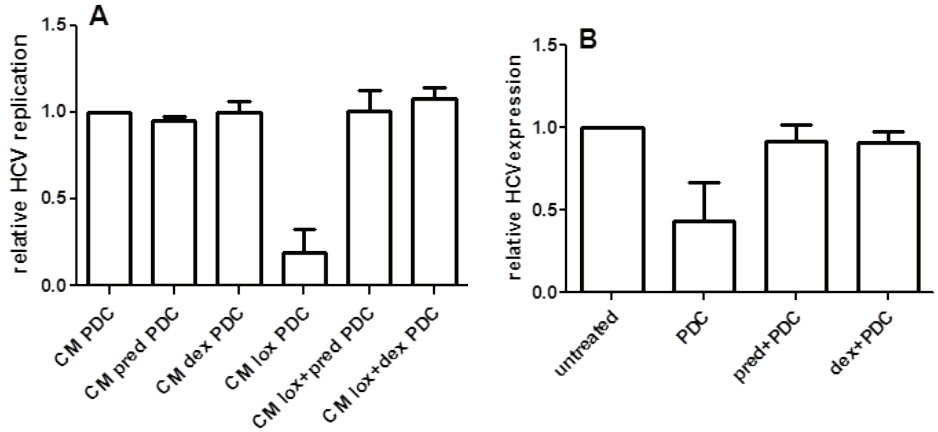

Figure 3. Glucocorticosteroids suppress the antiviral activity of plasmacytoid dendritic cells
(A) Huh7-ET cells were incubated for 24h with 25% PDC-CM of PDC that were not stimulated or stimulated with loxoribin (lox) in the presence or absence of 1 µM PRED or DEX. DEX and PRED completely reversed the antiviral capacity of lox stimulated PDC. No inhibition of HCV replication was observed with PDC-CM from unstimulated PDC cultured in the presence or absence of glucocorticosteroids. Shown are the mean±SEM of three independent experiments with duplicate incubations. (B) HCV replication in Huh7-ET was inhibited by co-culture with $2x10^4$ PDC. This could be completely reversed by PRED and DEX. Shown are the mean±SEM of three independent experiments with duplicate incubations.

lated human PDC with TLR-7 agonist loxoribin in the presence or absence of PRED or DEX and collected the conditioned medium after 24h of culture (PDC-CM). Subsequently Huh7-ET cells were incubated overnight with PDC-CM, and replication of HCV was measured as relative luciferase activity (figure 3). The results show that conditioned media from TLR-7 activated PDC suppress HCV-replication by more than 80%, and that the antiviral activity of PDC-CM was completely abrogated by PRED or DEX (figure 3A). When Huh7-ETluc cells were cultured in the presence of PDC, we observed a 60% reduction of HCV replication that also could be completely reversed by PRED or DEX (Figure 3B).

To determine whether the antiviral activity of PDC is mediated via type I IFN, we blocked the IFN-α/β receptor on the surface of the Huh7-ETluc cells with neutralizing antibodies before adding PDC-CM from loxoribin-stimulated PDC, or from PDC that were co-cultured with Huh7-ETluc cells. By blocking the IFN-α/β receptor, the antiviral activity of the CM from lox-stimulated PDC was partially inhibited, and the antiviral activity of CM from replicon-stimulated PDC was completely abrogated (figure 4).

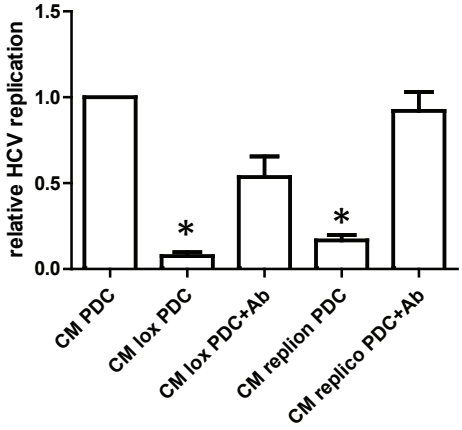

Figure 4: Antiviral action of PDC-CM can be abrogated with IFN-receptor blocking antibodies. HCV replication in Huh7-ET was inhibited with conditioned media from PDC that were stimulated with loxoribin (CM lox PDC) or stimulated with Huh7-ET replicon co-culture (CM replicon PDC). The inhibition of HCV replication by CM lox PDC could be partially prevented, and inhibition of CM replicon PDC was fully abrogated by pre-incubation with IFN receptor blocking antibodies. Conditioned media from unstimulated PDC (CM PDC) served as control. Shown is the mean±SD of three independent experiments. *p<0.05 compared to CM PDC.

DISCUSSION

A major challenge in organ transplantation is to find the right balance between suppression of the immune system and the maintenance of defense against viral and other infections. The use of immunosuppressants in HCV-infected LTX-patients is associated with a more severe recurrence of HCV. The contribution of maintenance therapy with glucocorticosteroids to HCV-recurrence after LTX still remains a matter of debate. Some clinical studies suggest that glucocorticosteroid maintenance therapy is associated with less severe disease or slower fibrosis progression rates, while others find that steroid avoidance is associated with less HCV recurrence (7). In order to solve this controversy, it is necessary to determine whether glucocorticosteroids can affect HCV-replication.

In this study we show that glucocorticosteroids do not affect HCV-replication in hepatocytes *in vitro*, and do not directly inhibit the antiviral activity of IFN-α and IFN-α induced gene expression in hepatocytes. The lack of effect of glucorticoisteroids in Huh7 cells can not be explained by the absence of glucorticoid receptor signaling, because we are able to detect induction of glucocorticoid regulated genes by DEX and PRED (data not shown). However, we show that glucocorticosteroids negatively affect the inhibition of HCV-replication by PDC. Conditioned media from TLR-7 stimulated PDC, as well as

co-culture with PDC, potently suppressed HCV-replication via secretion of type I IFN. Glucocorticosteroids in a concentration that is reached in serum during maintenance therapy (18-19) abrogated the anti-viral activity of PDC in both experimental conditions. This observation can be explained by the strong suppressive effect of glucocorticosteroids on IFN-α production by TLR7-stimulated PDC (15, 20-21). Glucocorticosteroids induce rapid apoptosis of unstimulated PDC, and we have previously observed that the numbers of circulating PDC decrease dramatically in glucocorticosteroid-treated LTX-recipients (15). Signaling of viruses and TLR-agonists however prevents the induction of apoptosis in PDC by increasing expression of anti-apoptotic molecules (Bcl-2 and Bcl-xL, BIRC3, CFLAR) via activation of the NF-κB pathway (15, 20-21). We have previously shown that the concentration of prednisolone used in the current study does not induce apoptosis of PDC, but does suppress IFN-α production. This indicates that the abrogation of the anti-viral effect of TLR-7 stimulated PDC by glucocorticosteroids is probably due to inhibition of IFN-α secretion. However, HCV infected cells do not activate the NF-κB pathway in PDC (22), and abrogation of the anti-viral effect of PDC in co-culture with replicon cells could therefore also be due to glucocorticosteroid induced apoptosis. The effect of glucocorticosteroids on the anti-viral effect of PDC may contribute to the increased HCV replication and accelerated recurrence of HCV infection after LTX. This notion is supported by the finding that treatment of rejection with high doses of glucocorticosteroids is associated with diminished survival in HCV-positive but not HCV-negative recipients (23). Although we can not exclude that the accelerated liver failure after transplantation of HCV-infected patients is caused by a more general effect of the immunosuppressive therapy, our data further support steroid minimization after LTX in HCV-infected patients.

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chapter 8

Rapamycin has suppressive and stimulatory effects on human plasmacytoid dendritic cell functions

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Revision submitted

ABSTRACT

Plasmacytoid dendritic cells (PDC) are involved in innate immunity by IFN-α production, and in adaptive immunity by stimulating T-cells and inducing generation of regulatory T-cells (Treg). Here we studied the effects of mTOR-inhibition by rapamycin, a commonly used immunosuppressive and anti-cancer drug, on innate and adaptive immune functions of human PDC. A clinically relevant concentration of rapamycin inhibited TLR7-induced IFN-α secretion potently (-64%) but TLR9-induced IFN-α secretion only slightly (-20%), while the same concentration suppressed pro-inflammatory cytokine production by TLR7-activated and TLR9-activated PDC with similar efficacy. Rapamycin inhibited the ability of both TLR7-activated and TLR9-activated PDC to stimulate production of pro-inflammatory (IFN-γ and IL-17) and anti-inflammatory (IL-10) cytokines by allogeneic T-cells. Surprisingly, mTOR-inhibition enhanced the capacity of TLR7-activated PDC to stimulate T-helper cell proliferation, which was caused by rapamycin-induced upregulation of CD80 expression on PDC. Rapamycin did not hamper the generation of suppressive $CD8^+CD38^+LAG\text{-}3^+$ Treg by TLR-stimulated PDC.
Conclusions: In general rapamycin inhibits innate and adaptive immune functions of TLR-stimulated human PDC, but it enhances the ability of TLR7-stimulated PDC to stimulate $CD4^+$ T cell proliferation, and leaves their capacity to generate functional $CD8^+$ Treg unaffected.

INTRODUCTION

Plasmacytoid dendritic cells (PDC) have important functions in innate and adaptive immunity. They are unique in rapidly producing massive amounts of type I interferon upon recognition of viral nucleotides or self-DNA-protein complexes by their Toll-Like receptors (TLR). In addition, after maturation in response to TLR-ligation or CD40-engagement they acquire dendritic cell morphology and capacity to present antigens to T cells. Mature PDC can activate as well as inhibit T cell responses. On the one hand mature PDC can prime productive $CD4^+$ and $CD8^+$ T cell responses (1), and on the other hand they possess a capacity to induce generation of $CD4^+$ and $CD8^+$ regulatory T cells (Treg) from naïve $CD4^+$ or $CD8^+$ T cells, respectively (2-7). Recently, we showed that human PDC preferentially induce generation of a unique type of $CD8^+$ Treg, but not $CD4^+Foxp3^+$ Treg, when both $CD4^+$ and $CD8^+$ T cells are present (8). Importantly, these $CD8^+CD38^+LAG\text{-}3^+CTLA\text{-}4^+$ Treg were not only able to inhibit naïve T-cells, but also memory T-cell responses. Indeed, *in vivo*, depending on the experimental animal model, PDC either induce effective T cell immunity (9-11) or inhibit T cell responses by driving differentiation of Treg *in vivo* (12-14). A recent study in which PDC were selectively eliminated from mice showed that PDC can simultaneously suppress and stimulate T cell responses *in vivo* (15).

Recently, is has been shown that the selective mTOR-inhibitor rapamycin inhibits production of IFN-α and pro-inflammatory cytokines by TLR-activated mouse PDC, and reduces their capacity to stimulate $CD4^+$ T cells. Rapamycin was found to block the interaction of TLR with MyD88, resulting in reduced IRF-7 phosphorylation (16). However, important questions regarding the effects of rapamycin on PDC functions have still be to be resolved. First, the effect of rapamycin on the ability of PDC to generate Treg has not been studied. Secondly, Cao et al studied mouse PDC, and, whereas they recapitulated the inhibitory effect of rapamycin on IFN-α secretion on human PDC, it remains to be established whether and how rapamycin affects the T-cell stimulatory capacity of human PDC.

These questions are clinically highly relevant, because the indications for rapamycin treatment are expanding. Originally used as immunosuppressive drug in transplant recipients, rapamycin and rapamycin analogs are now increasingly being evaluated as anti-proliferative drug in cancer treatment (17). Moreover, studies have been initiated to determine its efficacy in auto-immune diseases like Systemic Lupus Erythematosus (SLE) (18), which are mainly caused by overproduction of IFN-α by PDC (19-20).

Therefore, the aims of the present study were to determine systematically the effects of a clinically relevant concentration of rapamycin on cytokine production, T-cell stimulatory capacity, and $CD8^+$ Treg-generating capacity of human PDC.

MATERIALS AND METHODS

Reagents

IL-3, anti-BDCA2-FITC, anti-BDCA4-PE, anti-BDCA1-PE mAb, CD15-microbeads, CD235-microbeads, CD14-microbeads, anti-PE-microbeads, MS, LS and LD-columns were obtained from Miltenyi Biotec, Bergisch Gladbach, Germany. 7-AAD, anti-HLA-DR-APC, CD3-PerCP, CD4-PerCP, CD45RO-APC, CD56-FITC and CD14-PE mAb were obtained from BD Biosciences, Erembodegem, Belgium. CD19-PE, CD45RA-FITC, CD38-FITC, CD45-FITC, CD80-FITC and CD123-PE mAb were purchased from Beckman Coulter, Immunotech, Marseille, France, and CD40-APC, CD45RA-PE, IgG1-FITC, IgG2a-FITC,

CD8-APC, functional grade IgG2a isotype control mAb and IFNα, IL-6, IL-10 and TNF-α ELISA-kits were obtained from eBiosciences, Vienna, Austria. CD86-APC, anti-HLA-ABC-FITC and IgG1-APC were obtained from Biolegend, London, UK. CpG A ODN2336 and loxoribin were purchased from InVivogen, San Diego, USA. Anti-LAG3-PE and IL-17 ELISA kit were purchased from R&D systems, Abingdon, UK. IFNγ, IL-4 and CXCL-10 (IP-10) ELISA-kits and CFSE were purchased from Life Technologies, Bleiswijk, the Netherlands. Neutralizing IFNαR2 mAb was obtained from Merck Millipore, Amsterdam, the Netherlands. Rabbit anti-phosphorylated S6 antibody was from Cell Signaling Technology, Danvers, USA and mouse-anti-β-actin antibody from SantaCruz, Heidelberg, Germany. GM-CSF was a kind gift of Schering-Plough, Kenilworth, USA, and neutralizing CD80 mAb (clone: B7-24) (21) was a kind gift of M. de Boer (Tanox Pharma BV, Amsterdam, The Netherlands), PHA was obtained from Murex, Paris, France. Rapamycin was purchased from Merck, Schiphol-Rijk, The Netherlands, and PTEN-inhibitor VO-OHpic trihydrate from Sigma-Aldrich, St Louis, MO.

Purification of PDC, T cells and monocytes from human blood, and generation of monocyte-derived DC

PBMC were isolated from buffy coats of healthy blood-bank donors by Ficoll density centrifugation. For isolation of PDC, PBMC were incubated with anti-BDCA4-PE mAb, washed, and incubated with anti-PE-microbeads. After a second wash, PDC were isolated in two rounds of separation over MS columns. Alternatively, BDCA-4 labeled PDC were isolated by enrichment over an LS-column, followed by flowcytometric sorting on a FacsAria II cellsorter. Purity of isolated PDC, as determined by staining with anti-BDCA2-FITC and flowcytometry, was >94%. T cells were purified from PBMC by negative selection upon labeling with PE-conjugated antibodies against BDCA1, CD14, CD19, CD56, CD123 as well as CD15- and CD235-microbeads followed by incubation with anti-PE microbeads. T cells were isolated over an LD column. The purity of the T cells analyzed after labeling with CD3-PerCP and CD45-FITC was >97%. In selected experiments the isolated T-cells were labeled with CD45RA-FITC and CD45RO-APC to isolate naïve and memory T-cells by flowcytometric sorting. Monocytes were isolated using CD14 microbeads over an MS column. Purity was >98%. For the generation of monocyte-derived DC (MoDC) monocytes ($1x10^6$/ml) were cultured in RPMI supplemented with 10% FCS, 50 ng/ml GM-CSF and 200 U/ml IL-4 for 7 days. On day 6, 500 ng/ml LPS was added to stimulate MoDC maturation.

Analysis of PDC immunophenotype and cytokine production

Purified PDC ($2x10^4$/200 µl RPMI supplemented with 10% FCS) were stimulated in round-bottom wells (Greiner Bio-one, Alphen a/d Rijn, the Netherlands) with 5 µg/ml CpG A ODN2336 or 400 µM loxoribin in the absence or presence of 20 ng/ml rapamycin. In all conditions 10 U/ml IL-3 was added as a survival factor. Rapamycin, or DMSO vehicle in case of non-stimulated cells, were added 1 hour before addition of the stimuli. After 18 hours supernatants were collected for quantification of cytokines, and PDC immunophenotype was analyzed. The following combinations of antibodies were used: CD80-FITC, anti-BDCA4-PE and CD86-APC, anti-BDCA4-PE and CD40-APC, and anti-HLA-ABC-FITC, anti-BDCA4-PE and anti-HLA-DR-APC. Dead cells were excluded with 7-AAD. Cells were analyzed on a Canto II flowcytometer using Diva 6.0 software (Becton Dickinson) or a Calibur flowcytometer with CellQuest Pro 5.2 software. Isotype-matched irrelevant mAb labeling was used to analyze expression of these molecules appropriately.

Western blot analysis
PDC were stimulated with CpG-A or loxoribin as described, and thereafter lysed in Laemmli buffer. The lysates were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and blotted on Immobilon-FL transfer membrane (Millipore, Billerica, MA, USA). The membranes were incubated with the appropriate antibodies, and for detection anti-rabbit or anti-mouse IRDye-conjugated secondary antibodies (Li-cor, Lincoln, USA) were used according to the manufacturer's directions. The blots were scanned by Odyssey infrared imaging (LI-COR Biosciences, Lincoln, NE, USA). Results were visualized with Odyssey 3.0 software.

Stimulation of T cells with allogeneic PDC
After stimulation of purified PDC ($2x10^4$/200 µl) for 18 hours with CpG A ODN2336 or loxoribin in the absence or presence of 20 ng/ml rapamycin, rapamycin was carefully washed away, and allogeneic $CD3^+$ T cells were added ($1x10^5$/200 µl RPMI supplemented with 10% FCS). The cells were cultured at 37ºC with 5% CO_2. Proliferation was determined after 5 days of culture by measurement of incorporation of 0.5 µCi/well [^{3}H]thymidine (Radiochemical Centre, Amersham, Little Chalfont, UK) during the last 18 hours or the culture. In all cultures, T cells stimulated with PHA (5 µg/ml) served as a positive control to assess their proliferative capacity. Alternatively, after 7 days cell-free supernatant was collected for cytokine analysis, total numbers of viable cells were counted using trypan blue exclusion and proportions of $CD3^+CD4^+$, $CD3^+CD8^+$, and $CD8^+CD38^+$LAG-3^+ T cells were analyzed. In another set of experiments, CFSE-labeled allogeneic $CD3^+$ T cells were added to PDC, and T-cell proliferation was determined by flowcytometric measurement of CFSE-dilution.

Cytokine measurement
The supernatants of the stimulated PDC were analysed for IFNα, interleukin-6, and TNFα concentrations by standard ELISA according to the manufacturer's instructions. The supernatants of T cells co-cultured with allogeneic PDC were analysed for IFNγ, interleukin-10, interleukin-4, interleukin-17 and CXCL-10 also by standard ELISA according to the manufacturer's instructions.

Suppression assay
To assess the suppressive capacity of $CD8^+CD38^+LAG3^+$ regulatory T cells generated during co-cultures with allogeneic PDC, $CD8^+CD38^+LAG3^+$ T cells were purified from cultured cells by flowcytometric sorting using a FacsAria Cell Sorter (Becton Dickinson), and added in graded doses to cultures of $CD3^+$ T cells ($1x10^5$/200 µl) that were stimulated with allogeneic irradiated (3000rad) donor-specific MoDC ($1.5x10^4$) in round bottom wells. In these experiments MoDC and PDC were derived from the same donor. After 5 days, proliferation was assessed by determination of [^{3}H]thymidine incorporation for 18 hours.

Statistical analysis
All experiments were performed n times, as indicated in the figure legends, with cells from different individuals, and mean values ± SEM were calculated. Significance of differences between paired observations was tested in the paired T test using Microsoft excel 2003 software. A p-value of less than 0.05 was considered significant.

RESULTS

Effects of rapamycin on cytokine secretion by human PDC

The effects of rapamycin were studied using purified human PDC stimulated with TLR9 ligand CpG type A oligodeoxynucleotide (CpG-A)-2336 or TLR7 ligand loxoribin, in the presence of interleukin-3 (IL-3) as essential survival factor. To determine whether a clinically relevant concentration of 20 ng/ml rapamycin, which is similar to the blood peak level reached during rapamycin treatment (Rapamune summary of product characteristics, Wyeth-Ayerst Pharmaceuticals Inc. Philadelpha, PA, USA), inhibits mTOR-signaling

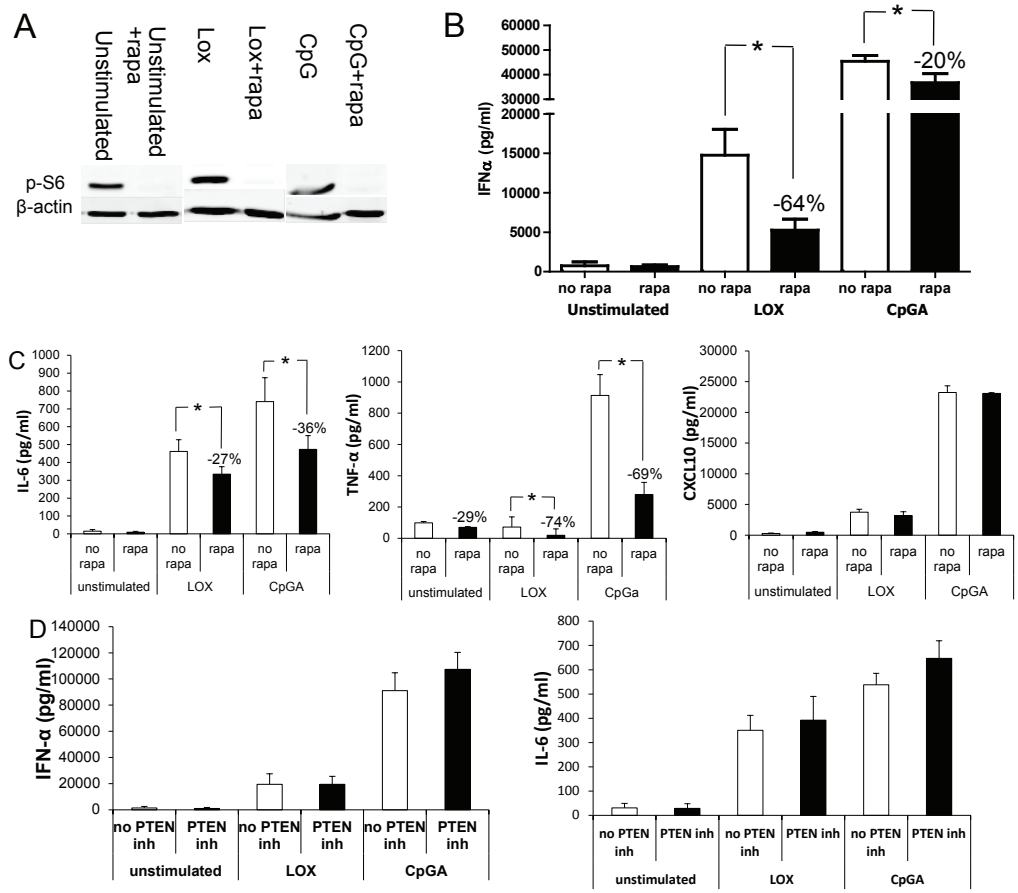

Figure 1: Rapamycin inhibits mTOR-signaling and cytokine production in human PDC

(A). Immunoblot analysis of phosphorylated S6 protein in lysates of purified unstimulated PDC, PDC stimulated for 15 minutes with 400 μM loxoribin or with 5 μg/ml CpG A ODN 2336, in the absence or presence of 20 ng/ml rapamycin. In all conditions 10 IU/ml IL-3 was added as a survival factor. Rapamycin, or DMSO vehicle in case of non-stimulated cells, were added 1 hour before addition of the stimuli. As a reference protein, β-actin expression was determined. (B, C). Two x 10^4 human PDC in 200 μl were stimulated with 400 μM loxoribin, or 5 μg/ml CpG A ODN 2336, or left non-stimulated, in the absence or presence of 20 ng/ml rapamycin. IL-3 was added as a survival factor. Rapamycin, or DMSO vehicle in case of non-stimulated cells, were added 1 hour before addition of the stimuli. After 18 hours cell-free media were harvested, and (B) IFN-α and (C) IL-6, TNF-α, and CXCL10 were determined. Data depicted are means±SEM of 6 independent experiments. *p<0.03. (D) Two x 10^4 human PDC in 200 μl were stimulated with 400 μM loxoribin, or 5 μg/ml CpG A ODN 2336, or left non-stimulated, in the absence or presence of 10 nM VO-OHpic trihydrate. IL-3 was added as a survival factor. Rapamycin, or DMSO vehicle in case of non-stimulated cells, were added 1 hour before addition of the stimuli. After 18 hours cell-free media were harvested, and IFN-α and IL-6 were determined. Data depicted are means±SEM of 3 independent experiments.

in PDC, we measured phosphorylation of the 40S ribosomal protein S6, which is a downstream phosphorylation target of mTOR (22). Figure 1A shows that S6 is phosphorylated in both non-stimulated and in stimulated PDC, and that 20 ng/ml rapamycin inhibits S6-phosphorylation completely in all conditions, indicating that this concentration of rapamcyin suppresses mTOR-signaling in PDC effectively.
CpG-A was a more effective stimulus to induce IFN-α secretion than loxoribin, (Figure 1B). While 20 ng/ml rapamycin inhibited loxoribin-induced IFN-α secretion by 64%, it inhibited CpG-A-induced IFN-α secretion only by 20% despite almost complete suppression of mTOR-signaling. In contrast, secretion of the pro-inflammatory cytokines IL-6 and TNF-α was inhibited by rapamycin with similar efficacy in both stimulation conditions (Figure 1C). The observed inhibitory effects of rapamycin were not due to general impairment of PDC function, because no inhibition of CXCL10 secretion was observed (Figure 1C) and rapamycin did not induce apoptosis as demonstrated by the absence of active caspase-3 (data not shown).
To test whether stimulation of mTOR-signaling would enhance cytokine secretion by PDC, we added VO-OHpic trihydrate, a specific inhibitor of phosphatase-and-tensin homolog (PTEN), during PDC-activition. The upstream signaling pathway that activates mTOR is initiated by phosphatidylinositol 3-kinase (PI3K), which generates 3-phosphorylated inositol lipids (PIP3) (23). PTEN is a negative regulator of PIP3K-signaling because it dephosphorylates PIP3 (24), and therefore inhibition of PTEN abrogates negative regulation of mTOR-phosphorylation. Addition of VO-OHpic trihydrate to TLR-activated PDC in a concentration that increased generation of PDC from human $CD34^+$ progenitor cells (25) did, however, not affect cytokine production by PDC (figure 1D), suggesting that PI3K-mTOR signaling is not a limiting factor in cytokine secretion by PDC. This is consistent with the constitutive mTOR-signaling in PDC that we observed, even in the absence of a stimulus.
Together, these data show that a clinically relevant concentration of rapamycin inhibits pro-inflammatory cytokine production by TLR7-activated PDC and TLR9-activated PDC, while it suppresses IFN-α secretion in TLR7-activated PDC but almost not in TLR9-engaged PDC.

Rapamycin promotes the T-cell stimulatory capacity of TLR7-activated PDC by enhancing CD80-expression

To study the effects of mTOR inhibition on the T cell stimulatory capacity of PDC, we activated PDC with TLR-ligands for 18 hours and than added allogeneic $CD3^+$ T cells. After activation in the presence or absence of rapamycin, PDC were carefully washed to remove rapamycin before T cells were added. Activation of PDC via TLR-7 in the presence of rapamycin increased their capacity to stimulate T-cell proliferation, while addition of rapamycin during TLR-9 activation did not (Figure 2A). The increased proliferation of T-cells upon mTOR inhibition in TLR-7 activated PDC was confined to enhanced expansion of the CD4 compartment (figure 2B), and was observed in both memory ($CD45RO^+$) and naïve ($CD45RA^+$) T-cells (figure 2C). Higher concentrations of rapamycin (up to 100 ng/ml) did not further enhance the increased T-cell proliferation after TLR7 ligation of PDC. T cells stimulated by PDC secreted pro-inflammatory (IFN-γ, IL-17) and anti-inflammatory (IL-10) cytokines (Figure 2D), but no Th2 cytokines (data not shown). Treatment of PDC with rapamycin suppressed the capacity of PDC to stimulate pro- and anti-inflammatory cytokine secretion by T-cells irrespective of the mode of PDC-activation.
To find an explanation for the observed increase in T-cell proliferation induced by ra-

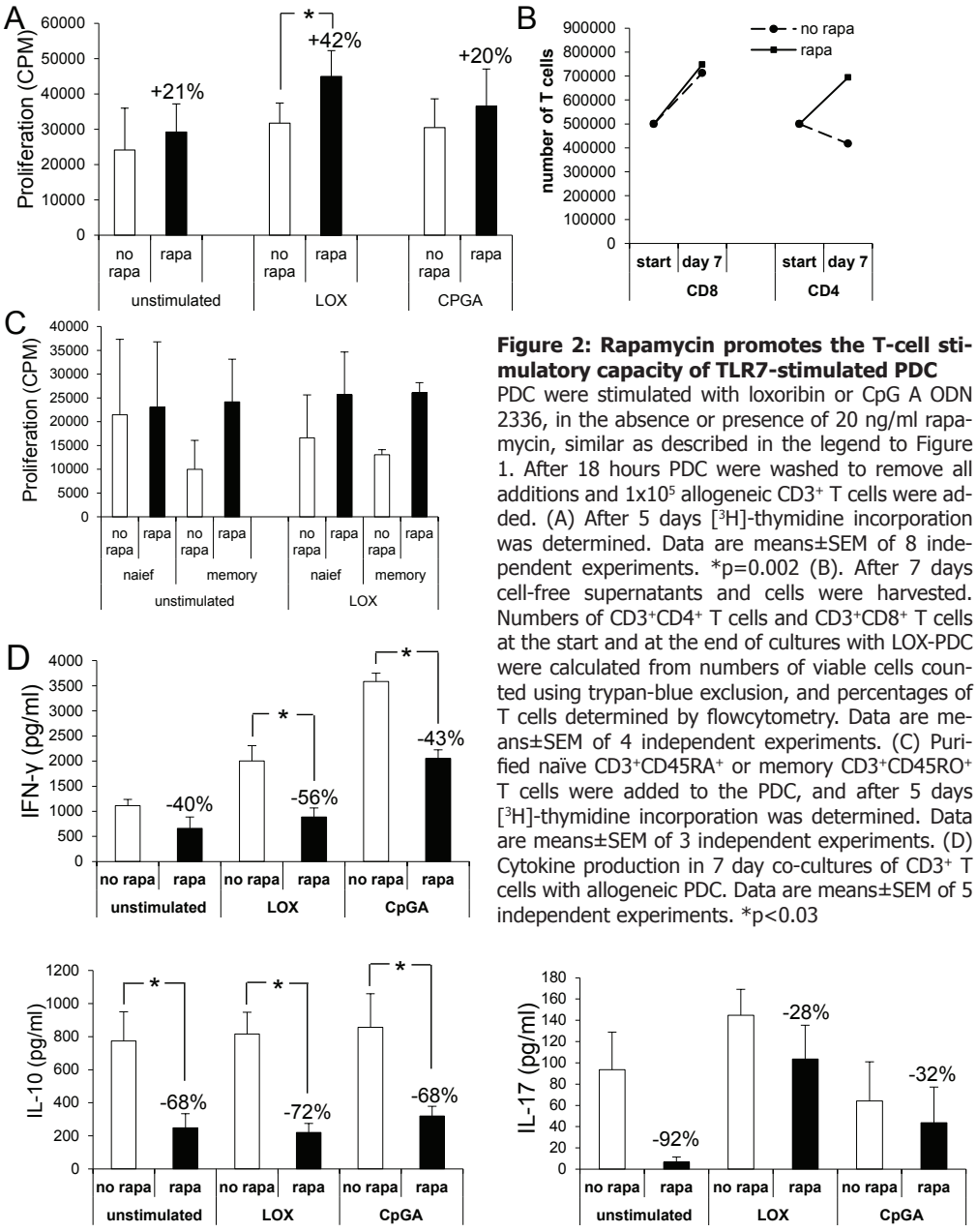

Figure 2: Rapamycin promotes the T-cell stimulatory capacity of TLR7-stimulated PDC
PDC were stimulated with loxoribin or CpG A ODN 2336, in the absence or presence of 20 ng/ml rapamycin, similar as described in the legend to Figure 1. After 18 hours PDC were washed to remove all additions and $1x10^5$ allogeneic $CD3^+$ T cells were added. (A) After 5 days [^{3}H]-thymidine incorporation was determined. Data are means±SEM of 8 independent experiments. *p=0.002 (B). After 7 days cell-free supernatants and cells were harvested. Numbers of $CD3^+CD4^+$ T cells and $CD3^+CD8^+$ T cells at the start and at the end of cultures with LOX-PDC were calculated from numbers of viable cells counted using trypan-blue exclusion, and percentages of T cells determined by flowcytometry. Data are means±SEM of 4 independent experiments. (C) Purified naïve $CD3^+CD45RA^+$ or memory $CD3^+CD45RO^+$ T cells were added to the PDC, and after 5 days [^{3}H]-thymidine incorporation was determined. Data are means±SEM of 3 independent experiments. (D) Cytokine production in 7 day co-cultures of $CD3^+$ T cells with allogeneic PDC. Data are means±SEM of 5 independent experiments. *p<0.03

pamycin-treated TLR-7 activated PDC, we determined the effects of rapamycin on expression of MHC and co-stimulatory molecules on PDC. Under none of the stimulation conditions rapamycin affected expression of MHC class I and II molecules on PDC (data not shown). CD40-expression on PDC was suppressed by rapamcyin in both stimulation conditions, while CD86 expression was not affected. Interestingly, rapamycin enhanced up regulation of CD80 on TLR7-ligated PDC, but not on TLR9-activated PDC (Figure 3A). In the absence of rapamycin a subpopulation of TLR7-stimulated PDC did not express CD80, while in the presence of rapamycin all PDC upregulated CD80-expression. To determine whether the increased CD80 expression might be responsible for the incre-

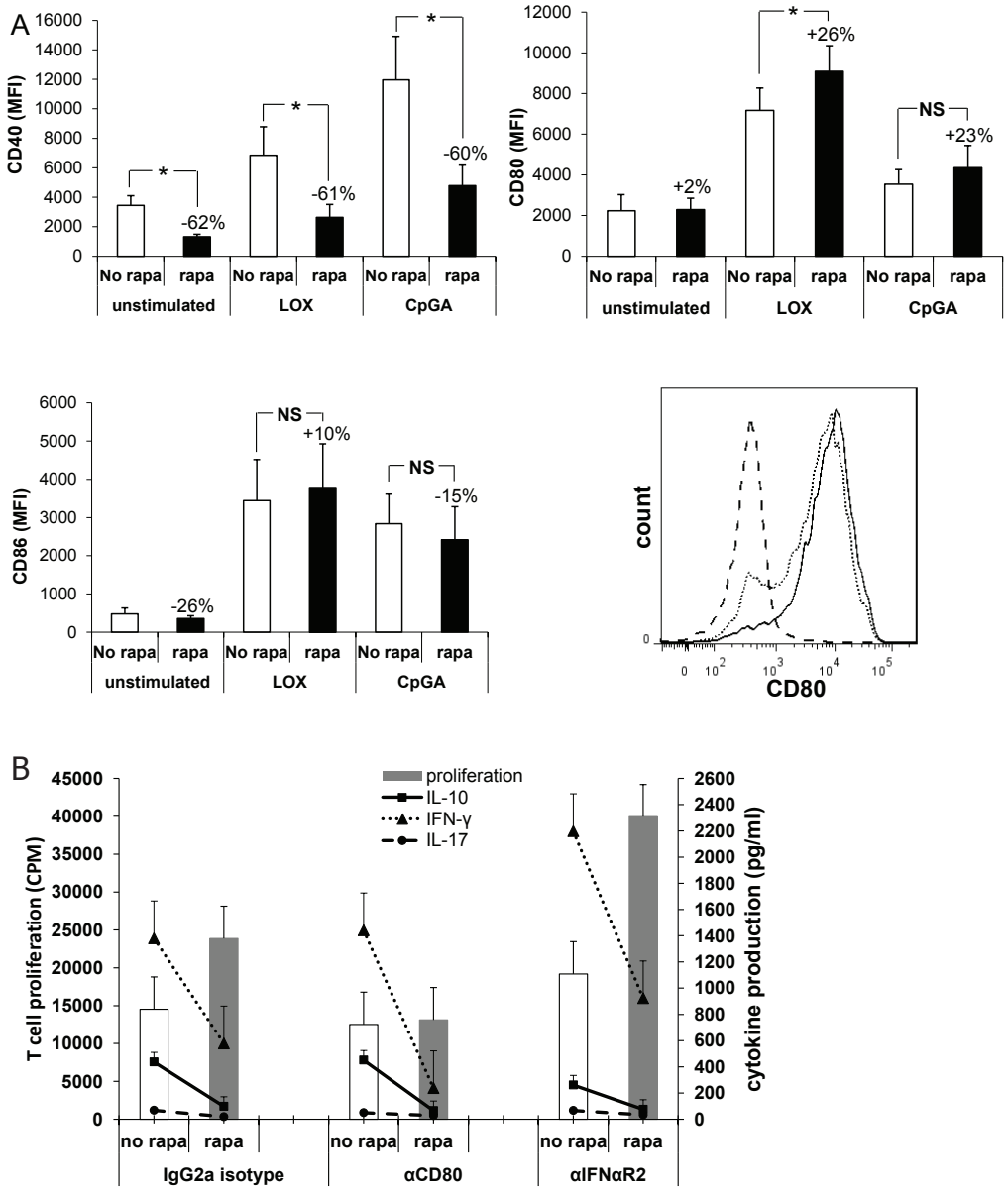

Figure 3: The enhanced T-cell stimulatory capacity of rapamycin-treated TLR7-activated PDC is caused by enhanced CD80-expression. (A) Effect of rapamycin on the expression of co-stimulatory molecules on TLR-activated PDC. Two x 10^4 human PDC in 200 µl were stimulated with loxoribin or CpG A ODN 2336, in the absence or presence of 20 ng/ml rapamycin, similar as described in the legend to Figure 1. After 18 hours PDC were harvested and expression of CD40, CD80, and CD86 was measured by flowcytometry. Bar plots show means geometric means of fluorescence (MFI) ± SEM of 8 independent experiments. *$p<0.05$. Histogram shows CD80 expression on PDC stimulated with loxoribin (dotted line), with loxoribin and rapamycin (solid line) and isotype control mAb (dashed line). (B) PDC were stimulated with loxoribin in the absence or presence of 20 ng/ml rapamycin, similar as described in the legend of Figure 1. After 18 hours PDC were washed to remove all additions and 1x10^5 allogeneic $CD3^+$ T cells were added together with neutralizing mAb against CD80 or IFNαR2, or an isotype-matched irrelevant mAb. After 5 days part of the supernatants was collected for IL-10, IFNγ and IL-17 measurement and [^{3}H]-thymidine incorporation was determined. Data are means±SEM of 4 independent experiments.

ased ability of rapamycin-treated TLR7-activated PDC to stimulate T-cell proliferation, a neutralizing antibody against CD80 was added to co-cultures of TLR7-stimulated PDC and allogeneic T-cells. Since rapamycin inhibits IFNα production by TLR7-activated PDC and IFNα has an inhibitory effect on T-cell proliferation (26-27), we also determined the effect of a neutralizing IFNα-Receptor 2 antibody on the T-cell stimulatory capacity of TLR7-activated PDC. Addition of the anti-IFNαR2 antibody did not abolish the difference in T-cell stimulatory ability between PDC that were treated or not treated with rapamycin (Figure 3B). However, blocking of CD80 on TLR7-activated PDC reduced their capacity to stimulate T-cell proliferation by ±15% and completely abrogated the increase in T-cell stimulatory ability of rapamycin-treated TLR7-activated PDC, indicating that this is caused by the enhanced CD80 expression. Blockade of IFNαR2 or CD80 did not abrogate the difference in ability between rapamycin-treated and non rapamycin-treated PDC to stimulate cytokine secretion by T cells, indicating that this was not due to reduced IFNα production or increased CD80 expression by rapamycin-treated PDC.

Inhibition of PTEN during PDC-activation did not enhance their capacity to stimulate allogeneic T cell proliferation or cytokine production (Figure 4A and B), suggesting that the mTOR-signaling pathway is also not a limiting step in induction of T-cell stimulatory ability in human PDC.

Together, these data show that rapamycin on the one hand promotes the ability of TLR7-activated PDC, but not of TLR9-activated PDC, to stimulate $CD4^+$ T cell prolifera-

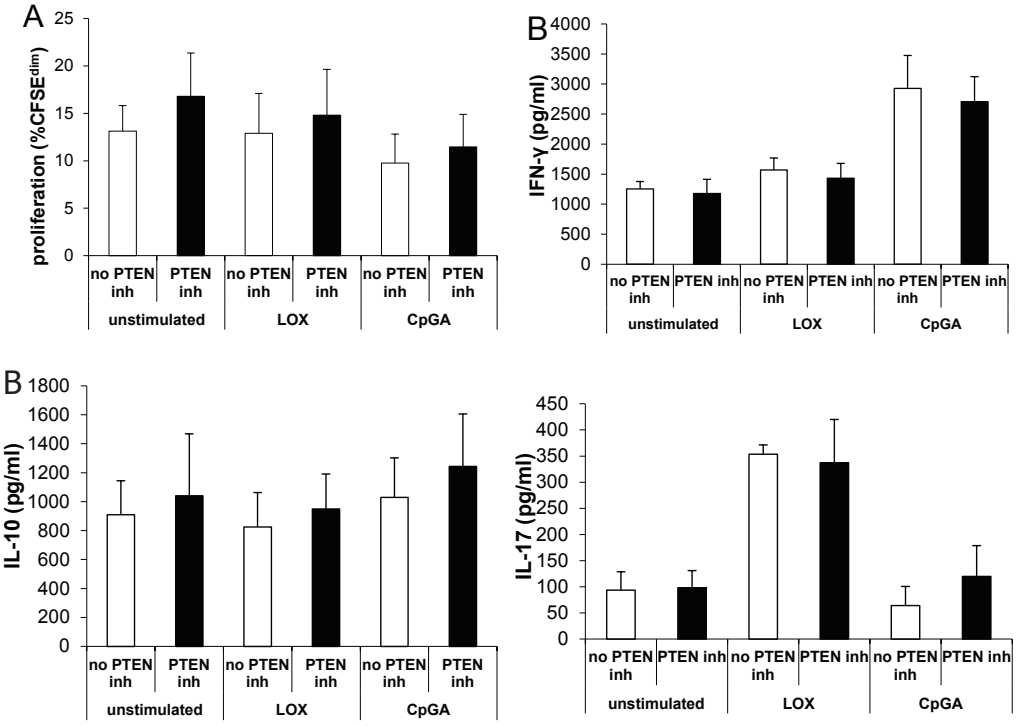

Figure 4: PTEN-inhibition during PDC-activation does not affect their capacity to stimulate allogeneic T cell proliferation or cytokine production. PDC were stimulated with loxoribin or CpG A ODN 2336 in the absence or presence of 10 nM VO-OHpic trihydrate. After 18 hours PDC were washed to remove all additions and $1x10^5$ CFSE-labeled allogeneic $CD3^+$ T cells were added. After 7 days cell-free supernatants and cells were collected. Data are means±SEM of 3 independent experiments. (A) Percentages of proliferating $CD3^+$ $CFSE^{dim}$ T cells were determined by flowcytometry. (B) Cytokine production in 7 day co-cultures of $CD3^+$ T cells with allogeneic PDC.

tion by increasing their expression of CD80, but on the other hand inhibits the capacity of PDC to stimulate cytokine production by T cells.

Rapamycin does not hamper the capacity of TLR7-activated PDC to generate functional CD8$^+$ Treg

Previously, we have shown that human PDC induce the generation of alloantigen-specific CD8$^+$CD38$^+$LAG-3$^+$CTLA-4$^+$ Treg from allogeneic CD3$^+$ T cells, and that activation of PDC by TLR-ligation enhances their ability to generate CD8$^+$ Treg (8). Here, we determined whether rapamycin affects the ability of TLR7-activated PDC to generate CD8$^+$ Treg. Seven-day co-cultures of CD3$^+$ T cells with loxoribin-stimulated PDC resulted in 32±7% of CD8$^+$ T cells showing the regulatory CD38$^+$LAG3$^+$ phenotype, while co-cultures with rapamyin-treated loxoribin-stimulated PDC generated 25±3% CD38$^+$LAG3$^+$ Treg within total CD8 T-cells (Figure 5A). In absolute numbers, the addition of rapamy-

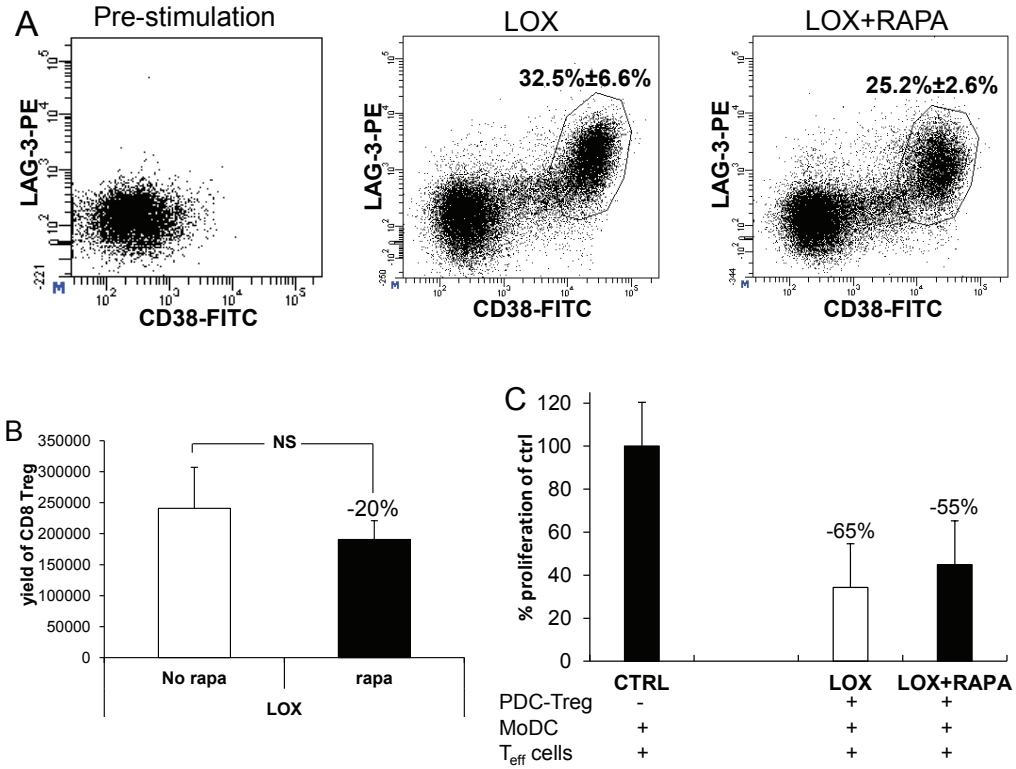

Figure 5: Rapamycin does not hamper generation of functional CD8$^+$ Treg by TLR7- stimulated PDC. Two x 10^4 human PDC in 200 µl were stimulated with loxoribin in the absence or presence of 20 ng/ml rapamycin, similar as described in the legend to Figure 1. After 18 hours PDC were washed to remove all additions and 1x10^5 allogeneic CD3$^+$ T cells were added. After 7 days of co-culture cells from several wells were pooled, and viable cells were counted. Percentages of CD8$^+$CD38$^+$LAG-3$^+$Treg were measured by flowcytometry. (A) Representative flowcytometry dot plots showing the absence of CD8$^+$CD38$^+$LAG-3$^+$Treg before co-culture with PDC and their presence after co-culture with TLR7-activated PDC treated or not treated with rapamycin. Figures in the dot plots show the average percentages (± SEM) of CD38$^+$LAG-3$^+$ cells within CD3$^+$CD8$^+$ cells at the end of the cultures in 4 independent experiments. (B) The calculated yields (± SEM) of CD8$^+$CD38$^+$LAG-3$^+$ Treg yielded in 4 independent experiments normalized to an input of 1x10^6 CD3$^+$ T cells. (C) To determine the suppressive capacity of CD8$^+$ Treg generated in the cultures, CD8$^+$CD38$^+$LAG-3$^+$ Treg were purified from the cultures by flowcytometric sorting and added in a 1:2 ratio to autologous CD3$^+$ T cells stimulated by moDC derived from the same donor as the PDC. [^{3}H]thymidine incorporation was measured after 5 days. Data depicted are means±SEM of 3 independent experiments.

cin to PDC during their activation with loxoribin did not significantly affect the yield of $CD8^+CD38^+LAG3^+$ Treg at the end of the cultures (Figure 5B). In addition, the suppressive function of the $CD8^+$ Treg was not affected by rapamycin (figure 5C). Thus, rapamycin treatment of TLR7 stimulated PDC does not hamper their capacity to generate functional $CD8^+$ Treg.

DISCUSSION

Rapamycin is an immunosuppressive and anti-proliferative drug, used as maintenance medication to prevent transplant rejection, and currently being evaluated for treatment of cancer and auto-immune diseases. We found that a clinically relevant concentration of rapamycin inhibits innate as well as adaptive immune functions of TLR-activated human PDC, but with two exceptions: 1. It enhances the ability of TLR7-stimulated PDC to stimulate $CD4^+$ T cell proliferation by enhancing CD80-expression; 2. It leaves their capacity to generate functional $CD8^+$ Treg unaffected.

Rapamycin inhibited IFN-α secretion by PDC effectively in case of TLR7-stimulation, but only a minor inhibitory effect was observed upon TLR9-stimulation despite effective suppression of mTOR-signaling in TLR9-stimulated PDC. This observation is of critical importance for emerging studies on rapamycin treatment of auto-immune diseases caused by chronic stimulation of IFN-α production by PDC, like SLE and psoriasis (18, 28). In these diseases, PDC are continuously stimulated by immune complexes comprising self DNA and RNA. While RNA complexes are sensed by TLR7, DNA-complexes are sensed by TLR9 in the early endosomes, like CpG-A (29). Our results predict that rapamycin treatment can ameliorate overproduction of IFN which is induced by self-RNA complexes, but not self-DNA-driven IFN production. Similarly, our findings suggest that rapamycin treatment may abrogate the early IFN-α response to RNA viruses which are sensed by TLR7, such as influenza virus, RSV and HCV, thereby enhancing susceptibility to these viruses, but not to DNA-viruses sensed by TLR9.

Cao et al (16) also reported that rapamycin in the same concentration as we used in the present study, inhibits CpG-A ODN 2336-induced IFN-α production by human PDC less efficiently compared to loxoribin-induced IFN-α production. Still, these authors reported a two-fold inhibition, while we observed only 20% inhibition of CpG-A ODN 2336-induced IFN-α secretion. One explanation for this difference may be related to the use of different IFN-α ELISA-kits with different sensitivities for IFN-α subtypes. The ELISA that we used detects the main subtypes IFN-α2a, IFN-α2b and IFN-α2c.

In addition to its well-known immunosuppressive effects, recent studies revealed immunostimulatory effects of rapamycin, such as stimulation of pro-inflammatory cytokine production in myeloid cells (30) and promotion of $CD8^+$ memory T cell differentiation (31-32). The data presented here add to the emerging contrasting effects of rapamycin on the immune system. Immunogenic functions of PDC that are inhibited by rapamycin include: pro-inflammatory cytokine production, IFN-α secretion induced by TRL7 ligation, and the capacity to stimulate pro-inflammatory cytokine production in allogeneic T cells. On the other hand rapamycin enhances the capacity of TLR7-activated PDC to stimulate $CD4^+$ T cell expansion, and inhibits the ability of TLR-engaged PDC to stimulate IL-10 secretion by T cells.

While rapamycin increased the ability of TLR7-activated PDC to stimulate $CD4^+$ T-cell proliferation, it did not modulate their capacity to stimulate $CD8^+$ T-cell proliferation and generate functional $CD8^+$ Treg. Rapamycin enhanced the T-cell stimulatory capacity of TLR7-activated PDC by stimulating the upregulation of the co-stimulatory molecule

CD80. Apparently, CD80 is less important in stimulating expansion of $CD8^+$ T cells and generation of $CD8^+$ Treg. Rapamycin enhanced also CD252 (OX40-ligand; ligand for the secondary co-stimulatory molecule CD134) and CCR7 expression on TLR7-activated PDC (data not shown). We do not know why mTOR-inhibition has opposite effects on CD40 and CD80/CD252/CCR7 expression. PDC-maturation, resulting in upregulation of co-stimulatory molecules, is thought to be mediated by NFκB-signaling (33) which is inhibited in PDC by rapamycin (16). PDC utilize an autocrine IFNα feedback loop that further enhances INF-α production (34) after stimulation with CpG or loxoribin. We tested if mTOR inhibition is involved in this autocrine IFN-α feedback loop to explain the reduced IFN-α production of the PDC after rapamycin treatment. This was done by blocking the IFNα-receptor2 with neutralizing antibodies during TLR9 or TLR7-activation. Blocking the IFNα-receptor reduced IFN-α production by PDC, but did not influence the effects of rapamycin on INF-α production, nor on IL-6 production. In addition, blocking of the IFNα-receptor had no effect on CD40, CD80 and CCR7 expression on PDC (data not shown). These data indicate that rapamycin does not affect the autocrine IFN-α feedback loop in PDC, and that this loop is not involved in the differential regulation of CD40 and CD80/CD252/CCR7 expression.

While rapamycin enhanced the capacity of loxoribin-activated PDC to stimulate $CD4^+$ T cell proliferation, we found no effect of rapamycin on the T-cell stimulatory capacity of CpG-A-stimulated PDC. Accordingly, rapamycin did not upregulate CD80-expression on TRL9-activated PDC. In contrast, Cao et al (16) reported that rapamycin suppresses the capacity of CpG-A-stimulated mouse PDC to stimulate antigen-specific proliferation by $CD4^+$ T cells. Apart from the species difference, it should be realized that Cao et al used a more artificial system by adding T cells which expressed a transgenic T cell receptor specific for an ovalbumin peptide to the PDC, while we used primary T cells.

We do currently not know how rapamycin inhibits the capacity of TLR-activated PDC to stimulate cytokine production by T cells. Neither blocking of CD80 nor blocking of IFNαR2 abrogated the difference in cytokine production of T-cells that were stimulated by PDC activated loxoribin in the presence or absence of rapamycin.

Previously, we have reported that corticosteroids induce apoptosis of resting human PDC and suppresses the functions of activated PDC (35). In the present study we show that rapamycin inhibits innate and most adaptive immune functions of human PDC, but can enhance their capacity to stimulate $CD4^+$ T cell proliferation and inhibit their capacity to stimulate IL-10 production by T cells. Therefore, together with other recent studies (31-32), these observations may help to understand why rapamycin monotherapy is not very effective in preventing graft rejection, and is sometimes even accompanied by inflammatory side effects, including pneumonitis and glomerulonephritis (36).

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chapter 9

Summary and discussion
Nederlandse samenvatting
Dankwoord
Curriculum Vitae
Portfolio
List of publications

SUMMARY AND DISCUSSION

Extensive evidence is available that proves that the liver is an immunotolerant organ, from the hypo-responsiveness to food-antigens and the low incidence of chronic rejection after liver transplantation to the chronicity of hepatitis B en C viral infections. The underlying mechanism(s) explaining the tolerogenic properties of the liver are not fully understood. Dendritic cells (DC) are thought to play an important role in maintaining the fine balance between immune responsiveness and unresponsiveness in the liver. Two main subtypes of DC can be distinguished: Myeloid DC (MDC) and plasmacytoid DC (PDC). 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 T-cell stimulatory capacity. Therefore in general, T-cell responses are not initiated in parenchymal organs, but in secondary lymphoid organs. While knowledge on murine DC in both lymphoid- and non-lymphoid tissues has dramatically increased during the last few decades, knowledge of human DC is largely derived from studies on MDC generated from monocytes *in vitro*, or from DC isolated from blood. Studies on DC in human tissues are much more limited, and only few reports are available on MDC in human LN.

Therefore in **chapter two** of this thesis we started to test an immunomagnetic technique to isolate MDC and PDC from human lymph node cell suspension. We found that is was feasible to isolate pure $CD1c^+$ MDC from inguinal LN using immunomagnetic selection with anti-BDCA1 antibody. Although their viability was moderate, these MDC appeared to be functional. They produced high amounts of cytokines after stimulation and were able to stimulate allogeneic T cell proliferation. Although fully functional PDC were isolated from LN by immunomagnetic selection with anti-BDCA-4 antibody, the resulting PDC-preparations were contaminated with about 40% T-cells and NK-cells.

In **chapter 3**, this method was also successfully applied to isolate $CD1c^+$ MDC from liver graft perfusates. It was shown that $CD1c^+$ MDC were present in the portal fields of human donor livers and that these resident donor liver MDC had an immature phenotype as demonstrated by low expression of CD80 and CD83. During vascular perfusion of the donor liver before transplantation, on average one million $CD1c^+$ MDC detached from the graft. These liver-derived MDC had an immature phenotype, but intermediate between blood MDC and hepatic lymph node MDC. In contrast to blood MDC, freshly isolated perfusate MDC were able to stimulate allogeneic T cell proliferation. Moreover, MDC from liver graft perfusate were responsive to stimulation with lipopolysaccharide (LPS) and produced significantly more of the anti-inflammatory cytokine IL-10 compared to blood MDC. However, in a subsequent study, which is not included in this thesis, we showed that MDC that detach from human liver grafts not only produce more IL-10, but also more pro-inflammatory cytokines compared to blood MDC. Moreover, we observed that $CD1c^+$ MDC from human liver upon activation with LPS acquire capacity to stimulate T-cell proliferation and pro-inflammatory, but not anti-inflammatory, cytokine production (1).Therefore, we conclude that $CD1c^+$ MDC in human liver are capable of producing both anti-inflammatory and pro-inflammatory cytokines and to stimulate T-helper 1 responses, and do therefore not show tolerogenic properties.

In **chapter 4** numbers, immunophenotype and functional capacity of DC subsets in human hepatic LN were compared with those in skin/muscle draining inguinal LN, spleen

and liver perfusate. Studying MDC in draining LN provides the opportunity to clarify whether MDC of the liver undergo a differential maturation process during *in vivo* migration via lymph compared to MDC which migrate into skin/muscle-draining LN on the one hand, or MDC that migrate from blood into spleen (2). The comparison between these types of LN were made because pathogens or toxic agents that enter the skin or blood normally result in strong immune responses, whereas immune responses to foreign compounds entering the liver are tightly controlled and in general more tolerogenic. We found that hepatic LN contain lower numbers of $CD1c^+$ MDC (2x) and PDC (7x) compared to inguinal lymph nodes. The numbers of $BDCA3^{bright}$ MDC were low in both hepatic and inguinal lymph nodes, ~6 times lower compared to spleen. Moreover, the numbers of $CD14^+$ monocytes/macrophages in hepatic lymph nodes were also lower compared to inguinal lymph nodes and spleen. Both $CD1c^+$ MDC and the $BDCA3^{bright}$ MDC displayed a more mature phenotype in the hepatic lymph nodes compared to inguinal lymph nodes and spleen, suggesting that they have stronger T-cell stimulatory capacity. Nevertheless, $BDCA1^+BDCA3^+$ MDC isolated from hepatic LN showed a weaker T-cell stimulatory capacity compared to those isolated from inguinal LN. In addition, upon stimulation via TLR3 or CD40, $CD1c^+$ MDC from hepatic LN hardly produced any cytokines, while inguinal LN MDC produced IL10, IL12, IL6, and TNFα. Whether the lack of cytokine production explains the reduced T-cell stimulatory capacity of $CD1c^+$ MDC from liver LN remains to be established. Further functional experiments are required to obtain insight whether secreted factors or stimulatory or inhibitory surface molecules are the prevailing factors in the difference in T-cell stimulatory capacity between liver and skin/muscle-draining LN.
Based on this study we suggest that the low numbers of antigen presenting cells in hepatic LN and the weak functional capacity of $CD1c^+$ MDC in hepatic lymph nodes both may contribute to the low immunogenicity of the liver environment.

Organ transplant recipients need life-long treated with immunosuppressive drugs to prevent rejection of their graft. These immunosuppressive drugs contributed tremendously to the success of transplantation medicine, but also have serious adverse effects. Since not only immunity against the graft is suppressed, but immunity in general, patients become susceptible for infections and cancer. Induction of specific transplant tolerance is therefore a major goal in transplantation research. In **chapter 5** we show co-culture of human T cells with allogeneic plasmacytoid dendritic cells (PDC), that have been pre-activated with TLR-7 or TLR-9 ligands, results in the generation of $CD8^+LAG3^+$ $Foxp3^+CTLA4^+$ Treg. These Treg are anergic and inhibit the proliferation of effector T cells in a donor-specific fashion. At least 50% of allo-reactive T-cells in humans are memory T-cells. They have been generated during microbial infections, but cross-react with allogeneic MHC-molecules (3-4). These memory allo-reactive T-cells are relatively resistant to suppression by $CD4^+CD25^+Foxp3^+$ Tregs (5-7). We showed that the $CD8^+$ Treg that are induced by PDC can potently inhibit memory T cells, rendering these $CD8^+$ Treg potentially a better candidate for cellular immunotherapy to prevent graft rejection than $CD4^+Foxp3^+$ Treg. The induction of the $CD8^+$ Treg by PDC is partly mediated by indoleamine-2,3 dioxygenase (IDO) activity, which is in agreement with findings of others (8-9). The inhibition of effector T cell proliferation by the $CD8^+$ Treg induced by PDC depends on expression of CTLA-4, since a neutralizing antibody against CTLA-4 could reduce their suppressive function. CTLA-4 is an inhibitory costimulatory molecule that competes with CD28 for binding to CD80 and CD86 on antigen presenting cells with an at least 20 times higher avidity than CD28 (10). It is reported that CLTA-4 is also

involved in the immune-supressive function of $CD25^+CD4^+$ Tregs (11-12), although the precise mechanism has not fully been elucidated. By reverse signalling via CD80 and CD86 CTLA-4 can induce IDO expression in antigen presenting cells (13). Degradation productions of IDO, such as kynurenine, can exert anti-proliferative signals to T cells (14). An alternative explanation could be that the $CD8^+$ Treg produce a soluble form of CTLA-4 (15-16) that blocks CD80 and CD86 expressed on the antigen presenting cells and thereby reduces the availability of costimulation necessary to mount efficient effector T cell activation. Additional experiments are required to clarify the exact mechanism of suppression of the PDC induced $CD8^+$ Treg.

Beside $CD8^+$ Treg, PDC also induced IL-10 producing $CD4^+$ T cells in our experiments. However in suppression assays these cells exerted only limited suppressive function, despite IL-10 production. A possible explanation for the lack of suppressive function in our assay system is that we stimulated monocyte-derived DC (MoDC) by LPS prior to their use as stimulatory cells in the suppression assays. IL-10 inhibits the upregulation of costimulatory molecules and proinflammatory cytokine production during stimulation of MoDC, but since the MoDC used in our suppression assays were stimulated with LPS prior to co-culture with T cells, IL-10 produced by $CD4^+$ T cells could probably not affect the maturation of the MoDC anymore. Thus the suppressive function of PDC-stimulated $CD4^+$ T cells we show in **chapter 5** is probably an underestimation of their real suppressive capability.

Our *in vitro* data on the generation of $CD8^+$ Treg by PDC are supported by a transplantation model, wherein treatment of rats with CD40Ig induced $CD8^+CD45C^{low}$ Treg, but not $CD4^+$ Treg *in vivo*. These $CD8^+CD45C^{low}$ Treg mediated tolerance to allogeneic cardiac transplants. The dynamics of co-localization of $CD8^+CD45C^{low}$ Treg and PDC in these animals, first in the allograft and subsequently in the spleen, suggested that PDC play an important role in the induction of $CD8^+$ Treg (17).

Flowcytometric stainings with CD3, CD8, CD38, Lag-3 and CTLA4 were performed to establish whether $CD8^+$ T cells with a similar immunophenotype were present in human tissues. We analyzed liver graft perfusate, inguinal LN, hepatic LN and spleen single cell suspensions for $CD8^+CD38^+LAG\text{-}3^+CTLA4^+$ T cells. Only in spleen tissue we could detect cells with this immunophenotype, which amounted to 5-6% of splenic $CD8^+$ cells (figure 1). Spleen $CD8^+CD38^+LAG\text{-}3^+CTLA4^+$ T cells still have to be purified and tested for functionality to establish if they possess T-cell suppressive capacity.

With regard to potential immunotherapeutic application of PDC to prevent organ graft rejection, we could think of two approaches. In the first approach PDC are purified from donor blood or spleen using clinical grade immuno-magnetic equipment, and are subsequently stimulated *ex vivo* and thereafter administered to transplant patients. In this approach it is hypothesized that donor PDC induce donor-specific $CD8^+$ Treg *in vivo*. Unfortunately, besides their tolerogenic properties, PDC also exert immunogenic functions. In **chapters 5** and **8** we show that activated PDC produce pro-inflammatory cytokines, like IFN-α, IL-6 and TNF-α, and stimulate T-cells to produce both anti-inflammatory and pro-inflammatory cytokines. Although the outcome of PDC-activation depends on the mode of PDC-activation, none of the investigated stimuli selectively activates pro-tolerogenic functions of PDC. Indeed, it is not well established under which conditions the tolerogenic properties predominate the immuno-activating properties (18). In addition,

PDC can interact with MDC, and this interplay synergizes in the induction of immune responses by MDC (19). Conversely, MDC promote PDC survival and enhance their T cell stimulatory capacity (20). These interactions depend on both soluble factors and cell-cell contact (21). Our conclusion is that, although infusion of autologous TLR-activated PDC into humans has proven to be safe (22), infusion of allogeneic PDC might evoke inflammatory responses on short term. Therefore in a second approach, donor-derived PDC could be used to induce $CD8^+$ Treg *ex vivo*, and the resulting $CD8^+$ Treg could be administered to the transplant recipient. Eventually, $CD8^+$ Treg should be further expanded in order to enable infusion of sufficient numbers. However, before a clinical trial could start, it is required to study whether infusion of PDC-induced $CD8^+$ Treg can prevent graft rejection in a clinically relevant experimental animal model. Moreover, the survival of $CD8^+$ Treg *in vivo*, and their effect on T-cell responses against pathogens should be determined in animal experiments.

In **chapter 6** the kinetics of the numbers of circulating PDC were determined in patients during the first year after liver transplantation. The relative numbers of PDC declined 4 fold immediately after transplantation. Between 3 and 12 months post-transplantation the numbers started to rise again but did not reach pre-LTX levels at 12 months post-LTX. The longitudinal kinetics of circulating PDC was associated with prednisone treatment. We showed that corticosteroids induce apoptosis of PDC. TLR-activation of the PDC protected them partially to apoptosis induction by corticosteroids, but affected their function. Cytokine production and up-regulation of costimulatory molecules were diminished, as well as their ability to stimulated T cells.

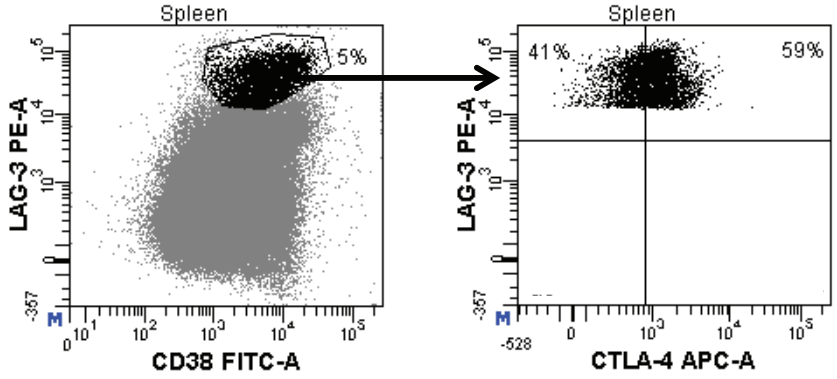

figure 1. In spleen are T-cells present that express CD8, CD38, Lag-3 and CTLA4. Spleen cell suspensions were stained with antibodies against CD3, CD8, CD38, Lag-3 and intracellular CTLA4. The populations in the dot plots, are gated on CD3 and CD8. One representative spleen sample out of three is shown.

In **chapter 7** the effects of corticosteroids on the anti-viral activity of PDC were shown using an HCV-replication model with Huh7 hepatoma cells transfected with a non-structural coding sequence of HCV coupled to a luciferase reporter gene. Corticosteroids did not directly inhibit HCV-replication, nor interfered with inhibition of HCV-replication by IFN-α. However, corticosteroids abrogated the anti-viral function of TLR-7 stimulated PDC. Moreover, co-culture of transfected Huh7 cells with PDC significantly reduced HCV replication, and this reduction was almost completely reversed by addition of steroids. We conclude that corticosteroids reduce the numbers of circulating PDC in liver transplant recipients and inhibit the anti-viral activity of human PDC. These mechanisms may

be responsible for the observed contribution of corticosteroid therapy to the recurrence of HCV-infection after liver transplantation. Therefore, corticosteroid-free immunosuppressive therapy might reduce the number and severity of viral infections after organ transplantation. A large systematic review combining 30 randomized controlled studies (5949 participants) showed that it is safe to avoid or withdraw corticosteroids in kidney transplant recipients. Recipients showed an increase incidence of acute rejection, but this is not associated with increased mortality or graft loss, and they have a lower infection rate (23).

Our results showing that corticosteroids inhibit apoptosis and functionality of PDC and demonstrating that TLR-activation protects against corticosteroid-induced apoptosis, were confirmed by others (24-25). Lepelletier et al showed that microbial stimulation made PDC less sensitive to corticosteroid-induced apoptosis and that this protection was caused by TLR-induced autocrine TNF-α and IFN-α production that up-regulated anti-apoptotic gene expression (24). Moreover patients with auto-immune diseases, like systemic lupus erythematosus (SLE) are often treated with corticosteroids. In SLE, recognition of self nucleic acids by TLR7 and TLR9 on PDC leads to IFN-α production and the formation of anti-nuclear antibodies. However, corticosteroids are not always successful in maintaining control over the SLE activity. Guiducci et al showed that immune complexes containing self nucleic acids activate PDC via TLR7 and TLR9, thereby increasing PDC survival and IFN-α production (25) and counteracting apoptosis of PDC induced by corticosteroids.

Activation via TLR rescues PDC from corticosteroid-induced apoptosis, but TLR-activated PDC are still hampered in their functions. The mechanism of action by which corticosteroids inhibit functions of TRL-activated PDC is still unknown. In macrophages IFN-α production depends on activation of IFN regulatory factor 3 (IRF3) downstream of TLR-3. IRF3 interacts with the cofactor Glucocorticoid Receptor Interacting Protein-1 (GRIP1). During corticosteroid treatment there is competition for GRIP1-binding between the glucocorticosteroid receptor (GR) and IRF3, leading to a block in IRF3-mediated transcription and IFN-α production (26). IFN-α production in PDC depends on IRF7 and not IRF3, however, GRIP1 binds strongly to IRF7 as well (26), suggesting that the same mechanism plays a role in suppression of IFN-α by PDC, provided that the GRIP1 concentration is equally low in PDC as in macrophages. Moreover GRIP1 is a cofactor in the STAT1-STAT2-IRF9 (ISGF3) transcription complex that forms when IFN-α binds to its receptor. This starts the IFN-α autocrine feedback loop that further enhances IFN-α production. Competition of the GR and IRF9 in the ISGF3 for GRIP1 leads to a reduced IFN-α positive autocrine feedback loop (27). This mechanism is only valid in cell types that express the GRIP1 protein at extremely low levels. Whether corticosteroids suppress IFN-α production by TLR-activated PDC via this molecular mechanism remains to be established.

In addition to the effects of corticosteroids, the effects of the immunosuppressive drug rapamycin on PDC were studied in **chapter 8**. A clinically relevant concentration of rapamycin inhibited TLR7-induced IFN-α secretion potently (-64%) but TLR9-induced IFN-α secretion only slightly (-20%), while the same concentration suppressed pro-inflammatory cytokine production by TLR7-activated and TLR9-activated PDC with similar efficacy. Rapamycin inhibited the ability of both TLR7-activated and TLR9-activated PDC to stimulate production of pro-inflammatory (IFN-γ and IL-17) and anti-inflammato-

ry (IL-10) cytokines by allogeneic T-cells. mTOR-inhibition enhanced the capacity of TLR7-activated PDC to stimulate T-helper cell proliferation, which was caused by rapamycin-induced upregulation of CD80 expression on PDC, since CD80 neutralizing antibodies could abolish the increased T-cell proliferation. Rapamycin did not hamper the generation of suppressive $CD8^+CD38^+LAG\text{-}3^+$ Treg by TLR-stimulated PDC.

The data presented in this chapter add to the emerging contrasting effects of rapamycin on the immune system. In addition to its well-known immunosuppressive effects, recent studies revealed immunostimulatory effects of rapamycin, such as stimulation of pro-inflammatory cytokine production in myeloid cells (28) and promoting $CD8^+$ memory T cell differentiation (29-30). Moreover, in stable kidney transplant patients treated with rapamycin monotherapy it was shown that they had increased frequencies of $CD4^+CD25^+Foxp3^+$ T cells, however this was accompanied by an increased number of effector memory cells, innate immune cells and by the enrichment of NF-κB related pro-inflammatory expression pathways. It does not seem to confer a more 'tolerogeneic' environment than that provided by calcineurin inhibitors in these patients (31).

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NEDERLANDSE SAMENVATTING

Uitgebreid bewijs toont aan dat de lever een immuuntolerant orgaan is, zo is er een lage immuniteit tegen voedsel-antigenen, er is een lage incidentie van chronische afstoting na levertransplantatie en virale hepatitis B en C infecties worden slecht geklaard. De onderliggende mechanismes voor deze tolerogene eigenschappen van de lever worden niet volledig begrepen. Dendritische cellen (DC) spelen een belangrijke rol in het evenwicht tussen immuunreactiviteit en tolerantie in de lever. DC kunnen worden opgesplitst in twee subtypes: myeloïde DC (MDC) en plasmacytoïde DC (PDC). Onder stabiele omstandigheden is er een voortdurende migratie van MDC in de richting van de drainerende lymfeklieren (LK) en dit proces wordt versneld na antigeen stimulatie. Tijdens de migratie naar de lymfeklieren, matureren MDC waardoor ze eigenschappen verkrijgen waarmee ze T-cellen kunnen activeren. Dus in het algemeen ontstaan T-cel responsen niet in parenchymale cellen van organen, maar in secundaire lymfeklieren. Hoewel de kennis van muizen DC in zowel lymfoïde-en niet-lymfoïde weefsels ontzettend is toegenomen in de loop van de laatste decennia, is de kennis van humane DC grotendeels ontleend aan studies over MDC gegenereerd uit monocyten, of van DC geïsoleerd uit het bloed. Studies over DC in humane weefsels zijn veel schaarser, en slechts enkele publicaties zijn beschikbaar over MDC in humane LK.

Hierom wordt in **hoofdstuk 2** van dit proefschrift een techniek beschreven om MDC en PDC te isoleren uit humane lymfeklieren. We vonden dat het haalbaar was om zuivere $CD1c^+$ MDCé te isoleren uit lies LK met behulp van een immunomagnetische selectie methode gebruikmakend van anti-BDCA1 antilichaam. Hoewel deze MDC een matige vitaliteit hadden, bleken ze functioneel. Na stimulatie produceerde ze grote hoeveelheden cytokines en ze konden allogene T-cellen stimuleren. PDC werden geïsoleerd uit LK door immunomagnetische selectie met anti-BDCA-4 antilichaam. Deze cellen waren functioneel, alleen was de zuiverheid niet optimaal. De verkregen PDC-preparaten bevatten ongeveer 40% T-cellen en NK-cellen.

In **hoofdstuk 3**, werd deze methode succesvol toegepast om $CD1c^+$ MDC te isoleren uit leverperfusaten en er werden met behulp van immunohistochemie, $CD1c^+$ MDC aangetoond in portale velden van humane donor levers. Deze residente donorlever MDC hadden een immatuur fenotype wat bleek uit lage expressie van CD80 en CD83. Tijdens vasculaire perfusie van de donorlever vóór de transplantatie, kwamen gemiddeld één miljoen $CD1c^+$ MDC los van het transplantaat. Deze MDC, die afkomstig waren van de lever hadden een immatuur fenotype, dat meer matuur was dan bloed MDC, maar minder matuur dan lever lymfeklier MDC. In tegenstelling tot bloed MDC, konden vers geïsoleerde perfusaat MDC allogene T-celproliferatie stimuleren. Bovendien reageerde MDC uit lever perfusaat op stimulatie met lipopolysaccharide (LPS) en produceerde significant meer van het anti-inflammatoire (ontstekkingsremmende) cytokine IL-10 in vergelijking met bloed MDC. Echter, in een andere studie, die niet is opgenomen in dit proefschrift hebben we aangetoond dat humane lever MDC uit perfusaten niet alleen meer IL-10 produceerden, maar ook meer pro-inflammatoire (ontstekkingsbevorderende) cytokines in vergelijking met bloed MDC. Bovendien hadden we waargenomen dat $CD1c^+$ MDC uit humane lever na activering met LPS het vermogen verwierven om T-cel proliferatie te induceren. Deze T-cellen produceerde pro-inflammatoire, maar geen anti-inflammatoire, cytokines (1). Daarom concludeerden wij dat $CD1c^+$ MDC uit humane lever zowel anti-inflammatoire als pro-inflammatoire cytokines produceerden en dat ze

T-helper 1 responsen stimuleerden en dat ze derhalve niet beschikten over tolerogene eigenschappen.

In **hoofdstuk 4** werden aantallen, immunofenotype en de functionele capaciteit van DC subsets in humane lever LK bepaald en vergeleken met die in huid/spier drainerende lies LK, milt en lever perfusaat. Het bestuderen van MDC uit drainerende LK biedt de mogelijkheid om inzicht te krijgen of MDC uit de lever een ander maturatie proces ondergaan tijdens hun *in vivo* migratie via de lymfevaten naar de LK dan MDC die migreren naar huid/spier LK, of die migreren vanuit het bloed naar de milt (2). Deze typen lymfeklieren werden vergeleken, omdat ziekteverwekkers of giftige stoffen die via de huid of het bloed binnenkomen normaal gesproken leiden tot een sterke immuunrespons, terwijl vreemde stoffen die via de poortader de lever binnenkomen niet direct tot een immuunreactie, maar eerder tot tolerantie leiden. We vonden dat hepatische LK lagere aantallen $CD1c^+$ MDC (2 x lager) en PDC (7 x lager) bevatten vergeleken met lies lymfeklieren. De aantallen $BDCA3^{bright}$ MDC waren laag in zowel de lever als de lies lymfeklieren, ze waren ~6 keer lager dan in de milt. Bovendien waren de aantallen $CD14^+$ monocyten/macrofagen in de lever LK ook lager dan in lies LK en in de milt. $CD1c^+$ MDC en $BDCA3^{bright}$ MDC hebben een meer mature fenotype in lever LK dan in inguinale LK en milt, wat suggereert dat ze een sterkere T-cel stimulerende capaciteit bezitten. Echter uit experimenten bleek dat uit lever LK geïsoleerde $BDCA1^+BDCA3^+$ MDC een zwakkere T-cel stimulerende capaciteit bezitten in vergelijking met die geïsoleerd uit lies LK. Bovendien produceren $CD1c^+$ MDC geisoleerd uit lever LK na stimulatie met Toll like receptor (TLR)-3-ligand of CD40-ligand nauwelijks cytokines, terwijl lies LK MDC IL10, IL12, IL6, en TNFa produceren. Of het gebrek aan cytokineproductie een verklaring is voor de beperkte T-cel stimulerende capaciteit van $CD1c^+$ MDC uit lever LK moet nog worden vastgesteld. Additionele experimenten zijn nodig om inzicht te krijgen of uitgescheiden factoren of stimulerende of remmende membraanmoleculen de overheersende factoren zijn om het verschil in T-cel stimulerende capaciteit tussen lever en huid/spier-drainerende LK te verklaren. Dit onderzoek suggereert dat zowel het lage aantal antigeen presenterende cellen in lever LK als de zwakke functionele capaciteit van $CD1c^+$ MDC in lever LK kan bijdragen aan de lage immunogeniciteit van het lever milieu.

Om afstoting van een getransplanteerd orgaan te voorkomen, worden orgaantransplantatie ontvangers een leven lang behandeld met medicijnen die het immuunsysteem onderdrukken. Deze immuunsuppressieve medicijnen hebben enorm bijgedragen aan het succes van de transplantatie geneeskunde, maar hebben ook ernstige bijwerkingen. Aangezien niet alleen de immuniteit tegen het transplantaat wordt onderdrukt, maar immuniteit in het algemeen, zijn deze patiënten extra gevoelig voor het krijgen van infecties en kanker. Inductie van tolerantie specifiek gericht tegen het donor orgaan is dan ook een belangrijk doel in transplantatie onderzoek. In **hoofdstuk 5** wordt beschreven dat humane allogene plasmacytoïde dendritische cellen (PDC), die vooraf geactiveerd zijn met TLR-7 en TLR-9 liganden, $CD8^+LAG3^+Foxp3^+CTLA4^+$ regulatoire T-cellen (Treg) genereren uit T-cellen afkomstig uit bloed. Deze Treg zijn anergisch en remmen de proliferatie van effector-T-cellen op een donor-specifieke manier. Tenminste 50% van alle allo-reactieve T-cellen (T-cellen die reageren op donor antigenen) in mensen zijn memory T-cellen. Ze zijn ontstaan tijdens microbiële infecties, maar kruisreageren met allogene MHC-moleculen (3-4). Deze memory allo-reactieve T-cellen zijn relatief resistent tegen onderdrukking door $CD4^+CD25^+Foxp3^+$ Tregs (5-7). Wij toonden aan dat deze door PDC geïnduceerde $CD8^+$ Treg memory T-cellen sterk kunnen remmen, waardoor deze $CD8^+$

Treg potentieel een betere kandidaat zijn voor cellulaire immunotherapie om afstoting te voorkomen dan $CD4^+CD25^+Foxp3^+$ Tregs.
De inductie van de $CD8^+$ Treg door PDC werd gedeeltelijk gemedieerd door indoleamine-2, 3 dioxygenase (IDO) activiteit, wat overeenkomt met de resultaten van anderen (8-9). De remming van effector T-celproliferatie door PDC geïnduceerde $CD8^+$ Treg is afhankelijk van de expressie van CTLA-4, aangezien een neutraliserend antilichaam tegen CTLA-4 de onderdrukkende functie van deze Treg verminderd. CTLA-4 is een remmend co-stimulatoir molecuul dat concurreert met CD28 voor binding aan CD80 en CD86 op antigeen presenterende cellen met een tenminste 20 maal hogere aviditeit dan CD28 (10). Het is beschreven dat CLTA-4 ook een rol speelt bij de immuun-supressieve functie van $CD4^+CD25^+Foxp3^+$ Tregs (11-12), echter het precieze mechanisme is nog niet volledig opgehelderd. Doordat CTLA-4 Bi-directioneel signaleert, kan binding van CTLA-4 aan CD80 of CD86 IDO expressie induceren in antigen presenterende cellen (13). Afbraak producten van IDO, zoals kynurenine kunnen de groei van T-cellen remmen (14). Een alternatieve verklaring kan zijn dat de $CD8^+$ Treg een oplosbare vorm van CTLA-4 produceren en uitscheiden (15-16) waarmee CD80 en CD86 moleculen op antigeen presenterende cellen worden afgeblokt, waardoor de beschikbaarheid van co-stimulerende moleculen wordt verminderd die nodig zijn om een efficiënte effector T cel activatie te induceren. Aanvullende experimenten zijn nodig om het exacte werkingsmechanisme van de PDC geïnduceerde $CD8^+$ Treg te achterhalen.

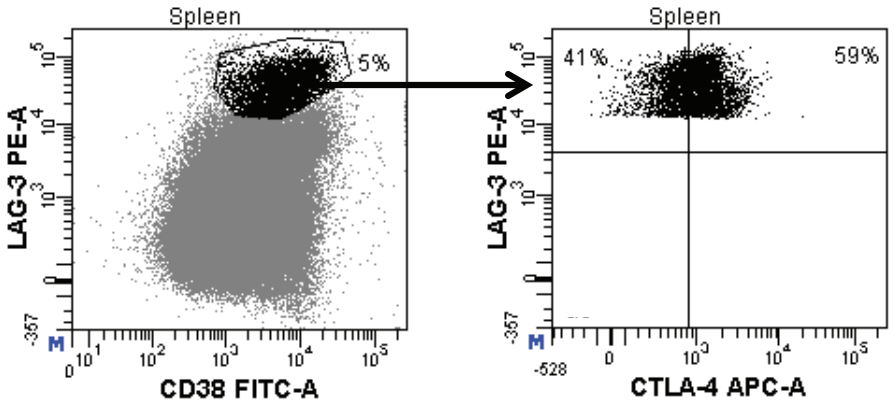
In onze experimenten worden behalve $CD8^+$ Treg ook IL-10-producerende $CD4^+$ T-cellen geïnduceerd door PDC. Ondanks hun IL-10 produktie beschikken deze T-cellen in experimenten om hun suppressieve vermogen te bepalen, maar een beperkte mate van suppressieve activiteit. Een mogelijke verklaring voor het gebrek aan onderdrukkende functie is dat we tijdens deze experimenten gebruik hebben gemaakt van uit monocyten opgekweekte DC (MoDC). Deze MoDC werden voor gebruik gestimuleerd met LPS, zodat ze beter in staat zijn om effector T-cellen te activeren. IL-10 remt de opregulatie van costimulerende moleculen en de productie van pro-inflammatoire cytokines van MoDC, maar omdat de MoDC in onze suppressie assays vooraf al werden gestimuleerd met LPS, heeft de IL-10 geproduceerd door $CD4^+$ T-cellen tijdens het experiment waarschijnlijk geen invloed meer op de rijping van de MoDC. Dus de onderdrukkende functie van de PDC-geinduceerde $CD4^+$ T-cellen zoals die is beschreven in **hoofdstuk 5** is waarschijnlijk een onderschatting van het werkelijke onderdrukkende vermogen van deze cellen.

Onze *in vitro* gegevens over de inductie van $CD8^+$ Treg door PDC wordt ondersteund door een transplantatie dierenmodel, waarbij in ratten die behandeld worden met CD40Ig, $CD8^+CD45C^{low}$ Treg ontstaan. Deze $CD8^+$ $CD45C^{low}$ Treg remmen de afstoting van allogene harttransplantaten. De dynamiek van co-lokalisatie van deze $CD8^+$ Treg $CD45C^{low}$ samen met PDC, eerst in het transplantaat en vervolgens in de milt, suggereert dat PDC een belangrijke rol spelen bij de inductie van deze $CD8^+$ Treg (17).

Om te bepalen of $CD8^+$ T-cellen met vergelijkbaar fenotype als de PDC geïnduceerde $CD8^+$ Treg aanwezig zijn in humaan weefsels, zijn er flowcytometrische kleuringen met CD3, CD8, CD38, Lag-3 en CTLA4 uitgevoerd. We analyseerden celsuspensies uit leverperfusaat, lever LK, lies LK, en milt voor $CD8^+CD38^+LAG\text{-}3^+CTLA\text{-}4^+$ T-cellen. Alleen in miltweefsel konden we cellen detecteren met dit immunofenotype. Van alle $CD8^+$ T-cellen bevatte ongeveer 5-6% dit fenotype. Deze T-cellen moeten nog uit de milt gezuiverd

worden om ze te kunnen testen op functionaliteit. Dan wordt duidelijk of ze effector T-celproliferatie kunnen remmen.

Wij kunnen twee manieren bedenken om mogelijk in de toekomst PDC toe te passen in een immunotherapie protocol om orgaanafstoting te voorkomen. In de eerste manier worden PDC gezuiverd uit donorbloed of milt met behulp van klinisch toepasbare immuno-magnetische beads. Vervolgens worden ze *ex vivo* gestimuleerd waarna ze toegediend kunnen worden aan transplantatiepatiënten. In deze benadering wordt verondersteld dat donor PDC donor-specifieke $CD8^+$ Treg *in vivo* induceren. PDC bezitten naast tolerogene eigenschappen ook immuno-stimulerende eigenschappen. In **hoofdstukken 5** en **8** laten wij zien dat geactiveerde PDC pro-inflammatoire cytokines, zoals IFN-α, IL-6 en TNF-α produceren en dat ze T-cellen activeren om zowel anti-inflammatoire en pro-inflammatoire cytokines te produceren. Hoewel de uitkomst van PDC-activering afhangt van de wijze waarop de PDC geactiveerd wordt, activeert geen van de onderzochte stimuli selectief pro-tolerogeen functies in PDC. Het is tot op heden onduidelijk bij welke manier van stimuleren de tolerogene eigenschappen domineren over de immuno-activerende eigenschappen (18). Bovendien zijn er ook interacties mogelijk tussen PDC en MDC en deze interacties versterken de immuunresponsen geïnduceerd door MDC (19). Omgekeerd verhogen MDC de overleving van PDC, alsook hun vermogen om T-cellen te stimulerend (20). Deze interacties zijn afhankelijk van zowel oplosbare factoren als cel-cel contact (21). Wij denken dat ondanks dat de infusie van autologe TLR-geactiveerde PDC in mensen veilig is gebleken (22), infusie van allogene PDC ontstekingsreacties kunnen veroorzaken. Daarom denken we dat een andere, tweede manier beter is. Hierin worden PDC afkomstig van de donor gebruikt om $CD8^+$ Treg *ex vivo* te induceren, en deze $CD8^+$ Treg kunnen worden toegediend aan de getransplanteerde patiënt. Eventueel zouden de $CD8^+$ Treg verder kunnen worden geëxpandeerd om toediening van voldoende aantallen mogelijk te maken. Maar voordat een klinische proef kan worden toegepast is het noodzakelijk te onderzoeken of infusie van PDC geïnduceerde $CD8^+$ Treg transplantaatafstoting in een klinisch relevant proefdiermodel kan voorkomen. Bovendien moet in een proefdier worden vastgesteld hoe goed de $CD8^+$ Treg overleven *in vivo* en wat hun effect is op T-cel responsen tegen pathogenen.



Figuur 1. Humane milt bevat T cellen die CD8, CD38, LAG-3 en CTLA4 tot expressie brengen. Suspensies van miltcellen werden gekleurd met antistoffen tegen CD3, CD8, CD38, Lag-3 en intracellulair CTLA4. De populaties in the dot plots zijn gegated op CD3 en CD8. Een representatief milt monster van drie is afgebeeld.

In **hoofdstuk 6** is de kinetiek bepaald van de aantallen circulerende PDC bij patiënten gedurende het eerste jaar na levertransplantatie. De relatieve aantallen PDC daalde met een factor 4 onmiddellijk na transplantatie. Tussen de 3 en 12 maanden na de transplantatie begonnen de aantallen weer te stijgen, maar na 12 maanden hadden ze nog niet het niveau bereikt van voor de transplantatie. De longitudinale kinetiek van circulerende PDC was geassocieerd met prednison behandeling. We toonden aan dat corticosteroïden apoptose induceren in PDC. TLR stimulatie van de PDC beschermde gedeeltelijk tegen inductie van apoptose door corticosteroïden, maar hun functie werd beïnvloed. Cytokine productie en opregulatie van costimulatoire moleculen werden verminderd, evenals hun vermogen om T-cellen te activeren.

In **hoofdstuk 7** worden de effecten beschreven van corticosteroïden op de antivirale activiteit van PDC op een HCV-replicatie model met Huh7 hepatoma cellen getransfecteerd met een niet-structureel coderende sequentie van HCV gekoppeld aan een luciferase reporter-gen. Corticosteroïden hadden geen direct effect op de HCV-replicatie en ook werd de remming van de HCV-replicatie door IFN-α niet direct verstoord. Echter corticosteroïden remden de anti-virale werking van TLR-7 gestimuleerd PDC. TLR-7 gestimuleerde PDC konden wanneer ze samen werden gekweekt met getransfecteerde Huh7 cellen de HCV replicatie aanzienlijk verminderen. Deze remming van de HCV replicatie kon volledig te niet worden gedaan door het toevoegen van steroïden. We concludeerden dat corticosteroïden de aantallen circulerende PDC in levertransplantatiepatiënten reduceren en dat corticosteroïden de anti-virale activiteit van humane PDC remmen. Deze mechanismen kunnen verantwoordelijk zijn voor de versnelde mate waarin een HCV-infectie na een levertransplantatie terugkeert.
Misschien dat een immunosuppressief behandelingsprotocol zonder steroïden het aantal en de ernst van virale infecties na orgaantransplantatie kan verminderen.
In een groot systematische review waarin 30 gerandomiseerde gecontroleerde studies (5949 patiënten) werden gecombineerd, werd aangetoond dat het veilig is om geen corticosteroïden toe te dienen of heel snel af te bouwen na niertransplantatie. Ontvangers toonde een verhoogde incidentie van acute afstoting, maar dit werd niet in verband gebracht met een verhoogde sterfte of verlies van het transplantaat en ze hadden minder infecties (23).

Onze resultaten, die laten zien dat corticosteroïden apoptose induceren en de functie van PDC remmen, maar dat TLR activatie beschermt tegen corticosteroïden geïnduceerde apoptose, werden bevestigd door anderen (24-25). Lepelletier et al liet zien dat microbiële stimulatie PDC minder gevoelig maakt voor corticosteroïden geïnduceerde apoptose en dat deze bescherming veroorzaakt wordt door TLR stimulatie geïnduceerde autocriene TNF-α en IFN-α productie die leidt tot opregulatie van anti-apoptotische genexpressie (24). patiënten met auto-immuunziekten, zoals systemische lupus erythematosus (SLE) worden vaak behandeld met corticosteroïden. In SLE leidt herkenning van lichaamseigen nucleïnezuren die binden aan TLR-7 en TLR-9 op de PDC tot IFN-α productie en de vorming van anti-nucleaire antilichamen. Corticosteroïden zijn niet altijd succesvol gebleken in het bestrijden van deze ziekte. Guiducci et al. Hebben aangetoond dat immuuncomplexen die lichaamseigen nucleïnezuren bevatten PDC kunnen activeren via TLR-7 en TLR-9. Deze geactiveerde PDC produceren IFN-α productie (25) en zijn relatief resistent tegen apoptose waardoor corticosteroïden behandeling geen of minder effect heeft.

Activering via TLR redt PDC van corticosteroïden geïnduceerde apoptose, maar corticosteroiden remmen TLR-gestimuleerde PDC nog steeds in hun functioneren. De manier waarop corticosteroïden de functies van TRL-geactiveerde PDC remmen is nog onbekend. In macrofagen is de IFN-α productie afhankelijk van de fosforylatie van IFN regulatory factor 3 (IRF3), een signaleringsmolecuul stroomafwaarts van TLR-3. Na activatie van IRF3 ontstaat er een complex dat ook de cofactor Glucocorticoid Receptor Interacting Protein-1 (GRIP1) bevat. Tijdens corticosteroïdebehandeling is er competitie voor GRIP1-binding tussen de glucocorticosteroïde receptor (GR) en IRF3, waardoor er minder IRF3 complexen gevormd kunnen worden, wat leidt tot verminderde IFN-α productie (26). Bij PDC is de IFN-α productie afhankelijk van IRF7 en niet van IRF3, echter GRIP1 is ook een cofactor in het complex dat ontstaat na IRF7 activatie (26). Het zou kunnen dat dit mechanisme ook een rol speelt bij de verminderde IFN-α productie van PDC, hiervoor moet de concentratie van GRIP1 in PDC echter even laag zijn als in macrofagen. GRIP1 is een cofactor in het STAT1-STAT2-IRF9 (ISGF3) transcriptie complex dat gevormd wordt nadat IFN-α bindt aan zijn receptor. Signalering van de IFN-α receptor via het ISGF3-complex leidt tot extra productie van IFN-α. Concurrentie van de GR en IRF9 in het ISGF3-complex voor GRIP1 leidt tot een verminderde IFN-α productie, doordat de vorming van de positieve autocriene feedback lus verstoord is (27). Dit mechanisme is alleen aannemelijk in celtypen die het GRIP1 eiwit in extreem lage hoeveelheden tot expressie brengen. Het moet nog worden vastgesteld of dit moleculaire mechanisme een rol speelt bij de door corticosteroïden onderdrukte IFN-α productie in TLR-geactiveerde PDC.

Naast de effecten van corticosteroïden, werden in **hoofdstuk 8** de effecten van het immunosuppressieve medicijn rapamycine op PDC bestudeerd. Rapamycine remt de activeren van het signaleringsmolecuul mTOR, dat onder andere een belangrijke rol speelt bij de celdeling. Klinisch relevante concentraties van rapamycine remde IFN-α productie geïnduceerd door TLR-7 stimulatie sterk (-64%), maar de IFN-α production geïnduceerd door TLR-9 stimulatie werd maar licht (-20%) geremd. Rapamycine onderdrukte de productie van pro-inflammatoire cytokines door PDC, die geïnduceerd werden via TLR-7 en TLR-9-activatie met vergelijkbare effectiviteit. Rapamycine remde het vermogen van zowel met TLR-7 en TLR-9 gestimuleerde PDC om pro-inflammatoire (IFN-γ en IL-17) en anti-inflammatoire (IL-10) cytokine productie te induceren in allogene T-cellen. Echter mTOR-remming vergrootte de capaciteit van TLR-7-geactiveerde PDC om T-helper-cellen te laten delen. Dit werd veroorzaakt doordat rapamycine de opregulatie van CD80 expressie op PDC versterkte. Neutraliserende antilichamen tegen CD80 konden de hogere T-celproliferatie weer opheffen. De generatie van $CD8^+$ $CD38^+$ $LAG\text{-}3^+$ Treg door TLR gestimuleerde PDC werd niet belemmerd door rapamycine.
De data die in dit hoofdstuk beschreven worden laten zien dat rapamycine contrasterende effecten heeft op het immuunsysteem. Naast de bekende immunosuppressieve effecten tonen wij en andere recente studies aan dat rapamycin ook immuunstimulerende effecten heeft. Zo is beschreven dat rapamycin pro-inflammatoire cytokine productie stimuleerd in myeloïde cellen (28) en dat rapamycine $CD8^+$ memory T-celdifferentiatie bevorderd (29-30). Bovendien is in stabiele niertransplantatie patiënten die behandeld zijn met rapamycine monotherapie aangetoond dat de frequentie van $CD4^+CD25^+Foxp3^+$ T-cellen weliswaar is verhoogd, maar dat dit gepaard ging met een verhoogd aantal effector memory cellen, naïeve immuun cellen en een toename van NF-κB gerelateerde pro-inflammatoire signaleringsroutes. Het lijkt niet dat rapamycine leidt tot meer tolerantie in deze patiënten in vergelijking met de standaard toegepaste

calcineurineremmers (31).

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CURRICULUM VITAE

Patrick Boor werd op 16 november 1973 geboren in Waalwijk. Hij voltooide zijn voortgezet onderwijs aan het dr. Mollercollege in Waalwijk. In 1996 werd de HLO opleiding afgerond aan de Hogeschool Westbrabant in Etten-Leur. Gedurende deze opleiding werd 1 jaar onderzoeksstage gelopen bij de unit moleculaire immunologie van de afdeling immunologie aan de Erasmus Universiteit in Rotterdam. Onder begeleiding van Prof dr. J.J.M van Dongen deed hij onderzoek naar de mogelijkheden om antistoffen gericht tegen Vβ-domeinen van T-celreceptoren te gebruiken bij het vaststellen van klonaliteit van rijpe T-celleukemieën. Hierna kreeg hij een baan aangeboden bij dezelfde afdeling om het onderzoek voort te zetten. Een jaar later ging hij werken bij de afdeling Interne geneeskunde aan het opsporen van genmutaties en polymorfismen bij patiënten met Peutz-Jeghers syndroom en porferie onder leiding van Prof. dr. J.H.P Wilson en Dr. F.W.M de Rooij. Tevens werkte hij hier aan een project om met behulp van microsatelliet analyse genen te identificeren die betrokken zijn bij de ziekte van Crohn en Colitus Ulcerosa. Vanaf 2002 werkte hij bij de afdeling maag, darm en leverziekten van het ErasmusMC in Rotterdam onder supervisie van dr. J. Kwekkeboom aan de rol van dendritische cellen in lever transplantatie en hepatitis B infectie. Vanaf 2006 tot heden combineerde hij een baan als PhD-student en research analist op dezelfde afdeling. Onder leiding van Prof dr. H.J Metselaar en Dr. J. Kwekkeboom werd het onderzoek verricht dat in dit proefschrift beschreven is.

PHD PORTFOLIO

Name PhD student:	Patrick Boor
ErasmusMC Department:	Gastroenterology and Hepatology
PhD Period:	2006 - 2012
Promotor:	Prof. Dr. H.J. Metselaar
Copromotor:	Dr. J. Kwekkeboom

PHD Training

General academic and research skills	
Basic radiation protection 5A/B	2005
In Vivo Imaging	2007
English biomedical writing and communication	2009

(inter)national conferences

- 16th Bootcongres, Nederlandse Transplantatie Vereniging	2004
- 18th Bootcongres, Nederlandse Transplanatie Vereniging	2006
- 12th International congress of ILTS	2006
- Annual Meeting NVVI	2006
- 19th Bootcongres, Nederlandse Transplantatie Vereniging	2007
- 13th International congress of ILTS	2007
- Annual Meeting NVVI	2007
- 20th Bootcongres, Nederlandse Transplantatie Vereniging	2008
- Voorjaarsvergadering, NVGE/NVH	2008
- 8th American Transplant congress	2008
-14th International congress of ILTS	2008
- Annual Meeting NVVI	2008
- III Basic Science Meeting, ESOT, Brussels, Belgium	2009
- Voorjaarsvergadering NVGE/NVH	2009
- 2nd European congress of Immunology	2009
- 22th Bootcongres, Nederlandse Transplantatie Vereniging	2010
- DC2010: Forum on Vaccine Science	2010
- Annual Meeting NVVI	2010
- 15th congress of the European society for organ transplantation	2011
- Voorjaarsvergadering NVGE/NVH	2012
- 3rd European congress of Immunology	2012

Presentations

- Oral, 16e Bootcongres, NTV, Rockanje, NL	2004
- Oral, 18e Bootcongres, NTV, Zeewolde, NL	2006
- Poster, World Transplant Congress, Boston, USA	2006
- Oral, 12th international congress of ILTS, Milan, Italy	2006
- Poster, Annual Meeting NVVI	2006
- Oral, 19th Bootcongres, NTV, Zeewolde, NL	2007
- Oral, 13th international congress of ILTS, Rio de Janeiro, Brazil	2007
- Poster, Annual Meeting NVVI, Noordwijkerhout, NL	2007

- Oral, 20th Bootcongres NTV, Zeewolde, NL 2008
- Oral, Voorjaarsvergadering NVGE/NVH, Veldhoven, NL 2008
- Poster, 8th American Transplant congress, Toronto, Canada 2008
- Oral, 14th International congress of ILTS, Paris, France 2008
- Poster, Annual Meeting NVVI 2008
- Poster, Voorjaarsvergadering NVGE/NVH, veldhoven, NL 2009
- Oral/Poster, European congress of Immunology, Berlin, Germany 2009
- Oral, 22th Bootcongres, Rotterdam, NL 2010
- Poster, DC2010, Lugano, Switzerland 2010
- Oral, Annual Meeting NVVI, Noordwijkerhout, NL 2010
- Oral, ESOT, Glasgow, Scotland 2011
- Oral/Poster Voorjaarsvergadering NVGE/NVH 2012
- Oral, European congress of Immunology, Glasgow, Scotland 2012

Teaching activities
- supervising intership MSc student S. de Jonge 2008
- supervising and lecture medical students 2009
- supervising students for Master of Science Infection and Immunity 2011
- supervising medical students 2012

Grants
- Travelgrant ILTS (t.b.v ILTS) 2006
- Travelgrant Nederlandse transplantatie vereniging (t.b.v Bootcongres) 2006
- Travelgrant Novartis B.V. (t.b.v ILTS) 2007
- Travelgrant Novartis B.V. (t.b.v ATC) 2008
- Travelgrant NVH (t.b.v ILTS) 2008
- Travelgrant NVVI (t.b.v ECI) 2009
- Travelgrant Novartis B.V. (t.b.v ESOT) 2011

Invited Speaker
Miltenyi Biotec GmbH, Bergisch Gladbach, Germany 2009

LIST OF PUBLICATIONS

1. Boor PP, Metselaar HJ, Jonge S, Mancham S, van der Laan LJ, Kwekkeboom J. Human plasmacytoid dendritic cells induce CD8(+) LAG-3(+) Foxp3(+) CTLA-4(+) regulatory T cells that suppress allo-reactive memory T cells. Eur J Immunol. 2011 Jun;41(6):1663-74.

2. Bosma BM, Metselaar HJ, Gerrits JH, van Besouw NM, Mancham S, Groothuismink ZM, Boor PP, et al. Migration of allosensitizing donor myeloid dendritic cells into recipients after liver transplantation. Liver Transpl. 2010 Jan;16(1):12-22.

3. van der Linde K, Boor PP, Mejissen MA, Sandkuijl LA, Houwing-Duistermaat JJ, Kuipers EJ, et al. Allele-sharing of cytokine genes in familial inflammatory bowel disease. Hepatogastroenterology. 2007 Jul-Aug;54(77):1467-71.

4. van der Linde K, Boor PP, Houwing-Duistermaat JJ, Crusius BJ, Wilson PJ, Kuipers EJ, et al. CARD15 mutations in Dutch familial and sporadic inflammatory bowel disease and an overview of European studies. Eur J Gastroenterol Hepatol. 2007 Jun;19(6):449-59.

5. Tha-In T, Metselaar HJ, Tilanus HW, Boor PP, Mancham S, Kuipers EJ, et al. Superior immunomodulatory effects of intravenous immunoglobulins on human T-cells and dendritic cells: comparison to calcineurin inhibitors. Transplantation. 2006 Jun 27;81(12):1725-34.

6. Bosma BM, Metselaar HJ, Mancham S, Boor PP, Kusters JG, Kazemier G, et al. Characterization of human liver dendritic cells in liver grafts and perfusates. Liver Transpl. 2006 Mar;12(3):384-93.

7. Boor PP, Metselaar HJ, Mancham S, Tilanus HW, Kusters JG, Kwekkeboom J. Prednisolone suppresses the function and promotes apoptosis of plasmacytoid dendritic cells. Am J Transplant. 2006 Oct;6(10):2332-41.

8. van der Linde K, Boor PP, van Bodegraven AA, de Jong DJ, Crusius JB, Naber TH, et al. A functional interleukin-10 mutation in Dutch patients with Crohn's disease. Dig Liver Dis. 2005 May;37(5):330-5.

9. Kwekkeboom J, Tha-In T, Tra WM, Hop W, Boor PP, Mancham S, et al. Hepatitis B immunoglobulins inhibit dendritic cells and T cells and protect against acute rejection after liver transplantation. Am J Transplant. 2005 Oct;5(10):2393-402.

10. Kwekkeboom J, Boor PP, Sen E, Kusters JG, Drexhage HA, de Jong EC, et al. Human liver myeloid dendritic cells maturate in vivo into effector DC with a poor allogeneic T-cell stimulatory capacity. Transplant Proc. 2005 Jan-Feb;37(1):15-6.

11. Boor PP, Ijzermans JN, van der Molen RG, Binda R, Mancham S, Metselaar HJ, et al. Immunomagnetic selection of functional dendritic cells from human lymph nodes. Immunol Lett. 2005 Jul 15;99(2):162-8.

12. van der Linde K, Boor PP, Sandkuijl LA, Meijssen MA, Savelkoul HF, Wilson JH, et al. A Gly15Arg mutation in the interleukin-10 gene reduces secretion of interleukin-10 in Crohn disease. Scand J Gastroenterol. 2003 Jun;38(6):611-7.

13. Linde K, Boor PP, Houwing-Duistermaat JJ, Kuipers EJ, Wilson JH, de Rooij FW. Card15 and Crohn's disease: healthy homozygous carriers of the 3020insC frameshift mutation. Am J Gastroenterol. 2003 Mar;98(3):613-7.

14. de Reuver P, Pravica V, Hop W, Boor P, Metselaar HJ, Hutchinson IV, et al. Recipient ctla-4 +49 G/G genotype is associated with reduced incidence of acute rejection after liver transplantation. Am J Transplant. 2003 Dec;3(12):1587-94.

15. Langerak AW, van Den Beemd R, Wolvers-Tettero IL, Boor PP, van Lochem EG, Hooijkaas H, et al. Molecular and flow cytometric analysis of the Vbeta repertoire for clonality assessment in mature TCRalphabeta T-cell proliferations. Blood. 2001 Jul 1;98(1):165-73.

16. van den Beemd R, Boor PP, van Lochem EG, Hop WC, Langerak AW, Wolvers-Tettero IL, et al. Flow cytometric analysis of the Vbeta repertoire in healthy controls. Cytometry. 2000 Aug 1;40(4):336-45.

17. Westerman AM, Entius MM, de Baar E, Boor PP, Koole R, van Velthuysen ML, et al. Peutz-Jeghers syndrome: 78-year follow-up of the original family. Lancet. 1999 Apr 10;353(9160):1211-5.

18. Westerman AM, Entius MM, Boor PP, Koole R, de Baar E, Offerhaus GJ, et al. Novel mutations in the LKB1/STK11 gene in Dutch Peutz-Jeghers families. Hum Mutat. 1999;13(6):476-81.

19. Derksen PW, Langerak AW, Kerkhof E, Wolvers-Tettero IL, Boor PP, Mulder AH, et al. Comparison of different polymerase chain reaction-based approaches for clonality assessment of immunoglobulin heavy-chain gene rearrangements in B-cell neoplasia. Mod Pathol. 1999 Aug;12(8):794-805.

20. Kluin-Nelemans HC, Kester MG, van deCorput L, Boor PP, Landegent JE, van Dongen JJ, et al. Correction of abnormal T-cell receptor repertoire during interferon-alpha therapy in patients with hairy cell leukemia. Blood. 1998 Jun 1;91(11):4224-31.

The liver is not only scientifically an interesting organ. For everyone who would like to explore the liver in a different way, here is a culinary recipe.

THAI SPICY LIVER SALAD

Ingredients

* 200 grams pork liver (or beef liver)
* 1 tablespoon ground roasted sticky rice
* 1 tablespoon ground chili
* 2 tablespoons lime juice
* 1 tablespoon fish sauce
* 1 tablespoon chopped scallion
* 1/2 cup mint leaves
* 4 shallots, thinly sliced

Food Preparations

1. Wash the liver in clean water and sliced into well pieces.
2. Heat water in a pot. Then scald sliced liver until nearly cooked. Remove and drain.
3. In a medium-sized bowl, add liver, sugar, lime juice, fish sauce, chopped scallion, ground chili, ground roasted sticky rice and shallots. Stir until all ingredients mixed well.
4. Transfer to a serving plate. Garnish with mint leaves and serve immediately with fresh vegetable (cabbage, cucumber, etc.) and hot steamed rice (or sticky rice).

(For 2 Serving)

bon appetit
