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Towards tolerance inducing strategies in kidney transplantation.

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Introduction

Towards tolerance inducing strategies in kidney transplantation

Chapter 1

Introduction

Towards tolerance inducing strategies in kidney transplantation

General introduction

The immune system is extremely important for the defence against pathogens, such as viruses and bacteria. Without a proper immune system, humans are not able to deal with these infections. Since there is an enormous variety of pathogens, the immune system has to recognize these different foreign intruders and to elicit a reaction against them in order to be able to eliminate these pathogens. The immune system consists of two major components: the innate or non-specific immunity and the acquired or specific immunity. The innate immune response forms the first line of defence and consists of physical barriers such as the skin and mucosa present in the gastrointestinal and reproductive tract and the respiratory system. Besides the complement system and natural killer (NK) cells, phagocytic cells are key players in the innate system. Among the phagocytic cells are monocytes, macrophages and dendritic cells (DC). These cells can in a non-specific way target invading pathogens by internalising them and subsequently killing them. Furthermore, these cells can present antigens to T lymphocytes that are crucial players in the acquired immune response. The acquired immune system is antigen specific and able to induce memory, which is associated with a fast and strong response when the immune system is re-exposed to the same pathogen, hereby preventing secondary infections. The acquired immune response consists of both a cellular immune response in which T lymphocytes can specifically recognize foreign antigen that is presented in the major histocompatibility complex (MHC) molecules on antigen

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presenting cells (APC) such as the dendritic cell (DC) and a humoral response in which B cells can eliminate pathogens by the production of antibodies (by plasma cells) that specifically bind independently of the MHC molecule to the antigen that initially activated the B cell.

The main function of the immune system is to discriminate between self and non-self. The thymus plays here an essential role with respect to T cell reactivity. During T cell development in the thymus T cells are selected by positive and negative selection mechanisms (1,2). Only T cells with T cell receptors (TCR) with a moderate affinity for self-MHC will survive whereas T cells with a high or very low affinity for self MHC undergo apoptosis. Deletion of T cells with a high affinity for self will prevent to a large extent the occurrence of immune reactions against autologous cells and tissues but additional regulatory mechanisms are necessary to prevent autoimmune responses.

Nowadays, transplantation of kidneys, heart, lung and liver is a common procedure in patients with end stage disease of these organs. The immune response that normally protects human beings is an unwanted feature in solid organ transplantation. A graft is seen as foreign and thus the patient that receives the organ will initiate an immune response that can cause rejection of the transplant by the recognition of foreign MHC antigens on the donor cells. In order to prevent rejection of the organ, patients have to use immunosuppressive drugs lifelong. These drugs have several serious side effects. Because these drugs are non-specific, there is an increased risk of infections and malignancies. Matching the MHC molecules, which are named human leukocyte antigens (HLA) in humans, of donor and recipient, is used as a tool to increase kidney graft survival (3-5). However, the chance of finding a well-matched graft is low because of the high degree of polymorphism of HLA (6,7).

Therefore, besides the improvement in selecting donor organs that are less likely to be rejected by the patient for example by investigating the differential immunogenicity of HLA mismatches (8), other strategies have to be explored to optimise graft survival.

The ultimate goal in transplantation is the induction and maintenance of donor specific tolerance. This means the induction of specific immunological unresponsiveness towards a donor organ that will lead to the lifelong survival of the graft without the use of immunosuppressive drugs and the maintenance of the immune response against pathogens and tumour surveillance.

In this thesis the induction of tolerance is explored via two ways. First, we examined the possibility of naturally induced tolerance based on observations that suggest that pre- and postnatal exposure to noninherited maternal HLA antigens (NIMA) can lead to tolerance towards these antigens. This naturally induced tolerance can have an influence on transplant outcome later in life. Chapter 2 gives

an overview of the current knowledge about the NIMA effect. In this thesis we investigated the influence of NIMA on the alloimmune response in both healthy individuals and patients transplanted with a NIMA haplotype mismatched kidney graft. Secondly, we investigated whether tolerance can be induced by a more artificial way by modulating dendritic cells (DC), which are the central cells between the innate and the acquired immune response. Finally, we examined the possibility to use in vitro tools to detect a possible tolerant status in a transplant recipient. Monitoring of transplant recipients may help to identify which patient is at risk of rejection and which patient is more predisposed to tolerance. Eventually, generating tolerance via natural or induced pathways in transplant recipients and monitoring of such patients can help to increase graft survival and to optimise immunosuppressive regimens leading to less side effects.

Transplantation and the immune response

Human Leukocyte antigens and the immune system

In transplantation, the antigens which are the primary targets for immunological rejection are the major histocompatibility antigens (MHC). The function of these molecules is to present antigens, i.e. peptides, to T cells. In humans the MHC system is known as the human leukocyte antigen (HLA) system and was identified in the 1950s by Dausset, Paine and Van Rood (9-11). A cluster of genes on the short arm of human chromosome 6 encodes the HLA molecules (12). The classical HLA molecules are divided into class I and class II molecules, based on differences in subunit structures, the intracellular pathways of biosynthesis of peptides and the group of T lymphocytes to which they present peptides.

The classical HLA class I molecules (HLA-A, -B and -C) are composed of one heavy α chain linked to β_2 -microglobulin that is required for a stable class I formation (13,14). Peptides presented by HLA class I are normally derived from endogenously synthesized proteins, such as viral antigens or self peptides. These intracellular antigens are presented in the context of HLA class I molecules to stimulate CD8⁺ cytotoxic T lymphocytes (CTL) (15). HLA class I is expressed on virtually all nucleated cells, as any cell may need to show that foreign viral antigen is present to trigger passing immune cells.

HLA class II molecules are encoded by the HLA-DR, -DQ and -DP genes and are composed of two chains, α and β that are linked together to form the complete class II molecule (16). HLA class II is mainly expressed on antigen presenting cells, like B lymphocytes, monocytes, macrophages and dendritic cells (DC). These cells capture proteins present in the surrounding environment and present the extracellular derived antigen in the context of HLA class II to the TCR of CD4⁺ T helper cells (17). Upon activation naive CD4⁺ T cells will polarize towards Th1 or

Th2 cells based on the type of activation (18,19). DC that produce high levels of IL-12 can induce Th1 cells that specifically secrete IL-2, interferon (IFN)- γ and tumour necrosis factor (TNF). Th1 cells are important to eliminate intracellular pathogens through the activation of cytotoxic functions of effector cells like CTLs, macrophages and NK cells. On the other hand, Th2 cells stimulate B cells to proliferate and to produce antibodies and are mainly effective towards extracellular pathogens (20,21). Furthermore, these cells mainly produce cytokines like interleukin (IL)-4, IL-5, IL-6, IL-10 and IL-13.

Transplant Rejection

Although the 1-year graft survival is in most cases excellent due to the improvement of immunosuppressive drugs, rejection still occurs. Several types of rejection can be identified.

Hyperacute rejection occurs very rapidly (within 24 hours) after transplantation. The cause of **hyperacute rejection** can be ABO incompatibility between donor and recipient or the existence of donor-specific anti-HLA-antibodies before transplantation (22). These preformed antibodies can be induced by prior blood transfusions, pregnancies or the rejection of a previous transplant. Antibodies fix complement and subsequently damage the endothelium of the blood vessels. This leads to aggregation of platelets thereby depriving the blood supply to the graft. By the introduction of a crossmatch with serum of the patient against cells of the donor before transplantation, hyperacute rejection can be avoided (23). In case of a positive crossmatch the transplantation will not be performed.

Acute rejection takes days or weeks to develop and is mainly due to primary activation of T cells (24). Some patients experience one or more rejection episodes that in most cases can be effectively treated with immunosuppressive drugs.

Chronic rejection is generally a slow process characterized by loss of graft function late after transplantation. In kidney transplantation, chronic rejection is associated with proteinuria, hypertension and interstitial fibrosis (25). The mechanism of chronic rejection is still poorly understood and several factors are probably involved. It can be very difficult to distinguish chronic rejection from other processes such as chronic cyclosporine A (CyA) nephrotoxicity and therefore the general term chronic allograft nephropathy (CAN) is often used, which is the development of fibrotic processes leading to progressive allograft dysfunction with variable proteinuria and hypertension (26).

Dendritic cells and T cell activation

Costimulation

Besides the recognition of the HLA peptide complex by the TCR (signal 1), also a costimulatory signal (signal 2) is necessary to induce activation of T cells (27-29). A first costimulatory signal is provided through the CD28 receptor after binding of CD80 (B7.1) or CD86 (B7.2) on the antigen presenting cell (APC). After binding of CD28 to CD80 or CD86, CD40 expressed on the APC binds CD40L on the T cell and this interaction provides further costimulation and thus T cell activation (30-32). In order to prevent an ongoing immune response T cell activation has to be regulated. CTLA-4 (CD152) is a homologue of CD28 that binds CD80 and CD86 with a higher affinity than CD28 (33). CTLA-4 plays a critical role as a negative regulator of T cell activation and is upregulated after activation leading to attenuation of the response. When antigen recognition occurs without a costimulatory signal, T cells become non-responsive (34). This can lead to the induction of tolerance (35,36). Indeed, several studies in animals showed that blocking of the costimulatory pathway leads to prolonged allograft survival (37,38). Blocking both the CD28 together with the CD40 pathway had a synergistic graft-prolonging effect (39).

Direct and indirect allorecognition

In a normal immune response T cells recognize foreign peptides in the context of self HLA. In transplantation the situation is different because of the introduction of cells expressing foreign or non-self HLA. T cells from the patient will recognize these allogeneic HLA molecules on the graft derived cells and subsequently evoke an immune response that can lead to allograft rejection. The allogeneic HLA molecules are recognized by T cells via two distinct pathways, the direct or the indirect pathway of allorecognition (Figure 1) (40-42). In the direct pathway, donor derived DC migrate out of the graft to the secondary lymphoid tissues of the recipient. The recipient T cells recognize directly the intact HLA-peptide complexes presented on the donor derived DC and subsequently activation will occur. The indirect pathway involves the uptake of donor-derived antigens by recipient-derived DC that infiltrate the graft. Subsequently, a peptide derived from the mismatched HLA is presented by recipient DC in the context of self MHC to recipient T cells.

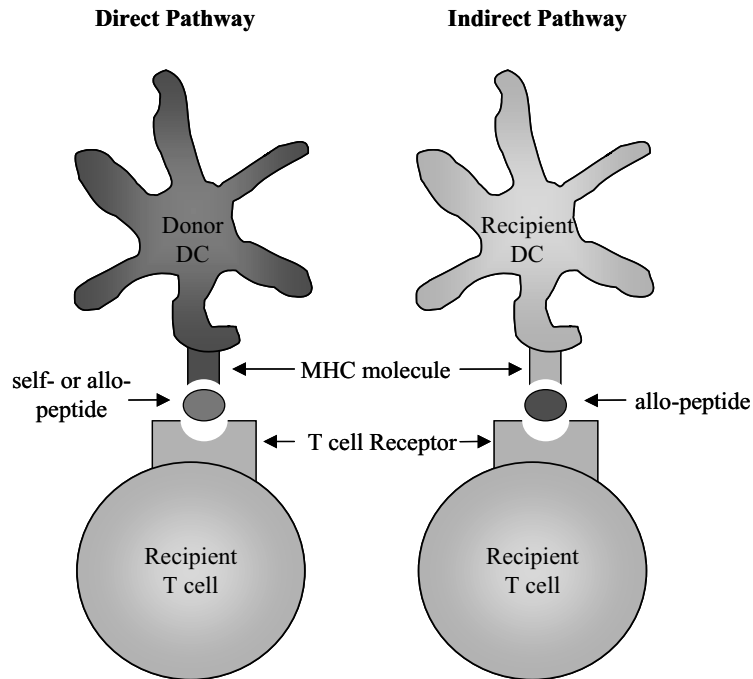


Figure 1. The direct and indirect pathway of allorecognition. The direct pathway (left) involves the direct recognition of donor MHC molecules on donor DC by recipient T cells. The indirect pathway (right) involves recognition of processed alloantigens of donor origin by recipient DC.

The involvement of direct and indirect recognition in the transplantation setting is not fully understood. The general thought is that directly after transplantation the direct pathway of allorecognition mainly influences the immune response and therefore is responsible for the initiation of acute rejection (43-45). Later after transplantation, when the donor derived DC disappear and are replaced by recipient derived DC, the indirect pathway of allorecognition plays a more prominent role and therefore is associated with chronic rejection (46). However, the exact role of both pathways in rejection remains not fully understood especially since there is also evidence for the indirect pathway to be involved in acute rejection (47).

Because of their role in rejection, targeting both the direct and indirect pathway may be important when designing strategies for the induction of tolerance. It may be possible to target both pathways by the use of HLA haploidentical recipient-donor combinations (Figure 2) (48,49). Then, the recipient will be exposed to donor cells that are tolerized for both pathways expressing both donor HLA (direct pathway) and "self" HLA (indirect pathway).

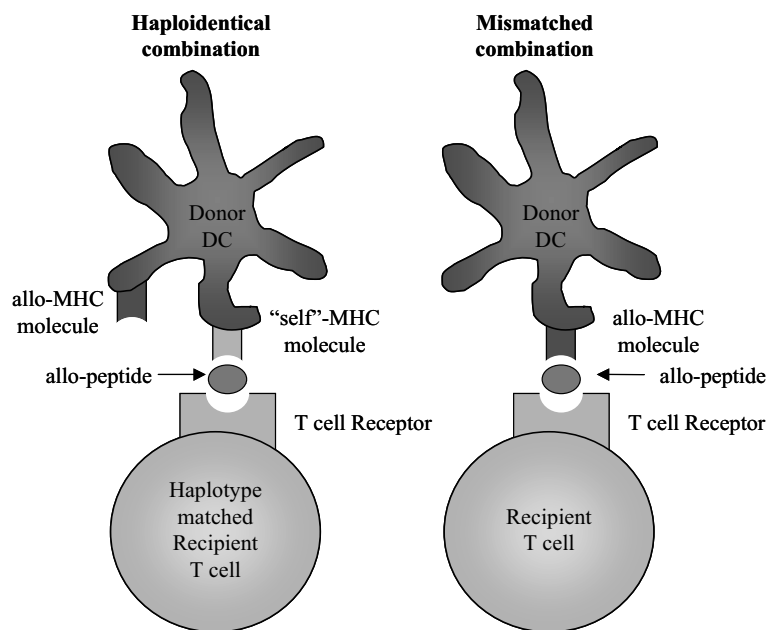


Figure 2. Targeting of the direct and indirect pathway by the use of haploidentical recipient-donor combinations. In case of a haploidentical combination (left), the recipient will be exposed to donor cells expressing both donor MHC (direct pathway) and "self" MHC (indirect pathway), whereas in a fully mismatched combination (right) only the direct pathway of allorecognition is present.

Dendritic cells and the alloimmune response

Dendritic cells are the most potent antigen presenting cells (APC) both in vitro and in vivo and are in fact the sentinels of the immune system. Since DC are able to activate naïve T cells and initiate an immune response, they are the central cells between the innate and acquired immune response (50,51). They reside as immature DC in the peripheral tissues, such as the skin, the kidney and other non-

lymphoid tissues. Here, they internalise and process both self and non-self antigens. Encounter with danger signals such as lipopolysaccharide (LPS) from pathogens, induces the maturation and migration of DC to the lymph nodes. During maturation they lose the ability to capture and process antigen and they start to express large amounts of MHC-peptide complexes and costimulatory molecules like CD80, CD86 and CD40 that are required for the induction of T cell responses (52). Once they arrive in the T cell areas of the secondary lymphoid tissues the mature DC can activate naïve T cells and hereby skew the immune response to a Th1, Th2 or even a regulatory type (53-55).

DC thus play a pivotal role in the initiation of the immune response and are therefore considered to be an important target for the induction of transplantation tolerance. When antigen presentation is present without costimulation, T cell function can be downregulated and eventually regulatory T cells can be induced (56-60). Thus, DC with a more immature (no or low expression of costimulatory molecules) phenotype are especially of interest for the induction of transplantation tolerance. Several biological agents, such as immunoregulatory cytokines (TGF- β or IL-10) (61-63), corticosteroids (64-66) or $1\alpha,25$ -dihydroxy vitamin D3 (67,68), are described to interfere with the maturation of DC and eventually these DC can be tolerogenic or comprise a suppressed immunogenicity. These modulated DC are also described to have a beneficial effect on allograft rejection (55,69,70).

A possible risk when using in vitro prepared immature DC for antigen-specific tolerization in vivo is that such DC can encounter inflammatory stimuli and/or helper CD4⁺ T cells in the host. Exposure to inflammatory stimuli or CD4⁺ T helper cells could lead to full activation instead of the desired suppression of the immune response towards the graft. Thus, it is essential to end up with stable, i.e. maturation resistant in vitro prepared DC.

Regulatory T cells

The concept of T cells that are capable of downregulating or suppressing the function of other cells, such as T cells and APC, was described more than 30 years ago (71). However, because identification of these "suppressor" cells failed and the soluble factors that were thought to be involved were absent, interest in this concept disappeared. In the 1990s Sakaguchi et al. showed that depletion of the CD4⁺CD25⁺ T cells induced the development of autoimmune disease in mice (72). Furthermore, mice that were thymectomized at day 3 after birth developed various organ-specific autoimmune diseases, due to activation of self reactive T cells that are produced before thymectomy (73). The interest in suppressor or regulatory T cells revived and since then several different subsets of regulatory T cells are described both in mice and in human (56,74,75). Most attention focused on the

cells described by Sakaguchi et al., the naturally occurring CD4⁺CD25⁺ regulatory T cells (72,76-78). However, CD4⁺ regulatory T cells can also be induced in vitro or in vivo, the so-called adaptive regulatory T cells (57,59,60) and in addition suppressive CD8⁺ T cells are described (79-81).

Naturally occurring Regulatory T cells

The naturally occurring regulatory T cells (Treg) subset is derived from the thymus and represents about 5%-10% of the total CD4⁺ T cell fraction in healthy individuals. Naturally occurring Treg are CD4⁺ and express high levels of CD25 (the IL-2 receptor α -chain) (82). These CD4⁺CD25⁺ cells are anergic, i.e. in response to T cell stimuli they show a low proliferative capacity and they do not produce cytokines in vitro. To exert their suppressive function T cell receptor ligation and direct cell-cell contact are required. Furthermore, suppression is not APC dependent (83,84) and the suppressive effect is not antigen-specific (85). Besides increasing evidence that naturally occurring Treg play a role in preventing autoimmune disease via the suppression of auto-reactive T cells (86,87), these cells are also described to play a role in transplantation tolerance (88) and in maternal tolerance towards the foetus (89).

Adaptive Regulatory T cells

Treg cells can also be induced in the periphery. Adaptive Treg cells can be induced both in vitro and in vivo (90-95). The most described peripherally induced or adaptive Treg are the Tr1 cells (90) and the Th3 cells. Tr1 cells are induced after exposure of T cells to antigen in the presence of the regulatory cytokine IL-10 (60,96). These CD4⁺ T cells produce mainly IL-10, but also TGF- β , IFN- γ and IL-5, low IL-2 and no IL-4. Furthermore, they have a low proliferative potential and suppress an immune response in an antigen specific way (59,97). Furthermore, addition of anti-IL-10 monoclonal antibody reverses the suppressive effect of Tr1 cells, indicating that IL-10 plays an important role.

Th3 cells can be induced in vivo following oral administration of antigen (91,92). These cells are CD4⁺ Treg cells that function mainly via TGF- β . Addition of anti-TGF- β antibodies abolishes the suppressive effect of Th3 cells. Th3 cells can be generated in vitro by the addition of TGF- β to the culture of T cells.

As already mentioned, Treg cells can also be induced by immature DC and by the lack of costimulation (56-60).

CD8⁺ suppressor T cells

Several subsets of CD8⁺ T suppressor (Ts) cells are described. Among them are the antigen specific CD8⁺CD28⁻ Ts cells that act via cell-cell contact with APC (79) and the CD8⁺CD28⁻ Ts cells that suppress antigen non-specific via soluble factors like IFN- γ and IL-6 (98). Recently, these cells were shown to mediate their

suppressive effect via IL-10, since anti-IL-10 antibodies abrogates suppression (81). Additionally, these non-specific CD8⁺CD28⁻ Ts cells do not express the marker CD27, whereas the antigen specific CD8⁺CD28⁻ Ts cells do.

The antigen non-specific Ts cells have been found to be functionally impaired in patients with multiple sclerosis and also in patients with systemic lupus erythematosus and thus these cells are associated with autoimmune diseases (81,98).

Recently, a higher percentage of CD8⁺CD28⁻ T cells was found in the maternal part of the placenta, suggesting a role in the regulation of maternal alloreactivity towards the foetus (99).

CD8⁺ Ts also have been found in a tolerant kidney transplant recipient (100). Human HA-1 (minor histocompatibility antigen) specific CD8⁺CD28⁺ exert their suppression via TGF- β and IL-10 and suppression was CTLA-4 dependent. Interestingly the Tregs coexisted with CD8⁺ T effector cells that had a higher affinity for the antigen. This indicates that lifelong peripheral tolerance to an organ allografts can be achieved without the loss of immunologic memory to donor antigen.

Phenotypic characteristics of regulatory T cells and suppressor T cells

Today, no good phenotypic markers are available to distinguish the different types of regulatory T cells or suppressor T cells. The naturally occurring Tregs are CD4⁺CD25^{hi} and express CTLA-4 (CD152) and glucocorticoid-induced tumour necrosis factor receptor family related protein (GITR). However, the induced CD4⁺ Treg cells also express CD25, CTLA-4 and GITR. Furthermore, expression of CD25, CTLA-4 and GITR is not specific for regulatory populations since activated T cells also express these markers (101,102). Recently, a new marker was proposed to be specific for CD4⁺ Tregs, namely the transcription factor FOXP3 (forkhead box P3) (103). This transcription factor is genetically defective in an inflammatory and autoimmune disease in both mice and humans. However, also this marker seems to be not completely specific for Tregs (104).

Even less is known about the phenotype of CD8⁺ Ts cells. They can express CD28, but most suppressor cells described do not express CD28. Furthermore, the absence or presence of CD27 is described (81,105) and finally, the CD8⁺CD28⁺ Ts cells found in a kidney graft recipient did not express CD62L and CCR7 (100). However, the diversity between the Ts cells described is too large to assign a marker specific for suppression.

Aim of this thesis

The ultimate goal in the transplantation field is the induction and maintenance of donor specific tolerance. Treg cells that control immune responses to alloantigens give opportunities for tolerogenic therapies in transplantation. However, it is important to investigate the mechanisms of tolerance induction in order to use the optimal strategy. Therefore, we explored both natural tolerance towards NIMA that can be induced during fetal life and induced tolerance by modulation of DC.

Naturally induced tolerance towards NIMA can have an influence on transplant outcome later in life. **Chapter 2** gives an introduction and overview of the current knowledge about the NIMA effect on the outcome of transplantation. In **chapter 3** we explored the influence of NIMA on the alloreactive T cell repertoire in healthy individuals and in **chapter 4** we focused on the NIMA effect in patients transplanted with a NIMA haplotype mismatched kidney graft.

In order to actively induce tolerance, we modulated DC to generate Treg cells, since this may be of clinical relevance in the future for patients that are on the waiting list for transplantation. In **chapter 5** we explored the possibility of using modulated DC for the induction of transplantation tolerance in a fully allogeneic setting in mice. **Chapter 6** describes an in vitro system for the use of human modulated DC to induce Treg cells. We show that two differentially modulated human DC can lead to different types of Treg cells.

Finally, we examined the possibility to use in vitro tools to measure a possible tolerant state in patients. Monitoring of e.g. Treg cells and/or cytokines may give an indication which patients are at risk for rejection and which patients are more predisposed to tolerance. **Chapter 7** describes the Elispot technique as a possible tool to monitor patients that received a renal allograft.

Finally, the results are summarized and discussed in **chapter 8**.

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