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

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CHAPTER 6

Summary and Discussion

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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

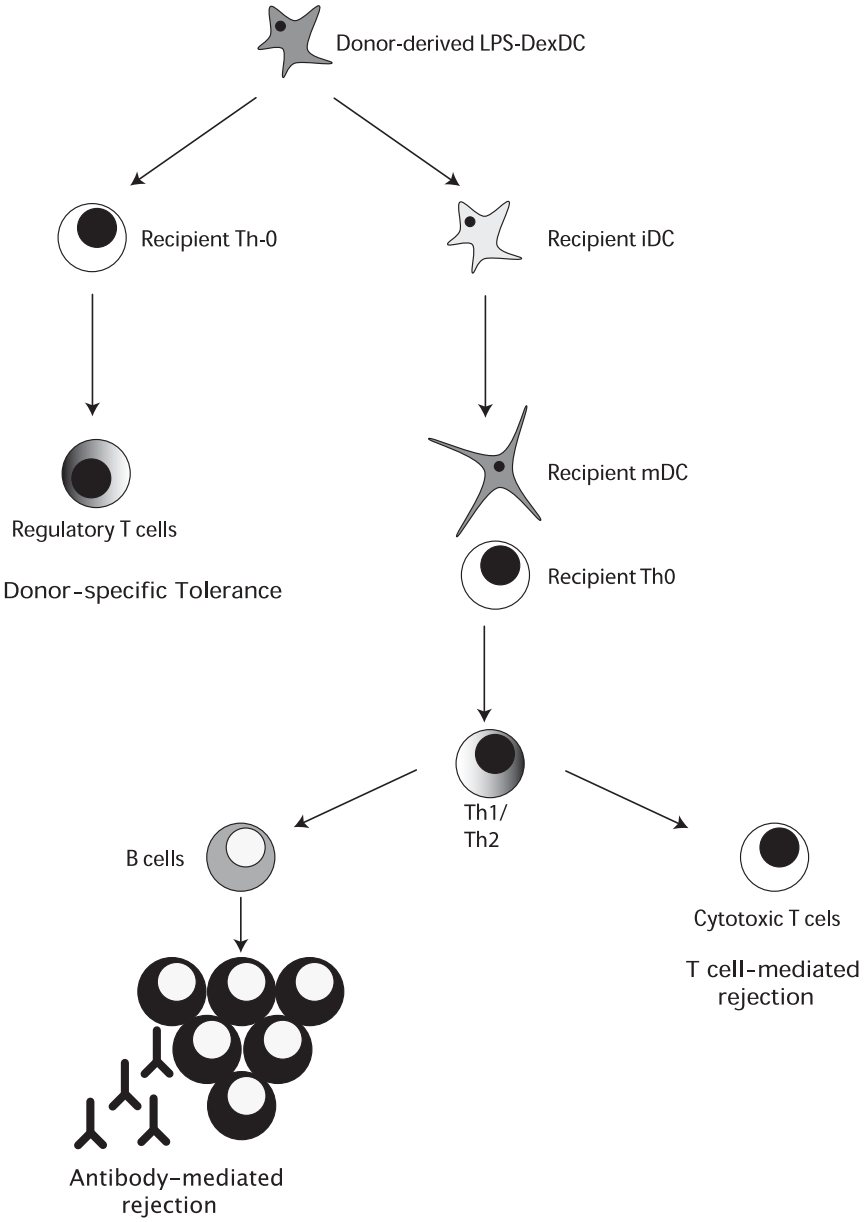

Figure 1. Immune regulation and immune activation of donor-derived LPS-DexDC.
Application of donor-derived LPS-DexDC will regulate recipient T cell responses in the direct pathway of antigen recognition. Low expression levels of MHC and co-stimulatory molecules on donor-derived LPS-DexDC together with 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).

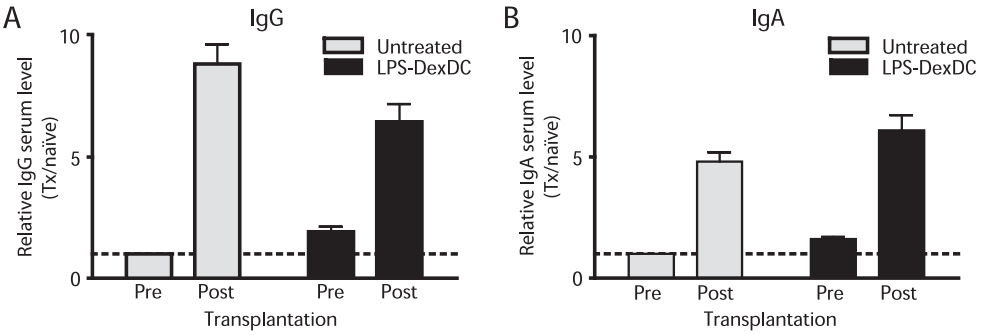

Figure 2. Anti-donor IgG and IgA levels in serum of PBS or LPS-DexDC treated recipients.
IgG and IgA levels were measured in serum of LEW recipients 7 days after PBS (gray) or LPS-DexDC (black) infusion (pre-serum) or at the time of rejection (post-serum) by ELISA. Serum was incubated on donor-derived 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.

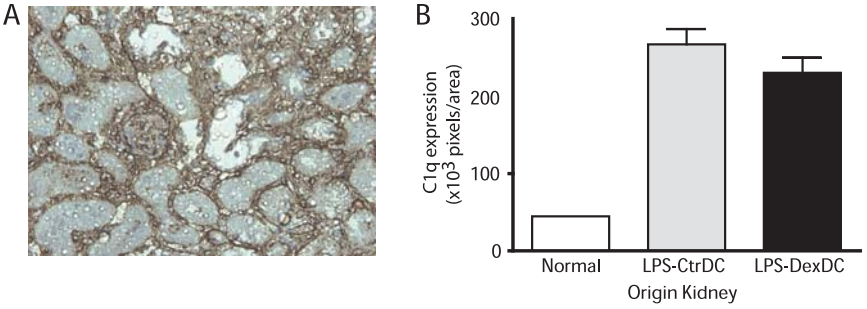

Figure 3. Increase of C1q in rejected kidneys.
A) Frozen sections from rejected allografts fro.m LPS-DexDC-treated recipients were stained for the presence of C1q. Depicted in color at page 79. B) Positive staining was quantified from normal kidneys (white) and rejected kidneys derived from LPS-CtrDC (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.

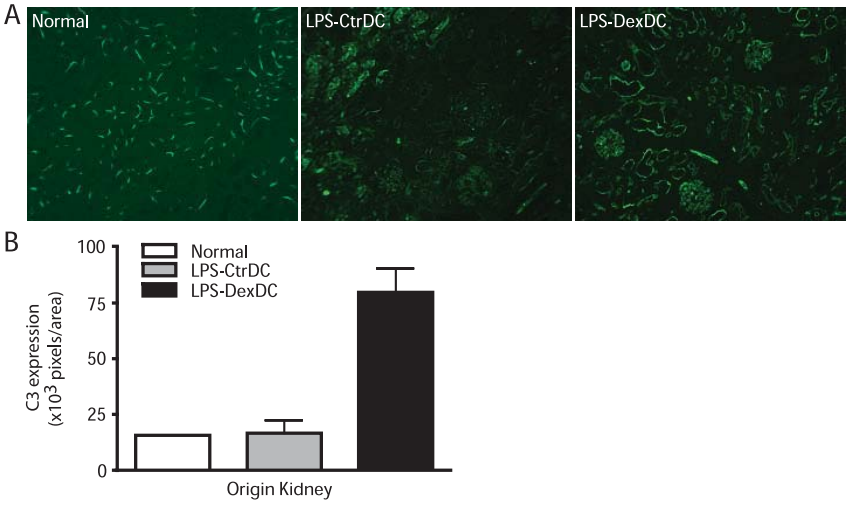

Figure 4. Increase of C3 in rejected kidneys.
A) C3 staining was performed on normal kidneys and on rejected kidneys derived from LPS-CtrDC or LPS-DexDC treated recipients. Depicted in color at page 79. B) Quantification of positive staining on frozen sections from normal kidney (white) and rejected 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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