Modulated rat dendritic cells in renal transplantation models : immune regulation and graft outcome

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Contents

CHAPTER 1

General Introduction

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Renal transplantation

Renal transplantation is currently the treatment of choice for patients with end stage organ failure. The majority of the recipients receive genetically non-identical donor-organs (allografts). Most of these transplants would be rejected if patients would not receive immunosuppressive treatment. Various types of rejection can be distinguished, including hyperacute rejection, which occurs immediately after establishment of the blood supply, acute rejection, which arises early after transplantation (mostly within the first 6 months) or chronic rejection, which takes place after more than 6 months. To prevent rejection of transplanted allogeneic organs, it is necessary to apply immunosuppressive therapy. Traditionally immunosuppressive drugs have been mostly directed against T cells. Over the years, powerful combinations of drugs have been developed, resulting in strongly improved short-term survival increases [1]. In contrast to the improved 1-year survival rates, less progress have been made to improve long-term survival. In addition, patients treated with immunosuppressive medication suffer from various side effects, including malignancy and infections, due to the non-specificity of this type of medication [2]. The development of novel therapeutic strategies is therefore important to improve long-term transplant survival.

Immunology of transplantation

The immune system distinguishes self from non-self and has the capacity to either maintain tolerance or to induce immunity to eliminate infectious agents and to minimize tissue damage. Since transplanted organs are recognized as non-self, immunity arises in most cases after transplantation leading to allograft rejection. Although various components of the immune system and a wide array of effector mechanisms are involved, most attention has been paid to the role of T cells.

Activation of alloreactive T cells

Activation of T cells requires a variety of signals to determine the antigen specificity, the strength of the response and the type of the response. The first signal is triggered by the interaction between the T cell receptor (TCR) and a MHC/peptide complex on antigen presenting cells (APC). Simultaneously, a second signal can be delivered when CD28, which is constitutively expressed by naïve T cells, binds to the co-stimulatory B7 molecules (CD80 and CD86), present on activated APC. In addition, a third signal is provided by proinflammatory cytokines, such as IL-12, which are produced by activated APC [3, 4]. This activation process is rapidly amplified when co-stimulation via the CD40-CD40L pathway takes place, due to elevated expression levels of MHC, B7 molecules and increased cytokine production. Together these signals lead to full activation of naïve T cells, resulting in proliferation and secretion of various cytokines, such as IL-2 and IFN-γ (**Fig. 1**) [5, 6]. Subsequent to T cell priming, the T cell displays an increasing transcription and expression of cytotoxic T lymphocyte antigen (CTLA)-4 on the cell surface. This molecule has a higher affinity for B7 molecules than CD28 and has been shown to exert a suppressive effect on T cell activation [7]. More recent

studies demonstrate the presence of additional co-stimulatory molecules, which are involved in “positive” or “negative” signaling. Many of these receptor-ligand pairs belong to the CD28-B7 or CD40-CD40L families. These interactions point to a role of APC in the regulation of T cell activation [8-10].

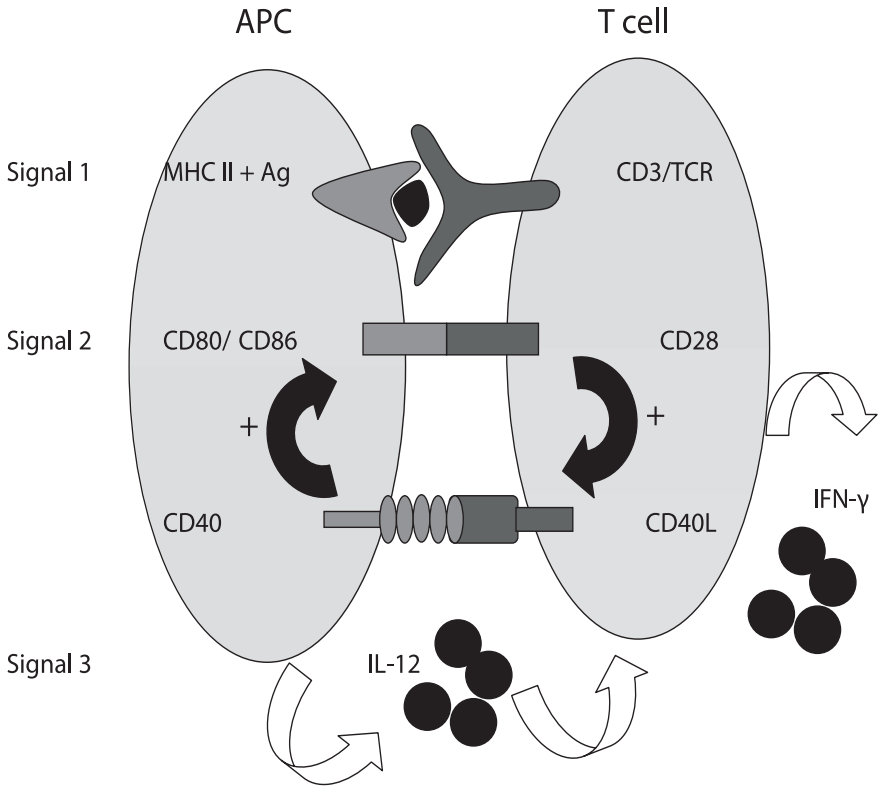

Figure 1. Signals involved in T cell activation.
Three signals are necessary to activate T cells. These signals are provided by matured antigen presenting cells (APC) and include presentation of foreign peptides to naïve T cells in the context of MHC molecules (signal 1). The second signal comprises the interaction between B7 molecules (CD80 and CD86), expressed by mature APC, and CD28, which is constitutively expressed on naïve T cells. Together with the secreted proinflammatory cytokines, such as IL-12 (signal 3), this will result in T cell activation and involves upregulation of CD40L. Subsequently, CD40L interacts with CD40, expressed by mature APC, and leads to an enhanced maturation status of the APC as shown by higher levels of MHC and B7 molecule expression together with elevated levels of proinflammatory cytokine secretion. Consequently, T cells become fully activated and produce high levels of cytokines, such as IFN-γ.

Dendritic cells in allograft rejection

One of the most potent APC are dendritic cells (DC). These are bone marrow-derived cells that populate all lymphoid- as well as non-lymphoid organs, including the kidney. They have a central role in immune regulation, ranging from tolerance induction and the prevention of autoimmunity to the induction of anti-tumor immunity and the protection against infectious agents. Although DC are a heterogeneous group of cells that represent differences in origin, anatomic location, cell surface phenotype, and function, they all have potent antigen presenting capacity to stimulate naive, memory,

effector and/or regulatory T cells (**Fig 2**). Therefore, DC serve as an essential link between innate and adaptive immune responses [11, 12].
Immature DC have specialized capacities for internalisation and processing of self or non-self antigens. DC express several Toll like receptors (TLR), which detect self and non-self products. In response to a broad spectrum of pathogen-associated molecular patterns (PAMPs) these TLR activate different signaling pathways, leading to maturation of DC [13]. Consequently, DC migrate towards secondary lymphoid organs and along the way, lose their capacity to capture and process antigens while gaining the capacity to stimulate T cells. Efficient T cell stimulation by DC requires antigen presentation to T cells, upregulation of co-stimulatory molecules and production of proinflammatory cytokines. Upon interaction with antigen-specific T cells, DC maturation is further ehanced via engagement of CD40 with CD40L, expressed by these activated T cells [14, 15]. In transplantation settings, both donor passenger DC and recipient DC can cause

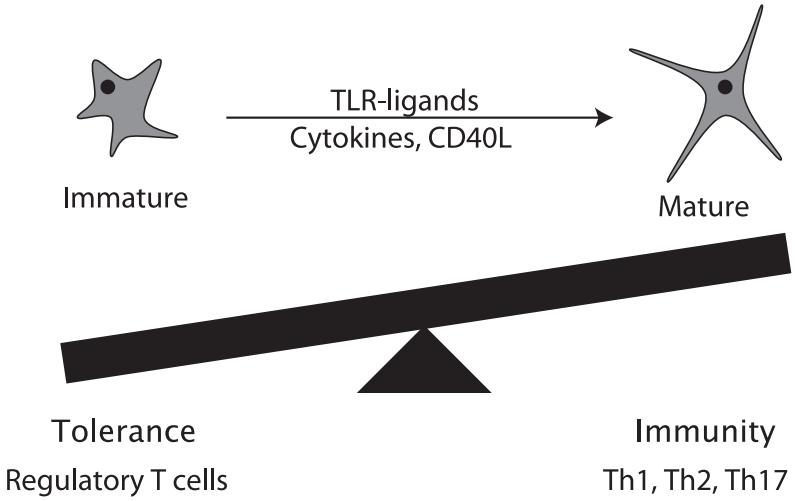

Figure 2. Immune responses induced by DC.
Immature DC express low levels of MHC and co-stimulatory molecules and have the capacity to induce tolerogenic responses. In the presence of danger signals, such as TLR ligands (either PAMPs or endogenous danger signals), certain cytokines or CD40L, immature DC will mature. Mature DC express high levels of MHC and co-stimulatory molecules and produce proinflammatory cytokines. In addition, mature DC have the capacity to induce immunogenic responses.

activation of alloreactive T cells, however this occurs via different routes of recognition. Recipient T cells either recognize the foreign MHC molecule on donor DC, or recognize foreign MHC peptides presented by recipient MHC molecules on recipients DC, known as the direct or indirect pathway of T cell activation, respectively (**Fig. 3**).
The type of T cell response is determined by the cytokines produced by activated DC [15, 16]. DC involved in specific allo-immune responses are programmed to produce high levels of IL-12, which subsequently induces $CD4^+$ T helper 1 cells (Th1). These Th1 secrete IFN-γ and promote cellular mechanisms of immunity through activation of macrophages, NK cells and $CD8^+$ T cells. Although most of the allo-responses are regulated via Th1, also Th2 can be involved in allograft rejection. Th2 provide help to B cells to produce allo-antibodies, which subsequently can mediating rejection [17-19].

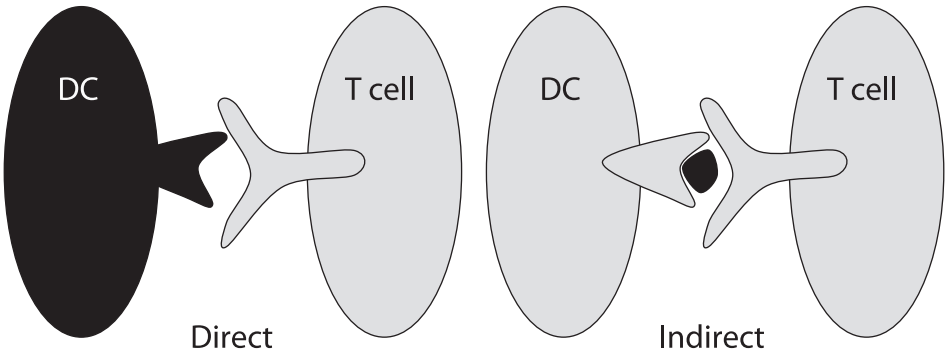

Figure 3. Antigen recognition by recipient T cells.
Recipient T cells can recognize donor antigen via direct or indirect presentation. In case of direct presentation recipient T cells recognize the full MHC molecule on donor APC (black), whereas via indirect presentation recipient APC (gray) present donor antigens (black) on recipient MHC molecules.ect presentation.

Next to Th1 and Th2, another T helper cell has been described recently. This “novel” T helper cell (Th17) has been shown to be important in the pathogenesis of autoimmunity and involves cytokines including IL-23 and IL-17 [20, 21]. Although increased levels of IL-17 have been detected in renal tissue derived from rejected biopsies [22], the role of Th17 cells in transplantation immunity is still unclear. Along with Th1 cells, Th17 cells may mediate allograft rejection, and their role may be more important when Th1 responses are suppressed (**Fig. 4**) [23].
Beside T cells involved in immunogenic responses, regulatory T cells (Treg) have been shown to be important negative regulators of immune responses. They have been identified as being CD4 positive with high levels of cell-surface expression of CD25. Various Treg subsets can be distinguished, including the naturally occurring Treg, which develop in the thymus, and the inducible Treg, which are generated in the periphery under various tolerogenic conditions. The latter subset can be classified in Tr1 and Th3 cells. Tr1 cells secrete IL-10 and TGF-β and are generated by antigenic stimulation in the presence of IL-10 [24], whereas Th3 cells are identified after the induction of oral tolerance and secrete TGF-β [25]. The suppressive capacity of Treg towards effector T cells plays a major role in immunopathology. Absence or dysfunction of Treg has been correlated with autoimmunity [26, 27], whereas their presence has been associated with tolerance as shown in transplantation models [28, 29].

Experimental models of transplantation

Experimental models of transplantation have been studied widely. These models differ in many aspects, including the donor-recipient strain combinations, the transplanted organ and treatments. There seems to be a hierarchy in the immunogenicity of transplanted organs, ranging from low levels of alloresponse in liver transplantation and increasing levels in kidney, heart, skin and bone marrow transplantations [30]. In addition, it has been proposed that transplantations performed in rats are more stringent than in

mice. Even within one species, some strains raise vigorous alloreactive responses and reject most grafts quickly, while in other strains grafts are accepted more rapidly. Consequently, treatments are more effective in models that are associated with less immunogenicity [30].

Kidney transplantation is preferentially studied in rats. The technical procedure in mouse models is very demanding resulting in low survival rates, whereas a higher survival rate is achieved when rats are used. To study various types of kidney rejection different models have been used. Chronic rejection has been examined in the strain combination Fisher (F344, $RT1^{lv1}$) to Lewis (LEW, $RT1^{l}$). Renal transplantation from donor F344 rats to recipients LEW rats demonstrates acute rejection episodes followed by chronic lesions, which ultimately result in kidney failure. In the reverse strain combination, a LEW kidney transplanted into a F344 recipient, acute rejection episodes can be observed, but lesions spontaneously resolve with maintenance of renal function [31, 32]. Other strain combinations, such as Brown Norway (BN, $RT1^{n}$) to LEW or Dark Agouti (DA, $RT1^{a}$) to LEW, demonstrate acute allograft rejection within 7 days [33, 34].

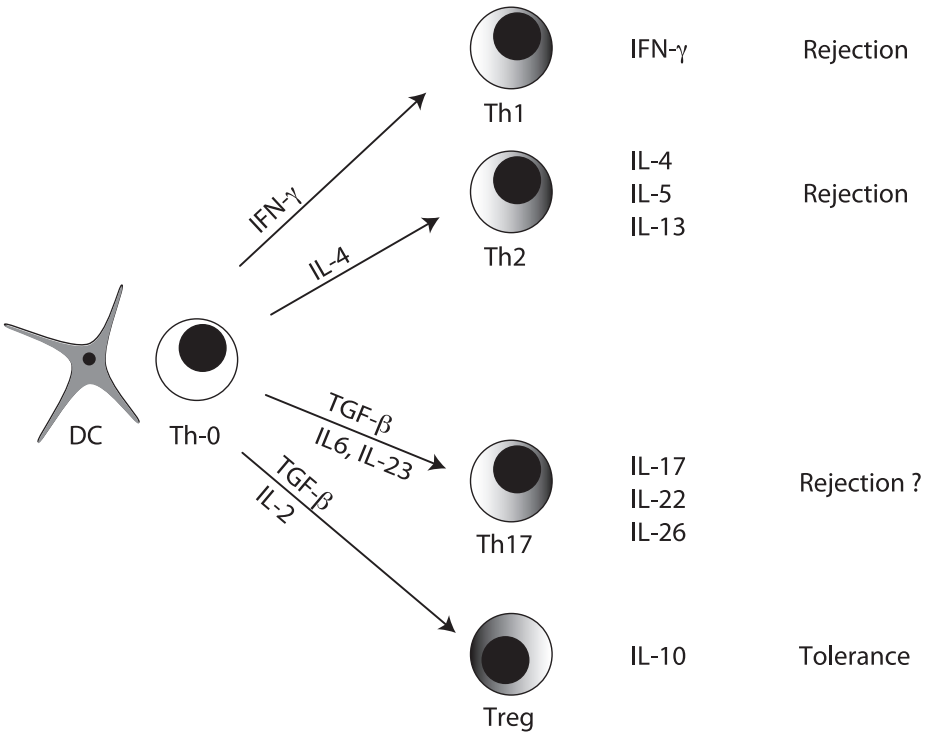

Figure 4. T cell lineage commitment in human.
Naïve T cells develop into T cell lineages defined by their cytokine profile when stimulated by antigen presenting cells (APC). Th1 cell development is induced when APC secrete IL-12 and the cells are characterized by their IFN-γ production. Th2 cells require IL-4 and upon activation produce IL-4, IL-5 and IL-13. Both Th1 and Th2 cells play a role in the graft rejection process. TGF-β is critical for the development of both Th17 and Treg cells. In conjunction with IL-6 or IL-23 inflammatory Th17 cells develop, while Treg development requires IL-2. Treg have been shown to induce tolerance, but the role of Th17 cells in transplantation settings is still unknown.

Depletion of $CD4^+$ cells, using RIB-5/2, prolonged allogeneic kidney survival in a DA to LEW model [35, 36], implying that interference with the immune response in a fully mismatched model can prolong graft survival.

Transplantation Therapies

The introduction of efficient and strong immunosuppressive strategies has significantly improved the short-term survival of allogeneic organs. However, this has come with the price that patients treated with these immunosuppressive drugs suffer from various side effects, including an increased rate of malignancy and infections. It is therefore necessary to develop more specific therapies, in which the ultimate goal is to induce donor-specific tolerance. At present, the various strategies that have been implemented are aimed to control allospecific T cells, including lymphocyte depletion, blockade of co-stimulatory molecules or cell-based therapies.

Therapeutic targeting of co-stimulatory molecules

The molecular identification and characterization of co-stimulatory molecules has provided new tools to target co-stimulatory molecules in rodents and in primates. Since co-stimulatory molecules are essential to induce full T cell activation and T cell activation is a crucial feature of graft rejection, various studies elucidated the effect of co-stimulatory blockade on graft survival. The most extensively studied interactions are CD40-CD40L and CD28-B7 [37, 38]. In rat models, rejection of various solid organs was prevented, and even donor-specific tolerance was induced, when either one of these interactions was blocked [39-41]. More recently it was shown that blocking one of the co-stimulatory pathways indeed resulted in prevention of acute rejection. However, histological signs of chronic rejection were still found at a later stage. Yet, blocking both pathways simultaneously resulted in 50% reduction of chronic rejection in these recipients [42, 43]. In clinical settings, the use of anti-CD40L antibodies in autoimmune disease and transplantation was terminated due to an unanticipated, elevated incidence of thrombo-embolic complications [44, 45]. In contrast, preliminary data from clinical trials using an optimized version of CTLA4-Ig (belatacept or LEA29Y) treatment, demonstrated improved renal function and reduced chronic allograft nephropathy at 1 year without thrombotic complications [46-48].

Donor-specific transfusion

Donor-derived blood transfusions (donor-specific transfusion, DST) have been shown to positively influence survival of allogeneic organs, at least under some conditions [49, 50]. Studies in rodents and non-human primates have demonstrated that a combination of DST and anti-CD40L blocking antibody (Ab), or DST, anti-CD40L blocking Ab and Sirolimus (immunosuppression) synergistically enhanced the survival rate of allogeneic kidneys or pancreatic islets [51, 52]. However, blood transfusions also hold the risk of sensitization and the mechanisms underlying the blood transfusion effect are incompletely understood. Studies in several rodent models indicate that clonal deletion or anergy may play an essential role in silencing the alloreactive T cells [53]. In addition,

rodent and human studies suggest that $CD4^+CD25^+$ regulatory T cells are induced after DST treatment and play a role in graft survival. In the latter studies, it has been shown that only in the case of HLA-DR matched-blood transfusion, these regulatory T cells are induced, whereas when mismatched-blood is administered regulatory T cells are not induced. Survival of allogeneic kidneys in this study was significantly higher in the matched-transfusion recipients compared to mismatched-transfusion recipients [54, 55].

Regulatory T cells

Both human and rodent studies have demonstrated that Treg contain the capacity to suppress effector T cells [29, 56, 57], making Treg an interesting target for cell-based therapy. Clinical application of Treg cells in transplantation settings can be realized by promoting the development of Treg in vivo by administration of immunosuppressive drugs, either alone or together with alloantigens. For example, mycophenolate mofetil has been shown to promote and increase the frequency of $CD4^+CD25^+$ Treg in mice and to induce transplantation tolerance when combined with 1α, 25-dihydroxyvitamin D3 [58]. Another approach is to isolate Treg cells from the recipient and expand these in vitro [59-61]. Infusion of these expanded $CD4^+CD25^+$ Treg has been shown to inhibit graft-versus-host disease [62], and prolong allogeneic skin and heart survival in irradiated mice [63]. To apply Treg in the clinic one has to confirm that they maintain regulatory activity after expansion ex vivo. Many other properties will also need to be examined, including the capacity of expanded Treg cells to survive and migrate appropriately in vivo when re-introduced into the transplant recipient. All of these factors will require careful evaluation in relevant models before such clonally expanded Treg can be used to treat transplant recipients effectively and safely [28, 29].

Tolerogenic dendritic cells

The broad range of powerful immune stimulatory as well as regulatory functions, has made DC targets for vaccine development strategies. This includes cellular vaccination for treatment of cancer or infectious diseases, as well as “negative vaccination” for the treatment of autoimmune diseases and prevention of allograft rejections. The latter can be accomplished by inhibiting the immunostimulatory capacity of DC or more importantly, used to exploit tolerogenic DC to specifically silence immune responses.

Since only low numbers of DC can be isolated from blood, researchers are required to make use of DC progenitor cells isolated from either blood or bone marrow and to differentiate these progenitor cells into DC in vitro. Generation of human-derived DC is realized by culturing monocytes, isolated from blood, in the presence of granulocyte monocyte colony stimulating factor (GM-CSF) and IL-4. Studies on rodent DC make use of bone marrow (BM) cells, which are cultured in the presence of GM-CSF and in some cases also IL-4, to generate DC.

Both human monocyte-derived DC (Mo-DC) and rodent BM-derived DC (BM-DC) have been shown to express MHC and B7 molecules and upon maturation they have the capacity to induce T cell stimulation. Phenotypically, there are some differences in marker expression, Mo-DC are characterised by the expression of CD1a and DC-SIGN [64, 65], murine BM-DC express CD11c [66] and no typical DC marker has been

determined for rat BM-DC, although OX62 has been shown to be expressed by some DC subsets [67]. Modulation of these generated DC will lead to tolerogenic properties as will be described in the next section.

Mixed chimerism

A powerful strategy to induce transplant tolerance is the induction of mixed chimerism. This is a situation where the immune system of donor and recipient are coexisting, thereby actively inducing central and peripheral tolerance. To induce mixed chimerism, recipients are pretreated with T cell depleting antibodies or with co-stimulatory blocking antibodies along with a sublethal dose of total body irradiation. This treatment results in the presence of hematopoietic cells from both the recipient and the donor in the thymus and will consequently delete both host-reactive and donor-reactive T cells, resulting in a peripheral T cell repertoire that is tolerant toward the donor and the host. Studies performed in experimental models have demonstrated that these treatments resulting in mixed chimerism can prolong graft survival [68]. However, this approach to induce transplant tolerance is highly toxic and therefore prohibited as a standard procedure in the clinic. Nevertheless, recently 2 studies described the induction of mixed chimerism in patients receiving a kidney and hematopoietic stem-cell transplantation [69, 70]. Another recent study described the induction of mixed chimerism in a patient who received a completely mismatched liver in the absence of stem-cell infusion. In this patient lymphopenia was detected, most likely caused by a viral infection, which persisted for a half year. In the mean time, passenger leukocytes from the graft largely replaced the recipient's leukocytes [71]. The allografts in patients described in the 3 studies have maintained good function for up to 5 years in the absence of immunosuppressive treatment. These studies demonstrate that tolerance induction in these patients was either caused by the stem cell co-transplantation or by replacement of recipient's leukocytes by donor passenger leukocytes due to lymphopenia and clearly show the power of mixed chimerism.

Generation and application of tolerogenic dendritic cells

In vitro generation and characterization of tolerogenic dendritic cells

One approach to promote the tolerogenicity of DC is to suppress their maturation by using anti-inflammatory cytokines or pharmacological agents or by using genetically engineered DC expressing immunosuppressive molecules, as recently reviewed by several groups [72-75]. One class of agents which has shown promising effects on prevention of DC maturation and which is widely applicable are glucocorticoids (GC). GC are among the most potent immunosuppressive and anti-inflammatory drugs currently available and are effective in the treatment of both Th1 and Th2 associated inflammatory diseases, including allograft rejection, rheumatoid arthritis and asthma [76]. The therapeutic effects of GC were initially ascribed to the strong inhibitory effect on T cells. At the moment, however, it is obvious that also antigen presenting cells (APC) are strongly affected by GC. In clinical practice, various derivatives of GC are used and, as far as we know, there are no differences in the functional effects on

DC between the different compounds, although the in vivo efficacy might be different. Most experimental studies have used dexamethasone (Dex), but other GC have shown similar effects.
A consistent finding has been the inhibitory effect of GC on the development of immature DC from monocytes or bone marrow precursors. The GC-treated human monocytes retain a monocyte/macrophage phenotype with high CD14 expression and no expression of CD1a, a typical DC marker, but also lack expression of CD68, a typical macrophage marker [77-79]. However, these cells do express the DC marker DC-SIGN [80]. Upon stimulation of Dexamethasone-treated DC (DexDC), these cells were strongly hampered in their upregulation of co-stimulatory and MHC molecules [77, 79] (**Fig. 5**). This reduced expression was observed with different modes of activation, including proinflammatory cytokines, LPS or CD40L and cannot be explained by a reduced receptor expression.
Next to reduced levels of co-stimulatory molecules, it was demonstrated that activated DexDC secrete reduced levels of proinflammatory cytokines, including IL-6, TNF-α, IL-1β, and IL-12 [77, 79, 81, 82]. Importantly, the same conditions result in an increased production of the anti-inflammatory cytokine IL-10. It is thought that the balance between IL-10 and IL-12 is important for the outcome of T cell activation. As a consequence, DexDC are poor stimulators of allogeneic T cells [77, 79]. Moreover, T cells recovered from this primary stimulation showed a hyporesponsiveness upon secondary challenge. Hyporesponsiveness was observed for proliferative responses, but especially IFN-γ production was strongly suppressed [80]. This points towards a role for cytokines produced by activated DC, since these are as a determining factor for the development of different functional T cell subsets.

In vivo use of immature dendritic cells

Phenotypically, iDC express low levels of co-stimulatory molecules and do not produce cytokines. Consequently, these cells are involved in the generation of tolerance. In transplantation research, iDC have been used for cell-based therapies to prolong allograft survival or even more importantly to induce donor-specific tolerance. Mice pretreated with donor-derived immature DC showed prolonged allograft survival in models of pancreatic islet and heart transplantation [83-85]. Similarly, the regulatory role of DC was shown by adoptive transfer of allopeptide-loaded recipient-derived lymphoid and myeloid DC that were able to prolong cardiac and islet allograft survival in rat transplantation models [86, 87].

In vivo use of Dexamethason-treated dendritic cells

In view of the need to control DC maturation, and the fear that use of immature DC could result in further maturation after administration, these cells have been treated with several of the modulating agents as described above, including Dex. The effect of DexDC has been studied in transplantation models such as the fully mismatched mice combination of C57BL/6 to Balb/c. Pretreatment of recipients with 10^6 Dex treated D1 cells (a DC cell line of C57BL/6 origin) resulted in prolonged skin graft survival from 17 to 35 days, whereas third party skins were rejected with the same speed

[88]. DC used for treatment were treated with Dex from day 6 onwards, whereas LPS was included at day 7 for the last 48 hours. A similar model and approach was used in a heart transplantation model. Pretreatment with alternatively activated LPS-Dex DC generated from donor bone marrow prolonged heart allograft survival from 10 to 20 days [89]. This prolongation was not observed when Dex-DC were used without LPS activation. The requirement for activation of the tolerogenic DC to obtain optimal effects was also demonstrated with other modes of DC modulation (IL-10 + TGF-β)

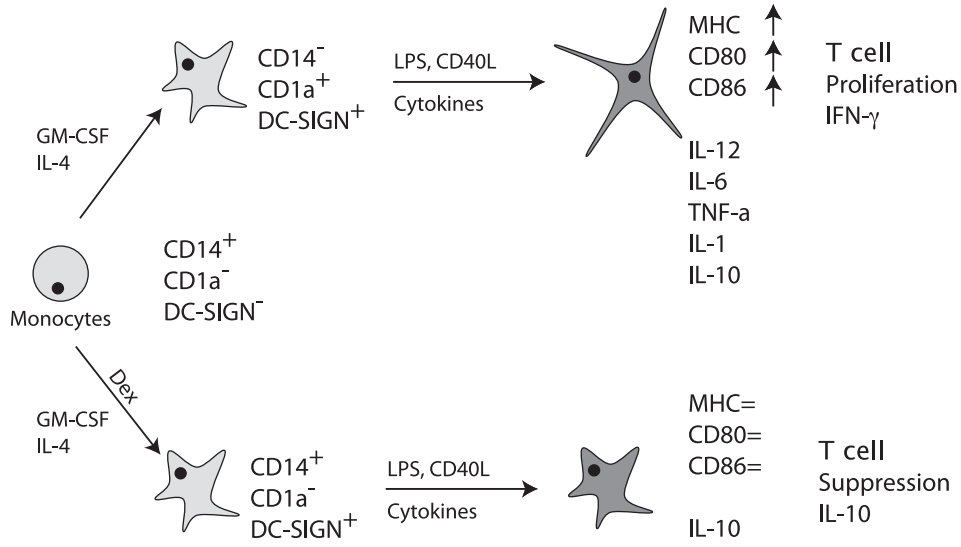

Figure 5. Phenotypic analysis of control DC versus dexamethasone-treated DC.
Human-derived monocytes cultured in the presence of GM-CSF and IL-4 differentiate into immature DC, expressing CD1a and DC-SIGN. Upon maturation signals, including LPS or CD40L, DC upregulate CD80, CD86 and MHC molecule expression levels and secrete IL-1, IL-6, IL-10, IL-12 and TNF-α. In addition, these mature DC have the capacity to induce T cell proliferation and IFN-γ production. In contrast, adding dexamethasone to the human-derived monocytes culture results in DC-SIGN expressing cells, which lack expression of the macrophage marker CD68. Stimulation of these cells with LPS or CD40L does not result in an upregulation of co-stimulatory or MHC molecules. In addition, compared to control DC, a reduced production of proinflammatory cytokines and an elevated level of IL-10 production can be observed. These matured Dex-DC suppress T cell proliferation and IFN-γ production, but induce IL-10 production by the T cells.

[90]. Interestingly, heart allograft prolongation was also obtained when immature DC were combined with Dex treatment in vivo. However, this was only successful when Dex was added after, but not before, the transplantation procedure [89]. In contrast, administration of LPS-Dex-DC of donor origin was not able to prevent rejection of fully mismatched or haploidentical stem cells [91].
In rats tolerogenic DC have been investigated in a August (AUG, RT1^c) to Lewis model. In this model, indefinite renal graft survival was induced using a DC vaccination strategy. In this case, F1 DC (LEW x AUG) were administered to LEW recipients in combination with CTLA4-Ig treatment 10 days before transplantation and recipients were treated with CsA (10 mg/kg/day) for the first 10 days after transplantation. This conditioning resulted in the development of T cell anergy and induction of allo-specific,

self-restricted Treg and a completely normal histology at day 100. Importantly, these results could not be observed when donor DC, treated and applied in the same way, or when mature non-Dex-treated F1 DC were used [92].

Scope of the thesis

The scope of this thesis is to obtain more insight into the effect of dexamethasone-modulated DC in rat kidney transplantation models. Some studies have demonstrated that immature or DexDC have the capacity to prolong allogeneic skin, heart or pancreatic islet survival in murine models. Kidney transplantation in mouse models are very demanding microsurgical procedures, and even in experienced hands only show survival rates between 40 and 70%. Kidney transplantations are therefore preferable performed in rat models. This requires the development of rat specific tools and methods.

To elucidate the effect of tolerogenic DC on allograft survival in rat kidney transplantation models, we assessed the optimal culture conditions to generate bone marrow-derived DC of rat origin. **Chapter 2** describes the phenotype and function of these DC and demonstrates the difference in cytokine production by DC after lipopolysaccharide (LPS) and CD40L stimulation.

Chapter 3 and 4 focus on the effect of tolerogenic donor-derived DC in transplantation models. First, we characterised the tolerogenic properties of DexDC in vitro and demonstrated that DexDC are maturation-resistant. Subsequently, we determined the effect of donor-derived LPS-stimulated DexDC in fully mismatched kidney transplantation models (BN to LEW and DA to LEW) on graft survival and histology (**chapter 3**). In addition, we examined the regulation of the recipient's immune response (**chapter 4**). Although treatment of recipients with LPS-DexDC induced no prolonged graft survival in the examined models, the clear donor-specific T cell hyporesponse and reduced number of infiltrating $CD8^+$ T cells into the graft, demonstrates the regulatory capacity of LPS-DexDC. We think that cell-based therapy can be improved when LPS-DexDC treatment is combined with co-stimulatory blocking antibodies. Co-stimulatory blocking antibodies specifically targeting rat antigens are not widely available. In **chapter 5** we describe the generation and characterisation of a novel anti-rat CD40L blocking antibody, providing new tools for the development of therapeutic strategies to induce long-term allograft survival.

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CHAPTER 2

CD40L stimulation of rat dendritic cells specifically favors the IL-12/IL-10 ratio resulting in a strong T cell stimulatory capacity

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Abstract

Dendritic cells (DC) play an important role in immune responses and have been studied extensively in human and mouse models. CD40 triggering of DC has a pivotal role in their maturation process, obtaining the unique capacity to induce strong CD4 and CD8 T cell activation. Although rat models are frequently used for the understanding of the underlying mechanism of human diseases, relatively little is known about rat DC. To investigate the effect of CD40 triggering on rat DC, we cloned the rat CD40L gene and generated murine fibroblasts with stable expression (L-rCD40L). DC stimulated by L-rCD40L cells exhibited a strong T cell stimulatory capacity, associated with higher amounts of IFN-γ as compared to LPS-stimulated DC. Analysis of cytokine production showed that LPS induced both IL-12 and IL-10 production, whereas CD40L induced high amounts of IL-12, but little IL-10 production by rat DC. This implies that the difference found in T cell stimulatory capacity by the stimulated DC is due to the cytokine profile of the DC at the time of T cell activation.

Introduction

Dendritic cells (DC) are professional antigen presenting cells that play a key role in the initiation and regulation of immune responses. DC have the capacity to capture and process antigens during their immature state. Internalization of foreign antigen triggers maturation of DC and their migration to lymphoid organs. In the lymphoid organs, DC present the antigens to T cells and induce immune responses [1, 2]. Under steady state conditions, DC presenting self-antigens are capable of maintaining tolerance by either inducing regulatory T cells or deletion/anergy of autoreactive T cells [3, 4]. The capacity of DC to induce either an immunogenic or a tolerogenic response may provide therapeutic potential in the form of cellular therapy in various clinical settings. DC inducing immune responses can be a useful tool in cancer research, whereas DC preventing/suppressing immune responses can be an important tool in autoimmune or transplantation research [5-7].

The functional capacity of DC is strongly dependent on its activation/maturation status [8]. At least three different classes of DC activation can be distinguished, including inflammatory cytokines (like IL-1 or TNF-α), TLR ligands (like LPS) or interaction with activated T cells (like CD40-CD40L). Although several of the functional consequences are similar between these modes of activation, and especially LPS activation has been used as the prototypic way of DC maturation, it is also clear that there are major quantitative and qualitative differences. CD40 is broadly expressed on all professional antigen presenting cells as well as many other hematopoietic and non-hematopoietic cells [9]. Cross-linking of CD40 on DC results in strong activation, including high levels of IL-12 production [10, 11] and equips the activated DC with some unique features [12]. Contact with CD40L expressed on activated T cells will mostly take place in lymphoid organs, when part of the DC might have already received signals from TLR ligands and inflammatory cytokines in the periphery. However, under inflammatory conditions CD40L can also be expressed in peripheral organs [13]. The central role of CD40-CD40L in DC biology may contribute to the important consequences of either inhibiting CD40L in cases of unwanted immunity [14] or using agonistic CD40 reagents to promote immunity [15].

The important role of DC in normal homeostasis and disease pathogenesis has been demonstrated in several clinical situations and in many animal models, mostly of mouse origin. Although many experimental models of autoimmunity [16, 17] and transplantation [18, 19] are operational in rats, less information is available on rat DC. It has been demonstrated that GM-CSF is essential to obtain rat DC from bone marrow (BM) [20-22]. This cytokine has also been shown to be essential to obtain DC from human $CD14^+$ cells or mouse BM. Additional studies showed that the presence of IL-4 and Fms-like tyrosine kinase 3 ligand (Flt3L) increased the recovery of rat BM-derived DC [23-25]. It was shown that culturing BM in the presence of various combinations of GM-CSF, IL-4 or Flt3L resulted in different subtypes of DC [23-27].

In view of the central role of DC maturation in immune regulation and the pivotal role of CD40-CD40L in this process, we set out to study the response of rat DC upon activation by CD40L. To this end murine fibroblasts were transfected with a construct containing rat CD40L and the effect on rat DC was examined. Compared to LPS, CD40L was

able to induce a unique cytokine profile, which could explain the high T cell stimulatory capacity of the CD40L stimulated DC. These findings show that CD40L is a powerful stimulator of BM-derived DC.

Materials and Methods

Animals

Seven to twelve week-old male Brown Norway (BN RT1^{n}), Albino Oxford (AO RT1^{u}), Lewis (LEW RT1^{l}) or Dark Agouti (DA RT1^{a}) rats were purchased from Harlan (Horst, the Netherlands). Animals had free access to water and standard rat chow. Animal care and experimentation were performed in accordance with the local ethic committee of Leiden University Medical Center

Generation of rat CD40L expressing L-cells

To generate a cell line expressing functional rat CD40L, the gene was cloned from cDNA of rat splenocytes using a PCR based method. Briefly, primers were designed based on the published sequences of rat CD40L (GI 4102613), spanning the whole gene including start and stop codon and introducing an Xho1 and Not1 site at the 5'and 3' end respectively (table 1). This allowed directional cloning into the expression vector pME-hygro, as also used for the generation of human CD40L transfectants [28]. After amplification using a proofreading DNA polymerase, full length clones were obtained and used for sequencing. A clone showing 100% identity with the published sequence was used for transfections. Murine L-cell fibroblasts were transfected by electroporation using standard techniques and hygromycine was used for selection.

Generation of rat dendritic cells

DC were prepared as described by Grauer *et. al*. [23] with some modifications. In brief, BM was isolated from tibia and femur from BN, AO, DA or LEW rats by flushing the bones with medium. Red blood cells were lysed with lysis buffer containing 155 mM NH_4Cl, 10 mM $KHCO_3$. The remaining BM cells were passed through a 80 µm sieve. Cells were cultured in 6 wells plates (Costar, Cambridge, MA) in 3 ml of RPMI+ medium containing RPMI 1640 (Invitrogen, Breda the Netherlands) 10% heat-inactivated FCS (BioWhittaker, Vervier, Belgium), penicillin/streptomycin (Gibco), Fungizone (Gibco), β-Mercapto-ethanol (50 µM, Merck, Darmstadt, Germany*)*, L-Glutamine (2mM, Gibco) in the presence of rat GM-CSF (2 ng/ml, Invitrogen), rat IL-4 (5 ng/ml, Invitrogen) and human Flt3L (50 ng/ml, kindly provided by Amgen), at a density of $1.5x10^6$ cells per well. At day 2 and 4 all medium was removed and replaced by fresh medium containing cytokines (see above). At day 7, non-adherent and semi-adherent cells were harvested and activated with LPS (500 ng/ml, Salmonella Typhosa, Sigma, St. Louis, MO, USA) or rat CD40L for 24 hours. CD40L-activation was performed with irradiated (80Gy) rat CD40L transfected L-cells (L-rCD40L) in a DC:L-cell ratio 2:1. Non-transfected L-cells (L-orient) were used as control cells. Cells were plated in 6 well plates as described above.

Generation of monocyte-derived human DC

Human peripheral blood mononuclear cells (PBMC) were isolated from buffy coats obtained from healthy blood donors by Ficoll density gradient centrifugation. Cells were positively selected by CD14 MACS microbeads (Miltenyi Biotech, GmBH Berisch Gladbach, Germany), and cultured in RPMI+ medium, human GM-CSF (5 ng/ml, Leucomax®, Novartis Pharma BV, Arnhem, the Netherlands) and human IL-4 (10 ng/ml, Peprotech, RockyHill, USA) at a density of $1.5x10^6$ cells/well in a 6 well plate (Costar).

Flow cytometric analysis

DC were stained with monoclonal antibodies against CD163 (ED2), αβ-TCR (R73), κ-light chain (HIS8), MHC class II (OX6) (all kindly provided by Dr. E. de Heer, LUMC, Leiden the Netherlands), CD40 (Santa Cruz Biotechnology, Heidelberg, Germany), CD80, CD86 (BD, Breda, the Netherlands), OX62 (Serotec, Dusseldorf, Germany) and CD11b/c (OX42, kindly provided by Dr. P. Kuppen, LUMC Leiden, the Netherlands) in Facs-buffer (1% BSA, 1% heat inactivated normal human serum and 0.02% sodium azide in PBS). Binding was visualized using phycoerythrin (PE)-conjugated goat anti-mouse Ig (Dako, Glostrum, Denmark) or goat anti-rabbit Ig (Southern Biotechnology, Birmingham, Alabama, USA).

L-cells were stained for CD40L expression by incubating human CD40-Ig (12 µg/ml) in Facs-buffer, followed by a mouse anti-human IgG (ATCC Hb43, 5 µg/ml American Type Culture Collection, Halifax, GA). Binding was visualized using PE-conjugated goat anti-mouse Ig (Dako, Glostrum, Denmark). The cells were assessed for fluorescence using a FACS Calibur (BD, Mountain View, CA). Data analysis was performed using WinMDI 2.8 software.

Cytokine analysis by ELISA

Cytokines were measured in supernatants of the activated cells. Rat IL-12p40 (Invitrogen), rat IL-10 (R&D Abingdon, UK), rat IFN-γ (BD, Breda, the Netherlands) and human IL-10 (Sanquin, Amsterdam, the Netherlands) were measured following the instructions provided by the supplier.

Allogeneic Mixed Lymphocyte Reaction

Splenocytes were derived from LEW spleen using a 70µm cell strainer (BD). For T cell isolation, splenocytes were incubated with anti-κ light chain (Clone HIS8) and anti-MHC class II (OX6) in a buffer containing 2% heat inactivated FCS and 2.5 mM EDTA. After incubation, cells were washed and incubated with BioMag® goat anti-mouse IgG particles (Polysciences Inc, Warrington, PA, USA). Cells binding to the beads were removed using a magnet (BD) and the negative population was used as responder cells. Stimulator cells were irradiated (40 Gy) and added in graded doses to $1x10^5$ allogeneic T cells in a 96-well U-bottom tissue culture plates (Costar) in RPMI+ medium in a final volume of 0.2 ml/well. Cell proliferation was quantified by incubating the cells during the last 16 hours of day 5 with 0.5 µCi (37kBq) of [methyl-^{3}H] thymidine (NEN™ Life Science Products, Inc., Boston, MA, USA). Results are presented as the mean cpm ±SD obtained from triplicate cultures.

Statistical analysis

Statistical significant differences were determined in Graphpad Prism® using the Mann-Whitney test. Differences were considered significant when $p<0.05$.

Results

Generation and phenotypic analysis of bone marrow derived rat dendritic cells

Rat bone marrow cells were cultured in the presence of GM-CSF and IL-4 with or without the addition of Flt3L. Under the conditions where Flt3L was added to the BM cultures, significantly higher numbers of viable cells were recovered at day 7 of culture (**Fig. 1A**), demonstrating the need of Flt3L to obtain high numbers of DC. Cell cultures were characterised with large clusters of cell aggregates as previously described [23] (**Fig. 1B**).

We studied the phenotype of the cells cultured with Flt3L at day 7 by FACS. The cells were found to have a characteristic immature BM-derived DC phenotype: expressing MHC class II and low levels of the co-stimulatory molecules CD80, CD86 and CD40 (**Fig. 2**). In addition the cells expressed NKR-P1A, CD11b/c and OX62, a marker known to be present on most, but not all, rat DC [29]. In contrast, the macrophage marker (CD163) and T cell markers such as CD4 and TCR were not detectable.

Generation of murine fibroblasts expressing rat CD40L

To be able to study the effect of CD40-mediated activation of rat DC, we utilized a system of transfected cells, as successfully used for human DC [30]. Initial experiments indicated that human CD40L is not efficient for the crosslinking of rat CD40 (data not shown). Therefore, full length rat CD40L was cloned into an expression vector (see materials and methods) and transfected into murine L-cell fibroblasts. After selection with hygromycin, stably transfected cells were selected, showing interaction with human CD40-Ig in a small percentage of transfected cells (5%). This population was enriched

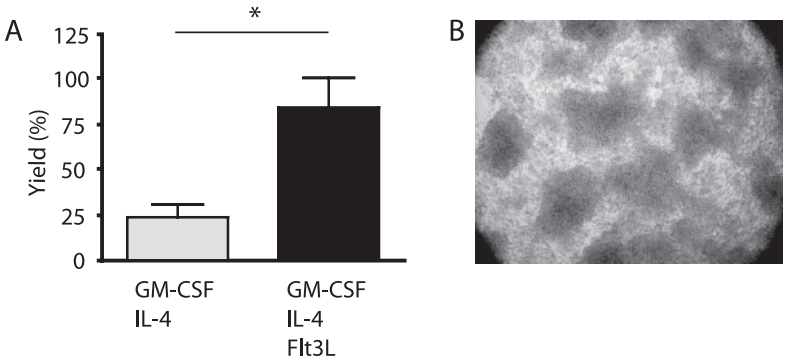

Figure 1. Generation of rat bone marrow derived dendritic cells.
A) Cell yield generated at day 7 of culture in the presence or absence of Flt3L. The input of bone marrow cells at day 0 was set on 100%. Results are the mean ± SD of 5 experiments ($p<0.05$). B) Cell aggregates present on day 5 of culture.

by cell sorting, resulting in a population with homogeneous and strong expression of CD40L (**Fig. 3A**). To confirm the rat origin of the CD40L molecule, we performed RT-PCR with species specific primers. Both in L-rCD40L (rat) and L-hCD40L (human), PCR products of the expected size were specifically amplified (**Fig. 3B**).

Stimulatory capacity of CD40L-DC is stronger than LPS-DC

To study the ability of the generated DC to stimulate allogeneic T cells, immature and LPS- or CD40L-matured DC were used as stimulators in a primary allogeneic mixed lymphocyte reaction (MLR). Increasing numbers of irradiated DC were added to $1x10^5$ allogeneic splenocytes. Both LPS-stimulated DC (LPS-DC) and CD40L-stimulated DC (CD40L-DC) induced a higher proliferation of the responder cells compared to immature DC (Fig. 4A). Remarkably, CD40L-DC appeared to induce a much stronger splenocyte proliferation, whereas LPS-DC and CD40L-DC induced similar amounts of IFN-γ by the responder cells (**Fig. 4B**). Additional experiments with CFSE labelled responder splenocytes showed that B cells were a major proliferating cell population when cultured together with CD40L-DC (data not shown).

Since CD40L is an important stimulator of B cell proliferation, carry over of L-CD40L used for DC maturation might be an explanation. Indeed direct activation of splenocytes with L-CD40L resulted in proliferation but no IFN-γ (**Fig. 5A**). Therefore we purified allogeneic T cells from spleen and used these as responder cells. Purified T cells did not respond directly to L-CD40L stimulation (**Fig. 5B**). In contrast, allogeneic T cells stimulated by CD40L-DC showed both proliferation and IFN-γ production.

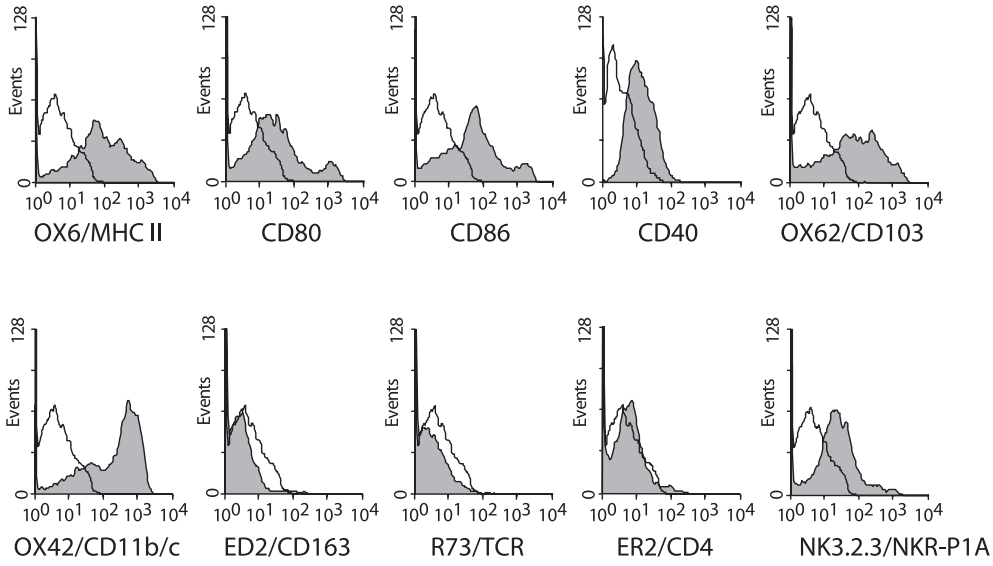

Figure 2. Phenotype of bone marrow derived dendritic cells.
Day 7 cells were stained with various monoclonal antibodies directed against surface molecules and analysed by flow cytometry. Specific staining is represented by filled histograms, open histograms represent basal fluorescence level after staining with only GaM-PE. Results shown are representative for 3-5 experiments.

Based on these findings we compared the stimulatory capacity of LPS-DC and CD40L-DC on purified responder T cells. Both LPS-DC and CD40L-DC induced increased proliferation compared to immature DC, showing highest proliferation on day 5 of culture, without significant difference between the mode of activation (**Fig. 6A**). Importantly, under these conditions where proliferative responses were similar, CD40L-DC induced a much stronger IFN-γ production by responder T cells (**Fig. 6B**).

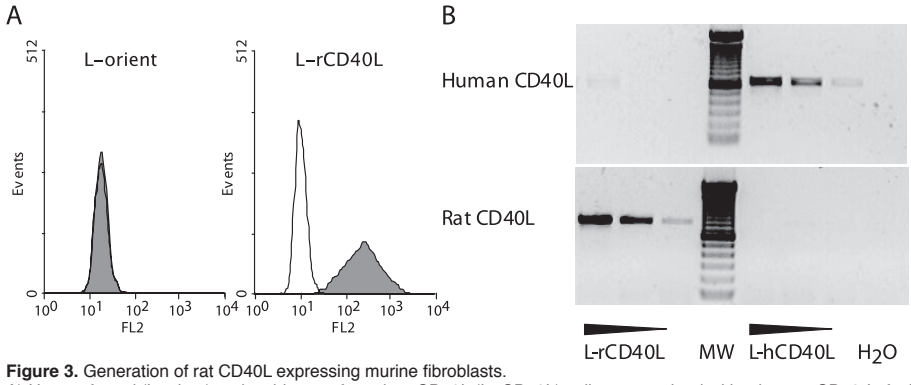

Figure 3. Generation of rat CD40L expressing murine fibroblasts.
A) Untransfected (L-orient) and stable transfected rat CD40L (L-rCD40L) cells were stained with a human CD40-Ig fusion protein and binding was visualized by flow cytometry. Specific staining is represented by filled histograms, open histograms represent basal fluorescence level after staining with only GaM-PE. B) Serial dilutions of cDNA from L- human CD40L or L- rat CD40L were amplified using specific human or rat CD40L primers. A 100 bp ladder was used to determine the sizes of the products.

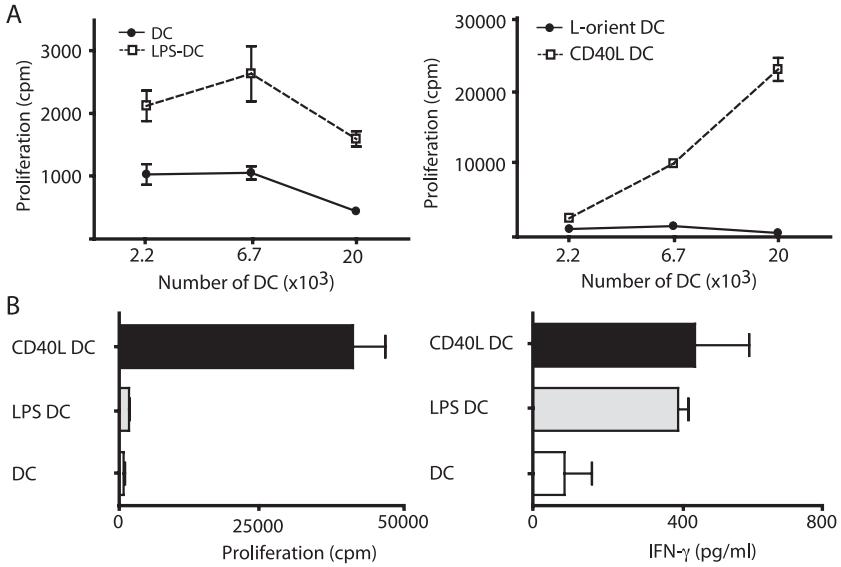

Figure 4. Stimulatory capacity of LPS or CD40L-activated DC of BN origin on allogeneic splenocytes isolated from LEW rats.
A) Splenocytes (1x10^5) were cultured with various numbers of irradiated (40 Gy) BN DC for 5 days. Solid line represents the immature DC, dotted line the stimulated DC showing the results as a mean ± SD of triplicate cultures. This figure is representative of 3 experiments. B) The bars represent 2x10^4 immature DC (white), LPS stimulated DC (gray) or CD40L stimulated DC (black). Cells were pulsed at day 5 of culture with ^{3}H-thymidine during the last 16 hr and IFN-γ production was measured in the supernatant. Results shown are the mean ± SD of three independent experiments.

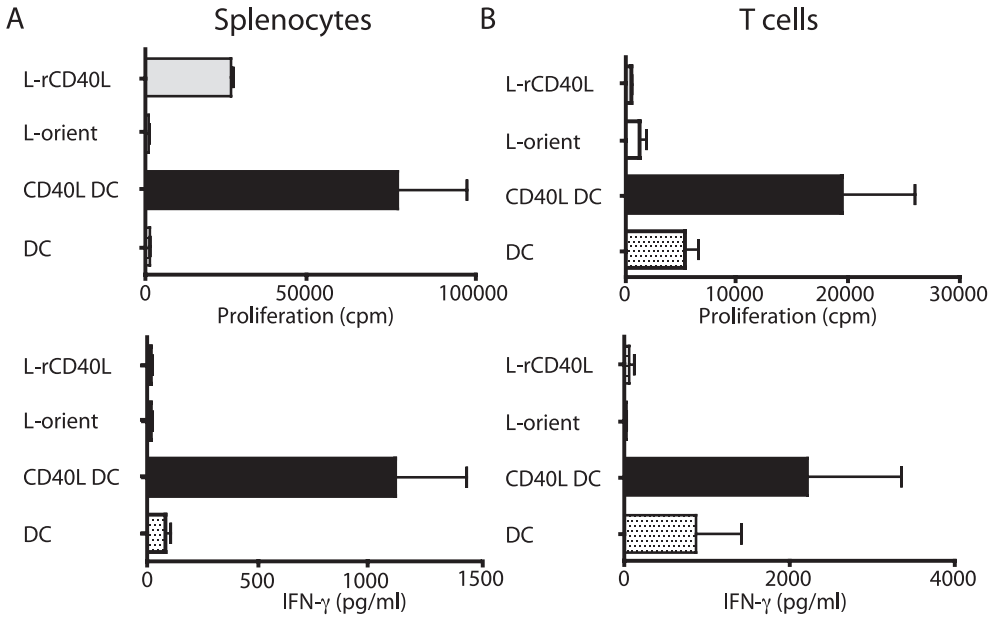

Figure 5. Comparison of the allogeneic response of splenocytes and purified T cells.
Allogeneic splenocytes (A) or T cells (B) were purified from LEW spleen, and $1x10^5$ cells were cultured in the presence of $2x10^4$ DC of BN origin, showing immature DC (dotted bar), CD40L-DC (black bar), $0.5x10^4$ L-orient (white bar) or L-rCD40L cells (gray bar) for 5 days. Cells were pulsed with ^{3}H-thymidine for the last 16 hrs and harvested. IFN-γ production was measured in the supernatant. Results are the mean ± SD of triplicate cultures, representative of three independent experiments.

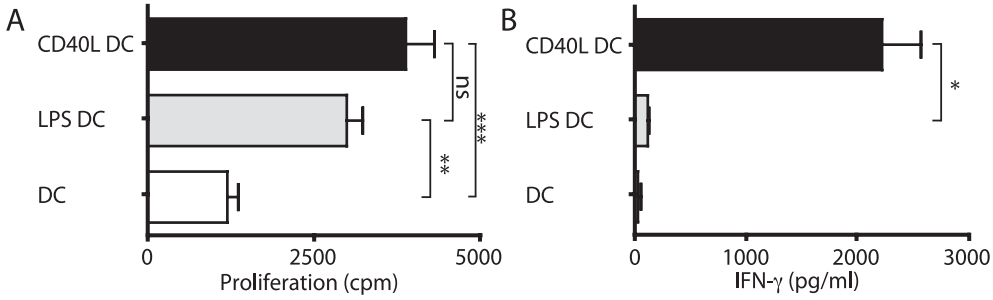

Figure 6. T cell stimulatory capacity of LPS or CD40L-activated DC.
Allogeneic T cells were purified from LEW rat. T cells ($1x10^5$) were cultured in the presence of $2x10^4$ DC of BN origin, showing immature DC (white bar), LPS-DC (gray bar) or rat CD40L-DC (black bar) for 5 days. A) Cells were pulsed with ^{3}H-thymidine for the last 16 hrs and harvested. B) IFN-γ production was measured in the supernatant. Results are expressed as the mean ± SD of three independent experiments (*p<0.05, **p<0.01, ***p<0.0005).

CD40L induces high levels of IL-12 and no IL-10 in contrast to LPS

To explain the superior IFN-γ production of T cells by CD40L-DC, we compared DC maturation induced by LPS and CD40L. In our rat DC cultures, both LPS and CD40L activation only resulted in a limited enhancement of CD86 and MHC class II expression, which was not different between the two modes of DC activation (**Fig.7**). As the balance between IL-12 and IL-10 has been proposed to be important for the IFN-γ production by T cells [31], we investigated the cytokine production by activated DC. Non-activated DC did not produce IL-12 or IL-10, confirming the immature status of the DC. Both LPS and CD40L induced the production of IL-12, but the production induced by CD40L was significantly higher (**Fig. 8A**). Strikingly, when the production of IL-10 was measured in the same supernatants, LPS-DC produced significantly more IL-10 (**Fig. 8A**). Experiments that focused on the effect of CD40L, showed that in 5 out of 8 experiments the IL-10 production was even non-detectable in supernatants of CD40L activated DC, despite the fact that these cells were efficiently activated as shown by their IL-12 production (data not shown).

To rule out the possibility that activation using L-rat CD40L is not suitable for induction of IL-10 production, we made use of the fact that rat CD40L is able to bind to human CD40 (Fig. 3A). In line with previous data using L-human CD40L, also L-rCD40L was able to induce IL-10 production by human monocyte-derived DC at a level comparable to LPS activation (**Fig. 8B**).

To exclude that this specific regulation of IL-12 and IL-10 was strain specific, cytokine production was also investigated from LPS- or CD40L-DC, generated from Lewis, Dark Agouti and Albino Oxford rats. Although the absolute levels of cytokines produced by activated DC of different rat strains were different, they all showed a similar pattern of regulation. LPS induced the production of IL-12 and high levels of IL-10. In contrast, DC produced especially high levels of IL-12 upon CD40L activation, with a limited production of IL-10 (**Fig. 8C**).

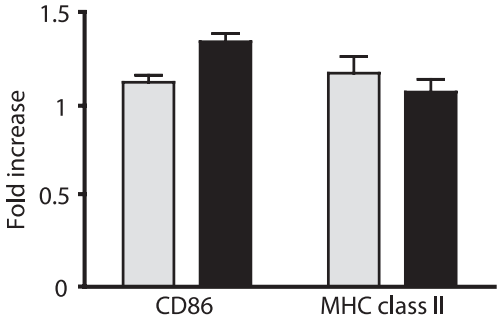

Figure 7 LPS or CD40L stimulation of rat DC results in limited enhancement of CD86 and MHC class II expression.
Day 7 immature DC were stimulated with 500 ng/ml LPS (gray) or CD40L (black) for 24 hr. Expression levels of CD86 and MHC class II were analysed by flow cytometry. Relative increase of expression levels on stimulated DC in relation to immature DC was calculated. Results are shown as the mean ± SD of three independent experiments.

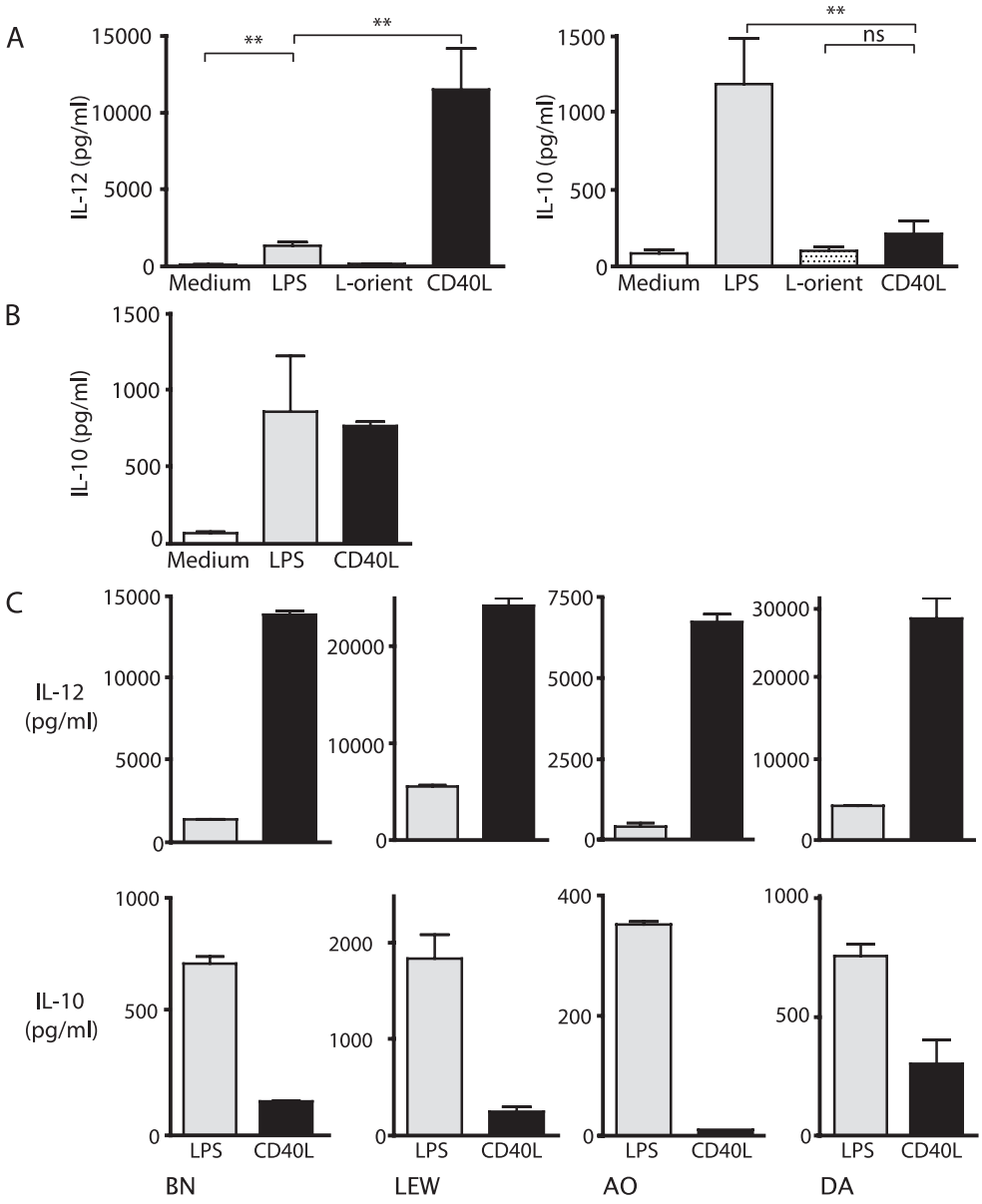

Figure 8. Cytokine profile of LPS or CD40L stimulated rat DC.
A) IL-12 and IL-10 production by BN derived DC cultured in the presence of medium (white), 500 ng/ml LPS (gray), L-orient (dotted) or L-rCD40L (black) was measured in supernatant 24 hr after stimulation using ELISA. Results are the mean ± SD of 3 independent experiments (**p<0.01). B) Cytokine profile of rat CD40L or LPS stimulated monocytes-derived human DC. After 24 hr stimulation of DC by either rat CD40L or 200 ng/ml LPS IL-10 production was measured in supernatant by ELISA. Results are the mean ± SD of three independent experiments. C) Cytokine profile from BN, LEW, AO or DA derived DC in the presence of 500 ng/ml LPS (gray) or L-rCD40L (black) in a 2:1 ratio. Results are the mean ± SD of duplicate cultures representative of three independent experiments. Data depicted for IL-10 and IL-12 production are derived from the same experiment.

Discussion

Since CD40 triggering plays an important role in the induction of immune responses in human and mouse models, in the present study we investigated the response of rat DC upon stimulation with CD40L. Previously it has been demonstrated that various culture conditions result in different rat DC types. Phenotypically DC generated in the presence of GM-CSF, IL-4 and Flt3-L, showed a typical expression profile of NKR-P1A, CD11b/c and OX62, which has been described before [12]. Upon stimulation with CD40L these DC produced high levels of IL-12 and little IL-10 and showed a strong capacity to stimulate allogeneic T cells associated with high levels of IFN-γ.

To investigate the stimulatory capacity of LPS-DC versus CD40L-DC both responder splenocytes and T cells were used in this study. Studies on the stimulatory capacity of rat DC have been performed both with allogeneic splenocytes and with purified T cells as responder cells [23, 24, 26, 32, 33]. Although in many of these cases this may not change the interpretation of the experiments, we observed that CD40L-activated DC induced a strong proliferative response of splenic B cells. A co-transfer of L-CD40L together with CD40L-activated DC into the MLR could explain this, as we found that L-CD40L could directly activate splenic B cell proliferation. In addition, also a direct effect of DC on B cells could contribute to the observed B cell response [34]. This indicates that it is important to use purified responder cells of interest, in our study T cells, to obtain reliable results.

In the present study we could show that the stimulatory capacity of CD40L-DC on allogeneic T cells was much higher than the activation induced by LPS-DC. This was especially true for the IFN-γ production. The cytokine production by activated T cells is influenced by a number of factors, in particular the balance between IL-12 and IL-10 produced by stimulated DC [31]. IL-12 is a potent driver of Th1 cell differentiation, whereas IL-10 blocks IL-12 secretion and impairs the ability of DC to generate Th1 responses [35-37]. The observation that CD40L-DC induce higher levels of IFN-γ production by the T cells compared to LPS-DC can thus be an effect of the cytokine profiles induced by the stimuli.

To elucidate the effect of CD40 triggering on rat DC it is important to use rat CD40L since human CD40L showed no cross-reactivity with rat CD40. Previous studies on the effect of CD40L on rat DC made use of soluble CD40L or a murine CD40L-CD8 fusion protein [23, 32]. Equal amounts of IL-12 production by the DC were detected upon stimulation by soluble CD40L or LPS, whereas IL-10 production was not shown [23]. Studies with the fusion protein showed that CD40L was able to induce more IL-12 secretion by the DC compared to LPS, however, both CD40L-DC and LPS-DC did not produce IL-10 [32]. When studying the effect of CD40L one should keep in mind that the kind of stimulator and the type of cross-linking can influence the strength of the response. It has been shown that the use of recombinant oligomeric CD40L is more effective than soluble CD40L, probably because this can engage multiple receptors and therefore induce a more effective signal [38, 39]. The L-rCD40L cells used in this study express high levels of CD40L on the membrane, potentially allowing a high level of cross-linking of CD40. We found that L-rCD40L was very efficient for the induction of IL-12 production, making the absence of IL-10 even more striking. Studies in which

murine BM-derived DC were stimulated with soluble α-CD40 demonstrated that CD40 triggering resulted in high IL-12 and low IL-10 production [40], reflecting the results found in the present study. On the other hand, human monocyte-derived DC produce both IL-12 and IL-10 after CD40 triggering and have a strong capacity to induce T cell proliferation. Interestingly, when the IL-10 production is neutralized, the T cell stimulatory capacity is enhanced [40].

Although human CD40L does not cross-react with rat CD40 (data not shown) or mouse CD40 [39], rat CD40L is able to bind human CD40. This enabled us to investigate the effect of rat CD40L on human DC. We found that the L-cells used in this study were able to induce IL-10 production by monocyte-derived human DC. Therefore, it is most likely the inability of the rat DC to produce IL-10 after CD40 triggering. Together, these results suggest that TLR4 activation of rat BM-derived DC induces signalling pathways resulting in IL-10 and IL-12 production, whereas CD40L especially favors the signalling pathway leading to IL-12 production. In addition, as both mouse and rat BM-derived DC show little production of IL-10, in contrast to human monocyte-derived DC, it is probably the progenitor cell chosen for the generation of DC that plays a role in this difference. This implies that when DC-based therapy is effective in disease models, it is important to understand the function of these in vitro generated DC. Only when this is known, the use of DC in therapeutic settings can be translated to the human situation.

CD40 signalling in monocyte-derived human DC or BM-derived mouse DC has been shown to induce p38 MAPK, ERK, JNK, Akt and NF-κB activation, which are regulated through various TNF-R associated factors (TRAF) [41, 42]. Until now, most research on the TRAF molecules has been performed in B cells [43, 44] and less is known about the role of TRAFs in DC. So far, TRAF6 has been shown to be important in controlling the IL-12p40 production by DC after CD40 activation [42]. In B cells TRAF3 has an inhibitory role in CD40-induced JNK activation and antibody secretion. Furthermore, it was shown that *Traf3*$^{-/-}$ BM-derived DC were hampered in their IL-10 production after LPS stimulation, whereas the IL-12p40 production increased [45]. The ratio TRAF6 versus TRAF3 recruitment may thus be important for the cytokine profile induced by TLR or CD40 triggering. This suggests that the absence of IL-10 production by CD40L-DC might be due to a signalling defect towards TRAF3. Alternatively, it is also known that IL-10 is able to suppress IL-12 production [46, 47], indicating that the high levels of IL-12 production by CD40L-DC in the present study might be partially related to the absence of IL-10.

In conclusion, LPS and CD40L differentially regulate IL-10 production by BM-derived rat DC. The results presented here show that CD40L-DC produce higher levels of IL-12 and are able to induce high levels of IFN-γ production by the T cells compared to LPS-DC. This suggests that signalling through CD40 involves different signalling pathways and may therefore induce stronger immune responses than signalling through TLR4. Like human and murine models, CD40L also plays an important role in the rat immune response. CD40L can therefore play a key role in therapeutic settings. In cancer research, CD40L stimulated DC can be used as a potential cellular therapy to induce a strong immune response against tumours. On the other hand, in the case of auto-immunity or transplantation it is important to prevent the induction of a strong immune response, which could be achieved by blocking the CD40-CD40L interaction.

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CHAPTER 3

Induction of donor-specific T cell hyporesponsiveness using dexamethasone-treated dendritic cells in two fully mismatched rat kidney transplantation models

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Abstract

Background Dendritic cells (DC) can exert powerful immune stimulatory as well as regulatory functions and are therefore important tools for therapeutic strategies. Dexamethasone (Dex) was previously shown to inhibit DC maturation and to induce regulatory properties both in vitro and in vivo. Here we investigated the immunoregulatory role of DexDC in two different rat acute rejection models of kidney transplantation.

Methods Rat DC were generated from BN and DA bone marrow in the presence of the corticosteroid, Dex. The function of Dex-modulated DC was analysed in vitro and in vivo, using a BN to LEW and a DA to LEW renal transplantation model in the absence of other forms of immunosuppression. T cells of transplanted rats were isolated and restimulated with donor mature DC (either LPS or CD40L activated). T cell responsiveness was analysed by proliferative capacity and IFN-γ production.

Results Stimulation of Dex-modulated rat DC with LPS resulted in normal IL-10 production, whereas synthesis of IL-12 was completely impaired. In accordance, the capacity of LPS-DexDC to stimulate T cell activation was decreased. In both renal transplantation models, treatment with donor-derived LPS-DexDC induced a significant donor-specific T cell hyporesponse. However pre-treatment did not result in a prolonged graft survival.

Conclusions In two fully mismatched kidney transplantation models, donor-derived LPS-DexDC induce a donor-specific T cell hyporesponse. However, in this setting allograft survival was not improved, suggesting an important role for T cells with indirect alloreactivity. Understanding the underlying mechanism involved in the rejection process will improve the development of a cell-based immunotherapy.

Introduction

Organ transplantation is one of the most important therapies for end stage organ failure. In the past, immunosuppressive therapy has led to significant improvement in short term survival rates for solid organ allografts. Unfortunately, the use of non-specific immunosuppression has many adverse side-effects like an increased rate of malignancy and infections [1, 2]. To prevent these side-effects and to improve transplantation outcome, the development of new treatment strategies is necessary. In this respect, development of a therapy, which can induce donor-specific tolerance in the absence of continuous immunosuppression, is the ultimate goal in transplantation research.

Dendritic cells (DC) are bone marrow-derived antigen presenting cells (APC) and have the potential to induce both immunity and tolerance, and are therefore a good candidate for cell-based therapy. Under steady-state conditions immature DC (iDC) have the capacity to internalise antigens. In the presence of pathogens, DC mature and acquire the capacity to induce an immunogenic response. In contrast, when self antigens are endocytosed in the absence of danger signals, DC induce a tolerogenic response [3, 4]. Previous studies have indeed shown that DC inducing tolerogenic responses are phenotypically immature or semi-immature [5].

The effect of iDC as a cellular treatment to induce transplant tolerance has been studied in different transplantation models [6]. Treatment of recipients with donor-derived iDC induced allogeneic T cell hyporesponsiveness and prolonged allograft survival in heart allograft models [7, 8]. These studies indicate that donor-derived iDC can modulate recipient's immune response. Since iDC can easily mature upon danger signals, there is a risk that, once injected into the recipient, donor-derived iDC will mature. Matured donor-derived DC may subsequently interfere or even counteract with tolerance induction. It may therefore be important to use donor-derived DC, which are blocked in the maturation process. Various compounds including Dexamethasone (Dex), IL-10, and Vitamin D_3 can influence the maturation status of DC and these modulated DC were strongly hampered in their T cell stimulatory capacity [9-14]. Different modes of activation, including proinflammatory cytokines, LPS or CD40L, demonstrated a hampered up-regulation of MHC and co-stimulatory molecules on Dex-treated DC (DexDC) [9, 10]. In addition, stimulated DexDC secrete reduced levels of proinflammatory cytokines, such as IL-6, IL-1β, IL-12 and TNF-α [9, 10, 15, 16]. Importantly, secretion of the anti-inflammatory cytokine IL-10 was shown to be increased. As a consequence, DexDC are poor stimulators of allogeneic T cells [9, 10].

The in vivo regulatory properties of modulated DC have been studied in various transplantation models. Contrasting data have been published concerning DC engineered to express IL-10 and TGF-β, demonstrating either their capacity to prolong renal allograft survival [17] or to exacerbate allogeneic heart rejection [18]. In case of Vitamin D_3-treated donor-derived DC it has been shown that allogeneic skin transplants survive longer when recipients are treated with these modulated DC [19]. The effect of Dexamethasone-treated donor-derived DC (DexDC) on transplant survival has been studied more widely in mouse and rat models. Alternatively activated DC (LPS-stimulated DexDC) prolonged transplant survival in a fully mismatched mouse model

[20, 21]. In rats, even more pronounced results were obtained using a semi-allogeneic transplantation model and applying DexDC in combination with CTLA4-Ig and cyclosporin [22]. It was shown that donor DC were not successful in graft prolongation and also that the CTLA4-Ig treatment was of critical importance. This suggests a contribution of re-presentation of injected cells and the necessity to control the indirect pathway of allorecognition.
In the present study we investigated the regulatory properties of donor-derived LPS-DexDC when applied in two different rat models of acute renal allograft rejection. We found that LPS-DexDC induced a donor-specific T cell hyporesponsiveness. However, in the absence of other immunosuppressive treatment, this did not result in prolonged graft survival. To improve DC-based therapies, it is important to understand the mechanisms involved in this rejection process.

Materials and Methods

Animals

Seven to twelve-week-old male Brown Norway (BN; $RT1^n$), Dark Agouti (DA; $RT1^a$) rats were used as donors and Lewis (LEW; $RT1^l$) rats were used as recipients. Rats were purchased from Harlan (Horst, the Netherlands). Animals had free access to water and standard rat chow. Animal care and experimentation were performed in accordance with the local committee of animal experiments of the Leiden University Medical Center.

Generation of rat dendritic cells

Bone marrow (BM) was isolated from tibias and femurs from BN or DA rats by flushing the bones with medium. DC were generated from BM as previously described [23]. Briefly, BM was cultured at a density of $1.5x10^6$ cells per well in 3 ml of RPMI 1640 (Invitrogen, Breda, the Netherlands) containing 10% heat-inactivated FCS (BioWhittaker, Vervier, Belgium), penicillin/streptomycin (Gibco), Fungizone (Gibco), β-Mercaptoethanol (50 μM, Merck, Darmstadt, Germany), L-Glutamine (2 mM, Gibco), rat GM-CSF (2 ng/ml, Invitrogen), rat IL-4 (5 ng/ml, Invitrogen) and human Flt3L (50 ng/ml, kindly provided by Amgen) in 6 wells plates (Costar, Cambridge, MA). At day 2 and 4 medium was replaced by fresh medium containing the cytokines. For the generation of DexDC, 10^{-6} M dexamethasone (Dex, Pharmacy LUMC, Leiden, the Netherlands) was added to the culture at day 4. At day 7 non-adherent and semi-adherent cells were harvested and activated with LPS (500 ng/ml, Salmonella Typhosa, Sigma, St. Louis, MO, USA) or CD40L, using L-rCD40L [23] or L-orient as a negative control. Cells were plated in new 6 well plates at a density of $1.5x10^6$ cells/well in the presence of GM-CSF (2 ng/ml), IL-4 (5 ng/ml) and Flt3L (50 ng/ml) and stimulated for 24 hours.

Kidney transplantation

LEW recipients were injected i.v. with PBS (untreated recipients) or $5x10^6$ LPS-stimulated DC. Seven days after DC infusion kidney transplantations were performed under isoflurane anesthesia. The left kidney from the donor was perfused using cold University of Wisconsin solution and kept on ice. The left kidney from the recipient

was removed and the donor kidney was transplanted in situ. A patch of the donor aorta and the inferior vena cava were anastomosed to the recipient donor aorta and inferior vena cava, respectively. The donor urether was anastomosed end-to-end to the urether of the recipient. Finally the native right kidney was removed. Postoperatively, the recipients received 1 mg/kg body weight of Temgesic (buprenorphine-hydrochlorid; Schering-Plough B.V., Amstelveen, the Netherlands) s.c. for pain relief. Blood samples were collected every other day by tail vein puncture and sera were stored at -80°C. Rats were placed in metabolic cages every other day to collect urine samples. Recipients were sacrificed when low amounts (<2.5 ml) urine was produced o/n. Sera and kidneys were collected. Creatinine levels were measured in sera.

Cytokine analysis by ELISA

Cytokines were measured in the supernatant of the activated cells. ELISAs detecting Rat IL-12p40, (Invitrogen), rat IL-10 (R&D Abingdon, UK) or rat IFN-γ (BD, Breda, the Netherlands) were performed following the instructions provided by the supplier.

Allogeneic Mixed Lymphocyte Reaction

LEW T cells were isolated from splenocytes by depletion of κ light chain and MHC class II expressing cells. In all cases, T cells were isolated from individual rats and tested individually in independent experiments as indicated. Briefly, splenocytes were incubated in phosphate buffered saline containing 2% heat inactivated FCS (BioWhittaker) and 2.5 mM EDTA and HIS8 (anti-κ light chain) and OX6 (anti-MHC class II) (kindly provided by Dr. E. de Heer, LUMC, the Netherlands). Where indicated NKR-P1A expressing cells were depleted by adding NK3.2.3 (kindly provided by Dr. P. Kuppen, LUMC, the Netherlands) to the buffer. After incubation, cells were washed and subsequently incubated with goat anti-mouse beads (Polysciences Inc, Warrington, PA, USA). The negative population was obtained using a magnet (BD) and used as responder cells. Irradiated (50Gy) stimulator cells were added in increasing doses to $1x10^5$ allogeneic T cells in 96-well U-bottom tissue culture plates (Costar) in RPMI 1640 containing 10% heat inactivated FCS in a final volume of 0.2ml/well. Cell proliferation was quantified by incubating the cells during the last 16 hours of cultures with 0.5 µCi (37kBq) of [methyl-^{3}H]thymidine (NEN™ Life Science Products, Inc., Boston, MA,USA). Stimulation Index (SI) was calculated (DC + T cells cpm/medium + T cells cpm). Results are presented as the mean (±SD) cpm obtained from triplicate cultures.

Flow cytometric analysis

LPS-stimulated DC were stained with CD86 (BD, Breda, the Netherlands) and MHC class II (OX 6) (kindly provided by Dr. E. de Heer, LUMC, Leiden the Netherlands). Expression levels were detected by making use of a phycoerythrin-conjugated goat anti-mouse Ig (Dako, Glostrum, Denmark).

The enriched T cell population was stained with antibodies directed to TCR (R73), CD4 (ER2), CD8 (OX8) (all kindly provided by Dr. E. de Heer, LUMC, the Netherlands) and NKR-P1A (NK3.2.3 IgG2b isotype) (kindly provided by Dr. P. Kuppen, LUMC, the Netherlands). Binding was visualized using phycoerythrin-conjugated goat anti-

mouse Ig (Dako, Glostrum, Denmark), or fluorescein isothiocyanate-conjugated goat anti-mouse IgG2b (Southern Biotechnology, Birmingham, Alabama, USA). The cells were assessed for fluorescence using a FACS Calibur (BD, Mountain View, CA). Data analysis was performed using WinMDI 2.8 software.

Statistical analysis

Statistical significant differences were determined with Graphpad Prism® using the Mann-Whitney test. Differences were considered significant when $p<0.05$.

Results

Dexamethasone blocks the maturation process of bone marrow-derived rat dendritic cells

To investigate whether Dex also blocks maturation of rat DC, rat bone marrow-derived DC from BN and DA rats were generated and cultured in the absence or presence of Dex, resulting in CtrDC or DexDC respectively. At day 7, cells were washed and stimulated with LPS, in the absence of Dex, and expression levels of CD86 and MHC class II molecules were determined as well as the cytokine profile. Upon LPS-stimulation, DexDC show reduced levels of CD86 and MHC class II molecule expression compared to CtrDC (**Fig 1A**).

Both BN- and DA-derived CtrDC produced IL-12 and IL-10 upon LPS activation. Stimulation of DexDC with LPS demonstrated that these DC secrete similar levels of IL-10 compared to non-treated DC. However, for both DC derived from BN or DA origin, treatment with Dex completely prevented the LPS-induced IL-12 production (**Fig. 1B&C**). Similarly, also CD40L-induced IL-12 was completely prevented (data not shown).

The T cell stimulatory capacity of CtrDC and DexDC was studied by culturing allogeneic T cells in the presence of increasing amounts of CtrDC or DexDC. LPS activation of CtrDC derived from BN and DA origin showed a strongly increased capacity to induce LEW-derived T cell proliferation (**Fig. 2A**) and IFN-γ production (**Fig. 2B**). In line with the altered cytokine production, both BN and DA derived LPS-DexDC were shown to be impaired in the capacity to stimulate allogeneic T cells (**Fig. 2**).

LPS-DexDC treatment does not prolong graft survival in a fully mismatched kidney transplantation model

The finding that LPS-DexDC were hampered in their capacity to stimulate T cells, prompted us to investigate the immunoregulatory potential of LPS-DexDC as a cellular therapy in transplantation. We used two rat models of acute cellular renal allograft rejection. LEW recipients were pretreated with PBS or $5x10^6$ donor DC, 7 days prior to orthotopic transplantion of either a BN or DA kidney. To circumvent potential interference with the induction of regulatory processes, transplantation was performed in the absence of any other form of immunosuppression. In both models, rejection occurred approximately 6 days after transplantation (for BN kidney at days 6,6,6,7,6, for DA

kidney at days 6,6,6,6). Pretreatment with LPS-DexDC did not result in a prolonged graft survival (for BN kidney recipients at day 6,7,7,7,7, for DA kidney recipients at day 8,6,6,6), as also demonstrated by a similar increase of serum creatinine levels in both groups (**Fig. 3A**). Histological analysis of renal tissue derived from LPS-DexDC treated recipients at the time of rejection demonstrated strong cellular influx and typical signs of acute rejection (**Fig. 3B**). In the DA to LEW model, LPS-CtrDC were used as a control cellular treatment, and as expected did also not result in prolonged graft survival (rejection was observed at day 4,6 after transplantation).

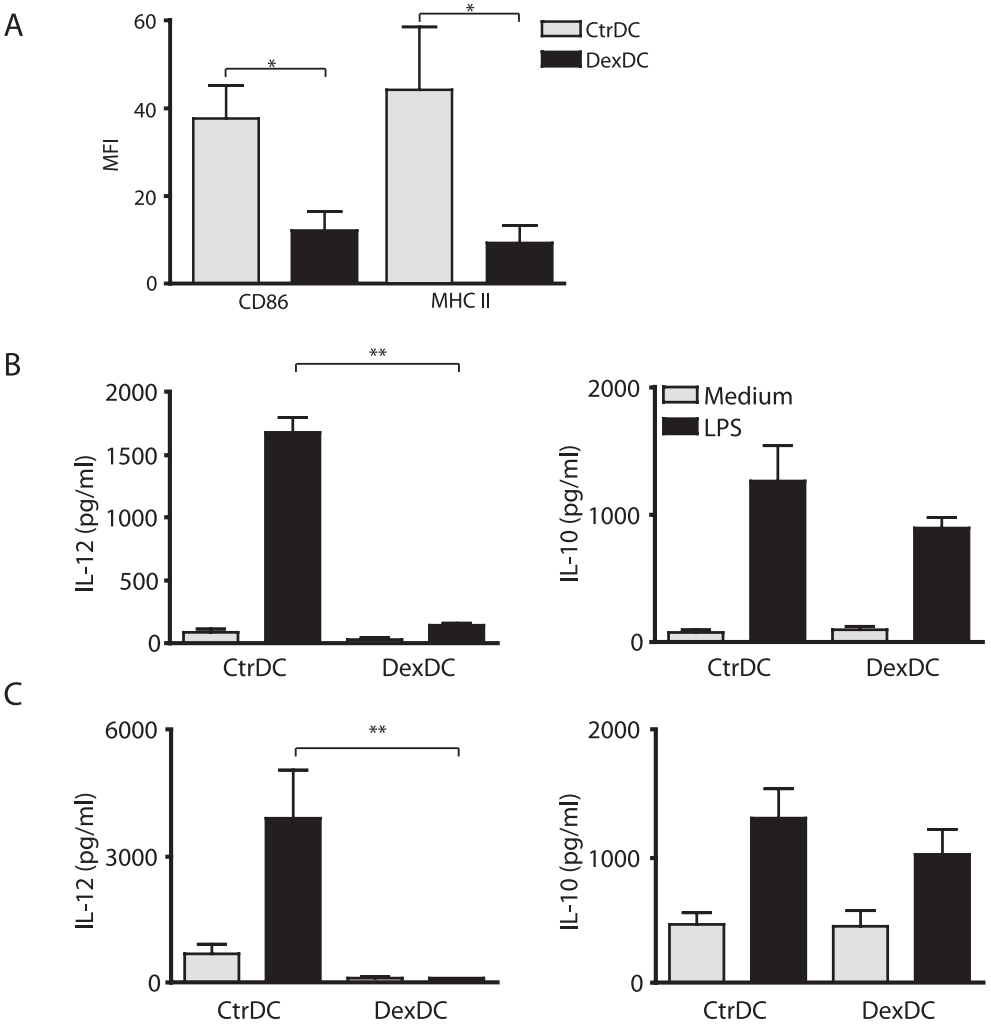

Figure 1. Dexamethasone alters the CD86 and MHC class II expression levels and the cytokine profile of activated rat DC. A) CtrDC or DexDC were generated from BN rats and stimulated with LPS for 24 hours. Expression levels of CD86 and MHC class II were determined by flow cytometry. Results shown are the mean ± SD of five experiments. CtrDC or DexDC derived from BN (B) or DA (C) origin were cultured in the presence of medium (gray) or LPS (black) for 24 hours. IL-12 and IL-10 were measured in supernatant. Results show the mean ± SD of three independent experiments.

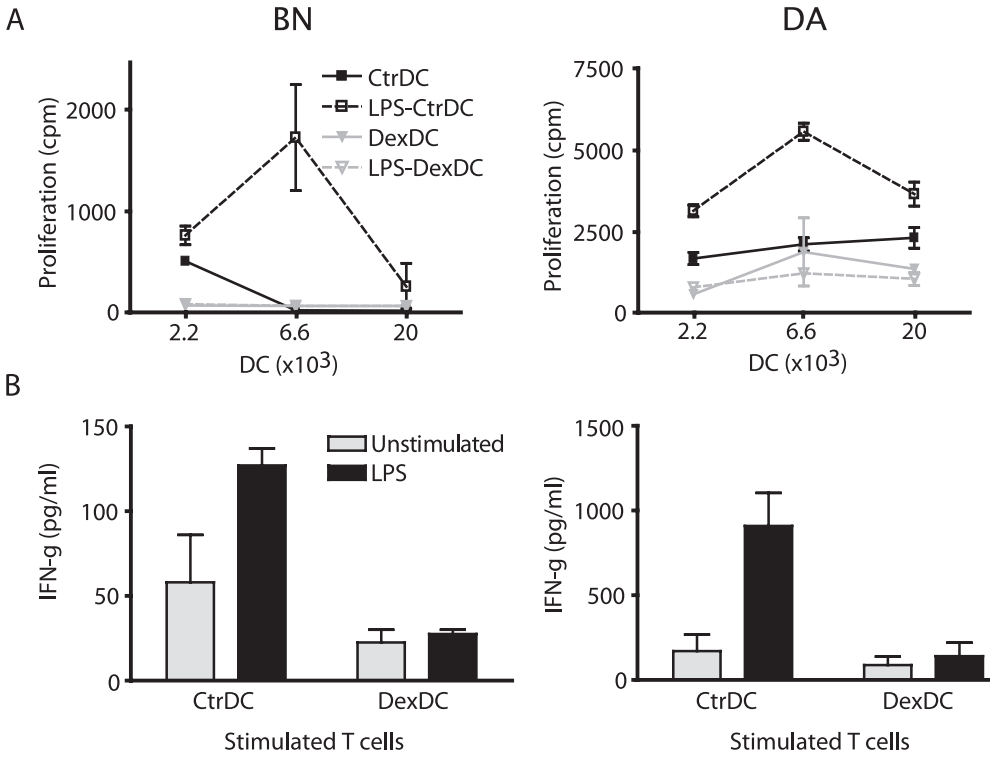

Figure 2. DexDC show a reduced T cell stimulatory capacity.
Increasing numbers of CtrDC (solid black line), LPS-CtrDC (dotted black line), DexDC (solid gray line) or LPS-DexDC (dotted gray line) were cultured with 1x10^5 LEW T cells for 5 days. A) Proliferation was determined by adding ^{3}H-thymidine for the last 16 hours of culture. B) Supernatant of unstimulated (gray) or LPS-stimulated (black) CtrDC or DexDC was used for measuring IFN-γ production. Results shown are mean ± SD of three independent experiments.

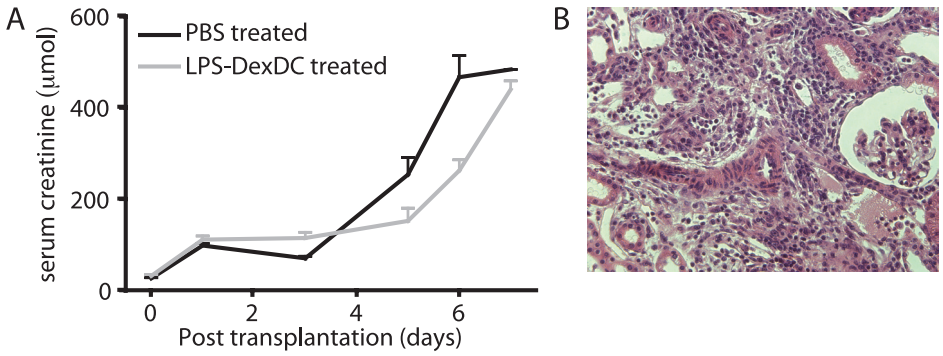

Figure 3. LPS-DexDC treatment does not prolong allograft survival.
Bilateral nephrectomised LEW recipients were transplanted with BN kidney. A) Creatinine levels were measured in serum from PBS treated (black) or LPS-DexDC treated (gray) recipients at various time points. Results shown are the mean ± SD of 5 recipients in both groups. B) Hematoxylin-Eosine staining on paraffin kidney section taken at the time of rejection from LPS-DexDC treated recipients, depicted in color at page 79.

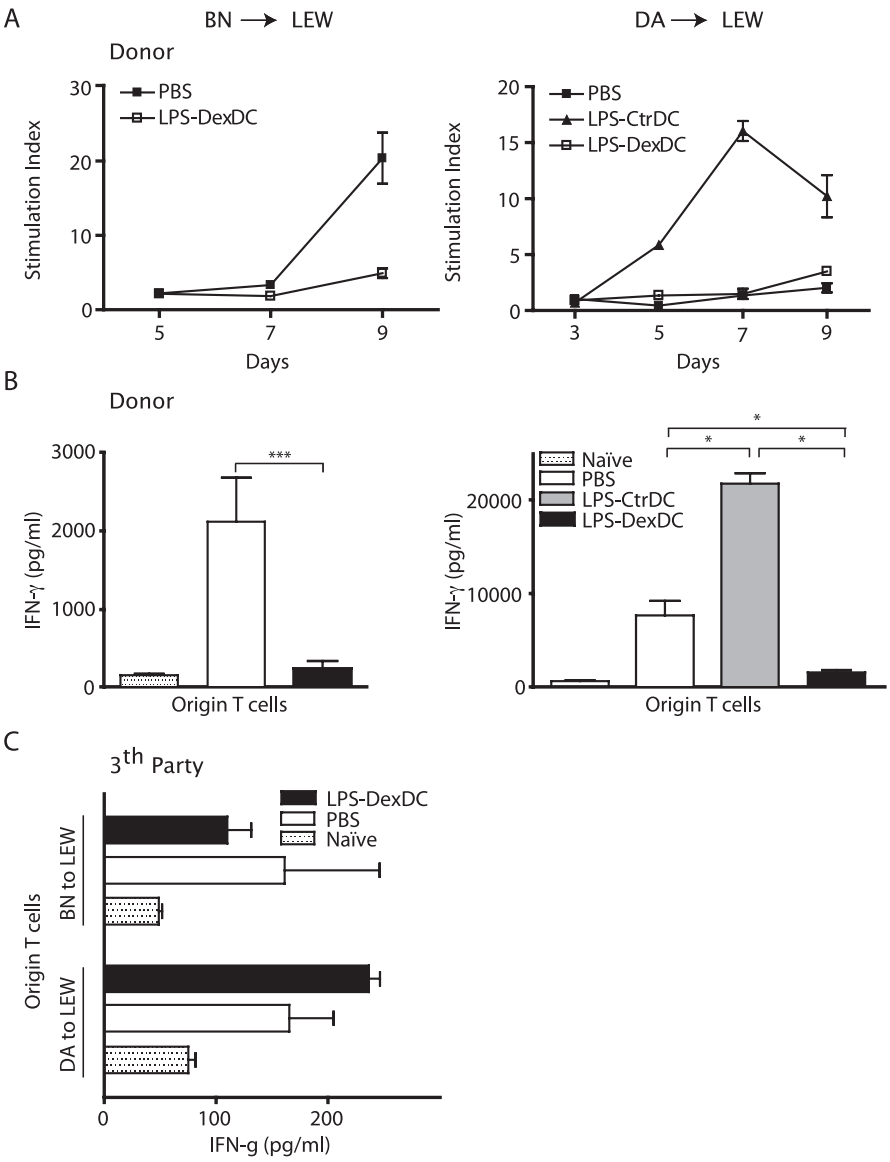

Figure 4. LPS-DexDC induces donor-specific T cell hyporesponse in vivo.
Enriched T cells were derived from naïve untransplanted rats or PBS treated, LPS-CtrDC or LPS-DexDC treated recipients of a BN kidney at the time of rejection. A) Proliferation of T cells derived from PBS treated (black square), LPS-DexDC treated (white square) or LPS-CtrDC treated (black triangle) recipients induced by LPS-stimulated donor DC was determined by adding ^{3}H-thymidine for the last 16 hours of culture and B) IFN-γ production by naïve (dotted), PBS treated (white), LPS-CtrDC (gray) or LPS-DexDC (black) derived T cells was measured in supernatant by ELISA after stimulation with LPS-stimulated donor-derived DC. Results from the BN to LEW model are depicted on the left and on the right are the results derived from the DA to LEW model are shown. C) Enriched T cells derived from naïve (dotted), PBS treated (white) or LPS-DexDC (black) recipients were cultured with LPS-stimulated third party DC (AO origin) and IFN-γ production was measured in supernatant by ELISA. Results shown are the mean ± SD of four independent experiments, except for the LPS-CtrDC group (n=2).

LPS-DexDC induce allogeneic T cell hyporesponsiveness in vivo

Although no effects on graft survival were observed, the fact that no other immunosuppression was used offered the possibility to directly investigate the in vivo regulatory effects of LPS-DexDC. T cells were isolated from spleen of renal allograft recipients and investigated for functional responsiveness to donor antigens. Restimulation of recipient T cells with LPS-matured donor-derived DC showed that the proliferative response of LPS-DexDC pretreated animals was reduced compared to PBS treated recipients (**Fig. 4A**). Although the proliferative responses were low in the DA to LEW condition, it clearly showed that Dex treatment prevented the specific priming observed with pretreatment with mature DC.

Experiments using human DexDC previously demonstrated that these cells especially induced donor-specific hyporesponsiveness at the level of IFN-γ production [24]. Restimulation with LPS-matured donor DC induced high levels of IFN-γ production by recipient T cells derived from PBS treated allogeneic transplantations. In both models, this production is much higher compared to naïve T cells, demonstrating that the transplantation has primed the immune system (**Fig. 4B**). Pretreatment with LPS-DexDC significantly reduced and almost completely prevented the production of IFN-γ by these recipient T cells. In contrast, pretreatment with LPS-CtrDC in the DA to LEW model even further increased levels of IFN-γ production compared to PBS treated recipients (**Fig. 4B**).

To exclude that observed IFN-γ hyporesponsiveness was due to a general immunosuppression, we investigated the effect of stimulation with third party DC. LPS-stimulated DC of AO origin induced comparable levels of IFN-γ production in T cells derived from PBS treated and LPS-DexDC treated transplant recipients (**Fig. 4C**), demonstrating that induction of T cell hyporesponsiveness was donor specific.

Previously we have shown that CD40L-stimulated DC have the capacity to induce a much stronger IFN-γ response compared to LPS-stimulated DC [23]. Comparable to the LPS-stimulated DC condition, T cells derived from PBS treated recipients showed

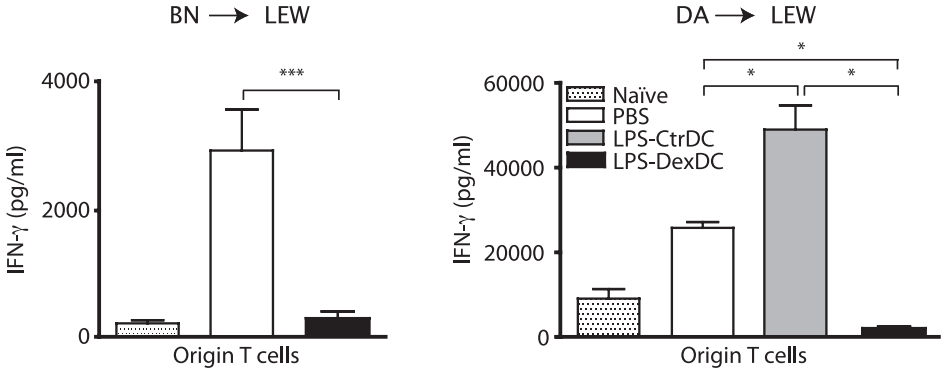

Figure 5. T cell hyporesponse remains after CD40L-stimulated donor DC.
Enriched T cells derived from naïve untransplanted rats (dotted), from PBS treated (white), LPS-CtrDC (gray) or LPS-DexDC (black) treated transplant recipients at the time of rejection were cultured with CD40L-stimulated donor-derived DC and IFN-γ levels were measured in supernatant by ELISA. Results shown are the mean ± SD of four independent experiments, except for the LPS-CtrDC group (n=2).

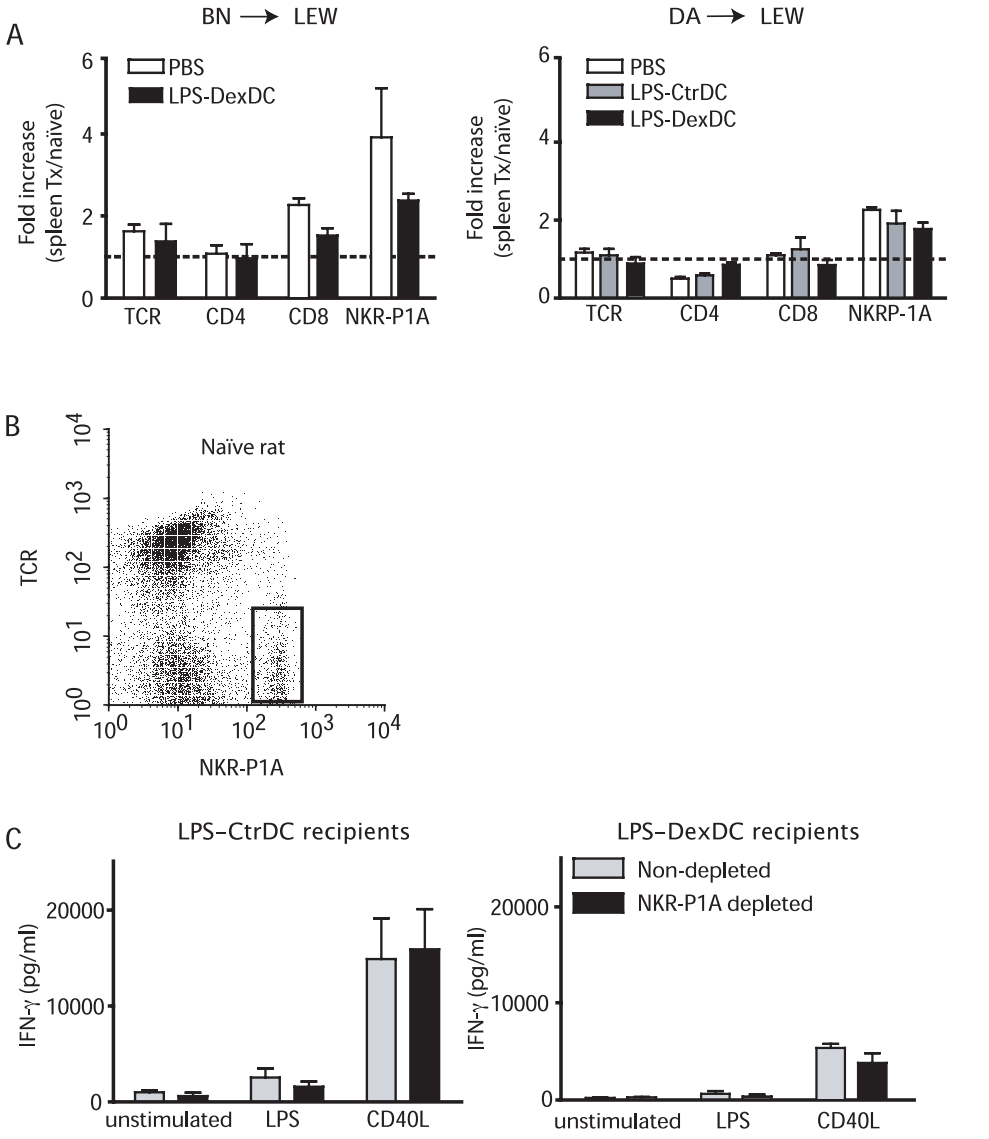

Figure 6. Increased NKR-P1A$^+$ population in recipient's spleen at time of rejection does not influence the IFN-γ response. Spleen was taken from BN and DA kidney recipients when rejection was observed and depleted from B cells and MHC class II$^+$ cells. A) Enriched cells derived from PBS treated (white), LPS-CtrDC (gray) or LPS-DexDC (black) treated recipients were stained for TCR, CD4, CD8 and NKR-P1A expression and detected by flow cytometry. Fold increase of the positive cells in recipient spleen in relation to naïve spleen, derived from untransplanted rats, was calculated, dividing the percentage of stained cells in recipient spleen by the percentage present in naïve spleen. Results shown are the mean ± SD of five independent experiments in BN to LEW model and four independent experiments in DA to LEW model with exception of LPS-CtrDC treated recipients (n=2). B) Enriched T cells derived from naïve rats were stained with TCR (R73) and NKRP-1A (NK3.2.3) and detected by flow cytometry. C) T cell enriched splenocytes derived from LPS-CtrDC or LPS-DexDC treated recipients (non-depleted in gray and NKRP1A depleted in black) were cultured with unstimulated, LPS or CD40L stimulated donor-derived DC. IFN-γ production was measured in supernatant by ELISA. Results shown are mean ± SD of two independent experiments

high production of IFN-γ upon culture with CD40L-stimulated DC, whereas T cells derived from LPS-DexDC treated recipients demonstrated only low levels of IFN-γ production in both models (**Fig. 5**). Furthermore, T cells derived from LPS-CtrDC treated recipients in the DA to LEW model produced higher levels of IFN-γ compared to T cells derived from PBS treated recipients.

Increased NKR-P1A^{+} cells in spleen taken from recipients not involved in IFN-γ response against donor-derived DC

Next to T cells, activated NK cells can contribute to the production of IFN-γ [25]. To investigate this contribution, splenocytes were isolated from either naïve LEW rats or recipients of DA or BN kidney allografts and depleted from B cells and MHC class II^{+} cells. Although the T cell composition of transplanted rats only showed minor changes compared to naïve rats, both models showed an increase in the number of cells expressing the NK cells marker NKR-P1A (**Fig. 6A**). Double staining with the TCR marker R73 and the expression level of NKR-P1A could be used to identify NK cells (**Fig. 6B**).

To determine the contribution of NK cells to the measured IFN-γ production, T cell-enriched splenocyte populations were depleted from NKR-P1A^{++} cells. IFN-γ levels were measured from the non-depleted T cell population and NKR-P1A depleted (at least 75% depletion of NKR-P1A^{++} cells) population after restimulation with donor DC (unstimulated, LPS-or CD40L-stimulated). Both non-depleted and NKR-P1A depleted T cells derived from LPS-CtrDC and LPS-DexDC treated recipients produced similar levels of IFN-γ in all conditions (**Fig. 6C**), demonstrating that NK cells do not play a major role in the T cell response detected in DC-treated recipients.

Discussion

In the present study, we investigated the immunoregulatory effect of donor-derived LPS-DexDC on rat kidney allograft survival. In the absence of any other co-treatments, this did not result in a prolonged graft survival in two different models of acute rejection. However, we established that in both models T cells from LPS-DexDC treated recipients were hyporesponsive to donor antigens while maintaining normal response to third party antigens, especially at the level of IFN-γ production. Although increased numbers of NKR-P1A^{+} cells were observed in the spleen following transplantation, these cells did not contribute to the observed IFN-γ production.

The presence of immunoregulatory cytokines and the absence of proinflammatory cytokines may be critical for the immunomodulatory effect of DC [5]. We observed that under all conditions, Dex treatment completely prevented the production of IL-12, without affecting IL-10 production. The level of IL-12 production is dependent on the mode of DC activation, and is superior upon CD40L activation. Interestingly, this is also reflected by the level of IFN-γ production when these CD40L-activated DC are used in a MLR. Moreover, we found that both in vitro and upon ex vivo restimulation, DC from DA origin, characterized by higher levels of IL-12, are also stronger inducers of IFN-γ production during MLR. Previously we have shown that rat DC produce IL-10

after LPS stimulation, in contrast to CD40L-stimulated DC [23]. This inability to produce IL-10 upon CD40L activation was also observed in DexDC. Therefore, in our in vivo experiments, we made use of LPS-stimulated DexDC, that do produce IL-10.
Administration of LPS-DexDC to recipients 7 days prior to transplantation did not result in prolonged transplant survival. Interestingly, we observed a significant induction of T cell hyporesponsiveness in recipients treated with LPS-DexDC in contrast to PBS treated recipients. In addition, T cells derived from recipients treated with LPS-CtrDC demonstrated an increased response to donor antigens. This confirms that Dex treatment is essential for the induction of donor-specific hyporesponsiveness and prevents priming of the immune system. Although LPS-DexDC possess the capacity to regulate T cell responses, in the present setting this treatment was not sufficient to downregulate all processes involved in renal transplant rejection. Antigen presentation in the transplantation setting can occur through the direct and indirect pathway. Pretreatment with donor cells and restimulation with donor-DC in MLR will both only involve the direct pathway and it is therefore tempting to speculate that the observed rejection might be caused by T cells with indirect allo-specificity.
Administration of donor-derived iDC prior to transplantation has been demonstrated to induce prolonged graft survival in several models [7, 8, 22, 26, 27]. Only a few studies investigated kidney transplantation survival upon DC treatment in rat models [22, 27]. DexDC have been shown to have the capacity to induce tolerance and mediate immune regulation via the indirect pathway and prolong kidney survival [22]. The latter study was performed with a semi-allogeneic rat model, in which LEW rats were treated with Dex-modulated (LEW x AUG) F1-derived DC and received kidneys derived from AUG rats. Recipients were co-treated with cyclosporin A and CTLA4-Ig and a unilateral kidney transplantation procedure was performed. It was shown that donor DC were not successful in graft prolongation and also that the CTLA4-Ig treatment was of critical importance. These experiments clearly demonstrate the importance in regulating the indirect pathway. In two fully mismatched transplantation models, where no additional co-treatment is given to recipients, we confirmed that regulation via the direct pathway is not sufficient. We think this might be a suitable model to investigate additional mechanisms involved in the rejection of allografts. To investigate whether Dex-DC pretreatment might affect humoral immunity, we measured development of donor-specific anti-MHC antibodies in the transplanted rats, pre-treated with Dex-DC or not. Strong antibody responses were detected in both groups, and there was no difference in the quantitative development of IgG responses between the two groups (data not shown).
NKR-P1A expressing cells were shown to be increased in the spleen of all recipients and NKR-P1A has been show to be a marker for NK cells [28]. Activated NK cells produce cytokines, such as IFN-γ, TNF-α or IL-5 and these signals can promote the generation of alloreactive T cells and are therefore associated with graft rejection [29, 30]. However, we show that the NKR-P1A^{++} population present in the spleen at the time of rejection did not contribute to the IFN-γ response against donor-derived DC.
In conclusion, despite the induction of donor-specific hyporesponsiveness by a single treatment of donor-derived LPS-DexDC, no prolonged graft survival was observed in two fully mismatched rat kidney transplantation models. The latter may be expected in such

a stringent model, but this model provides the opportunity to unravel all mechanisms involved in the response on DC-mediated cellular therapy in detail. It will be important to understand the mechanisms involved in the remaining rejection process to improve the effectiveness of such a cell-based therapy.

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CHAPTER 4

Dexamethasone-treated dendritic cells reduce the influx of $CD8^+$ T cells, but not of NK and myeloid cells, in a rat renal transplantation model

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Submitted

Abstract

Background Recently we described that pretreatment with dexamethasone-modulated rat DC (DexDC) did induce donor-specific T cell hyporesponsiveness, but no prolongation of graft survival in two rat models of acute renal allograft rejection. Here, we further characterized this rejection process and performed a quantitative analysis of the cellular composition before and after transplantation.

Methods Lewis recipient rats were pretreated either with PBS or donor-derived (DA) LPS-stimulated control (Ctr) or DexDC prior to kidney transplantation. Renal tissue at the time of rejection was compared with normal rat kidney and analysed for the presence of T cells, myeloid and NK cells using the following markers: TCR, CD4, CD8, CD11b/c, CD68, CD163 and NKR-P1A.

Results Analysis of tissue derived from DC-treated recipients at the time of rejection demonstrated a strong increase of all cell populations investigated. Treatment with LPS-DexDC significantly reduced the infiltration of CD8 T lymphocytes into the graft, but did not affect influx of myeloid cells. Moreover, rejection was also characterized by a strong influx of NK cells, and low numbers of NKT cells and plasmacytoid DC.

Conclusions In the absence of any other co-treatment, pretreatment with LPS-DexDC showed a reduced influx of $CD8^+$ T cells in a fully mismatched rat renal transplantation model. However, the massive infiltration of myeloid cells and NK cells was not significantly reduced, suggesting that additional cellular targets should be controlled to improve the efficacy of cellular therapy.

Introduction

Organ transplantation between non-identical individuals leads to a vigorous allo-immune response. Infiltration of recipient leukocytes is a hallmark of allograft rejection and is initiated and regulated by local production of chemokines. $CD8^+$ cytotoxic T cells have been considered as important effector cells infiltrating the graft, and therefore have been a key target for immunosuppressive strategies [1, 2]. However, it has become clear that the composition of the infiltrate is more complex and also includes $CD4^+$ T cells, B cells, NK cells, macrophages and dendritic cells (DC) [3-8]. Specific composition of the infiltrate, for instance the presence of B cells, has been associated with steroid resistant rejections [9], and has very swiftly opened new avenues for immunosuppressive strategies.

To prevent transplant rejection and to control alloreactive T cells, several strategies have been employed, including immunosuppressive drugs and specific antibody therapies. More recently, it has been postulated that also cellular therapy might be applicable in transplantation [10]. Immature DC (iDC) play an important role in tolerance induction and maintenance, whereas mature DC are critical for immune and inflammatory responses [11]. It has been demonstrated that treatment of recipients with immature donor-derived DC indeed prolonged solid organ graft survival in fully mismatched models [12-14]. Since iDC will encounter maturation signals once infused into the recipient, treatment efficacy and safety may be improved by the use of DC that are fixed in their immature state.

Various human, mouse and rat studies have shown that DC treated with dexamethasone (Dex) remain immature upon activation and show impaired production of pro-inflammatory cytokines as well as a decreased capacity to stimulate T cells [15-21]. Thus Dex-treated DC (DexDC) are promising candidates as cellular therapy. Some in vivo models have demonstrated the positive effect of donor-derived DexDC on transplant outcome in fully mismatched mouse models [22, 23] and in semi-allogeneic rat models [20]. The latter study demonstrated that infusion of Dex-treated (LEW x AUG) F1-DC into LEW recipients prolonged AUG-derived kidney survival.

Recently, in two fully mismatched rat kidney transplantation models, we observed that pretreatment with LPS-DexDC was able to induce donor-specific T cell hyporesponsiveness. However, this did not result in a prolongation of graft survival [24]. In the present study we further characterized this rejection process and performed a detailed analysis of the cellular composition before and after transplantation. We demonstrate that less $CD8^+$ T lymphocytes infiltrate grafts from LPS-DexDC treated recipients compared to LPS-CtrDC treated recipients, whereas no difference was found in the large influx of myeloid and NK cells.

Materials and Methods

Animals

Seven to twelve-week old male Dark Agouti (DA; $RT1^a$) rats were used as donors and Lewis (LEW; $RT1^l$) rats were used as recipients. To quantify passenger leukocytes,

kidneys were also harvested from normal Brown Norway (BN; $RT1^n$) and Albino Oxford (AO, $RT1^u$) rats. Animals were purchased from Harlan (Horst, the Netherlands). Animals had free access to water and standard rat chow. Animal care and experimentation were performed in accordance with the local committee of animal experiments of the Leiden University Medical Center.

Kidney transplantation

Orthotopic kidney transplantation from DA donors to LEW recipient rats was performed as described previously [25]. PBS (untreated) or $5x10^6$ LPS-stimulated DA-derived DC (with or without Dex treatment) were administered i.v. to recipient LEW rats, 7 days prior to the transplantation procedure [24].
Blood samples were collected every other day by tail vein puncture and rats were housed in metabolic cages every other day to collect urine samples. Recipients were sacrificed when low amounts (<2.5 ml o/n) urine was produced. In some experiments, rats were sacrificed at day 3 after transplantation. In all cases kidneys were harvested and used for histological and immunohistochemical analysis.

Histology and immunohistochemistry

Tissue samples were either fixed in 4% paraformaldehyde, embedded in paraffin, sectioned and stained with haematoxylin and eosin (HE), or frozen in isopentane. Acetone fixed frozen sections (3µm) were used to determine the presence of various cell types and were performed in parallel stainings. Endogenous peroxidase activity was blocked using 0.1% H_2O_2 and 0.1% NaN_3 for 30 minutes at room temperature (RT). Slides were washed and blocked with phosphate-buffered saline (PBS), 1% bovine serum albumin (BSA) and 5% heat inactivated normal rat serum (NRS). Next, sections were incubated with primary antibody in a humid atmosphere overnight at RT, using the monoclonal antibodies ER2 (anti-CD4), R73 (anti-TCR), NK3.2.3 (anti-NKR-P1A) (kindly provided by Dr. E. de Heer, LUMC, Leiden, the Netherlands) and OX42 (anti-CD11b/c) (kindly provided by Dr. P. Kuppen, LUMC, Leiden, the Netherlands). Antibody binding was detected with horseradish peroxidase (HRP) labelled goat anti-mouse Ig (DAKO, Glostrum, Germany). After washing, sections were incubated with tyramide-fluorescein isothiocyanate in tyramide buffer (NEN™ Life Science Products, Boston, MA, USA), washed and incubated with HRP-labelled rabbit anti-fluorescein isothiocyanate (DAKO) and developed with DAB (Sigma, St Louis, MO, USA). Sections were counterstained with haematoxylin (Merck, Darmstadt, Germany) and mounted with imsol (Klinipath, Duiven, the Netherlands).
Fluorescent stainings were performed as described above using R73 IgG1, NK3.2.3 IgG2b antibodies (kindly provided by Dr. P. Kuppen, LUMC, the Netherlands) and OX38 IgG1 (anti-CD4)(kindly provided by E. de Heer, LUMC, the Netherlands). Binding of these antibodies was visualised using HRP-labelled goat anti-mouse IgG1 (Nordic, Tilburg, the Netherlands) and tyramide-fluorescein isothiocyanate or using rabbit anti-mouse IgG2b (Nordic) followed by biotine-labelled goat anti-rabbit (Dako), AP-labelled streptavidin (Dako) and fast red TR/naphtol ASTR (Sigma).

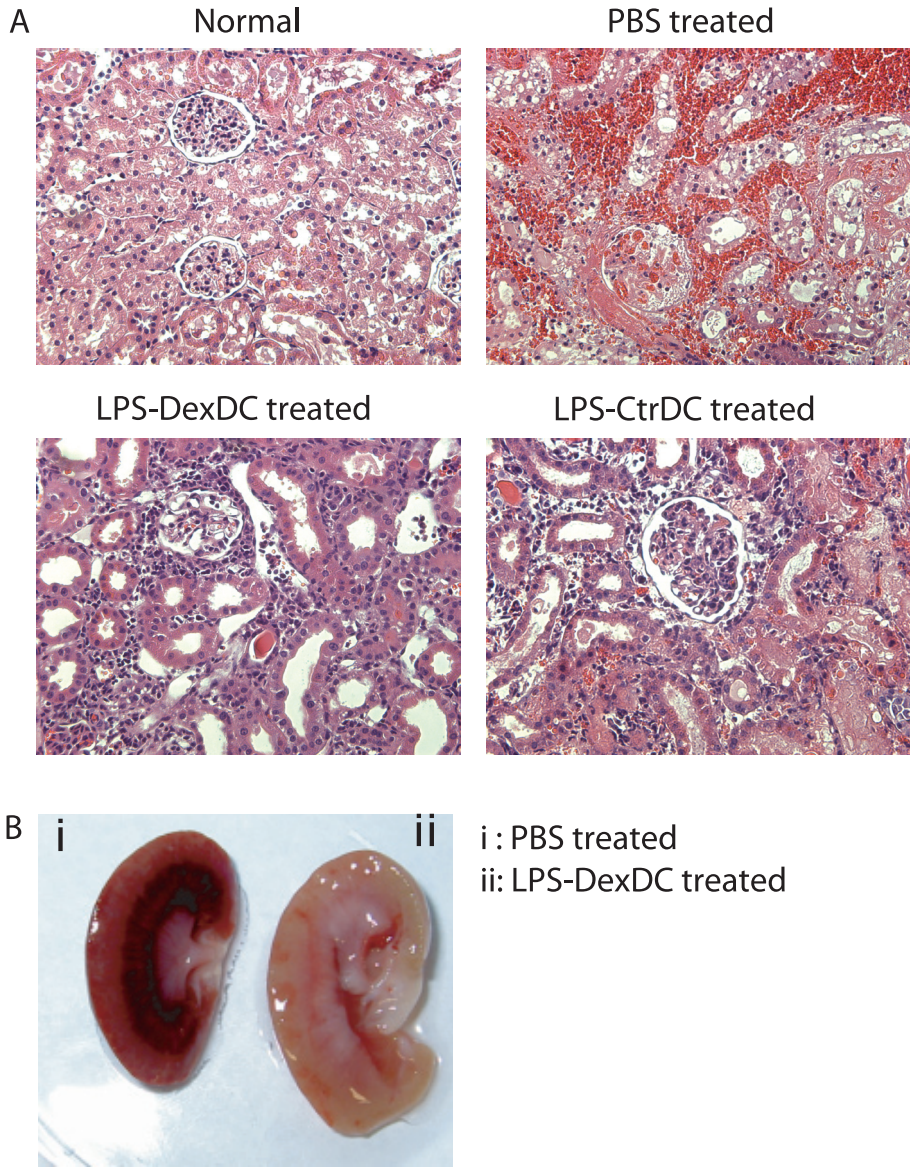

Figure 1. Modification of the rejection process by donor-derived LPS-DexDC
Bilateral nephrectomised LEW recipients were transplanted with DA kidney A) Hematoxylin-Eosine staining on paraffin kidney sections taken from naïve, non-transplanted, DA rats (normal) or from PBS, donor derived LPS-CtrDC or LPS-DexDC treated recipients at the time of rejection. B) Macroscopic image of kidneys at time of rejection derived from donor-derived PBS (i) or LPS-DexDC (ii) treated recipients. Results shown are representative for 4 untreated and LPS-DexDC treated recipients and 2 LPS-CtrDC treated recipients.

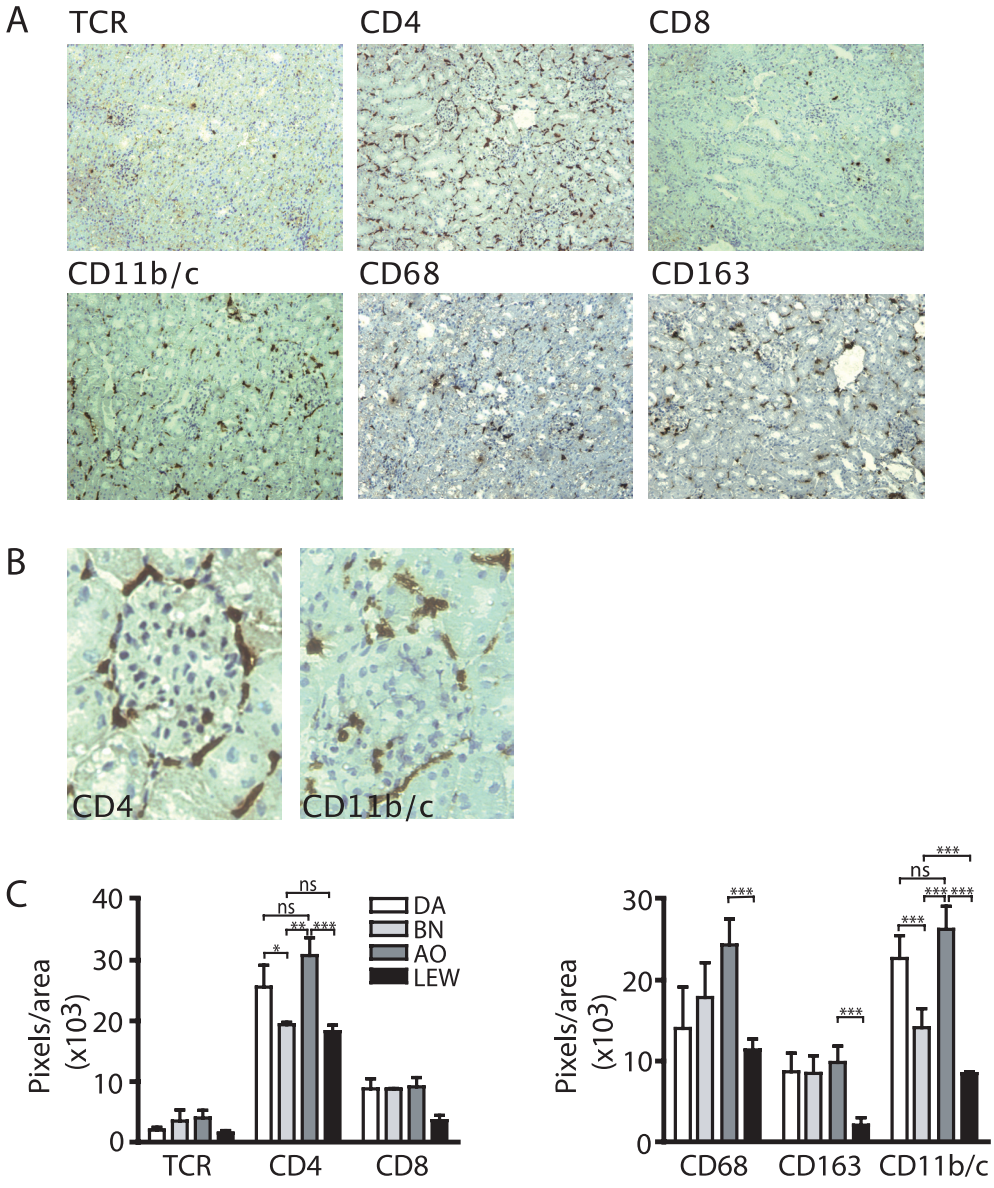

Figure 2. Quantification of resident leukocytes in normal kidneys of different rat strains.
Naïve, non-transplanted, DA, BN, AO and LEW frozen kidney sections were stained in parallel for TCR, CD4, CD8, CD11b/c, CD68 and CD163. A) Representative image (100x) taken from stained DA renal tissue. B) Close-up image of DA renal tissue stained for CD4 and CD11b/c. C) Quantification of positive staining for all examined rat strains. Results shown are the mean ± SD of 3 rats.

Quantitative analysis of stained kidney sections

The amount of positive staining present in normal kidneys or transplanted kidneys was determined by surface area measurement. Pictures were taken from complete biopsies using a 100x magnification. Positively stained areas were measured in 8 to 15 pictures using the digital image analysis program ImageJ (website: http://rsb.info.nih.gov/ij/). For each staining a macro was designed in which the colour signal was split into red, green and blue. The values of the blue channel were used for further analysis. Positively stained areas are expressed in pixels per area.

Statistical analysis

Statistical significant differences were determined using the Student's T test. Differences were considered significant when $p<0.05$.

Results

Kidneys of recipients pretreated with donor-derived LPS-stimulated DexDC show signs of acute cellular rejection

We recently showed that in two rat models of acute renal allograft rejection, pretreatment with tolerogenic donor DC (LPS-activated DexDC) did result in a donor specific T cell hyporesponsiveness [24]. However, both in the BN to LEW and DA to LEW fully mismatched strain combinations, this did not result in a prolonged graft survival. This prompted us to study the rejection process at the level of the renal tissue in more detail.

Kidneys were harvested at the time of rejection and used for histological and immunohistochemical analysis. When comparing paraffin sections, stained with HE, between PBS- and LPS-DexDC-treated animals, we found that the latter were characterized by a typical acute cellular rejection (**Fig 1A**). In contrast, PBS-treated animals showed dilated vessels and an accumulation of erythrocytes. This was compatible with the observation that these kidneys were difficult to perfuse and showed a distinct macroscopic image (**Fig 1B**). This difference was observed both in the BN to LEW and DA to LEW model and this obstruction was not observed in conditions of cellular pretreatment.

To exclude that these results were caused by early technical failure, we sacrificed rats at day 3 after transplantation. Kidneys of both PBS-treated and LPS-DexDC-treated animals showed a normal perfusion and histology, compatible with the normal renal function and serum creatinin levels at this time point (data not shown). Since obstructed kidneys did not allow a quantitative analysis of the rejection process, we concentrated our analysis on the comparison between LPS-CtrDC and LPS-DexDC treated recipients.

Number of resident leukocytes in normal kidneys varies between different rat strains

Before analysis of rejection biopsies, we first characterized the presence of lymphocytes and myeloid cells in normal rat kidneys before transplantation. Cells were stained and quantified in renal tissue derived from 2 rat strains used for transplantation (BN and DA) and compared with kidneys from AO and LEW rats. Frozen sections were stained to quantify lymphocytes, using TCR (R73), CD4 (ER2), CD8 (OX8) or myeloid cells, using CD11b/c (OX42), CD68 (ED1) and CD163 (ED2). As expected, low numbers of T lymphocytes (TCR and CD8) were detected within renal tissue and showed a lymphocytic morphology (**Fig 2A**). In contrast, much higher numbers of CD4 positive cells were detected, which in addition showed a more myeloid appearance. Staining pattern for CD4 was very similar to the staining as observed for CD11b/c, CD163 or CD68 (**Fig. 2A**), typically being present in the tubulointerstitial area and a high frequency of cells surrounding glomeruli (**Fig 2B**). Quantification of positively stained area revealed high numbers of myeloid cells in normal rat kidney.

Comparing the results of the different rat strains demonstrated that normal kidneys derived from LEW rats contain lower numbers of myeloid and lymphoid cells compared to DA, AO and BN rats. Furthermore, between DA, AO and BN rats there was a clear heterogeneity in the number of $CD68^+$, $CD11b/c^+$ and $CD4^+$ cells being present in the kidney (**Fig. 2C**). Interestingly, there was a similar expression profile for all kidneys of $CD11b/c^+$ and $CD4^+$ cells, again pointing towards CD4 as a marker of myeloid cells in the rat kidney.

Grafts from LPS-DexDC treated rats show reduced numbers of infiltrating $CD8^+$ T cells

To address the effect of LPS-DexDC compared to LPS-CtrDC on immune cell infiltration, we characterized and quantified infiltrating cells in renal tissue from LPS-CtrDC and LPS-DexDC-treated LEW recipients transplanted with DA kidneys. Both LPS-CtrDC and LPS-DexDC treated recipients showed a significantly increased number of infiltrating T cells and myeloid cells, compared to the normal kidney, when stained with anti-CD11b/c, CD4, CD8 and TCR monoclonal antibodies (**Fig 3A**). However, recipients treated with LPS-DexDC showed significantly less infiltrating TCR^+ and $CD8^+$ lymphocytes compared to LPS-CtrDC (**Fig. 3B**). No reduction in the number of $CD4^+$ cells was observed, however we were not able to discriminate between $CD4^+$ T cells and $CD4^+$ myeloid cells. Quantification of CD11b/c cells showed that pretreatment with LPS-DexDC did not result in a reduced influx of myeloid cells (**Fig 3B**).

NK cells infiltrate grafts of DC-treated recipients

Since both NKT and NK cells can mediate rejection [26-29], and we observed an increase in NKR-$P1A^+$ (CD161) cells in spleen of transplanted rats [24], we studied the presence of NKR-$P1A^+$ cells in renal tissue. In normal DA kidneys (**Fig 4A**), as well as kidneys from other strains (data not shown), low numbers of NKR-$P1A^+$ cells were detected. Renal tissue obtained from LPS-CtrDC or LPS-DexDC treated recipients at the time of rejection demonstrated a strong increase of NKR-$P1A^+$ cells (**Fig. 4A**).

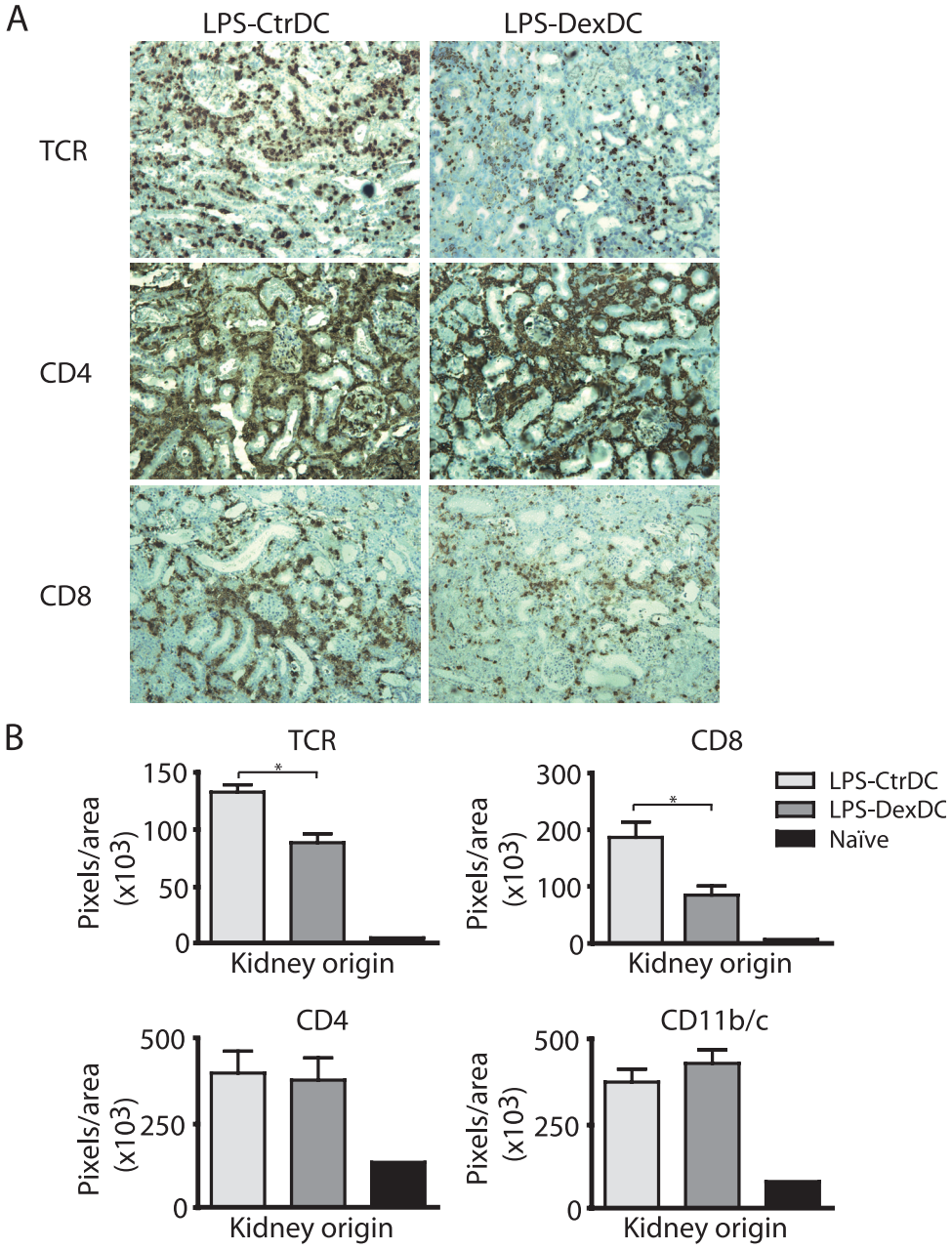

Figure 3. Reduced TCR$^+$ and CD8$^+$ lymphocytes influx into the grafts derived from LPS-DexDC.
LEW recipients treated with donor-derived LPS-CtrDC or LPS-DexDC were transplanted with a DA kidney. A) Frozen kidney sections taken from naïve, non-transplanted DA rats or from LPS-CtrDC or LPS-DexDC treated recipients at the time of rejection were stained for TCR, CD4 and CD8 expression. B) Positively stained areas were quantified and the pixels per area are shown. Results shown are the mean ± SD of 4 LPS-DexDC treated recipients and 2 LPS-CtrDC treated recipients.

Although there was a trend towards a lower influx after pretreatment with LPS-DexDC, this difference was not statistically significant (**Fig 4B**).
NKR-P1A is expressed by NK, NKT or plasmacytoid DC (pDC) [30-32]. To distinguish between NK and NKT cells a double staining was performed, using anti-NKR-P1A and TCR monoclonal antibodies. FACS analysis of splenocytes showed that this combination could be used to discriminate between NK cells and NKT cells [24]. Double staining of renal tissue demonstrated the presence of NKR-P1A or TCR single positive cells and only low numbers of double positive cells (**Fig. 5A**). Semi-quantitative analysis showed that the percentage of infiltrating NK cells was comparable to the amount of T cells infiltrating the graft (**Fig 5B**).
Double staining of CD4 together with NKR-P1A gave us the opportunity to discriminate between NK cells and NKT or pDC [30]. These stainings revealed the presence of many NKR-P1A or CD4 single positive cells and low numbers of CD4/NKR-P1A double positive cells (**Fig. 5C**), thereby excluding a major contribution of infiltrating pDC.

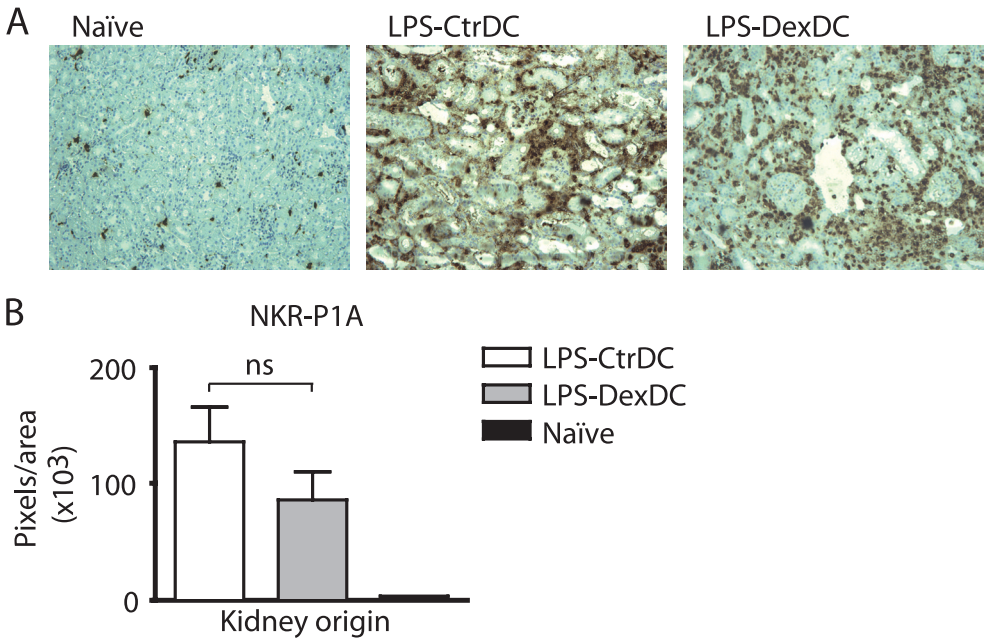

Figure 4. Influx of NKR-P1A$^+$ cells into grafts derived from DC-treated recipients.
LEW recipients were treated with donor-derived LPS-CtrDC or LPS-DexDC and transplanted with a DA kidney. A) Frozen kidney sections were taken at the time of rejection and naïve, non-transplanted, DA kidneys were included as a control. Sections were stained for NKR-P1A. B) The amount of positively stained area was quantified. Results are shown as a mean ± SD of 4 rats in LPS-DexDC group and 2 rats in LPS-CtrDC group.

Discussion

In the present study we demonstrate that pretreatment with LPS-DexDC in a fully mismatched renal transplantation model results in reduced numbers of $CD8^+$ T lymphocytes infiltrating the graft, compared to LPS-CtrDC-treated recipients. In contrast, the large influx of myeloid cells and NK cells, characteristic for this rejection, is similar for both treatment groups. This suggests that LPS-DexDC-treatment has an effect on T cell infiltration but not on myeloid and NK cell infiltration. Previously we showed that T cells isolated from these LPS-DexDC treated recipients showed a donor specific hyporesponsiveness [24]. Nevertheless, these kidneys showed no prolonged survival, and were rejected with typical histological signs of acute rejection. This underlines the importance of further characterizing the cellular infiltrate and determining non-T cell-mediated mechanisms of allograft rejection.

In allograft transplantation, donor passenger leukocytes, which reside in the transplanted organ, play an important role in determining graft outcome [33]. The presence of leukocytes in normal kidneys of various species has been demonstrated previously [4, 5, 34, 35]. These studies showed that the cortex of rat kidneys contains MHC class II^+ cells and $CD68^+$ cells in the interstitium and glomeruli. In the present study we confirmed the presence of a dense myeloid network in the normal rat kidney. Moreover, we showed that CD4 positive cells in normal renal tissue mainly showed a myeloid morphology. The presence of DC subsets has been demonstrated in mouse and human kidney [34, 36], but we have no indications that CD4 expression is characteristic for human renal DC (data not shown). In rats, CD4 has been shown to be expressed by peritoneal macrophages [37], a subset of rat spleen mDC [38] and by rat pDC [30]. More detailed studies will have to be performed to determine whether the CD4 expressing cells in rat kidney are DC, macrophages or both. Quantification of stainings using different markers on renal tissue from various rat strains, all stained in parallel, showed a clear difference in number of donor passenger leukocytes in normal tissue of BN, DA and especially LEW kidneys. However, differences in passenger leukocyte number in normal kidneys from BN and DA donor rats did not affect the observed rejection time in non-treated animals.

Studying the effect of LPS-DexDC treatment on graft outcome, unexpectedly revealed a major macroscopic difference between grafts derived from untreated and donor DC-treated recipients. Grafts from untreated recipients were difficult to perfuse with UW solution, suggesting the presence of an obstruction within the graft. This characteristic was observed in both BN to LEW and DA to LEW acute rejection models investigated. The histologically observed accumulation of erythrocytes within the capillaries of the graft would be compatible with such an obstruction. In xenotransplantation models, this has been described as acute vascular rejection [39], whereas in kidney transplantation models using the same strains as in this study, these findings have not been described [40-43]. It is important to note that early technical failure is an unlikely explanation since function and histology was normal at day 3 post transplantation. A contributing factor might have been the fact that in our procedure, to be able to monitor renal function immediately, we have removed both native kidneys during the transplantation procedure. In addition, and in contrast with other studies using semi-

allogeneic Dex-treated DC [20], we have not used other forms of immunosuppressive agents that will be beneficial to protect against early inflammatory processes. Infiltration of recipient leukocytes is the hallmark of allograft rejection. $CD8^+$ cytotoxic T cells have been considered important effector cells infiltrating the graft, and therefore have been a key target for immunosuppressive strategies [1, 2]. A significantly lower influx of $CD8^+$ T cells was detected in LPS-DexDC treated recipients compared to the LPS-CtrDC treated recipients. Due to the high numbers of infiltrating myeloid $CD4^+$ cells, no estimation could be made on the effect of LPS-DexDC on infiltrating $CD4^+$ T cells. Moreover, functional analysis had shown that donor-reactive T cells in LPS-DexDC treated recipients were hyporesponsive [24]. Nevertheless, no graft prolongation was observed, suggesting that other processes may promote rejection.

In both LPS-CtrDC and LPS-DexDC treated recipients high numbers of myeloid cells and NK cells infiltrated the graft. Both cell types have been implicated in T cell-independent rejection processes, especially in the context of xenografts [44, 45]. Macrophages have several cytotoxic effector mechanisms [46], and also DC with cytotoxic capacity have been described [47]. Both macrophages and DC (myeloid and plasmacytoid) are a prominent feature of human renal allograft rejection [8, 34]. Recent reports also showed NK cell infiltration into solid organ allografts in various models [7, 48-50] , whereas infiltrating NKT cells are rarely described [48]. In the present study we confirmed that acute cellular rejection is characterized by a large number of NKR-P1A positive cells. Using double staining with the TCR or CD4 marker, we also found low numbers of NKT or pDC, but we identified the major part of these infiltrating cells as NK cells.

Compelling evidence of a role for allogeneic NK cells in allogeneic rejection was shown in a cardiac transplant model. In this study MHC-haploidentical ($H\text{-}2^{b/d}$) hearts were tolerated in $CD28^{-/-}$ $H\text{-}2^b$ mice without immunosuppression, whereas fully mismatched hearts ($H\text{-}2^d$) were rejected acutely. Depletion of NK cells in the $CD28^{-/-}$ $H\text{-}2^b$ recipient resulted in the acceptation of both $H\text{-}2^{b/d}$ and $H\text{-}2^d$ hearts [48]. The explanation for the specific rejection of $H\text{-}2^d$ graft is that NK cells in the $CD28^{-/-}$ $H\text{-}2^b$ recipients sense the absence of self $H\text{-}2^b$ molecules on the $H\text{-}2^d$ hearts, but not on the $H\text{-}2^{b/d}$ hearts. Although NK cells alone might not be sufficient to induce rejection, they are certainly able to contribute to this process, possibly by influencing DC and T cells [51-53]. However, the functional diversity of NK cells might be more complex, since recently also tolerogenic activities have been attributed to this cell type [54].

In conclusion, in the absence of any other form of co-treatment, donor-derived LPS-DexDC treatment are able to reduce the influx of $CD8^+$ T cells into the graft. However the rejection process is characterized by a strong influx of myeloid cells and NK cells, and low numbers of NKT and pDC. Influx of these populations is not affected by pretreatment with LPS-DexDC. Therefore, to improve the development of cellular therapies, next to a short course of immunosuppression or blockade of co-stimulatory molecules, it will be necessary to expand our knowledge about the role of myeloid cells and NK cells in transplant rejection.

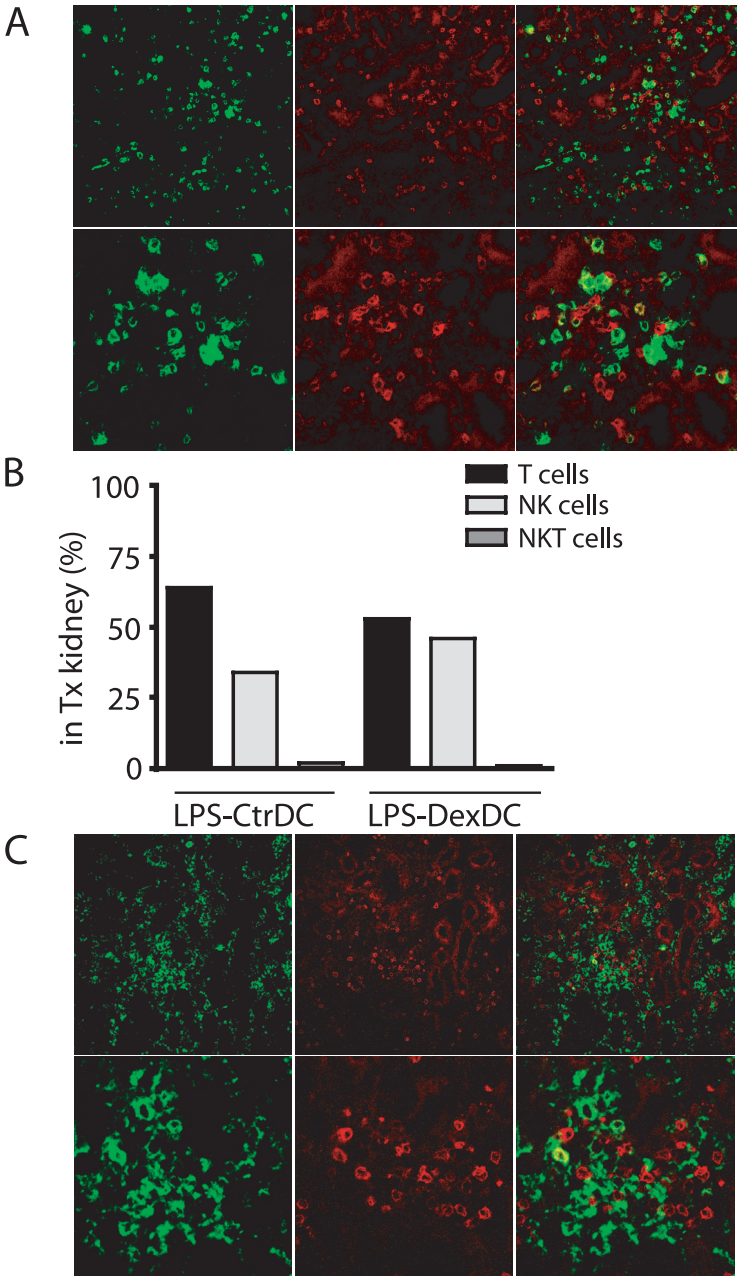

Figure 5. NK cells infiltrate grafts from DC-treated recipients.
Frozen sections from renal tissue were derived from donor-derived LPS-CtrDC and LPS-DexDC treated recipients. A) sections were stained for TCR (green) and NKR-P1A (red) and B) the percentage of single or double positive cells (yellow) was determined. C) Sections were stained for CD4 (green) and NKR-P1A (red). Results shown are representative of 4 rats in LPS-DexDC group and 2 rats in LPS-CtrDC group.

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Color figures

Chapter 3

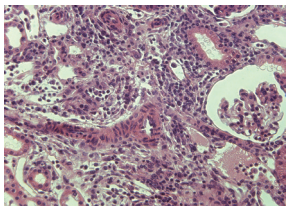

Figure 3B. LPS-DexDC treatment does not prolong allograft survival.
Bilateral nephrectomised LEW recipients were transplanted with BN kidney. Hematoxylin-Eosine staining on paraffin kidney section taken at the time of rejection from LPS-DexDC treated recipients.

Chapter 6

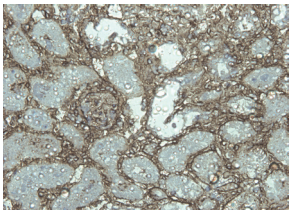

Figure 3A. Increase of C1q in rejected kidneys
Frozen sections from rejected allografts from LPS-DexDC-treated recipients were stained for the presence of C1q.

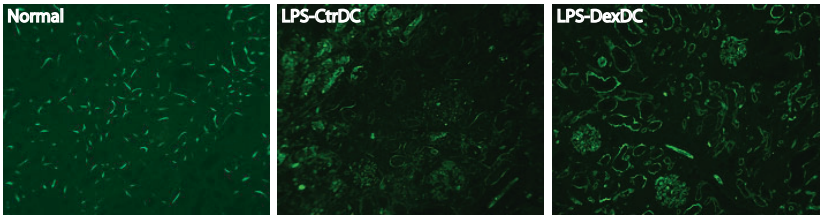

Figure 4A. Increase of C3 in rejected kidneys
C3 staining was performed on normal kidneys and on rejected kidneys derived from LPS-CtrDC or LPS-DexDC treated recipients.

CHAPTER 5

Generation and characterization of a novel anti-rat CD40L antibody with inhibitory activities in vitro and in vivo

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Abstract

The CD40-CD40L interaction plays a critical role in cell mediated immune responses. Blocking this interaction has been shown to be beneficial in the treatment of various diseases studied in murine models. Although rats are widely used to test therapeutic strategies in several disease models, a monoclonal antibody (mAb) to block the CD40-CD40L interaction in rats is not broadly available. In the present study we generated Armenian hamster fibroblasts expressing rat CD40L and used these to generate a novel anti-rat CD40L mAb (AS1). In vitro studies showed that AS1 was able to block CD40L-induced DC maturation and B cell proliferation. Most importantly, in vivo, AS1 inhibited B cell responses in a dose-dependent fashion, as measured by the production of OVA specific antibodies after subcutaneous immunization with OVA. Taken together, AS1 was shown to be a powerful tool to modulate Ag presentation in vitro and in vivo. Elucidating the effect of AS1 in various rat models for human diseases will provide more insight into blocking CD40-CD40L interaction as a therapeutic strategy to prevent human diseases.

Introduction

CD40L (CD154) is a 33 kDa type II transmembrane protein and is a member of the TNF family of ligands. CD40L is expressed on activated T cells and platelets and interacts with CD40, a membrane molecule expressed on various cell types, including B cells, macrophages, dendritic cells, fibroblasts and endothelial cells [1].
Several signals are involved to induce full T cell activation. Resting naïve $CD4^+$ T cells are triggered by MHC/peptide complexes in cooperation with co-stimulatory molecules on the surface of dendritic cells (DC) and the presence of pro-inflammatory cytokines [2, 3]. As a consequence the T cells become activated and express several surface molecules, amongst which CD40L. Binding of CD40L to CD40 results in an enhanced expression of MHC class II and co-stimulatory molecules on DC, resulting in fully mature DC that is capable to induce strong activation of T cells [4, 5].
Studies in various in vivo models have shown that interference of the CD40-CD40L interaction abrogates cell mediated immune responses [6], implying its beneficial effect in therapeutic strategies of several disease models such as transplantation or autoimmunity. In agreement with these observations, studies in mouse models have shown that the anti-CD40L blocking antibody MR1 is able to prevent the induction of experimental autoimmune encephalomyelitis (EAE) [7], collagen induced arthritis [8] and lupus nephritis [9]. Furthermore, blocking the CD40-CD40L interaction in murine transplantation models has been shown to prolong transplant survival [10].
Besides mouse models, also rat models are frequently used to study the underlying mechanism of human diseases. However, relatively little information is available concerning the inhibition of CD40-CD40L interactions in rat disease models. A few studies have demonstrated that transplant survival could be prolonged when recipients were treated with an anti-rat CD40L mAb together with a co-treatment [11-13]. We believe that availability of a reagent that interferes with CD40-CD40L interactions in rats will provide more insight and help in the design of new therapeutic strategies.
The aim of the present study was therefore to generate a CD40L blocking mAb specific for rat. We generated a novel anti-rat CD40L antibody (AS1) and found that this antibody is able to block DC and B cell activation after CD40L stimulation. In vivo, AS1 was shown to be functional because it prevented an induced OVA response. The use of the novel anti-rat CD40L mAb provides the opportunity to obtain more insight into the effects of CD40-CD40L interactions in rat models for human diseases.

Materials and Methods

Animals

Seven to twelve week-old male Lewis (LEW RT-$1A^l$) rats purchased from Harlan (Horst, the Netherlands). Armenian hamsters (*Cricetulus migratorius*) were purchased from Cytogen (Boston, MA). The animals had free acces to water and standard chow. Experimental studies with animals were performed in accordance with local ethical committee guidelines of Leiden University Medical Center.

Generation of anti-rat CD40L mAb

Using electroporation Armenian hamster fibroblasts (ARHO 12, kindly provided by Dr. Hamann, AMC, Amsterdam, the Netherlands) were transfected with the expression plasmid pMErCD40L, which was generated by cloning the full length CD40L gene from cDNA of rat splenocytes using PCR based method. Transfected cells were selected with 400 ng/ml hygromycin B (Invitrogen, Breda, the Netherlands) and stably transfected clones were analyzed for rat CD40L expression by flow cytometry. ARHO-12 cells expressing high levels of rat CD40L (ARHO-rCD40L) were injected into Armenian hamsters. Four injections with $2x10^6$ irradiated cells (50 Gy) in PBS were given at weekly intervals. One day after the last injection, serum was tested for the presence of anti-rat CD40L antibodies by incubating serum on mouse fibroblasts expressing rat CD40L (L-rCD40L). The presence of hamster antibodies (Ab) against rat CD40L were detected by flow cytometry.
Two days later, animals with a strong anti-rat CD40L response were sacrificed and splenocytes were fused with mouse myeloma SP2.0 cells by standard hybridoma technology. Hybridoma supernatants were tested for binding to L-rCD40L cells by flow cytometry. Selected hybridomas were subcloned to obtain a stable clone (AS1). Monoclonal Ab produced by this clone were purfied on a protein A column (Amersham Biosciences AB, Uppsala, Sweden).

Flow cytometric analysis

Cells were harvested and washed in FACS-buffer containing 1% BSA, 0.02% NaN_3. Cells were stained with human CD40-Ig (12 μg/ml) followed by a mouse anti-human IgG (ATCC Hb43, 5 μg/ml, American Type Culture Collection, Halifax, GA, USA) or with purified AS1, hamster anti-mouse CD40L (MR1, kind gift from Dr. Flierman, LUMC, Leiden, the Netherlands), mouse anti-human CD40L (LL48) (10 μg/ml) or sera derived from AS1 injected rats. Binding was visualized with phycoerythrin (PE)-conjugated goat anti mouse Ig (Dako, Glostrum, Denmark) or PE-conjugated goat anti-hamster Ig (Southern Biotechnology, Birmingham, Alabama, USA). Fluorescence intensities were assessed with a FACS Calibur (BD, Mountain View, CA). Data analysis was performed using WinMDI 2.8 software.

Culture of rat dendritic cells

Bone marrow (BM) was isolated from tibias and femurs from LEW rats by flushing the bones with medium. Red blood cells were lysed with lysis buffer (Pharmacy, LUMC, Leiden, the Netherlands). The remaining BM cells were passed through an 80 μm sieve. Cells were cultured in 6 wells plates (Costar, Cambridge, MA) in 3 ml of RPMI+ medium containing RPMI 1640 (Invitrogen), 10% heat-inactivated FCS (BioWhittaker, Vervier, Belgium), penicillin/streptomycin (Gibco), Fungizone (Gibco), β-Mercapto-ethanol (50 μM, Merck, Darmstadt, Germany), L-Glutamine (2mM, Gibco). Rat GM-CSF (2 ng/ml, Invitrogen), rat IL-4 (5 ng/ml, Invitrogen) and human Flt3L (50 ng/ml, kindly provided by Amgen) was added and cells were cultured at a density of $1.5x10^6$ cells per well. All medium was removed and replaced by fresh medium containing cytokines at day 2 and 4. Non-adherent and semi-adherent cells were harvested at day 7 and stimulated with LPS (500 ng/ml, Salmonella Typhosa, Sigma, Zwijndrecht, the Netherlands) or

L-rCD40L in a 2:1 ratio. Non-transfected L-cells (L-orient) were used as control cells. Cells were plated in 6 well plates at a density of $1.5x10^6$ cells/well in the presence of GM-CSF (2ng/ml), IL-4 (5ng/ml) and Flt3L (50ng/ml) and stimulated for 24 hours in the presence of medium, AS1, MR1, LL48, CD40Ig or hamster IgG (Biomeda, Foster city, CA, USA).

Generation of monocyte-derived human DC

Human peripheral blood mononuclear cells (PBMC) were isolated from buffy coats obtained from healthy blood donors by Ficoll density gradient centrifugation [14]. Cells were positively selected by CD14 MACS microbeads (Miltenyi Biotech, GmBH Berisch Gladbach, Germany), and cultured at a density of $1.5x10^6$ cells/well in a 6 well plate (Costar) in RPMI 1640 containing 10% heat inactivated FCS, penicillin/streptavidin, human GM-CSF (5 ng/ml, Leucomax®, Novartis Pharma BV, Arnhem, the Netherlands) and human IL-4 (10 ng/ml, Peprotech, RockyHill, USA).

Cytokine analysis or Ab detection by ELISA

ELISA's for the detection of rat IL-12p40, (Invitrogen) and human IL-12p70 (R&D, Abingdon, Oxon UK) were performed following the instructions provided by the supplier in supernatants of the stimulated cells. To detect a rat anti-hamster antibody response, 96 wells flat bottom plates (NUNC, Roskilde, Denmark) were coated with AS1 (10μg/ml). After 30 min. incubation with dilutions of rat sera, rat anti-hamster levels were detected using a horseradisch peroxidase conjugated rabbit anti-rat IgG (DAKO) and developed with 2,2-Azino-bis-3-ethylbenzothiazoline-6-sulfonic acid.

B cell proliferation

Splenocytes were derived from LEW spleen using a 70 μm cell strainer (BD, Breda, the Netherlands). $1x10^5$ splenocytes were cultured in RPMI+ together with irradiated (80Gy) L-orient or L-rCD40L cells in a 96-wells U-bottom plate (Costar) in a 10:1 ratio. On day 5, cells were pulsed with 0.5 μCi (37kBq) of [methyl-^{3}H] thymidine (NEN™ Life Science Products, Inc., Boston, MA,USA) for 16 hr to quantify cell proliferation. For inhibition studies AS1, MR1, LL48 or hamster IgG was added (5 μg/ml) at day 0. Results are presented as the mean counts per minute (cpm) ±SD obtained from triplicate cultures.

T cell stimulation

LEW splenocytes were incubating with anti-κ light chain (HIS8) and anti-MHC class II (OX6) in a buffer containing 2% heat inactivated FCS (BioWhittaker) and 2.5 mM EDTA. After incubation, cells were washed and subsequently incubated with goat anti-mouse beads (Polysciences Inc, Warrington, PA, USA). The negative population was isolated using a magnet (BD) and used as responder cells. Isolated T cells ($1x10^5$) were stimulated in 96-well flat-bottom plates coated with anti-CD3 (10 μg/ml, G4.18, BD) together with soluble anti-CD28 (1 μg/ml, JJ319, BD) for 24 hours. Cells were harvested and used for FACS analysis.

Blocking capacity of AS1 in vivo

LEW rats (n=5) were treated i.p. with 1.5 mg hamster IgG and 0.75 or 1.5 mg AS1 at day -1 and 3. All rats were immunized on day 0 with 50 μl of an emulsion of ovalbumine in PBS (OVA; 100 μg, Sigma) and an equal volume of complete Freunds adjuvant (Difco, Detroit, USA) subcutaneously in the tail. Serum was obtained at day 0 and at day 10 after immunization to measure IgG anti-OVA levels by ELISA. For this purpose OVA (10 μg/ml) was coated on a flat bottom ELISA plate (NUNC). Serum dilutions were incubated and IgG anti-OVA responses were detected by horseradish peroxidase conjugated rabbit anti-rat IgG (DAKO) and developed with 2,2-Azino-bis-3-ethylbenzothiazoline-6-sulfonic acid.

Statistical analysis

Statistical significant differences were determined using the Mann Whitney test. Differences were considered significant when $p<0.05$.

Results

Generation and characterization of an anti-rat CD40L specific monoclonal antibody

We recently cloned and expressed rat CD40L in murine L-cell fibroblasts and showed the potential of these cells for effective activation of CD40 expressing cells of rat origin. We used these cells to immunize mice but were unable to obtain a specific response recognizing rat CD40L. Alignment of the CD40L sequences showed a high homology between rat and mouse CD40L (95%), possibly explaining the lack of a specific response. We therefore tested the widely used hamster anti-mouse CD40L mAb, MR1, but although there was some recognition of rat CD40L, it was not able to inhibit CD40L induced responses (data not shown).

We thus decided to develop a hamster anti-rat CD40L mAb. Therefore we generated Armenian hamster fibroblasts expressing rat CD40L (ARHO-rCD40L) (**Fig. 1**). Subsequently, Armenian hamsters were immunized with these ARHO-rCD40L cells to generate AS1, a hamster mAb directed against rat CD40L (details described in materials and methods).

Next we examined the binding capacity of AS1, MR1 and LL48 (mouse anti-human CD40L) to various cell lines expressing rat or human CD40L. All cell lines transfected with either rat or human CD40L showed specific binding of human CD40-Ig, which was not observed with non-transfected cell lines. AS1 and MR1 showed binding to rat CD40L, whereas human CD40L was not recognized by either mAb. Monoclonal Ab LL48 exclusively bound to human CD40L (**Fig. 1**).

Functional characterization of AS1

To test the functional consequence of binding of AS1, rat DC were stimulated with rat CD40L in the presence or absence of various concentrations of AS1 for 24 hours. DC stimulated by rat CD40L showed strong production of IL-12. AS1 was able to

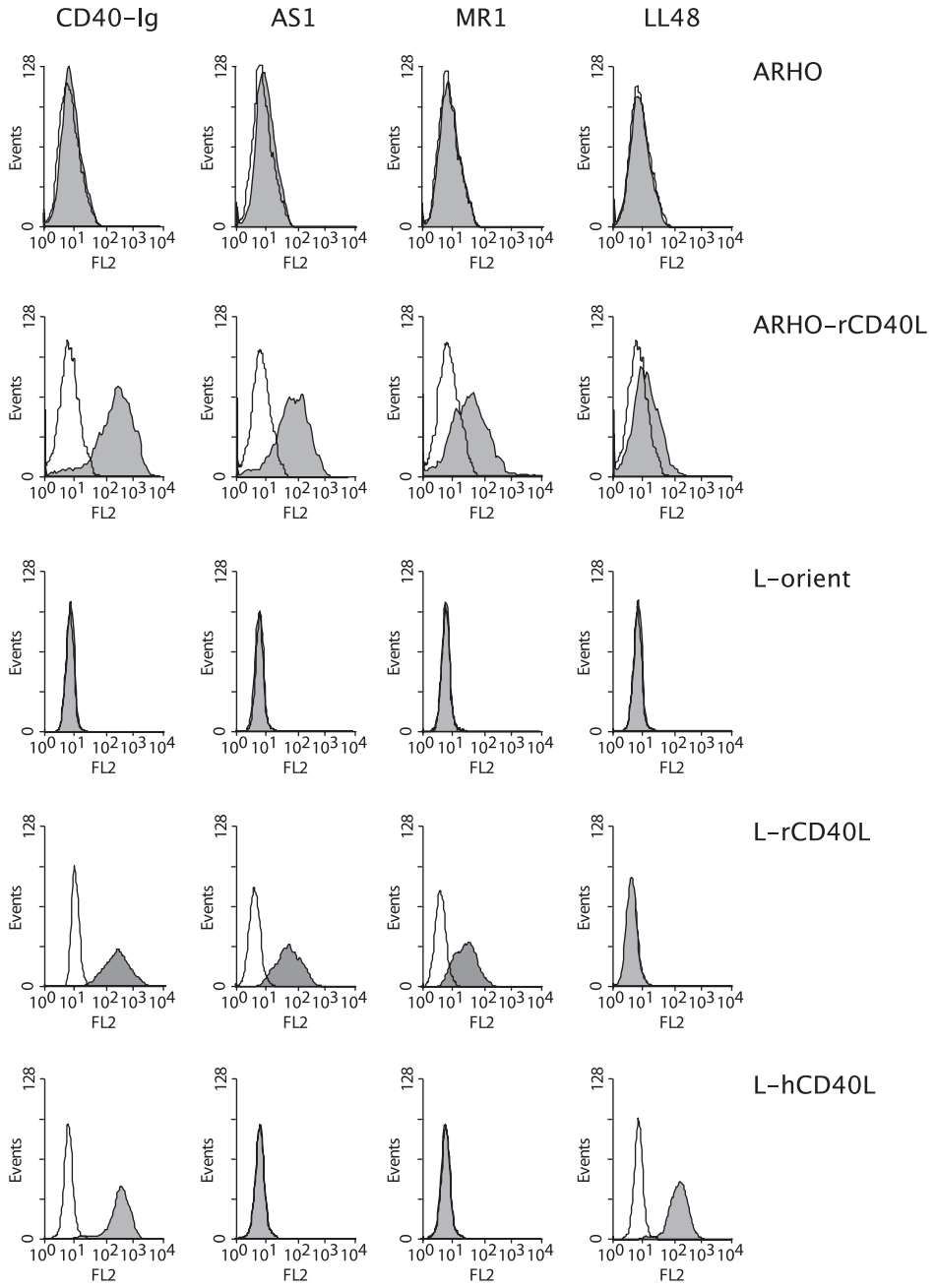

Figure 1. AS1 and MR1 bind to rat CD40L.
Binding of CD40-Ig, AS1, MR1 and LL48 was examined on ARHO-rCD40L, L-rCD40L expressing rat CD40L or L-hCD40L expressing human CD40L. As a control, non-transfected cell lines (ARHO or L-orient) were used. Binding capacity was detected by flow cytometry. Shown is a representative experiment out of 4.

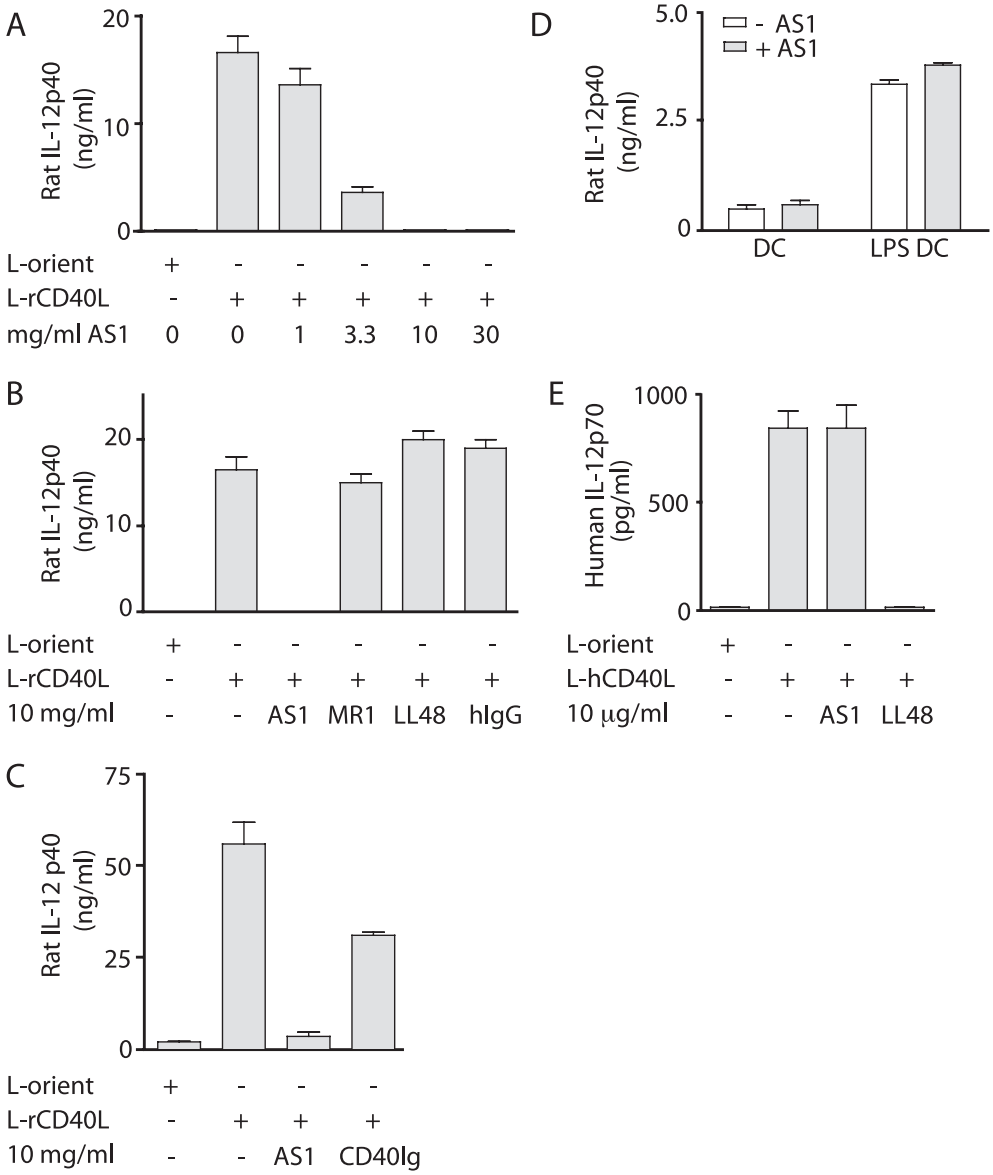

Figure 2. Only AS1 inhibits IL-12p40 production by CD40L stimulated DC.
A) LEW derived DC were stimulated by CD40L and cultured in the presence of various AS1 concentrations. L-orient was used as a negative control and L-rCD40L as a positive control B) LEW-derived DC were cultured in the presence of L-rCD40L and in the presence of 10 μg/ml AS1, MR1, LL48 or hamster IgG. C) LEW-derived DC were cultured in the presence of L-rCD40L in the presence or absence of 10 μg/ml AS1 or CD40Ig. D) LEW-derived DC matured with 500 ng/ml LPS were cultured in the absence (white bars) or presence (gray bars) of AS1. E) Human DC were stimulated by L-hCD40L in the presence or absence of AS1 or LL48 (10 μg/ml). Rat IL-12p40 or human IL-12p70 production was determined by ELISA. Results are mean ± SD of duplicate cultures representative of three independent experiments

completely inhibit this IL-12 production in a dose dependent fashion. Maximal inhibition of IL-12 production was observed with 10 μg/ml of AS1 (**Fig. 2A**). Similar concentrations of MR1, LL48 or hamster IgG were unable to inhibit the IL-12 production by CD40L-stimulated DC (**Fig. 2B**). In addition, human CD40-Ig partially inhibited the IL-12 production by DC when cocultured with L-rCD40L cells (**Fig. 2C**).

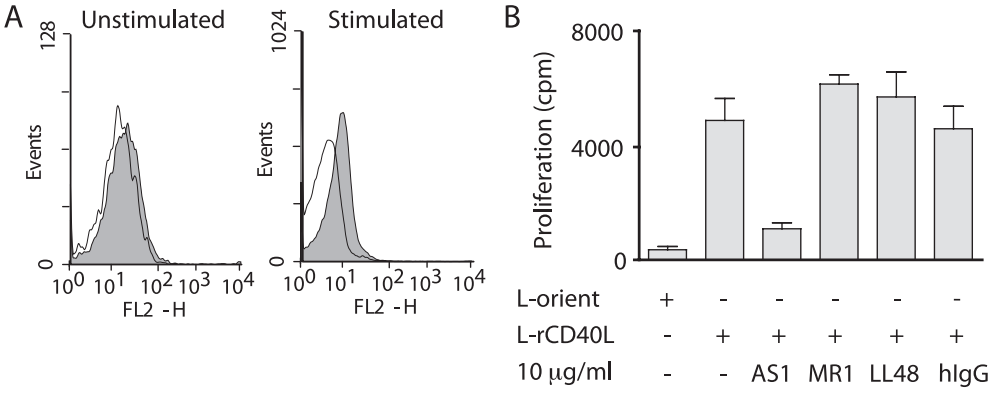

Figure 3. Inhibition of CD40L-induced B cell proliferation by AS1.
A) LEW T cells (1x10^5) were stimulated in 96-well flat-bottom plates using plate-bound anti-CD3 and soluble anti-CD28 for 24 hours. Stimulated T cells were analyzed for their binding of AS1 using flow-cytometry, as a control unstimulated T cells were used. Specific staining is represented by filled histograms, open histograms represent basal fluorescence level after staining with only PE-labelled goat anti-hamster secondary antibody. B) LEW-derived splenocytes (1x10^5) were cultured for 5 days in the presence of irradiated L-orient or L-rCD40L in a 10:1 ratio in the presence or absence of 10 μg/ml AS1, MR1, LL48 or hamster IgG. Proliferation was measured by incorporation of ^{3}H thymidine at day 5. Results shown are the mean ± SD of three experiments

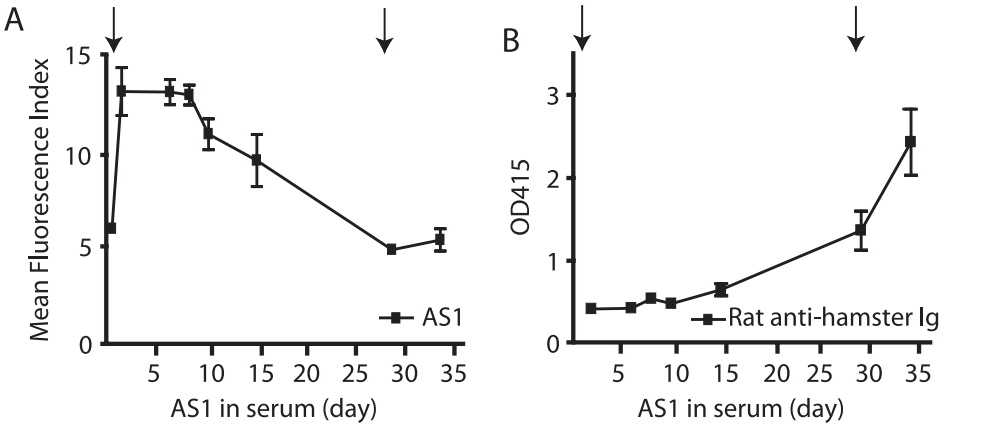

Figure 4. High levels of AS1 in serum during the first week after injection.
A) Sera derived from LEW rats (n=4) injected i.p. with AS1 (0.75 mg) on day 0 and 29 as indicated with an arrow, were collected at indicated time points and incubated with L-rCD40L cells. Day 29 serum was collected 6 hours after second AS1 injection. Binding of AS1 to rat CD40L was determined by flow cytometry. B) Presence of rat anti-hamster Ab were measured by ELISA. The same sera were used for the detection of AS1 and rat anti-hamster response.

To determine whether AS1 was specifically blocking the rat CD40-CD40L interaction, the effect of AS1 was examined on DC activated by another stimulus. DC stimulated by LPS showed production of IL-12, which was not inhibited in the presence of AS1 (**Fig. 2D**). In addition, CD40L-stimulated human monocyte-derived DC were not inhibited in their IL-12 production in the presence of AS1 whereas the anti-human CD40L Ab LL48 completely prevented IL-12 production (**Fig. 2E**). Thus AS1 specifically inhibits rat CD40-CD40L interaction.

Next we wanted to study whether AS1 exerted similar blocking capacities in vivo. It is known that in vivo interaction of CD40 on B cells with CD40L on T cells results in proliferation and production of Ab. We first explored whether AS1 could recognize native CD40L on stimulated T cells. Unstimulated and CD3/CD28 stimulated T cells were stained with AS1 and binding of AS1 was only detected on stimulated T cells (**Fig. 3A**). To investigate whether the CD40-CD40L interaction could be blocked, we measured B cell proliferation after CD40L stimulation in the presence or absence of AS1. Rat splenocytes were stimulated with rat CD40L and the effect of AS1, MR1, LL48 or hamster IgG was studied. CD40L induced strong B cell proliferation, which was inhibited by AS1. In contrast, similar concentrations of MR1, LL48 or hamster IgG showed no effect on the proliferation of CD40L-stimulated B cells (**Fig 3B**).

Blocking capacity of AS1 in vivo

To study the effect of AS1 in vivo we first investigated the time frame in which AS1 can be used in vivo. AS1 was measured in sera derived from rats injected i.p. with AS1 at day 0 followed by a second injection at day 29. L-rCD40L cells were incubated with sera obtained from various time points to measure the presence of AS1 by flow cytometry. AS1 levels were indeed detected in serum after the first injection, these levels were relatively stable until 8 days post injection. After 8 days AS1 levels started to decrease in serum (**Fig. 4A**). Interestingly, no detectable levels of AS1 were measured in sera taken 6 hours after a second injection on day 29.

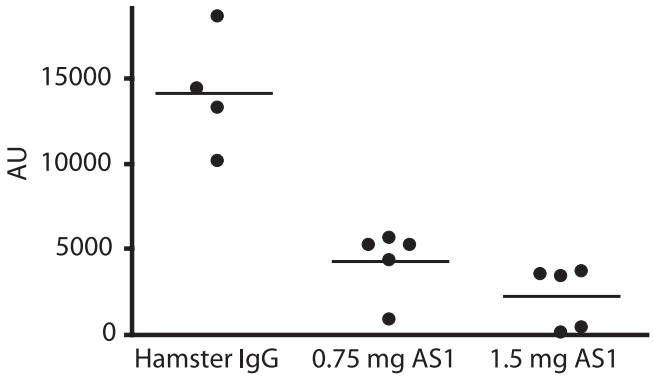

Figure 5. AS1 blocks OVA induced B cell response in vivo.
LEW rats were immunized s.c. with OVA in CFA. At day -1 and 3 various doses of AS1 or 1.5 mg hamster IgG were injected. Anti-OVA IgG levels were detected in day 10 serum using ELISA. Each dot represents one rat (* $p < 0.05$).

Since AS1 can be detected in serum 5 hours after injection, a rapid elimination of AS1 from the circulation after the second injection could have occurred. The induction of an anti-hamster response might explain these results. The same sera were used to measure the induction of a rat anti-hamster antibody response. We found a detectable increase in rat anti-hamster immunoglobulins from day 14 onwards (**Fig. 4B**).
To elucidate the ability of AS1 to inhibit the CD40L stimulatory capacity in vivo, rats were immunized with OVA in CFA on day 0. Groups (n=5) of rats were treated with 1.5 mg hamster IgG, 0.75 mg or 1.5 mg AS1 at day -1 and 3. At day 10 rats were sacrificed and IgG anti-OVA levels were determined in serum. Rats immunized with OVA and hamster IgG exhibited high levels of IgG anti-OVA in their serum. Treatment of rats with different doses of AS1 inhibited the IgG anti-OVA response dependent on the dose of AS1 given (**Fig. 5**). Similar results were obtained after immunization with keyhole limpet hemocyanin (KLH) (data not shown).

Discussion

In previous studies it has been shown that mAb against a number of murine molecules, including anti-mouse CD40L (MR1), are successfully generated using Armenian hamsters [6, 15, 16]. As the homology between mouse and rat CD40L is very high, we used Armenian hamsters for the generation of an anti-rat CD40L mAb. We identified a positive clone, AS1, which was expanded and characterized. In vitro and in vivo experiments showed that AS1 was able to specifically block CD40L induced activation.
We showed that both AS1 and MR1 were able to bind to rat and not to human CD40L. However, functional experiments demonstrated that although MR1 was able to bind to rat CD40L, it was not able to block the CD40-CD40L interaction in vitro, even not in high concentrations (data not shown). In contrast to MR1, AS1 completely blocked CD40L-induced maturation of DC and CD40L-induced B cell proliferation. These in vitro data imply that AS1 is able to block the CD40-CD40L interaction in rats. To determine the therapeutic window of AS1 in vivo, we monitored the presence of AS1 after injection and investigated the development of the rat anti-hamster response. We found that AS1 is present in serum at least until day 8. From day 14 an anti-hamster response was detectable, which lead to a rapid decrease of AS1 levels in serum. A second injection of AS1 at day 29 resulted in a rapid clearance of AS1 from circulation. Together, this indicates that AS1 can be used in vivo and will be most effective during the first week after injection.
To confirm the blocking effect of AS1 in vivo, we studied the effect of AS1 on the induction of OVA or KLH-specific Ab, which in mice were shown to be dependent on the interaction between CD40-CD40L [6]. We showed that OVA injected into rats resulted in high levels of anti-OVA IgG. Treatment of rats with AS1 inhibited the OVA response, indicating that the AS1 mAb is not only functionally blocking the CD40-CD40L interaction in vitro, but also in vivo.
In rat kidney transplantation models it has recently been shown that pretreatment of recipients with a hamster anti-rat CD40L mAb (AH.F5) prolonged survival [11-13, 17]. In addition, in more stringent primate transplantation models blockade of the CD40-

CD40L pathway was shown to be more promising than CTLA4-Ig, indicating that CD40L specific antibodies may play an important role in the induction of tolerance in humans [18, 19]. The mechanism of action of CD40L specific antibodies including AH.F5 and AS1 remains to be elucidated. It has been demonstrated that in mice binding of the hamster antibody MR1 to CD40L results in a selective depletion of activated T cells via a Fc-dependent mechanism rather than by co-stimulation blockade [20]. Since mice and rats are closely related, it is likely that a similar mechanism might be involved when AH.F5 or AS1 are applied in rats, although interaction of these hamster antibodies with rat Fc receptors still has to be demonstrated. The depletion of activated T cells could have a beneficial outcome for the immune response by creating a new balance in favor of regulatory versus effector T cells [20, 21].

In conclusion, we showed that the generated anti-rat CD40L mAb AS1 was able to block the CD40-CD40L interaction in vitro and in vivo. Studying this mAb in various rat models may therefore provide more insight into the role of CD40-CD40L interaction in these models and further support the identification of CD40-CD40L as therapeutic targets in human diseases.

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CHAPTER 6

Summary and Discussion

Summary and Discussion

Following allograft transplantation, the immune system is triggered to induce an immunogenic response against the non-self organ. To prevent the induction of this immunogenic response, recipients are treated with immunosuppressive medication. The majority of these medications target T cells, which play a key role in the rejection process, and thereby prevent acute rejection in most of the recipients. Non-specific targeting of these T cells not only prevents acute rejection, it also prevents responses against pathogens or tumor growth. In addition, long-term use of immunosuppressive agents may cause organ failure due to toxic effects on the organ [1]. Therefore, the ultimate goal is to develop a therapy, which targets alloreactive T cells, allowing a normal response against pathogens and tumors, in the absence of chronic use of immunosuppressive agents. Various strategies have been employed to induce such a donor-specific tolerance, amongst which treatment with immature DC [2]. These immature DC have, in contrast to mature DC, the capacity to induce tolerogenic responses and are therefore an attractive candidate for cellular therapy.
In the present thesis, we investigated the effect of modulated donor-derived DC on allograft survival in rat kidney transplantation models. To study this, we established a method to generate rat BM-derived DC and characterized the phenotype and function of these rat DC (**Chapter 2**). Since Dexamethasone was shown to block DC in their immature state, we studied the effect of DexDC in renal transplantation models (**Chapter 3 and 4**). In addition, we generated and characterized a novel anti-rat CD40L mAb, with inhibitory properties (**chapter 5**). This novel antibody can provide more insight in the effect of using co-stimulatory blocking Ab in transplantation models and in combination with infusion of modulated DC it may improve cell-based therapy in transplantation settings.

Bone marrow-derived rat dendritic cells

In contrast to human and mouse DC, rat DC have not been studied extensively until recently [3-5]. In our studies we confirmed that, under similar culture conditions as described before [5], immature DC express MHC class II and low levels of the co-stimulatory molecules CD80, CD86 and CD40. Furthermore, the cells express NKR-P1A, CD11b/c and OX62, a marker known to be present on most, but not all, rat DC [6]. In contrast, the macrophage marker (CD163) and T cell markers such as CD4 and TCR were not detectable (**chapter 2**).
Stimulation of DC of human or murine origin with LPS or CD40L has been demonstrated to result in an enhanced CD80 and CD86 expression level and the production of proinflammatory cytokines such as IL-12 [7, 8]. In contrast to human- and murine-derived DC, rat DC showed only limited enhancement of CD80 and CD86 after stimulation with LPS or CD40L. Nevertheless, both LPS and CD40L induced IL-12 production by DC. The level of IL-12 secretion was dependent on the type of stimulus given. CD40L-mediated activation was found to induce higher levels of IL-12 compared to LPS. In contrast, IL-10 production was only produced after LPS stimulation and no detectable levels were measured upon CD40L stimulation. The balance between IL-12 and IL-10 plays an important role in the outcome of the T cell response. DC producing

high levels of IL-12 have the capacity to induce high levels of IFN-γ by allogeneic T cells [9]. We indeed showed that CD40L-stimulated rat DC induced higher levels of IFN-γ by allogeneic T cells compared to LPS-stimulated DC (**chapter 2**).
Our data demonstrate that, although there are some differences with human and murine DC, stimulated rat DC also have the capacity to induce T cell activation.

Application of modulated dendritic cells in transplantation models

Evidence that DC can be used to prolong allograft survival has come initially from pancreatic islet and heart transplantation studies [10-12]. These studies made use of donor-derived immature DC, which were infused into recipient mice 7 days prior to transplantation. More recent studies have focused on applying maturation-resistant donor-derived DC, to prevent maturation of infused immature DC and improve the efficacy of the treatment. Various compounds have been shown to freeze DC in their immature state, including Vitamine D3, IL-10 and Dexamethasone (Dex) [13-18]. We confirmed that bone marrow-derived rat DC, as described in **chapter 2**, are impaired in their IL-12 production and in their capacity to stimulate allogeneic T cells when cultured in the presence of Dex and stimulated with LPS (**chapter 3**).
In vivo application of modulated DC has been shown to prolong allograft survival in mouse models [19-21]. In rat models, prolonged allograft survival was induced when recipient rats were treated with Dex-treated (donor x recipient) F1-derived DC together with a short course of immunosuppression and blocking co-stimulatory molecules [3]. This conditioning resulted in the development of T cell anergy and induction of allo-specific, self-restricted regulatory T cells and a completely normal histology at day 100. In addition, application of donor-derived DexDC did not result in graft survival [3].
High levels of IL-10 and low levels of IL-12 favor the induction of regulatory T cells. We therefore explored the effect of LPS-stimulated DexDC (LPS-DexDC) on allogeneic kidney transplant survival in rats. Since immunosuppressive drugs can interfere with the induction of regulatory T cells, the recipients received no other treatment besides LPS-DexDC. In addition, to mimic the clinical setting, where the transplanted kidney is the only functional kidney in patients, recipients were bilateral nephrectomised prior to transplantation.
In **chapter 3** we explored the effect of LPS-DexDC treatment on the recipient's immune response. A significant donor-specific T cell hyporesponsiveness was induced by this treatment, in contrast to untreated or LPS-CtrDC treated recipients. In the latter treatment recipient T cells were shown to be primed to donor antigen. This indicated that only modulated DC have the capacity to regulate the recipient's immune response (**chapter 3**). Nevertheless, despite the induction of donor-specific T cell hyporesponsiveness, LPS-DexDC treatment did not prolong allograft survival. This suggests that LPS-DexDC do not regulate all pathways that are involved in allograft rejection.
Treatment of recipients with donor-derived DC (either LPS-CtrDC or LPS-DexDC) revealed modulation of the rejection process compared to untreated recipients. Rejected kidneys from untreated recipients were unable to allow perfusion with UW solution and demonstrated a red colour inside, whereas kidneys from all DC treated animals could be normally perfused (**chapter 4**). This observation has not been described before in

these models and is at present unexplained.
Next to an induced donor-specific T cell hyporesponsiveness, LPS-DexDC treatment reduced the influx of $CD8^+$ T cells into the graft compared to LPS-CtrDC treated recipients. No difference, however, was found in infiltrating myeloid and NK cells between the two treatments (**chapter 4**). NK cells are cytotoxic to target cells mismatched for MHC class I molecules and can act as both an effector/mediator of rejection. Activated NK cells can provide signals, such as IFN-γ, TNF-α or IL-5, which promote the generation of alloreactive T cells, induce the differentiation and activation of alloantibody producing B cells and recruit macrophages and thus mediate graft rejection [22-27]. In addition, in a fully mismatched transplant model where recipient's T cell activation is hampered, such as $CD28^{-/-}$ mice, NK cells can mediate graft rejection [28]. This suggests that activated NK cells may provide T cells with co-stimulation signals, in situations where these are absent. However, it has become clear that NK cells may also provide a pivotal role in inducing tolerance in transplantation settings. In mouse models it was shown that NK cells destroy passenger APC. This may result in less or no dissemination of passenger DC to lymph nodes, and reduces direct recognition of donor antigens [29].
Together, these data suggest that treatment of recipient rats with LPS-DexDC results in a donor-specific T cell hyporesponsiveness and a reduced influx of $CD8^+$ T cells into the graft. Despite these regulatory capacities, LPS-DexDC do not have the capacity to prolong graft survival. We observed that LPS-DexDC treatment did not reduce infiltration of myeloid and NK cells. Since both myeloid and NK cells have the capacity to mediate graft rejection, these cells may have influenced the rejection process.

Alloantibody formation due to DC application

Studies described in this thesis mainly focused on the cellular rejection process of allografts. Next to T cells also B cell-mediated pathways can be involved in allograft rejection [30]. Recently, antibody-mediated mechanisms have been recognized to contribute to immune-mediated rejection and can occur in both early and late transplant rejection [31]. In case of cell-based therapy, cells are infused into recipients and induce immune responses in which T cells and B cells will play a role. In our setting, donor-derived LPS-DexDC were applied in vivo. Theoretically, these donor-derived LPS-DexDC regulate recipient T cells via the direct pathway. However, DC of recipient origin may phagocytose cells which are dying upon infusion of donor-derived LPS-DexDC and present these donor antigens to recipient T cells in the presence of co-stimulatory molecules and proinflammatory cytokines (**Fig. 1**). Thus, activation of recipient T cells via the indirect pathway can still occur and may result in the activation of alloreactive T cells or B cell activation with subsequent secretion of alloantibodies. Depending on the cytokines present, various types of immunoglobulines will be secreted. For example, IgG is produced when IL-4 or IFN-γ are present, whereas IgA seems to require the presence of TGF-β [32-34]. To test whether recipients pre-treated with DC are sensitized to donor antigens and produce alloantibodies, we explored the presence of donor-specific IgG and IgA levels in serum 7 days after DC application, at the day of transplantation, and 7 days after transplantation, when rejection occurred. Seven days after DC infusion we detected higher levels of donor-specific IgG than donor-specific IgA, but in both cases almost a 2-fold increase in comparison to naïve rats was detected (**Fig 2**). At the time

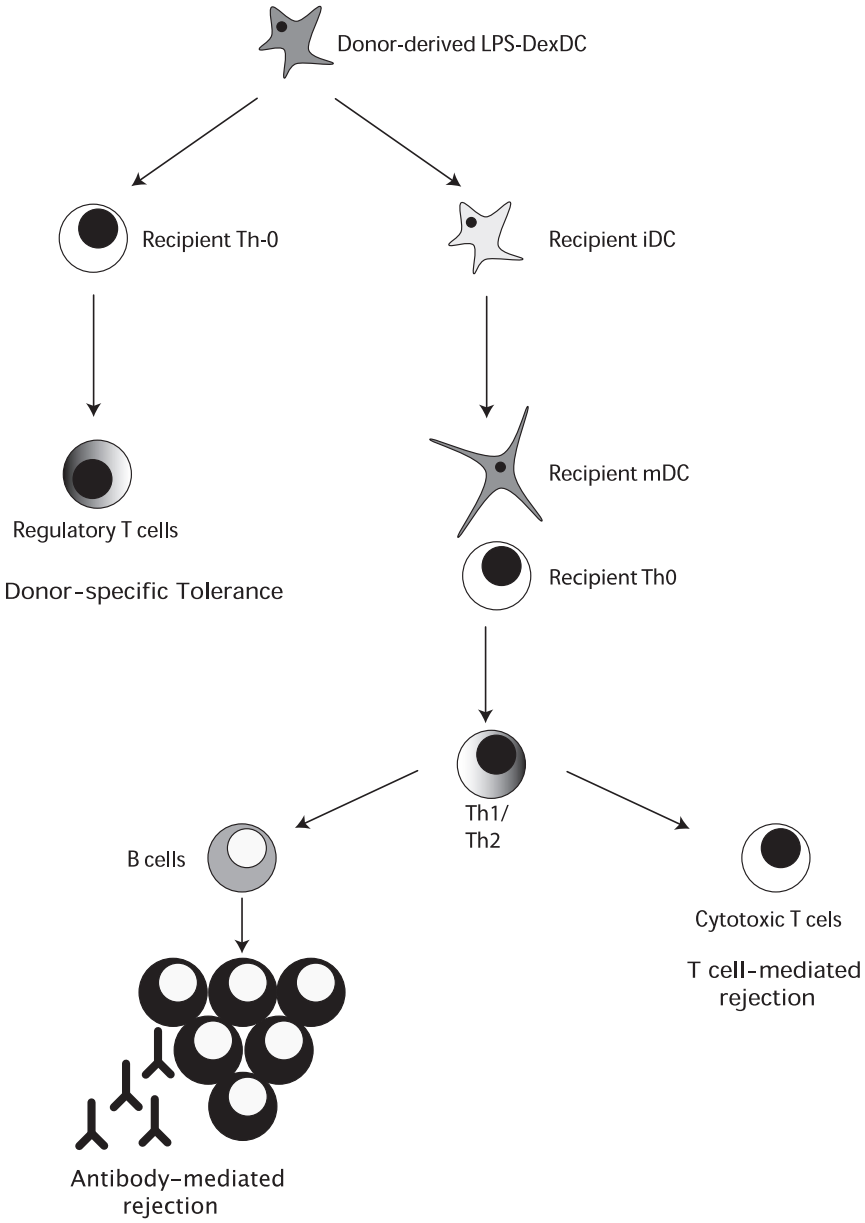

Figure 1. Immune regulation and immune activation of donor-derived LPS-DexDC.
Application of donor-derived LPS-DexDC will regulate recipient T cell responses in the direct pathway of antigen recognition. Low expression levels of MHC and co-stimulatory molecules on donor-derived LPS-DexDC together with the presence of IL-10 and absence of IL-12 secretion give rise to regulatory T cells. In contrast, recipient DC can take up and process the infused LPS-DexDC and present donor peptides in the context of recipient MHC molecules to recipient T cells in the presence of co-stimulatory molecules and proinflammatory cytokines. These signals are capable to induce allogeneic T cell activation. Subsequently, cytotoxic T cells (CTL) are activated, which have the capacity to destroy the allograft, but also B cells can be activated, which will produce alloantibodies. These alloantibodies are important for the induction of humoral-mediated graft rejection.

of rejection, both donor-specific IgG and IgA levels were strongly increased. However depending on the treatment (untreated or LPS-DexDC), a difference was detected in the fold increase of these levels. Untreated recipients were shown to have a 9-fold increase in IgG levels and a 5-fold increase in IgA levels in comparison to naïve rats, whereas LPS-DexDC treated recipients demonstrated in both IgG and IgA a 6.5-fold increase (**Fig. 2**) (Stax et. al. unpublished data).

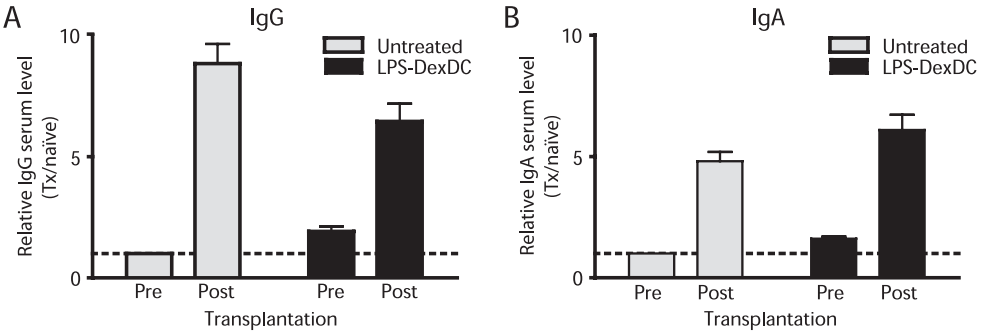

Figure 2. Anti-donor IgG and IgA levels in serum of PBS or LPS-DexDC treated recipients.
IgG and IgA levels were measured in serum of LEW recipients 7 days after PBS (gray) or LPS-DexDC (black) infusion (pre-serum) or at the time of rejection (post-serum) by ELISA. Serum was incubated on donor-derived DC and binding of anti-donor IgG or IgA was detected by flow cytometry. Fold increase of IgG (A) and IgA (B) levels in recipients was determined in relation to serum from naïve rats. Results shown are mean ± SD of 4 rats per group.

Complement components in rejection process

In clinical transplantation, deposition of the complement component C4d in the graft is considered a diagnostic indicator of antibody-mediated rejection, particularly in renal allografts [35]. Since no antibodies are available to specifically detect C4d in rats, we determined presence of the classical route component C4 in rejected renal tissue. C4 staining was performed on renal sections derived from LPS-DexDC and LPS-CtrDC treated recipients. Unfortunately, interpretation of these stainings was difficult due to high background levels.

To resolve the question whether antibody-mediated rejection occurred in recipient rats, we therefore stained for complement components other than C4. We focused on C1q, which is known to bind immunoglobulins, and C3, the central component of all complement activation pathways. Normal renal tissue showed only low amounts of C1q in the tubulo-interstitial compartment. However, in rejected renal tissue elevated amounts of C1q were detected, where C1q was localized both in the glomerulus and in the interstitium (**Fig. 3**). C1q present in the interstitium showed co-localization with infiltrating cells. Since iDC have been shown to produce C1q [36], infiltrating iDC may be the main source of C1q in rejected kidneys from LPS-DexDC treated recipients. The contribution of the C1q produced by iDC to the rejection process will have to be elucidated.

A more central component of the complement system is C3. C3 is required for all pathways of complement activation. In normal renal tissue low amounts of locally produced C3 can be detected in the peritubular region. C3 amounts were shown to be elevated specifically in rejected kidneys derived from LPS-DexDC treated recipients and

detected in the peritubular region and the glomeruli (**Fig. 4**). Although quantification of C3 in rejected kidney derived from LPS-CtrDC treated recipients revealed no increase of C3 deposition or synthesis, the localization of C3 is different compared to C3 detected in normal renal tissue (Stax *et. al.* unpublished).
Although there may be some co-localization of C1q and C3 in the glomeruli of rejected kidneys, most of the C1q and C3 do not co-localize, indicating that not only the classical pathway of complement activation is involved in the observed rejection process.
Depending on the presence of effector molecules or regulatory molecules and receptors, responses of the adaptive immune system can be stimulated or inhibited by the complement cascade [37]. For example, immune regulation is induced when apoptotic blebs are cleared, due to the presence of regulatory molecules. However in case of ischemic tissue, complement regulatory proteins are decreased and consequently result in immune activation [38]. The effect of C3 on T cell responses has been demonstrated in $C3^{-/-}$ mice. Renal allografts derived from $C3^{-/-}$ donors survive significantly longer than those from C3 expressing wild-type mice. In addition, a decreased T cell response was detected in recipients receiving allografts from $C3^{-/-}$ mice compared to the wildtype allograft [39]. The increased level of C3 detected in renal tissue derived from LPS-DexDC treated recipients may therefore not only have been activated via the classical pathway, but may also have been induced by ischemic injury. Since C3 has been shown to play a crucial role in the rejection process, the increased C3 levels may have mediated allograft rejection.

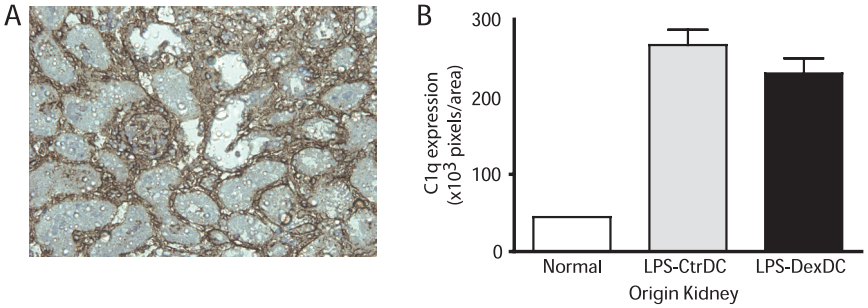

Figure 3. Increase of C1q in rejected kidneys.
A) Frozen sections from rejected allografts fro.m LPS-DexDC-treated recipients were stained for the presence of C1q. Depicted in color at page 79. B) Positive staining was quantified from normal kidneys (white) and rejected kidneys derived from LPS-CtrDC (gray) and LPS-DexDC (black) treated rats. Results shown are the mean ± SD of 3 rats, the LPS-CtrDC group contained 2 rats.

Improvements for DC-based therapy

As shown in **figure 1**, presentation of infused donor-derived DexDC by recipient DC may induce recipient's T cell activation. To prevent this route of T cell activation, it will be necessary to apply co-treatments, such as blockade of co-stimulatory molecules. The effect of blocking B7-CD28 and/or CD40-CD40L has been studied widely in transplantation models. It has been demonstrated that blockade of either one interaction prevents acute rejection in rat models [40, 41], while blocking both interactions decreases the occurrence of chronic rejection with 50% [42, 43]. Treatment of recipients with anti-mouse CD40L mAb (MR1) together with donor splenocytes, induced long term graft

survival (>100 days) in a murine fully mismatched cardiac transplant model [44], but also in rat models [45-47]. Since the hamster anti-rat CD40L mAb (AH.F5) used in these rat studies is not widely available, we generated a novel hamster anti-rat CD40L mAb (AS1). Both in vitro and in vivo, AS1 was shown to have inhibitory activities and may therefore improve cell-based therapy in rat models (**chapter 5**). Although the mechanism of action of AH.F5 and AS1 are to be elucidated, studies with MR1 have demonstrated that the mechanism of action is Fc-dependent depletion of activated T cells, rather than by co-stimulatory blockade [48]. Mice and rats are closely related, and it is therefore possible that a similar mechanism is involved when AH.F5 or AS1 are applied, but this remains to be examined. Starting from the idea that the mechanism of action of AS1 is via depletion of activated T cells, AS1 treatment may create a new balance in the immune response, favoring regulatory T cells (Treg) versus effector T cells.

Another approach to improve cell-based therapy is the use of a short course of immunosuppression to suppress immunity and inflammation at the time of transplantation. It has to be taken into account that immunosuppressive drugs not only affect effector T cells, but may also target Treg. Calcineurin inhibitors for example, reduce the number of Treg in kidney transplant patients, whereas in patients receiving rapamycin this was not observed [49]. In mouse models rapamycin has been shown to delete alloreactive T cells, while preserving Treg when applied in combination with agonist IL-2/Fc, and an antagonistic mutant IL-15/Fc [50]. However, in a rat heart transplantation model, co-treatment with rapamycin prevented no allograft survival induced by immature recipient-derived DC [51]. The impact of other immunosuppressive drugs on the development and function of regulatory T cells needs to be assessed.

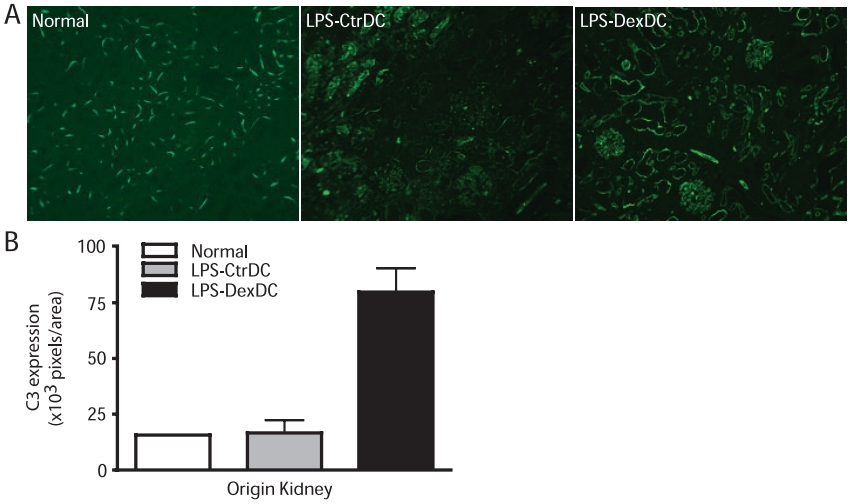

Figure 4. Increase of C3 in rejected kidneys.
A) C3 staining was performed on normal kidneys and on rejected kidneys derived from LPS-CtrDC or LPS-DexDC treated recipients. Depicted in color at page 79. B) Quantification of positive staining on frozen sections from normal kidney (white) and rejected allografts from LPS-CtrDC (gray) or LPS-DexDC (black) treated recipients. Results shown are the mean ± SD of 3 rats, the LPS-CtrDC group contained 2 rats.

Concluding remarks

The studies presented in this thesis demonstrate that in fully mismatched kidney transplantation models, administration of modulated donor-derived DC to recipient's results in regulation of recipient's immune response. Both the donor-specific hyporesponsiveness of recipient T cells and the reduced influx of $CD8^+$ T cells into the graft of LPS-DexDC treated recipients indicate a positive effect of this treatment. However, optimization of this treatment is necessary, since no prolonged allograft survival was induced. Several mechanisms, which are not regulated by LPS-DexDC, may be responsible for the observed rejection, amongst which the preformed alloantibodies, increased levels of C3 in the graft and the increased influx of NK cells. Additional studies are required to explore the modulating effects of AS1 and/or short courses of immunosuppressive drugs as a co-treatment in these settings.

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Nederlandse Samenvatting

Samenvatting

Niertransplantatie

Patiënten met eindstadium nierfalen zijn voor hun overleving afhankelijk van nierfunctievervangende therapie zoals nierdialyse of het ondergaan van een niertransplantatie. Aangezien dialyse niet de gehele nierfunctie kan overnemen en deze behandeling in hoge mate de manier van leven beperkt, wordt in het algemeen de voorkeur gegeven aan een niertranplantatie. Hierbij ontvangt de patiënt een nier van een donor, die in de meeste gevallen genetisch niet identiek is aan de patiënt. Daarom zal het immuunsysteem van de patiënt een specifieke reactie op gang brengen, die ervoor kan zorgen dat het lichaamsvreemde orgaan afgestoten wordt. Om dit te voorkomen is het voor transplantatiepatiënten noodzakelijk om levenslang immuunonderdrukkende medicatie in te nemen.

Witte bloedcellen spelen een belangrijke rol bij de afweer tegen lichaamsvreemde cellen. Van deze witte bloedcellen zijn dendritische cellen (DCs) in staat om lichaamsvreemde cellen te herkennen en vervolgens andere witte bloedcellen, T cellen, te informeren over de aanwezigheid van deze cellen. Zodra T cellen deze informatie krijgen, zijn ze in staat om de lichaamsvreemde cellen te verwijderen.

Dendritische cellen kunnen zowel lichaamseigen als lichaamsvreemde eiwitten opnemen en afbreken in kleine stukken, peptides. Deze peptides, die in het geval van een transplantatie afkomstig zijn van donorweefsel, worden gepresenteerd op eiwitten, HLA (Human Leukocyte Antigen) moleculen, die aanwezig zijn op het celoppervlak van DCs en voor elk individu uniek zijn. Vervolgens migreren DCs naar een gespecialiseerd lymfoïd orgaan, zoals de milt of lymfeklieren. Dit orgaan bevat T cellen, die de peptides afkomstig van de donor op het HLA-molecuul van ontvanger DCs herkennen. Dit is de indirecte route van antigeen presentatie. Daarnaast, gebaseerd op een vorm van kruisreactie, zijn sommige T cellen ook in staat om de vreemde HLA-moleculen op het oppervlak van donor DCs direct te herkennen. Deze zogenaamde directe route van antigeen presentatie, is specifiek voor transplantatie en lijkt vooral in de eerste periode na transplantatie een rol te spelen. De T cellen raken geactiveerd en vermenigvuldigen zich. Vanuit de lymfoïde organen migreren ze via de bloedbaan naar de getransplanteerde nier. Hier herkennen T cellen de lichaamsvreemde cellen en vernietigen deze, uiteindelijk resulterend in afstoting van de getransplanteerde nier.

De mate waarin T cellen geactiveerd raken, wordt bepaald door DCs en is afhankelijk van de aan- of afwezigheid van signalen. De chirurgische schade en het gebrek aan zuurstof, die ontstaat gedurende de transplantatie, induceert allerlei “gevaar-signalen”, welke leiden tot infiltratie van ontvanger DCs en activatie/maturatie van zowel ontvanger als donor DCs. Deze laatst genoemde cellen zijn al in het transplantaat aanwezig op het moment dat de transplantatie uitgevoerd wordt. Maturatie wordt gekenmerkt door de aanwezigheid van co-stimulatoire moleculen op DCs en productie van cytokinen. De combinatie van co-stimulatoire moleculen, cytokinen en de “directe” herkenning van vreemde HLA-moleculen op donor DCs of de “indirecte” herkenning van vreemde peptides gepresenteerd op HLA-moleculen van ontvanger DCs resulteert in de volledige activatie van T cellen. In tegenstelling tot de reactie op “gevaar-signalen”, vindt er geen maturatie plaats wanneer er lichaamseigen eiwitten worden opgenomen

door DCs. Dendritische cellen zijn dan in staat om het immuunsysteem te reguleren. De stimulatoire capaciteit van DCs is laag, T cellen ontvangen geen activatiesignalen en raken niet geactiveerd.
Zoals hierboven genoemd kan een afstotingsreactie grotendeels voorkomen worden door gebruik te maken van immunosuppressiva. Door sterke verbeteringen op dit gebied is tegenwoordig de kans op overleving van het transplantaat in het eerste jaar groter dan 90%. In tegenstelling tot de verbeterde 1ste jaars overleving, is er relatief weinig vooruitgang geboekt op de overleving van het transplantaat op de lange termijn. Verder blijkt ook dat patiënten die langdurig immunosupressiva gebruiken veel bijverschijnselen vertonen, zoals ontwikkeling van maligniteiten en verhoogde kans op infecties. De ontwikkeling van nieuwe therapieën is daarom van belang om de lange termijn overleving van transplantaties te verbeteren. Een strategie, die zich de afgelopen jaren aangediend heeft, is het gebruik van DCs als cellulaire therapie.
De capaciteit van DCs om het afweersysteem te stimuleren of te reguleren maakt het gebruik van DCs interessant voor cellulaire therapie. In proefdiermodellen is aangetoond dat voorbehandeling van ontvangers met immature DCs, afkomstig van de donor, in staat is om tolerantie te induceren voor een getransplanteerd hart of getransplanteerde eilandjes van Langerhans. Een nadeel van het gebruik van immature donor DCs is dat deze DCs in het lichaam van de ontvanger ook “gevaar-signalen” kunnen tegenkomen. Deze cellen kunnen dus geactiveerd raken, matureren, en uiteindelijk via de directe route T cellen activeren, een proces dat alsnog kan leiden tot een afstotingsreactie. Om dit laatste te voorkomen is het gewenst om de immature staat van DCs te behouden en te voorkomen dat ze matureren.
Studies met humane DCs hebben aangetoond dat in aanwezigheid van de corticosteroïd Dexamethason (Dex), DCs immatuur blijven. In het huidige proefschrift worden de studies beschreven waarin een methode is opgezet om rat DCs in vitro te genereren en te moduleren tot regulatoire DCs met behulp van Dex. Vervolgens is het effect van Dex behandelde DCs, afkomstig van de donor, op niertransplantaat overleving bestudeerd in een ratmodel.

Dendritische cellen als therapie tegen het afstotingsproces

Aangezien de hoeveelheid DCs, die direct uit het bloed verkregen kunnen worden, laag is, wordt voor toepassing van deze cellen veel gebruik gemaakt van cellen welke uitgaande van monocyten of beenmergcellen gedifferentieerd zijn tot DCs. In **hoofdstuk 2** wordt het fenotype beschreven van rat DCs, die gegenereerd zijn uit beenmergcellen. Vergelijkbaar met in vitro gegenereerde humane en muis DCs, expresseren de in dit proefschrift gebruikte rat DCs MHC moleculen (Major Histocompatablity Complex), die verwant zijn aan HLA-moleculen. Daarnaast expresseren ze ook de co-stimulatoire moleculen CD80, CD86 en CD40. Andere markers die aanwezig zijn op de rat DC zijn OX62, een molecuul dat op de meeste rat DCs aanwezig is, NKR-P1A en CD11b/c. Stimulatie van humane en muis DCs met Lipopolysaccharride (LPS), een component van een bacteriële celwand, welke een gevaar-signaal nabootst, of CD40L, een molecuul op T cellen dat de maturatie van DCs versterkt, verhoogt de expressie van de co-stimulatoire moleculen en de uitscheiding van cytokinen. Hierdoor neemt de stimulatoire capaciteit van DCs toe. In onze studie konden we geen verhoogde

expressie van de co-stimulatoire moleculen detecteren op rat DCs na stimulatie met LPS of CD40L. Vergeleken met niet geactiveerde cellen waren de gestimuleerde rat DCs echte wel beter in staat om T cellen te activeren. De uitscheiding van cytokinen door rat DCs bleek afhankelijk te zijn van het type stimulus. Zowel LPS als CD40L induceerden de uitscheiding van IL-12 door rat DCs. Opvallend hierbij was dat CD40L in staat was om veel meer IL-12 productie te induceren dan LPS. Verder werd IL-10 alleen geproduceerd wanneer de DCs gestimuleerd werden met LPS en niet met CD40L.

Hoofdstukken 3 en 4 beschrijven het effect van LPS-gestimuleerde Dex-gemoduleerde rat DCs, afkomstig van de donor, in een niertransplantatie model. In **hoofdstuk 3** wordt beschreven dat Dex de LPS-geïnduceerde maturatie van DCs voorkomt. LPS stimulatie van DCs, gegenereerd in aanwezigheid van Dex (LPS-DexDC), vertoonden een verlaagde productie van het proinflammatoire cytokine IL-12, terwijl de productie van het anti-inflammatoire cytokine IL-10 niet veranderde. Samen met een verlaagde expressie van co-stimulatoire moleculen leidde dit tot een verminderde capaciteit om T cellen te activeren. De in vivo experimenten demonstreerden dat T cellen, afkomstig van LPS-DexDC behandelde ontvanger ratten, in aanwezigheid van donor cellen zich niet vermenigvuldigden en minder IFN-γ produceerden. IFN-γ is een cytokine dat een rol speelt bij de vernietiging van donor cellen. Daarentegen bleken deze T cellen hiertoe wel in staat te zijn wanneer ze gekweekt werden met cellen afkomstig van een andere rattestam. Dit betekent dat LPS-DexDC, afkomstig van de donor, ook in vivo in staat zijn om T cellen te reguleren en dus een donor-specifieke T cel hyporesponsiviteit induceren. Ondanks de aanwezigheid van deze hyporesponsieve T cellen werd de getransplanteerde nier afgestoten.

Om meer inzicht te krijgen in het afstotingsproces werd deze studie in **hoofdstuk 4** vervolgd door de transplantaten histologisch te bestuderen. Gezonde rattennieren bevatten relatief meer myeloïde cellen dan lymfoïde cellen en het aantal myeloïde cellen verschilt per rattenstam. Getransplanteerde nieren, afkomstig van LPS-DexDC behandelde ontvangers, vertoonden een sterke toename van myeloïde en lymphoïde cellen op het moment dat afstoting werd geconstateerd. Opvallend was dat het aantal infiltrerende $CD8^+$ T cellen een stuk lager was in transplantaten van LPS-DexDC behandelde ontvangers vergeleken met controlebehandelingen. Ook dit wijst erop dat LPS-DexDC in staat zijn om in vivo T cellen te reguleren. Andere immuuncellen, zoals macrophagen, DCs en NK cellen, worden daarentegen niet gereguleerd door LPS-DexDC en zouden een verklaring kunnen zijn voor het afstotingsproces dat in de getransplanteerde dieren plaatsvond.

Verbetering van therapieën gebaseerd op dendritisch cellen

Ondanks de inductie van T cel hyporesponsiviteit in twee rattenmodellen door donor immature DCs, vond in beide modellen afstoting plaats. Dit zou ook verklaard kunnen worden door de herkenning van de toegediende donor DexDCs als vreemd door ontvanger DCs. Als gevolg hiervan zullen de ontvanger DCs deze ingespoten cellen opnemen en presenteren en uiteindelijk in staat zijn om T cellen te activeren via de indirecte route van antigeen presentatie. Hierdoor kan afstoting alsnog plaatsvinden. Een belangrijke route waarin ingegrepen kan worden om DC-gebaseerde therapie te

verbeteren, is het voorkomen van T cel activatie via de indirecte route. Een mogelijkheid is het blokkeren van co-stimulatoire moleculen, die noodzakelijk zijn voor een goede T cel activatie. Verschillende transplantatiemodellen hebben aangetoond dat het gebruik van alleen blokkerende antilichamen, gericht tegen co-stimulatoire moleculen, acute rejectie voorkomt en de kans op chronische rejectie met 50% verlaagt. Het gebruik van dit soort blokkerende antilichamen, in combinatie met het toedienen van donor afkomstige DCs, kan een toegevoegde waarde hebben. De beperkte beschikbaarheid van een blokkerend antilichaam tegen rat CD40L, heeft ons doen besluiten een nieuw antilichaam tegen rat CD40L te ontwikkelen.
Hoofdstuk 5 beschrijft de constructie van een nieuw hamster-anti rat CD40L antilichaam, genaamd AS1. Het monoclonale antilichaam bond aan CD40L en was zowel in vitro als in vivo in staat om activatie van cellen via CD40-CD40L te voorkomen. Het blokkerende karakter van AS1 kan een toegevoegde waarde hebben voor de inductie van tolerantie voor niertransplantaten in combinatie met immature donor DCs. Zoals reeds beschreven, induceerde een behandeling met immature donor DCs donor-specifieke hyporesponsieve T cellen. De mogelijkheid dat ontvanger DCs gematureerd raken door de toegediende donor DCs en op die manier T cellen kunnen activeren zou voorkomen kunnen worden door de ontvanger naast de immature donor DCs ook met AS1 te behandelen.

Conclusie

De huidige therapieën die gebruikt worden tijdens en na transplantatie zijn vaak niet in staat om chronische rejectie te voorkomen en bij langdurig gebruik ontstaan er allerlei bijwerkingen. De ontwikkeling van nieuwe therapieën om dit te verbeteren is daarom van groot belang.
De belangrijke rol van DCs in het stimuleren of reguleren van immuunresponsen en de mogelijkheid om deze cellen regulatoire eigenschappen te geven, maakt ze interessant voor gebruik als cellulaire therapie. In dit proefschrift wordt aangetoond dat gemoduleerde DCs, afkomstig van de donor, zowel in vitro en in vivo in staat zijn om donor-specifieke T cel hyporesponsiviteit te induceren. Daarnaast resulteert deze behandeling in een verlaagde infiltratie van $CD8^+$ T cellen in de getransplanteerde nier. Ondanks deze effecten is behandeling met alleen gemoduleerde donor DCs niet genoeg om transplantaat afstoting te voorkomen. Deze therapie zou verbeterd kunnen worden door een extra behandeling met blokkerende antilichamen gericht tegen co-stimulatoire moleculen, zoals AS1. Een andere mogelijkheid is het toedienen van immunosuppressie voor een korte periode vlak na transplantatie. Zoals beschreven in dit proefschrif hebben LPS-DexDC geen effect op infiltrerende macrophagen, DCs en NK cellen. Ook deze cellen kunnen een rol spelen in het initiëren van transplantaatafstoting. Een extra behandeling met immunosuppressiva gericht op deze cellen zou ervoor kunnen zorgen dat er minder macrophagen, DCs en NK cellen het transplantaat infiltreren als gevolg van de transplantatieprocedure en daarmee de kans op afstoting verkleinen. Het is te verwachten dat een behandeling met gemoduleerde donor DCs samen met een blokkerend antilichaam tegen co-stimulatoire moleculen, of met immunosuppressiva de overleving van een transplantaat kan verbeteren.

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Dankwoord

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Publicaties

Publicaties

Stax AM, Crul C, Kamerling SWA, Schlagwein N, van der Geest R, Woltman AM, van Kooten C. CD40L stimulation of rat dendritic cells specifically favors the IL-12/IL-10 ratio resulting in a strong T cell stimulatory capacity. *Mol. Immunology 2008 May 45 (9):2641-50*

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Curriculum Vitae

Curriculum Vitae

De auteur van dit proefschrift, Annelein Stax, werd geboren op 18 november 1976 in Voorburg. In 1994 behaalde zij het diploma Hoger Algemeen Voortgezet Onderwijs aan het Dalton scholengemeenschap te Voorburg en begon zij in september van datzelfde jaar aan haar studie medische biotechnologie aan de Hogere Laboratorium Opleiding in Utrecht. Tijdens deze studie liep zij stage bij de afdeling Medische Genetica aan het Universitair Medisch Centrum Utrecht (Dr.R. Sinke). In januari 1999 behaalde zij haar diploma en aansluitend trad zij in dienst bij het Centraal Bureau voor Schimmelcultures als analist. In september 1999 begon zij de studie biologie aan de Universiteit Utrecht. Als afronding van deze studie liep zij stage op de afdeling Infection and Immunology aan het Walter and Eliza Hall Institute in Melbourne, Australië (Dr. E. Handman). In maart 2003 behaalde zij haar doctoraal examen. Vanaf september 2003 begon zij als assistent in opleiding bij de afdeling Nierziekten aan het Leids Universitair Medisch Centrum, om onder begeleiding van Dr. C van Kooten en Prof. M.R. Daha aan het in dit proefschrift beschreven onderzoek te werken. Gedurende deze periode was zij bestuurslid van het Platform AIOs Nefrologie. Sinds mei 2008 is zij werkzaam als postdoc bij de afdeling Pathology, in de groep van Dr. P. van den Elzen aan de University of British Columbia in Vancouver, Canada.