# Immunosuppressive Drugs and Immune Regulation in Organ Transplantation

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# Immunosuppressive Drugs and Immune Regulation in Organ Transplantation

## Immuunsysteem onderdrukkende Medicatie en Immuunregulatie bij Orgaantransplantatie

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# Chapter 1

## General Introduction and Outline of this Thesis

## CONTENTS GENERAL INTRODUCTION

## 1.1 ORGAN TRANSPLANTATION

Solid organ transplantation is during the past two decades the finest and most suitable treatment with the best quality of life for patients with end stage organ failure. The first documented 'unrelated' kidney transplantation was performed on June 17, 1950 in Chicago, United States on a 44-year-old woman with polycystic kidney disease[2]. Unfortunately, the donated kidney was rejected because no adequate immunosuppressive therapy was available at the time and the development of effective anti-rejection drugs was years away. The first successful kidney transplantation was performed on December 23rd in 1954 from one healthy identical twin to his twin brother who was almost dying of renal disease[3]. The operation succeeded and renal function was restored in the recipient and resulted in enormous excitement in the media and among medical professionals. One decade further, the first human liver transplantation was performed in 1963 by a surgical team led by dr. Thomas Starzl[4].

The discovery and availability of potent immunosuppressive drugs that were able to prevent rejection of the transplanted donor graft, was followed by an era of many successful solid organ transplantations. The use of these immunosuppressive drugs resulted in acceptable graft-survival rates in the Netherlands. The graft survival rate over 2007 was 94% after clinical kidney transplantation with living donors, 85% after clinical kidney transplantation with deceased donors and 76% after clinical liver transplantation (Nederlandse Transplantatie Stichting). Nevertheless, acute rejection may occur in the first 3 to 6 months after transplantation, but this can be well treated with (steroid) anti-rejection therapy.

Although, the use of new classes of drugs and new combinations of immunosuppressive drugs have been shown to improve short-term outcomes as they improve graft survival and decrease the rate of acute allograft rejection in the first year of transplantation, a dark side of immunosuppressive drug therapy has emerged. There are two major caveats with prolonged immunosuppression after organ transplantation for the patient. First, the considerable clinical load that comprises numerous adverse effects conveyed to the patient and results in increased morbidity as e.g. they increase the susceptibility for infections and malignancies, but these also include non-immunological complications as osteoporosis, diabetes and an increase in cardiovascular risk factors, (nephro)toxicity and even mortality[5, 6] Secondly, the relatively poor efficacy in preventing immunologically driven chronic allograft rejection[7] resulting in graft failure after long-term usage of immunosuppressive drugs.

The side effects of immunosuppressive medication are due to lack of specificity as they do not only affect the cells of the immune system but also affect non-immune cells like parenchymal cells, smooth muscle cells, etc. Therefore, therapy that specifically targets immune cells involved in the reactivity against the donor graft without affecting cells of the immune system that provide immunity against infections and non-immune cells is severely needed.

## 1.2 THE IMMUNE SYSTEM

The immune system has been evolved to protect us from pathogens such as viruses and bacteria. There are two types of immune responses: 1) the innate immune response, which is the first line of defense against pathogens by macrophages and phagocytic cells and 2) the adaptive immune response, which is a specific immune response against particular foreign antigens (Box 1) or a specific pathogen. Adaptive immunity occurs during the lifetime of an individual as an adaptation to infection with that antigen or pathogen and offers life-long protection against re-infection with the same pathogen. Adaptive immune responses are initiated in peripheral lymphoid tissues (Figure 1B) after antigen presentation (Box 1).

Two major types of **peripheral lymphoid tissues**:

1. **the spleen**, that collects antigens from the blood;
2. **the secondary lymph nodes**, which collect **antigens** from sites of infection in the tissues.

Two major phases of any **immune response**:

1. Recognition of **antigens**; molecules recognized by receptors on **lymphocytes** in lymphoid tissues.
2. A reaction to eradicate the antigens.

**Lymphocytes** circulate in the **peripheral blood** and can mediate immunity (20% of white blood cells in adults)**.** They have specialized functions. **Lymphocytes** comprise:

1. **B cells** that differentiate in the fetal liver and the postnatal bone marrow and produce antibodies;
2. **T cells** that develop in the thymus (Fig. 1A) and recognize antigens with their T-cell receptor. **T cells** comprise:
   - ***CD8$^+$ cytotoxic T cells** kill virally affected cells;
   - ***CD4$^+$ helper T cells** coordinate the immune response by direct cell-cell interactions and release cytokines which help B cells to produce antibodies.
   - ***Regulatory T cells** can control immune responses to self-antigens.
3. **Natural Killer cells** can lyse certain tumour cells and virus-infected cells.

**Antigen-presenting cells (APCs)** are required by T cells to enable them to respond to antigens.
Dendritic cells, macrophages and B cells recognize native antigens not processed and presented by other cells.

The cells of the **innate immune system** (natural killer cells, macrophages, phagocytes) play a crucial part in the initiation and subsequent direction of **adaptive** immune responses by T and B lymphocytes.

**Mixed lymphocyte reactions (MLR**): in vitro assay to imitate immune responses from to patient to donor.

**Major histocompatibility Complex (MHC) - Human Leucocytes Antigens (HLA, Human Variant of MHC)**
The function of the MHC molecules on T cells is to bind peptide fragments derived from pathogens and display them on the cell surface for recognition by the appropriate T cells.

Large numbers of T cells are specifically reactive against particular **non-self or allogeneic MHC molecules** resulting in a **cell-mediated immune response.**

**Alloreactivity:** reactivity of immune cells of one individual against antigens from another individual.

**Clonal selection** is the central principle of **adaptive immunity** and involves recognition of antigen by a particular lymphocyte; leading to **proliferation** (expansion) and cytokine secretion by activated T cells and **differentiation** into **effector T cells**. The clonal expansion of these effector T cells can cause **acute cellular rejection**.

**Cytokines** are the soluble mediators of immunity and defined as a large group of molecules (proteins or peptides) involved in signaling between cells during immune responses.

**Box 1.** Introduction into the Immune System
Adapted from the textbooks 'Immunology'[13] and 'Immunobiology The immune system in health and disease'[14].

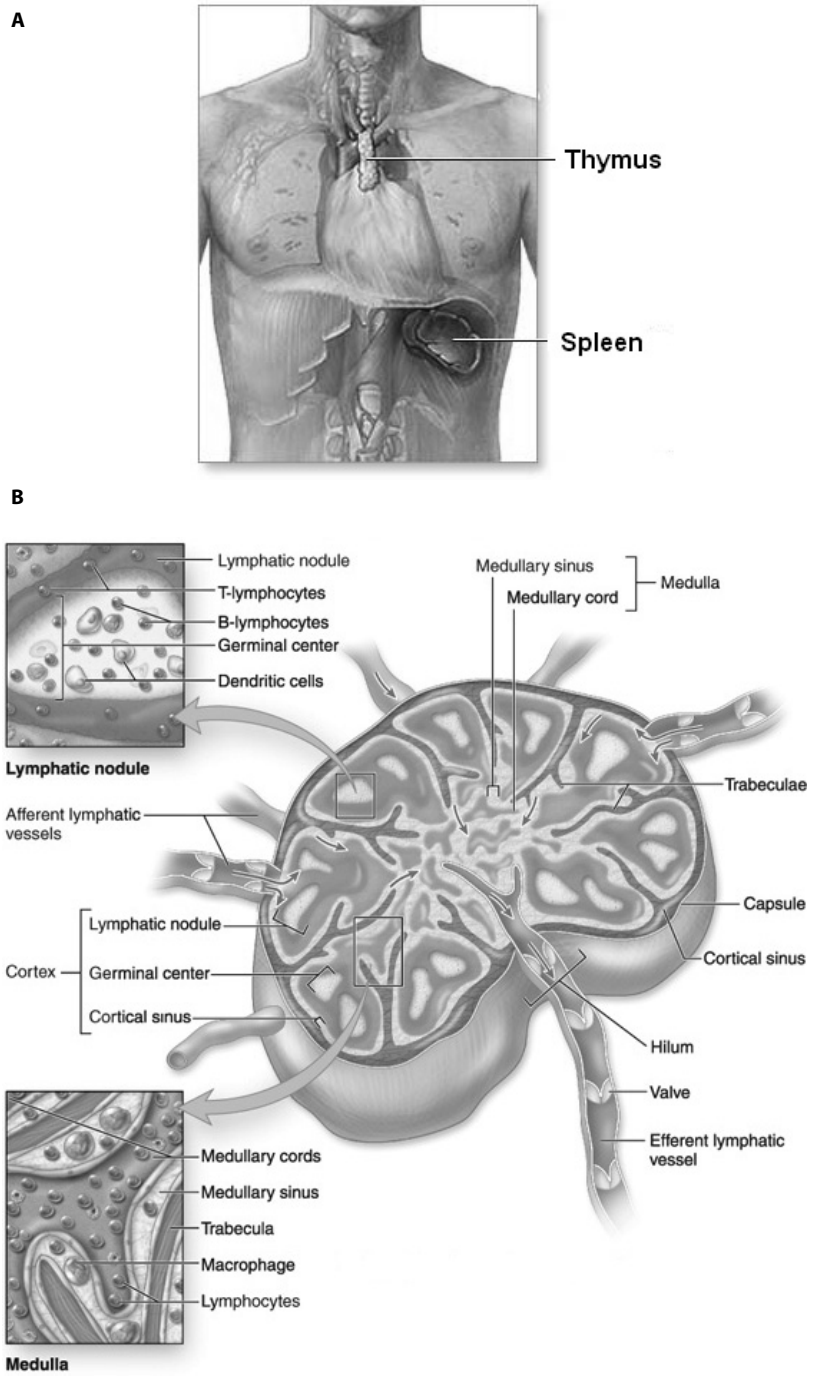


**Figure 1.** Primary and secondary lymphoid organs

**(A)** Location of the thymus and the spleen (primary lymphoid organs) in the human thorax, ©ADAM. **(B)** Structure and function of a lymph node and its components, where antigen recognition takes place (secondary lymphoid organ), adapted from the textbook 'Human Anatomy'[1].

*1.2.1 Transplantation immunobiology*

Immediately after solid organ transplantation, the immune system will respond to the foreign antigens of the donor graft in order to eliminate the graft by causing rejection. Professional antigen-presenting cells (APCs), mainly dendritic cells (DCs) from the donor will migrate from the donor graft towards the secondary lymphoid organs in the recipient (Box 1 and Figure 1B), where they can activate naïve T cells and resting/central memory alloreactive T cells from the recipient (Box 1 and Figure 3A). Immune responses (Box 1) are triggered after recognition of the allogeneic major histocompatibility complex mismatched antigens (MHC) or the human MHC, which is called Human Leukocyte Antigens (HLA), by receptors on T cells of the recipient (Box 1). This mode of T-cell activation by APCs of the recipient is called the direct pathway of allorecognition. Upon activation by alloantigens, $CD4^+$ helper T cells produce massive amounts of the T-cell growth factor Interleukin (IL)-2 which is required for their clonal expansion and for their differentiation into effector T cells (Teff). The direct pathway of antigen presentation is imitated *in vitro* by allogeneic mixed lymphocyte reactions (MLR) (Box 1). The major role of APCs in rejection has been demonstrated in experimental models showing that depletion of donor APCs can sometimes prolong graft survival[8].

A second mechanism for initiating an immune response and T-cell activation is the indirect pathway of allorecognition, which is stimulation of recipient T cells by recipient APCs that can present peptides of donor origin[8]. Evidence has been provided to demonstrate the contribution of the indirect pathway to rejection in animal experimental models[9-11] e.g. allogeneic skin grafts in mice that lack MHC class II antigens are rejected rapidly[11]. In humans, data about the relationship between reactivity of T cells with indirect allospecificity and graft rejection are lacking[12]. Thus, the role of the indirect pathway in rejection remains to be elucidated.

Whether via the direct or indirect pathways, both mechanisms of the immune system are activated after organ transplantation and can mediate acute or chronic cellular graft rejection respectively. These mechanisms that can cause an activated immune system of the recipient indicate the need for immunosuppressive drug therapy to eliminate alloreactive effector T cells from the recipient in order to prevent graft rejection.

## 1.3 REGULATORY T CELLS (TREG)

The immune system does not only have a defense mechanism to eliminate everything that is foreign. It has also built-in mechanisms to maintain immune homeostasis and to prevent the attack of healthy self-tissues. The first line of self-tolerance is the elimination of self-reactive T cells during negative selection in the thymus (Figure 1A) and B cells in the bone marrow. However, some self-reactive T cell clones 'escape' central tolerance and enter the peripheral compartment. There are several important mechanisms known by which the immune system can achieve peripheral tolerance. Except for other mechanisms to achieve tolerance concerning

Teff cells as clonal deletion, ignorance, activation-induced cell death and anergy, the induction of regulatory T cells may play an important role in the acquisition of peripheral tolerance[15].

Treg are able to control unwanted immune responses and have been shown to play a pivotal role in controlling autoimmunity. Their function has also been implicated in inflammatory diseases such as asthma and inflammatory bowel disease[16-19]. A growing body of evidence suggests that $CD4^+$ T cells that highly express the IL-2 receptor-α chain (CD25) on their surface have specificity for the direct pathway and play a role in transplant 'tolerance'. The Holy Grail in transplantation is to achieve long-term tolerance. Tolerance refers to a state of sustained specific non-responsiveness of the recipients' immune system to donor alloantigens, allowing long-term allograft survival in the absence of potential harmful chronic immunosuppressive drugs. Immunological tolerance is a state of antigen-specific T-cell unresponsiveness' or an immunologic blind spot for a specific antigen, whereas the responses to all other antigens are completely intact. Immunologic tolerance in the organ transplantation context is defined as a durable state of antigen-specific unresponsiveness, induced by exposure to donor antigens from the graft, in a patient who is otherwise fully immunologically competent[20].

When there is a state of minimal immune responsiveness by active regulation of the immune responses that are directed against the donor graft, this will automatically lead to little requirement for immunosuppressive drugs. Transplanted patients may benefit from a reduction in the dose and time of usage of the immunosuppressive drugs and even cessation after transplantation. 'Operational tolerance' is defined as long term (more than 12 months) independence from all immunosuppression in patients with normal graft functions. Mechanisms and protocols to actively induce 'operational tolerance' have already been investigated for more than 50 years in animal experimental models[21], but few papers report (liver) transplant patients that are tolerant for their graft[22-24]. Therefore, skewing of the immune system to a more donor-antigen specific therapy might be more promising.

### *1.3.1 Regulatory T-cell subsets*

Myriad surface expression profiles have revealed several subpopulations of Treg (Figure 2). The naturally occurring $CD4^+CD25^+$ T cells have their origin in the thymus (Figure 1A) and comprise 5-10% of total $CD4^+$ T cells in the human peripheral blood. Their function is to control the proliferation of $CD4^+$ and $CD8^+$ Teff cells.

In 1971, Gershon and Kondo were the first to report the importance of suppressor T cells as they showed the immunosuppressive effect of the presence of thymocytes during antigen pre-treatment in mice[25]. Hall et al. showed that a subset of T 'helper/inducer' T cells were responsible for the onset of specific unresponsiveness in rats treated with cyclosporine[26]. Five years later, the same group demonstrated in cyclosporine treated rats that $CD4^+$ T suppressor cells with inhibitory capacities were induced for this unresponsiveness[27]. After a period of quiescence, Sakaguchi and his colleagues provided evidence that $CD25^+$ T cells, of which the majority is $CD4^+$, prevent autoimmune disease and that depletion of these cells induces

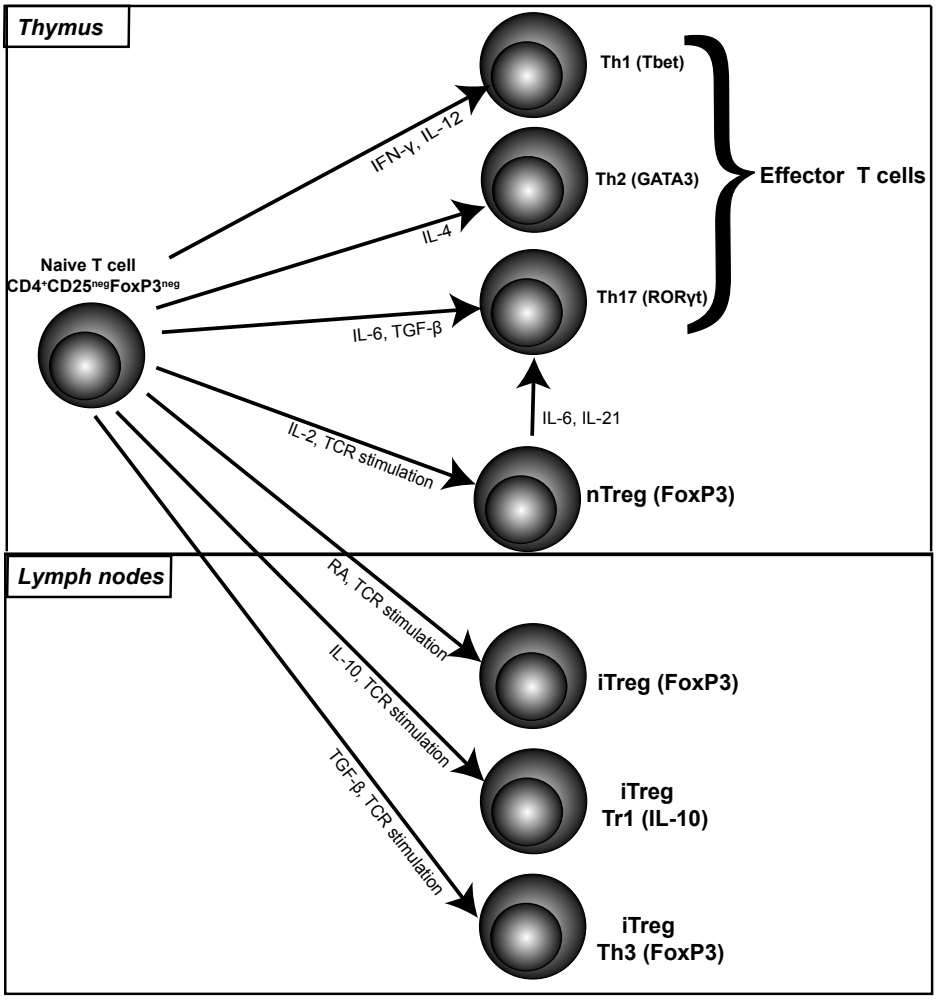


**Figure 2.** $CD4^+$ T cell differentiation and conversion

Differentiation factors are depicted next to the arrows. Transcription factors are shown between brackets. The factors that are required for thymic and peripheral generation of regulatory T cells are shown. Th; T helper, nTreg; natural regulatory T cell, iTreg; induced regulatory T cell, Tr; T regulatory cell type, TCR; T-cell receptor, RA; retinoic acid. It is important to note that this is a simplified model and that there is a great plasticity of $CD4^+$ T-cell differentiation than described in this figure[68].

autoimmunity[16]. They showed that when allogeneic skin grafts were transplanted at the time of $CD25^{neg}$ cell injection into immune deficient mice, there were heightened immune responses, but when $CD25^+$ T cells were reconstituted, these immune responses were immediately dampened[16]. A decade after this publication, it was reported that the severe Immune dysregulation, Polyendocrinopathy, Enteropathy, X-linked (IPEX) syndrome was caused by a mutation in the FOXP3 (forkhead/winged-helix box protein 3, Scurfin) gene[28]. Thereafter, it was demonstrated that over-expression of Scurfin FoxP3 in $CD4^+$ T cells attenuated activation-induced cytokine production and proliferation by these cells[29]. The FoxP3 protein acted as a repressor of

transcription and in this way regulated T-cell activation[29]. The breakthrough came when the association between the $CD4^+CD25^+$ T cells and FoxP3-expression was drawn by demonstrating that FoxP3 plays an important role in guiding the differentiation and function of $CD4^+CD25^{bright}$ T cells as they highly express this transcription factor[30, 31]. Now it has been revealed that FoxP3 binds to the promoters of over 700 genes and has both activating and inhibitory activities[32, 33]. Furthermore, it can interact with NFAT, the key regulator of T-cell activation and anergy[33, 34]. A few years later, human $CD4^+CD25^{bright}FoxP3^+$ T cells were shown to downregulate the IL-7 receptor α-chain (CD127) that is highly expressed by Teff cells and memory T cells[35].

Recently, it was reported that the level of FoxP3 expression and the extent of post-translational FoxP3-acetylation seems to be important factors in governing the suppressive activity of naturally occurring Treg[36, 37].

As $CD4^+CD25^{bright}$ Treg require IL-2 for their expansion, homeostasis and function[38], the mechanism of suppression by Treg takes place via several mechanisms. The most well-known mechanism is that they inhibit the proliferation of Teff cells by 1) the inhibition of the IL-2 production by Teff cells or 2) via scavenging of IL-2, which is one of the crucial mechanisms of suppression to control alloreactivity[39, 40]. Other notorious mechanisms of suppression may occur via interactions mediated through cell-surface molecules such as TGF-β, CTLA-4 (cytotoxic T lymphocyte-associated antigen-4) that can cause signaling through B7-1 and B7-2 on DCs leading to the production of indoleamine 2,3-dioxygenase[41], ICAM (intracellular adhesion molecule), and modulators of Treg function GITR (glucocorticoid-induced TNFR-related protein) and OX40. TGF-β seems to correlate with the maintenance of FoxP3-expression. However, FoxP3-expression by itself is not sufficient to confer suppressive function[42, 43]. Moreover, it is postulated that IFN-γ and IL-35 play a role in the paracrine suppressive mechanisms of regulatory T cells[44, 45]. However, the contribution of IL-35 to regulatory T cells function remains controversial[46]. Other factors include granzyme B that can be secreted by $CD4^+CD25^{bright}$ T cells and predominantly acts as an effector molecule to directly lyse autologous Teff cells and B cells after activation[47-49].

Treg are not only discharged into the periphery by the thymus, a small group of Treg has its origin in the periphery, and is known as induced (i) Treg[15] (Figure 2). Though, the contribution of iTreg in restraining immune responses to allo-antigen *in vivo* is unknown, the antigen-specific Treg are supposed to have potent suppressive properties in the inhibition of immune responses against donor-antigens as has been shown *in vitro*. It has been demonstrated that human naïve $CD25^{neg}$ T cells can differentiate into induced $CD4^+CD25^+FoxP3^+$ T cells and $CD8^+CD25^+FoxP3^+$ T cells with regulatory activities *in vitro* after stimulation with antigen in the presence of $CD14^+$ monocytes[50, 51]. CD3/CD46-induced regulatory T cells have been shown to mediate granzyme and perforin mediated lysis of activated Teff cells[47].

Other regulatory T cell types include the CD4 and CD8 double negative ($CD4^{neg}CD8^{neg}$) T cells[52], $CD3^+\gamma\delta TCR^+$ T cells[24], the natural killer T cells[53], IL-10-producing Tr1 T cells[54] (Figure 2) and the TGF-ß-producing Th3 T cells[55] (Figure 2).

$CD4^{neg}CD8^{neg}$ Treg comprise 1-2% of peripheral blood mononuclear cells (PBMC) and are associated with graft acceptance. They are capable of inhibiting immune responses via directly killing Teff cells in an antigen-specific fashion via FASL and are $CD27^{+}$[56, 57]. The $CD3^{+}\gamma\delta TCR^{+}$ T cells (Vδ1-type) produce massive amounts of IL-10 and were found to have a higher activation state in tolerant patients than in healthy controls[24]. Levels of $CD3^{+}\gamma\delta TCR^{+}$ T cells (Vδ1-type) were increased in the peripheral blood of tolerant recipients as compared with immunosuppression- dependent patients or healthy controls, suggesting that these cells may have an important function in establishing tolerance after transplantation[23]. They play an important role in controlling the development of various immune pathologies and enhance allo- and xeno-transplant survival. Moreover, in comparison with $\alpha\beta\text{-}TCR^{+}$ T cells, the $CD3^{+}\gamma\delta TCR^{+}$ T cells are insensitive to the immunosuppressive drug cyclosporine A[58]. The invariant natural killer T cells lyse autologous T and B cells via perforin and produce Th1 cytokines (IFN-γ and TNF-α) and Th2 cytokines (IL-4, IL-5, IL-10 and IL-13) (Box 1). In experimental models using knockout mice that lack iNKT cells, it has been demonstrated that they play an important immune regulatory role in the maintenance of transplant tolerance[59]. NKT levels can also be influenced by immunosuppressive drugs. The combination of several classes of drugs seems to enhance the levels of NKT cells and $CD4^{+}FasL^{+}$ T cells. In primates that received immunosuppressive therapy consisting of both the calcineurin inhibitor tacrolimus and the lymphocyte proliferation inhibitor sirolimus after kidney transplantation, increased levels were detected compared with the tacrolimus and sirolimus treated groups alone[60].

The IL-10-producing Tr1 Treg also harbor the $CD4^{+}CD25^{+}$ phenotype and have their immunoregulatory effect on APCs and T cells as they downmodulate the co-stimulatory molecules CD80 and CD86 on APCs as well as MHC class II, indispensable for T-cell activation[15].

As tolerance to food antigens (oral tolerance) might be achieved by the administration of high antigen dose and may lead to hyporesponsiveness mediated by anergy or deletion, low doses of antigen lead to the generation of antigen-specific regulatory Th3 cells that produce TGF-β[61, 62]. Furthermore, TGF-β-production by Th3 cells does not necessarily always correlates with the expression of other anti-inflammatory cytokines as IL-4 and IL-10[63]. Th3-cells have been shown to transfer tolerance *in vivo* and to suppress antigen-specific responses *in vitro*[64].

Treg are not only present within the $CD4^{+}$ T cell population, other subsets within the $CD8^{+}$ T cell population that also have (antigen-specific) regulatory capacities are the $CD28^{neg}$ T cells[65]. These cells share several similarities in their molecular markers with the $CD4^{+}CD25^{bright}$ T cells[65] e.g. they can also express FoxP3[67].

To summarize, there are different Treg subsets that have a different origin (Figure 2) and diverse mechanisms of action with the common purpose to regulate immune responses. Finally, it is important to take into account that there is a great degree of flexibility in the differentiation options of $CD4^{+}$ T cells i.e. the expression of the transcription factor Foxp3 by iTreg may not be that stable[68].

*1.3.2 Generation of regulatory T cells for therapeutic application*

Although the continued use of immunosuppressive drugs provides a degree of long-term maintenance of allograft function, as mentioned earlier, finding new protocols to target the activated T cells directed against donor-antigens to release the patient from the severe side effects is a challenging task for transplantation immunologists.

Treg are able to restrain donor-directed immune responses *in vitro* and may for that purpose be critical inducers of graft acceptance. However, natural occurring Treg are not specific as they are not directed against donor-antigens and those natural occurring Treg that cross-react with the Teff cells are present at low frequency.

The ultimate goal of transplantation may be established by increasing the frequency or enhancing the suppressive activities of regulatory T cells. To accomplish operational tolerance, expansion protocols have been developed to increase their number *in vitro* and ultimately re-infuse them into the patient by adoptive transfer. There are two protocols of *in vitro* expansion; 1) antigen-specific[69] and 2) polyclonal[70]. Alloantigen-specific Treg are supposed to provide higher therapeutic benefits in solid organ transplantation compared with polyclonal Treg, because they suppress immune responses that are directed against alloantigens and will diminish the overall immunity against pathogens[71]. Unfortunately, there are major drawbacks with the isolation, expansion and purity of these T cells *in vitro*. First, it is only possible to study the suppressive capacities of $CD25^{bright}CD127^{-/low}$ T cells. Although the majority of these cells are $FoxP3^{+}$, $FoxP3^{neg}$ cells are present in this population[35]. Furthermore, it has been demonstrated that Treg may lose their CD25 and FoxP3-expression *in vitro*[72, 73]. It has been shown that only the CD45RA (naïve) $CD4^{+}CD25^{bright}$ population gives rise to a homogenous population[74]. Moreover, when Treg are expanded *in vitro*, it is unknown whether they still contain their migratory capacities *in vivo* and reach the place where immune activation takes place; the peripheral lymph nodes (Figure 1B).

Other *in vivo* experimental transplantation studies have shown that Treg can be generated from naïve T cells that are recruited to the allograft by conversion of $CD25^{neg}$ precursors into $CD25^{+}$ T cells (Figure 2)[75, 76]. The induction of FoxP3 expression can only take place in the presence of TCR triggering and cytokines (Figure 2) or by immunosuppressive drugs as has been shown in experimental models[50, 77-80]. Whether Treg induction occurs *in vivo* depends on the context in which the T-cell encounters antigen dose in terms of antigen form, antigen dose, co-stimulation and the APC subtype[71]. More research needs to be done to fully characterize the natural and adaptive regulatory T cells and to reveal their role in transplantation tolerance.

## 1.4 T-CELL ACTIVATION AND TARGETS OF IMMUNOSUPPRESSIVE DRUGS

T cells require three distinct signals for most optimal activation to initiate an immune response. Signal 1 is provided by the CD3 complex on T-cell receptors that recognizes antigen on the

surface of dendritic cells and transduce this signal across the cell membrane (Figure 3)[81]. Signal 2 is the co-stimulatory signal that is provided by dendritic cells and is delivered when CD80 and CD86 on the surface of the dendritic cells engage CD28 on T cells (Figure 3). The combination of signal 1 and 2 activates three signal transduction pathways: the calcium-calcineurin pathway, the RAS-mitogen-activated protein (MAP) kinase pathway and the protein kinase C-nuclear factor-κB pathway which in turn can activate i.e. the nuclear factor of activated T cells (NFAT), activating protein 1 (AP-1), and NF-κB respectively (Figure 3). Activation of the NFAT, AP-1 and NF-κB transcription factors trigger the expression of many molecules such as the immunomodulator IL-2, CD154 (CD40L) and CD25[81]. APCs do not only trigger T-cell activation via cell-cell contact. They produce many cytokines as e.g. IFN-γ and IL-4 that can provide signal 3 and trigger T-cell activation and differentiation. Next to APCs, Th cells function as a source for cytokine production. IL-2, -4, -7, -9, -15 and 21 (cytokines of the IL-2 family) share the common γ-chain ($\gamma_c$) and can recruit Janus Kinase 1 and 3 (Jak3) upon autophosphorylation (Figure 3). These cytokines, in particular IL-2 and IL-15 activate the mammalian 'target of rapamycin' (mTOR) pathway and deliver growth signals through the phophoinositide-3-kinase that is the major trigger for T-cell proliferation[81] and differentiation into Teff cells. Immunosuppressive drugs that are currently in clinical practice act on distinct pathways for T-cell activation (Figure 3) and will be outlined in the next paragraphs.

## 1.5 SUBCLASSES OF IMMUNOSUPPRESSIVE DRUGS

### *1.5.1 Induction therapy*

Induction therapy consists of polyclonal or monoclonal antibodies directed against epitope(s) on T cells. The purpose of induction therapy is to deplete or modulate T-cell responses at the time of antigen presentation.

Rabbit anti-thymocyte globulins (rATG) are comprised of polyclonal immunoglobulins that are purified from the serum of rabbits after immunizing these animals with human thymocytes. Induction therapy using rATG is given to patients during the first days after organ transplantation to decrease the incidence of delayed graft function and to reduce the dosage of calcineurin inhibitors during the first days after transplantation. After two days of rATG-induction therapy, transplanted patients receive maintenance therapy consisting of a calcineurin inhibitor, mycophenolate mofetil (MMF) and prednisone. By binding to epitopes on the surface of lymphocytes and monocytes, rATG depletes these cells from the circulation and secondary lymphoid tissues, thereby preventing acute rejection of the graft (Figure 3)[82-84]. Toxic side effects include thrombopenia, the cytokine-release syndrome, and occasional serum sickness or allergic reactions.

The IL-2 receptor is also a potential target for immunosuppressive drugs. Monoclonal antibodies that bind to the CD25 antigen at the surface of activated T cells inhibit IL-2 mediated T cell activation, which is a crucial phase in the cellular immune response of allograft rejection (Figure 3).

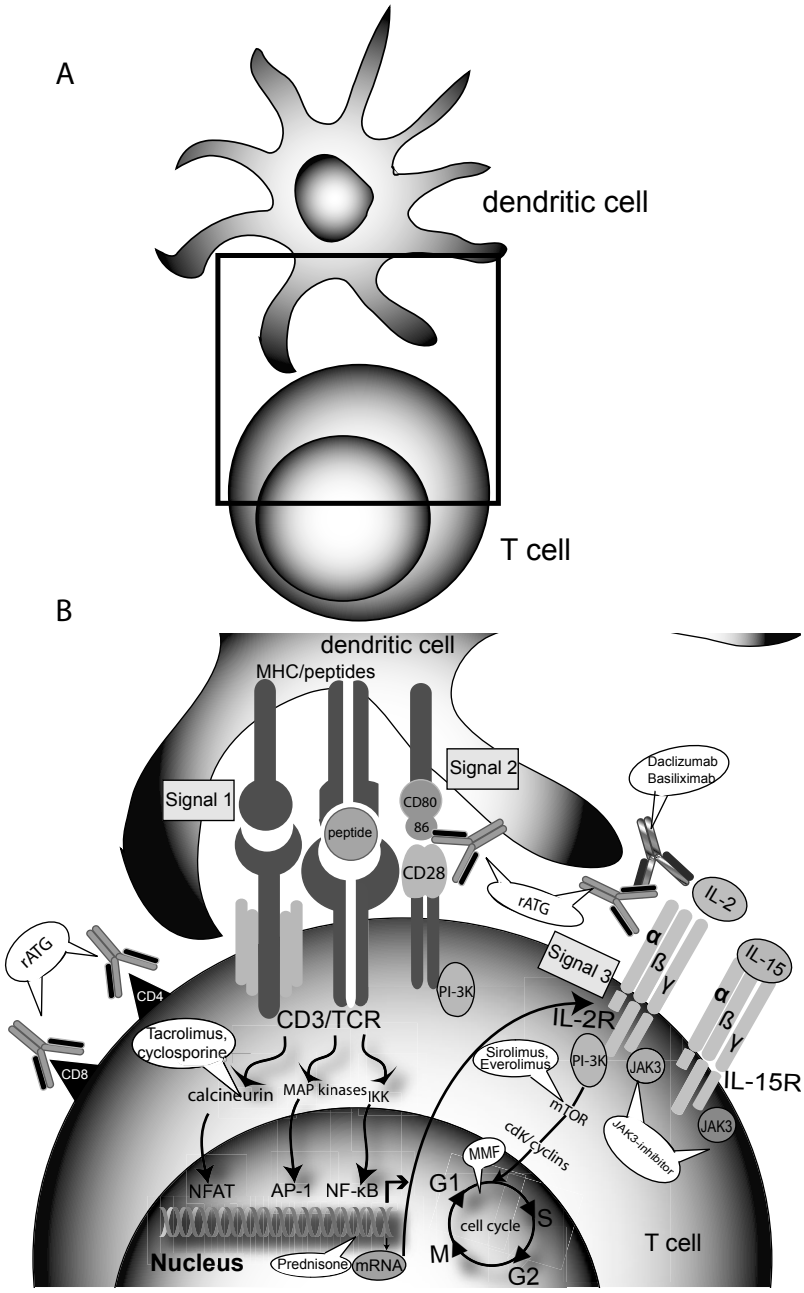


**Figure 3.** T-cell activation

**(A)** Dendritic cell encounters T cell and forms an immunological synapse **(B)** Signals for T-cell activation and individual targets of immunosuppressive drugs as described in this dissertation. Antigens ('peptides') trigger T-cell receptor (TCR) activation (signal 1). CD80 and CD86 on the dendritic cell engage CD28 on the T cell to provide signal 2. Signal 1 and 2 activate three signal transduction pathways. These pathways trigger the transcription of the IL-2Rα chain CD25. Jak3 is activated by the third signal which is mediated via cytokines of the γc. The mode of action of every group of immunosuppressants is depicted in white textballoons[81].

Therefore, anti-IL-2Rα antibodies are used as induction therapy at times before but generally after transplantation. In contrast to rATG that exist in two mixtures with a lymphocyte non-depleting and depleting effect, anti-IL-2Rα antibodies are only non-depleting. Daclizumab (Zenapax-Hoffmann-La Roche) was the first humanized mAb (~90% human and 10% murine) to the IL-2-receptor (Figure 3). The current commercially available preparation of anti-IL-2Rα monoclonal antibodies is the chimeric mAb (~75% human and 25% murine protein) basiliximab (Figure 3)[85].

*1.5.2 Calcineurin inhibitors*

The introduction of cyclosporine A by Sir Roy Calne in 1980 was a revolution in the transplantation field as it markedly improved patient outcomes. Cyclosporine A was and still is the cornerstone of immunosuppression in transplantation for more than two decades. Cyclosporine A binds to cyclophilins, which are cytoplasmic chaperone proteins of the immunophilin family, forming a complex that engages calcineurin leading to blockade of NFAT that is required for the transcription of the gene encoding IL-2 (Figure 3)[86]. Adverse effects of cyclosporine A include nephrotoxicity, hypertension, hyperlidemia, gingival hyperplasia, hirsutism and tremor. Moreover, it can also induce the hemolytic-uremic syndrome and post-transplantation diabetes mellitus[81] and increases the growth of facial and body hair.

Tacrolimus engages another member of the immunophilin family, FK506 (tacrolimus)-binding protein 12 to create a complex that inhibits calcineurin with greater molar potency than cyclosporine A[81]. Although there are no chemical similarities, cyclosporine A and tacrolimus act in the same way via the prevention of translocation of the signal evoked by antigen from the surface to the nucleus of the T cell (Figure 3). However, tacrolimus prolonged the life of experimental organ grafts[87, 88] and treatment with tacrolimus resulted in less rejection than with cyclosporine, as indicated by several trials[89, 90]. Minute quantities of this compound have extremely powerful immunosuppressive properties. Side effects of tacrolimus are nephrotoxicity and toxic effects on the central nervous system and the islets of Langerhans.

*1.5.3 Jak inhibitors*

Upon binding of cytokines and growth factors to the cell, intracellular signaling is conducted via the Janus family of tyrosine kinases (Jak) which consists of four kinases. Binding of cytokines of the IL-2 family to their receptors that signal via the $\gamma_c$ (as mentioned above) can activate the phosphorylation of Jak3 which then phosphorylates the tyrosine residues on the receptor[91]. Mutations of the $\gamma_c$ or Jak3 have profound effects on the immune system and result in severe combined immunodeficiency (SCID) in mice. In humans, these mutations can cause X-linked severe combined immunodeficiency (X-SCID) syndrome[92, 93] in which the T and NK-cells are absent. When Jak3 associates with the $\gamma_c$, and is phosphorylated, signal transducers and activators of transcription (STAT) proteins are recruited to the Jak-$\gamma_c$ receptor complex. Following binding of a cytokine to its receptor, STATs are phosphorylated by activated Jaks and dimerize. Subsequently STAT dimers translocate into the nucleus leading to gene transcription. Jak3

recruits STAT5, that can bind to the promoter region of several downstream target genes as FOXP3 and IL-2Rα. STAT5A and STAT5B play essential roles in orchestrating immune regulation and the development of immune cells[94].

Signal 3, the cytokine-signal that initiates signaling via the Jak-STAT pathway can be blocked by the Jak inhibitor CP-690,550 (Figure 3). It is being developed as an alternative immunosuppressive drug to calcineurin inhibitors in order to serve as the primary immunosuppressive agent for preventing acute rejection in kidney allograft recipients. Currently Phase II clinical trials with this compound are running.

### *1.5.4 Proliferation inhibitors*

MMF and the mTOR-inhibitors sirolimus (rapamycin) and everolimus are the most commonly used lymphocyte proliferation inhibitors. MMF is derived from mycophenolic acid (MPA) from the penicillum molds. For their replication, lymphocytes require synthesis of purine and pyrimidine nucleotides that are regulated by iosine monophosphate dehydrogenase (IMPDH) and dihydro-orotate dehydrogenase (DHODH), respectively. MMF inhibits the synthesis of guanosine monophosphate nucleotides by IMPDH and thereby blocks the purine synthesis, preventing proliferation of T and B cells[95]. Its main non-immune related toxicity is gastrointestinal symptoms (mainly diarrhea), neutropenia, and mild anemia. In addition, absorption of this drug may be reduced by cyclosporine.

Sirolimus and everolimus can -just as tacrolimus- bind to FK506 binding protein 12, the complex that inhibits the calcineurin phosphatase and T-cell activation. The most common adverse effects are hyperlipidemia, increased exposure to the toxicity effects of calcineurin inhibitors, thrombocytopenia, delayed graft function and mouth ulcers. Sirolimus and everolimus both have anti-neoplastic and arterial protective effects.

### *1.5.5 Corticosteroids*

In the 1950s, hormones that can bind to glucocorticoid receptors on virtually all cells and exert pleiotropic effects on multiple signaling pathways were discovered, named corticosteroids[96]. Within the cell, the cortisol-glucocorticoid receptor complex moves to the nucleus and binds as a homodimer to DNA-sequences, thereby facilitating or inhibiting transcription. Corticosteroids inhibit transcription factors as NF-κB and IL-2, TNF-α and IFN-γ. Furthermore, they inhibit protein synthesis to ultimately result in the inhibition of the release of numerous cellular mediators, such as interleukins, prostaglandins, leukotrienes, etc. that intervene in immunological reactions, thereby affecting the concentration, distribution and function of leucocytes. Because of the diverse mechanisms of actions of the corticosteroids, they are probably one of the most potent immunosuppressive drugs used in the setting of solid organ transplantation, as they inhibit the function of both APCs and (predominantly $CD4^+$) T cells at the level of proliferation and cytokine production[86].

Corticosteroids were seen as 'miracle drugs' by physicians that offered a relief for a diverse group of diseases. In 1950, dr. Philip Hench was awarded with the Nobel Prize for treatment of

a patient with rheumatoid arthritis with corticosteroids. In the early 1960s, corticosteroids were used to reverse acute rejection in a living donor kidney transplant recipient[97] and physicians all over the world quickly adopted the routine use of corticosteroids, in particular prednisone, prednisolone or methylprednisolone into the cocktail already consisting of a calcineurin inhibitor and a proliferation inhibitor that became the 'standard' therapy for all kidney transplantation patients[98]. However, therapy with this class of immunosuppressive drugs is accompanied with various chronic toxicities. They cause major complications as water and salt retention leading to swelling and edema, high blood pressure, diabetes, showing that this two-edged sword has its impact on solid-organ transplantation.

## 1.6 AIM AND OUTLINE OF THIS THESIS

$CD4^+CD25^{bright}$ Treg require IL-2 for their expansion, homeostasis and function[38]. Inhibition of the IL-2 pathway may therefore hamper the frequency and suppressive activities of regulatory T cells. However, to date, the effect of the calcineurin inhibitor cyclosporine on the function of $CD4^+CD25^{bright}$ Treg remains controversial. On one hand, several publications[99-102] demonstrate a negative influence on the number and suppressive capacities of $CD4^+CD25^{bright}$ Treg e.g. cyclosporine has been shown to inhibit FOXP3 mRNA expression in MLR[103]. On the other hand, evidence suggests that it does not seem to affect the frequency and suppressive capacity of $CD4^+CD25^{bright}FoxP3^+$ T cells[5, 104, 105]. Therefore, a study in human organ transplant recipients in which the effect of calcineurin inhibitors on the suppressive activities of human peripheral $CD4^+CD25^{bright}$ Treg is investigated would be enlightening.

RATG has been shown to induce $CD4^+CD25^{bright}FoxP3^+$ Treg *in vitro* and in peripheral cells of healthy individuals[78]. Moreover, data showing that horse ATG does not have the capacity to induce Treg[106] demonstrate that Treg-induction is a species-specific characteristic of rabbit ATG. Thus, the mechanisms of action of T-cell depletion agents does not seem to concentrate fully on T cell depletion but also on the immune regulatory component that may contribute to donor-specific hyporesponsiveness after solid organ transplantation. These findings create new potential for rATG to manipulate the immune system of organ transplant recipients by inducing (donor-specific) Treg, as has been shown before for the mTOR inhibitors everolimus and sirolimus[107-110].

Data with regard to the effect of MMF on Treg are scarce. Few groups reported that MMF does not interfere with the suppressor function of Treg[100, 111]. MMF has been shown to decrease the CD25-expression on lymphocytes[112]. Considering that MMF does not interfere with the IL-2 signaling pathway, one can only speculate that it will not negatively affect Treg function. As the cytokine signal and the downstream Jak-STAT5 pathway both are important for FoxP3-expression and the function of Treg, the Jak inhibitor CP-690,550 might affect Treg.

The scarce and controversial data from (animal) experimental models and studies describing the effect of immunosuppressive drugs on Treg, urges the quest for the analysis of regulatory T cells in immune suppressed organ transplant patients. This thesis focuses on the effects of various immunosuppressive drugs on the cell surface marker expression, frequency and function of human Treg in the peripheral blood.

**Chapter 2** describes the effect of triple therapy, consisting of cyclosporine, MMF and prednisone on the suppressive capacity of natural $CD4^+CD25^{bright}$ T cells in kidney transplant patients at 6-24 months after transplantation. In **Chapter 3,** we address the influence of two different immunosuppressive protocols; 1) tacrolimus/rapamycin and 2) tacrolimus/MMF in the first year after clinical kidney transplantation on the generation of donor-specific regulatory T-cell function. In this prospective study, we monitor the phenotype, frequency and function of $CD4^+CD25^{bright}FoxP3^+$ T cells in the peripheral blood of kidney transplant patients who received these immunosuppressive therapies. The **chapters 4, 5 and 6** are dedicated to the rabbit anti-thymocyte globulins. The effect of rATG-induction therapy on peripheral blood cells of kidney transplant patients is shown in **chapter 4**. In a prospective and controlled study, that included kidney transplant patients who received rATG-induction therapy and triple therapy consisting of tacrolimus, MMF and prednisone and kidney transplant patients in the control group that received triple therapy only, we measure the phenotype and frequency of $CD4^+CD25^{bright}FoxP3^+CD127^{-/low}$ T cells and analyze their function. To elaborate on the results of Treg induction by rATG in cells from healthy individuals as has been shown before[78], **chapter 5** questions whether rATG can also induce Treg in peripheral blood cells of patients with end-stage renal disease, who are candidates for kidney transplantation and rATG-induction therapy. **Chapter 6** investigates whether tacrolimus influences the induction of $CD25^+$ T cells by rATG. **Chapter 7** describes the impact of the Jak inhibitor CP-690,550 on peripheral $CD4^+CD25^{bright}$ T cells and $CD25^{-/dim}$ Teff cells of healthy individuals according to their STAT5 phosphorylation and their suppressive and proliferative function respectively. Furthermore, we investigate the suppressive activities of peripheral $CD25^{bright}$ T cells of kidney transplant patients who received CP-690,550 therapy, whereas CP-690,550 is present in the suppression assays. These results will be compared with that of the $CD25^{bright}$ T cells from patients in the cyclosporine (comparator) group. **Chapter 8** draws attention to the influence of immunosuppressants on regulatory T cells in liver transplantation. The effect of conversion from a calcineurin inhibitor based immunosuppressive treatment to a MMF based treatment on the frequency of $CD4^+CD25^{bright}FoxP3^+$ T cells in liver transplant patients and calcineurin inhibitor associated side effects are described.

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# Chapter 2

## Functional Analysis of $CD4^{+}CD25^{bright}$ T Cells in Kidney Transplant Patients: Improving Suppression of Donor-Directed Responses after Transplantation

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## ABSTRACT

The role of $CD4^+CD25^{bright}$ regulatory T cells (Treg) in controlling alloreactivity is established, but little is known whether antigen-specific Treg are induced in fully immunosuppressed kidney transplant patients. The frequency and function of $CD25^{bright}$ T cells of 9 stable kidney transplant patients before and 0.5-2 yr after transplantation were measured. Patients received triple therapy consisting of cyclosporine, MMF and prednisone. To investigate the influence of transplantation and immunosuppression on Treg function, we compared their suppressive capacities pre- and post-transplantation using mixed lymphocyte reactions (MLR) and kept the $CD25^{-/dim}$ effector T-cell (Teff) population constant. After transplantation, the percentage of $CD4^+CD25^{bright}$ T cells significantly decreased from 8.5% pre-transplant to 6.9% post-transplant (median, p=0.05). However, the lower percentage of post-transplant $CD4^+CD25^{bright}$ T cells was not associated with reduced, but rather improved suppressor function of these cells. The proliferative response of pre-transplant Teff to donor-antigens was more profoundly suppressed by post-transplant Treg than by pre-transplant Treg (pre-transplant 18% vs. post-transplant 55%, median, p=0.03) and was comparable against third party antigens at a $CD25^{bright}$ : $CD25^{-/dim}$ ratio of 1:20. In immunosuppressed kidney transplant patients, the donor-directed suppressive capacity of $CD4^+CD25^{bright}$ regulatory T cells improved, which may contribute to the development of donor-specific hyporesponsiveness against the graft.

## INTRODUCTION

After kidney transplantation, patients receive immunosuppressive therapy to prevent and to treat rejection of the allograft. Triple therapy, consisting of CNIs, MMF and steroids has contributed to low rates of acute rejection[1]. These immunosuppressants are able to inhibit alloreactivity by suppressing the donor-directed immune responses of activated effector T cells[2]. Unfortunately, the current prescribed drugs interfere with T-cell responses in a nonspecific manner, resulting in adverse effects, e.g., they increase the susceptibility for infections and malignancies but also non-immunological complications such as osteoporosis, diabetes and cardiovascular problems[2, 3]. To decrease or even to prevent these side effects, a more specific approach to target the donor-specific T cells is necessary. An option could be cell-based therapy, where immunosuppression is mediated via cells already present in the patient. FoxP3$^{+}$CD4$^{+}$CD25$^{bright}$ regulatory T cells (Treg) are prime candidates comprising a distinct subpopulation of T cells that have the ability to suppress the activation of other T cells and their cytokine production (i.e., IL-2 or IFN-γ)[4-6]. The contribution of Treg in the induction and maintenance of tolerance of the graft after organ transplantation has been demonstrated in several murine models[7-9] but studies showing their involvement in operational drug-free tolerance in patients are rare[10-12]. In clinically tolerant recipients the number of Treg is normal, yet decreased in kidney transplant patients with chronic rejection[10].

Although we and others previously reported that Treg may mediate donor-directed hyporesponsiveness in kidney-transplant patients[13-16], there is significant concern that immunosuppressants may influence the frequency or the suppressive activities of Treg, and thus hinder the development of hyporesponsiveness[2, 17, 18]. The function of CD25$^{bright}$ T cells is investigated by measuring the level of suppression of a Teff population in a proliferation or cytokine suppression assay[19]. In such proliferation suppression assays, the read-out of Treg function is the response of the Teff population. However, both the Treg and Teff population itself may be affected by several factors such as kidney failure, uremia, dialysis, the transplant, immunosuppressants and other various factors. These factors may affect the read-out of Treg function, which is disregarded in the suppression assay. Therefore, to analyze changes in Treg function, the Teff population should remain constant in a suppression assay.

In the present study, we investigated whether kidney transplantation influences the frequency and allosuppressive function and specificity of peripheral CD25$^{bright}$ regulatory T cells after transplantation. To study the changes in the function of CD25$^{bright}$ T cells, we performed a suppression assay in which we determined the suppression of the pre-transplant Teff population in the presence of pre- or post-transplant Treg.

## MATERIALS AND METHODS

*Patients*

Patients were asked by informed consent for their willingness to donate blood, a procedure that was approved by the Medical Ethical Commission (METC) of the Erasmus Medical Centre. Heparinized peripheral blood was obtained before and 5-24 months after transplantation from kidney transplantation patients (n=9) treated with triple therapy consisting of cyclosporine (whole blood trough level 178 ng/mL median, ranging from 85 to 250 ng/mL), MMF and low-dose prednisone. The median age of the patients was 59 yr, ranging from 43 to 68 years. All patients received a kidney from a deceased donor. The serum creatinine level decreased to 147 μM/L after transplantation (median, ranging from 99 to 252 μM/L). The mismatches between donor and recipient for HLA A-B-DR were 1.1 ± 0.6 / 1.3 ± 0.7 / 1.1 ± 0.3 (Mean ± SD), respectively. Patients (n=18) of an additional cohort for FoxP3 staining received kidneys from a deceased donor and were treated with triple therapy consisting of tacrolimus, MMF and prednisone.

*Isolation of Peripheral Blood Lymphocytes*

Peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation over Ficoll-paque (density 1.077 g/mL) (Amersham Pharmacia Biotech, Uppsala, Sweden). PBMC were washed twice with RPMI 1640 medium (BioWhittaker, Verviers, Belgium) and frozen in RPMI 1640-DM (Gibco BRL, Scotland, UK) supplemented with 2 mM/L L-glutamine (Gibco BRL), 100 IU/mL penicillin (Gibco BRL), 100 μg/mL streptomycin (Gibco BRL), 10% fetal bovine serum (FBS) (BioWhittaker), and 10% dimethylsulfoxide (Merck, Schuchardt, Germany) and stored at -140˚C until analysis.

*Isolation of human $CD25^{bright}$ cells and flow cytometric analysis*

Pre- and post transplantation PBMC of each patient were thawed on one day and washed twice with FBS and RPMI-1640 supplemented with DNase (20 μg/mL; Roche Molecular Biochemicals, Mannheim, Germany) to prevent aggregation of cells. PBMC were suspended in 10 % Human Culture Medium (HCM), which consisted of RPMI 1640 medium with L-glutamine (Bio Whittaker) supplemented with 10% pooled human serum (Blood Bank, Rotterdam, the Netherlands) and 100 IU/mL penicillin (Gibco BRL), 100 μg/mL streptomycin (Gibco BRL). The $CD25^{bright}$ T cells were isolated from PBMC after incubation with anti-CD25 microbeads, Miltenyi Biotec, Bergisch Gladbach, Germany) followed by a positive selection (POSSEL-program) on the autoMACS (Miltenyi Biotech). The untouched residual fraction consisted of $CD25^{-/dim}$ T cells and was used as responder population in the MLR. The isolated and the residual fractions were washed and resuspended in HCM for functional analysis. The PBMC population and the isolated and residual fractions were characterized by flow cytometry. The purity of the fractions used for functional analysis was determined by first measuring the percentage of $CD4^+CD25^{bright}$ in the PBMC population and subsequently in the isolated and residual fractions. The purity of $CD25^+$

T cells in the $CD25^{bright}$ isolated fraction was >90%, a typical example is depicted in Figure 1A. To determine the CD25 expression per cell, we measured the geometric mean fluorescence intensity (gMFI) of CD25 of the $CD4^{+}CD25^{bright}$ population in the PBMC and in the $CD25^{bright}$ isolated fraction. Therefore, we used CD4-PERCP (BD Biosciences, San Jose, CA, USA) and CD25-PE epitope B (clone M-A251, BD Biosciences). Flow cytometry was performed on a four color FACS Calibur machine with Cell Quest Pro software (BD Biosciences).

In the additional cohort consisting of 18 kidney transplant patients, PBMCs pre- and post-transplant were isolated and flow cytometry was performed with the same monoclonal antibodies as described above, supplemented with FoxP3-APC (clone PCH101; eBioscience, San Diego, CA, USA). Calibration was performed at least once a week calibration with reference CaliBRITE™ beads (unlabeled/FITC/PE/PERCP/APC catalog No. 349502, BD Biosciences). Instrument settings of the FACS Calibur machine were comparable in the period when flow cytometry was performed.

*Proliferation assay*

The proliferation capacity of the Teff, the thawed donor and third party PBMC or spleen cells (100 µL/$5x10^4$ cells per well, in triplicate) was tested by adding phytohemagglutinin (PHA) at a final concentration of 1 µg/mL in each well. Proliferation was assessed after 72 hr incubation at 37°C in a humidified atmosphere of 5% $CO_2$ of which $^3$H-thymidine (0.5 µCi/well: Amersham Pharmacia Biotech) was added for the last 8 hr before harvesting. $^3$H-thymidine incorporation into DNA was assessed using a Betaplate counter (LKB-Wallac, Turku, Finland).

Treg function was determined by MLR in which the suppressive capacity of pre- or post-transplant $CD25^{bright}$ cells was measured by their ability to inhibit the proliferative response of the pre-transplant Teff. Pre- and post-transplant Treg were co-cultured in triplicate with pre-transplant Teff. Irradiated (40 Gy) donor spleen cells and HLA mismatched (2-2-2) third party PBMC were used as stimulator cells ($1x10^5$ cells/100 µL) and co-cultured with $5x10^4$ cells/100 µL of a mixture of $CD25^{bright}$ : $CD25^{dim/-}$ at 1:10, 1:20 and 1:40 ratios in triplicate wells in round-bottom 96-well plates (Nunc, Roskilde, Denmark). After 7 days of incubation at 37°C in a humidified atmosphere of 5% $CO_2$, proliferation was measured after $^3$H-thymidine (0.5 µCi/well: Amersham Pharmacia Biotech) incubation for the last 16 hr before harvesting.

*Calculations and statistical analysis*

The median counts per minute (cpm) for each triplicate was determined and the level of suppression of the Teff population by Treg at different ratios was expressed as the percentage inhibition of the Teff population. The percentage inhibition is calculated by applying the following formula: [(cpm Teff) – (cpm Teff + Treg)] / (cpm Teff) x 100.

Statistical analysis of the flow cytometry and MLR data was performed using Graphpad Prism (version 4). For the determination of levels of statistical significance, the two-sided probability values according to the Wilcoxon matched pairs test or Mann Whitney U-test were used. P values ≤ 0.05 were considered statistically significant.

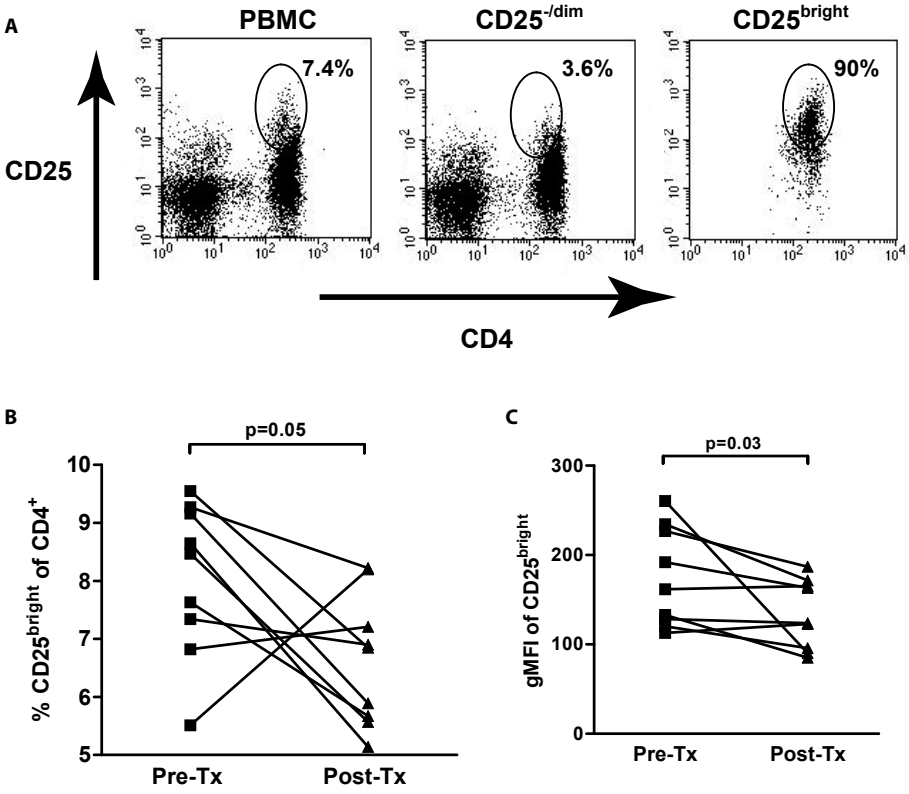


**Figure 1.** Phenotyping of $CD4^+CD25^{bright}$ T cells by flow cytometry

**(A)** Representative example of phenotyping of $CD4^+CD25^{bright}$ T cells. $CD4^+CD25^{bright}$ T cells are located in the oval gate and are expressed as the percentage of the $CD4^+$ lymphocytes in the PBMC population. The purity of the isolated $CD25^{bright}$ and the residual $CD25^{-/dim}$ fractions is determined by gating the $CD25^{bright}$ T cells. **(B)** The percentage of $CD4^+CD25^{bright}$ cells of the $CD4^+$ in PBMC samples before- and after transplant was measured, p=0.05. **(C)** For the CD25-expression per cell, the gMFI of CD25 of the $CD25^{bright}$ cells was assessed in the PBMCs. The gMFI significantly decreased after transplantation, p=0.03. Post-Tx, post-transplantation; pre-Tx, pre-transplantation.

## RESULTS

### *Flow cytometric analysis of the $CD4^+CD25^{bright}$ regulatory T cells before and after transplantation*

The percentage of $CD4^+CD25^{bright}$ cells of total $CD4^+$ lymphocytes in PBMC was significantly lower after than before transplantation (pre-transplant 8.5% vs. post-Transplant 6.9% median, p=0.05, Figure 1B). To define the level of CD25 expression per cell, the gMFI of CD25 of the $CD25^{bright}$ T cells was determined in the PBMC population. This was lower after transplantation, suggesting that the IL-2α receptor (CD25) is down-regulated (p=0.03, Figure 1C). However, instead of CD25, FoxP3 is considered to be the most reliable Treg marker[20-23]. In the current

study, we were unfortunately not able to measure the FoxP3 levels because of limited material available for flow cytometry. Therefore, in an additional new cohort we stained PBMC of 18 kidney transplant patients for FoxP3 and we found that 70% (median) of the $CD4^+CD25^{bright}$ T cells were $FoxP3^+$ pre- and post-transplant (data to be published). We also found that 95% of the $CD4^+FoxP3^+$ cells were located in the $CD4^+CD25^{bright}$ population.

*$CD25^{bright}$ T cells of both pre- and post-transplant are able to regulate allogeneic immune responses*

The function of Treg and Teff before and after transplantation was studied in the MLR. In response to irradiated donor- or third party cells, the isolated $CD25^{bright}$ T cells did not proliferate, a characteristic feature of Treg, data not shown. When stimulated with donor antigens, we observed a significant difference in the proliferative response of $CD25^{-/dim}$ T cells before compared with after transplantation (Figure 2A), suggesting that the anti-donor responses of the Teff population before and after transplantation are not the same (pre-transplant 13071 ± 4114 cpm vs. post-transplant 7309 ± 4777cpm, mean ± SEM, p=0.08). When pre-transplant Treg were reconstituted to pre-transplant Teff, it resulted in a dose-dependent inhibition of the proliferation of $CD25^{-/dim}$ T cells. In contrast, when post-transplant Treg were reconstituted to post-transplant Teff this dose-dependent inhibition was less clear (Figure 2A).

After stimulation with third party antigens, the proliferation of $CD25^{-/dim}$ T cells was comparable before and after transplantation (39647 ± 13846cpm; 35941 ± 9894cpm, mean ± SEM respectively), as shown in Figure 2B. The isolated Treg pre- and post transplant suppressed the third party allogeneic response of both $CD25^{-/dim}$ T cells pre- and post-transplant respectively in a dose-dependent manner (Figure 2B).

The decrease in anti-donor reactivity after transplantation, while the third party reactivity remained comparable, suggests that the Teff population is downregulated by one or more factors including Treg.

*Post-transplant Treg inhibit the donor-directed response of pre-transplant Teff stronger than pre-transplant Treg*

To control for the variability in the proliferation capacity of the Teff in the read-out of Treg function, we subsequently determined the inhibition of the proliferative response of the pre-transplant Teff by Treg isolated before and after transplantation. At a $CD25^{bright}$ : $CD25^{-/dim}$ ratio of 1:10, both the pre- and post-transplant Treg were able to suppress the alloreactivity of pre-transplant Teff when stimulated with donor- and third party antigens (Figures 3A and 3B, respectively). However, the pre-transplant Teff seemed more suppressed by post-transplant Treg than by pre-transplant Treg (Figures 3A and 3B). As depicted in Figure 3A, when diluting the Treg at a $CD25^{bright}$ : $CD25^{-/dim}$ ratio of 1:20, the donor-directed proliferation of the pre-transplant Teff was significantly more suppressed in the presence of the post-Transplant Treg than in the presence of the pre-transplant Treg (p=0.05). When comparing the proliferation of the pre-transplant Teff

in response to third party alloantigens, again a comparable pattern of improved suppression by post-transplant Treg was observed, but this did not reach statistical significance (Figure 3B). These results suggest that Treg isolated after transplantation are more potent at suppressing the Teff of before transplantation than Treg isolated before transplantation.

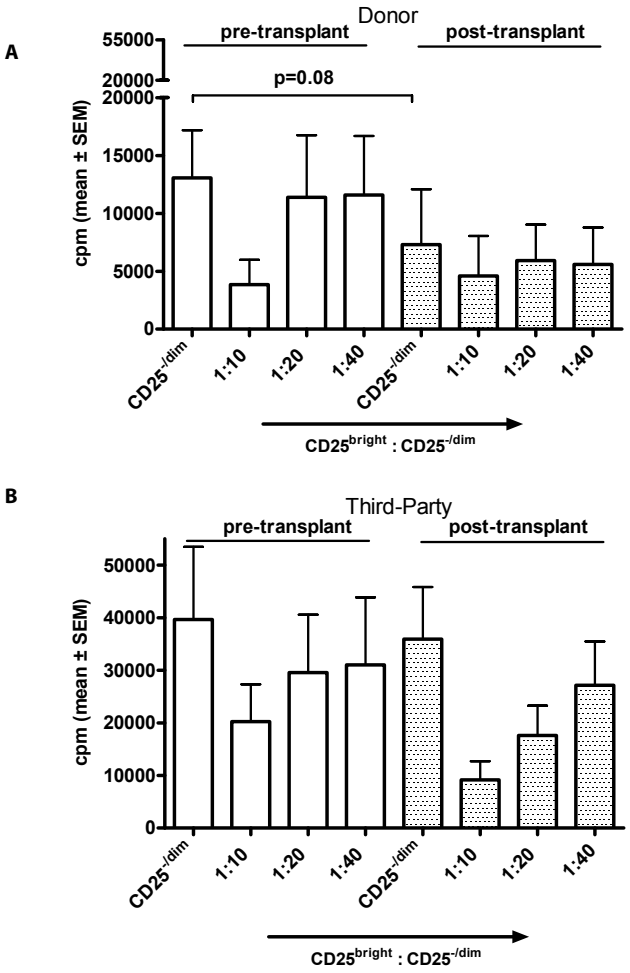


**Figure 2.** Suppression assay

**(A)** MLR stimulated with donor-antigens before and after transplantation. Note the difference in the anti-donor response by the pre-transplant Teff (white bars; mean ± SEM) and post-transplant Teff (dotted bars; mean ± SEM). The proliferation capacity of the post-transplant Teff cells is lower after transplantation; therefore, inhibition by post-transplant Treg cannot be measured. **(B)** MLR stimulated with third party alloantigens before and after transplantation. The proliferation of the pre- and post-transplant Teff is inhibited after reconstitution of pre- and post-transplant Treg respectively at a $CD25^{bright}$ : $CD25^{-/dim}$ ratio of 1:10, 1:20 and 1:40 in a dose-dependent manner. Error bars represent mean ± SEM

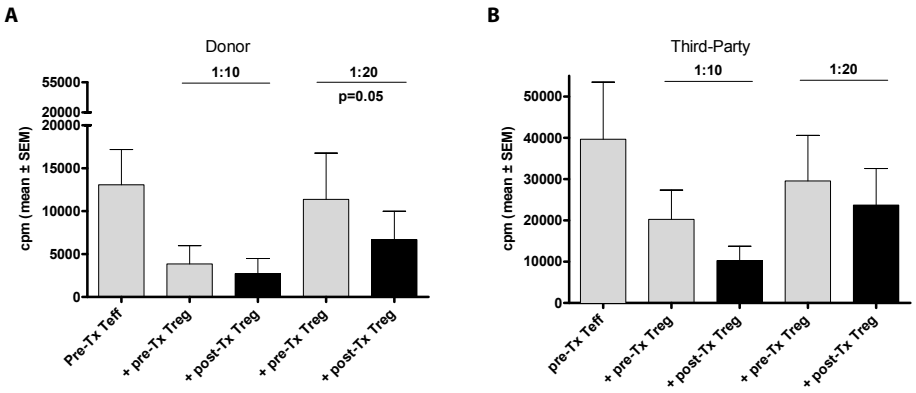


**Figure 3.** Suppressive capacity of the CD25$^{bright}$ T cells on pre-transplant Teff

**(A)** MLR in which the suppression of the pre-transplant Teff is determined upon stimulation with donor-antigens in the presence of pre-transplant Treg (gray bars; mean ± SEM) and post-transplant Treg (black bars; mean ± SEM) at a CD25$^{bright}$ : CD25$^{-/dim}$ ratio of 1:10 and 1:20, p=0.05. **(B)** MLR stimulated with third party antigens in the presence of pre-transplant (gray bars; mean ± SEM) and post-transplant Treg (black bars; mean ± SEM) at a CD25$^{bright}$ : CD25$^{-/dim}$ ratio of 1:10 and 1:20.

Subsequently, we determined the suppressive capacity of the Treg by calculating the percentage of inhibition of the proliferating Teff population for the individual patient. As illustrated by Figure 4A indeed, a larger percentage of proliferating pre-transplant Teff was inhibited in the presence of post-transplant Treg than in the presence of pre-transplant Treg at a CD25$^{bright}$ : CD25$^{-/dim}$ ratio of 1:20 (pre-transplant 18% vs. post-transplant 55%, median p=0.03). When stimulated with third party alloantigens, the regulation of the pre-transplant Teff by pre- and post-transplant Treg was comparable (pre-transplant 49% vs. post-transplant 37%, median, Figure 4B).

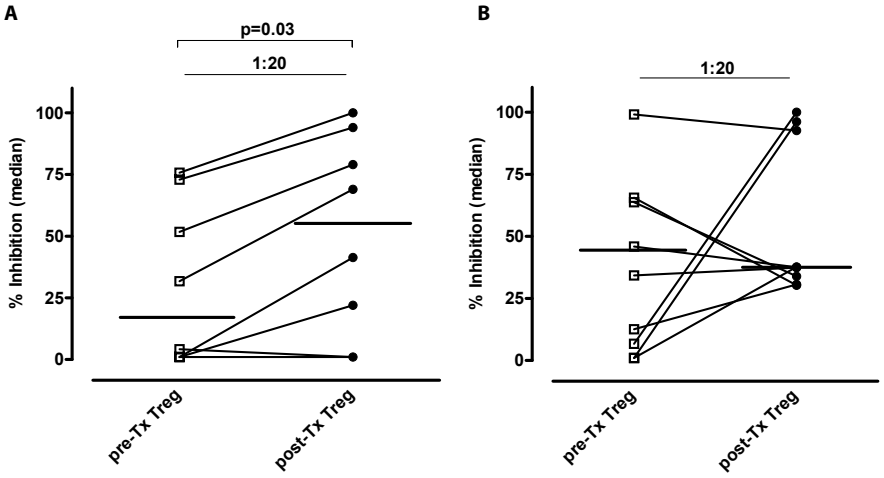


**Figure 4.** Percentage inhibition of post-transplant Teff by pre-transplant and post-transplant Treg for the individual patient **(A)** Percentage inhibition of the anti-donor proliferative response of pre-transplant Teff by pre- and post-transplant Treg at a CD25$^{bright}$ : CD25$^{-/dim}$ ratio of 1:20, p=0.03. Median is depicted pre- and post transplant. **(B)** Percentage inhibition of third party proliferative response of pre-transplant Teff by pre-transplant and post-transplant Treg at a CD25$^{bright}$ : CD25$^{-/dim}$ ratio of 1:20. Median is depicted pre- and post transplant.

## DISCUSSION

After clinical kidney transplantation, $CD4^+CD25^{bright}$ regulatory T cells are able to control the anti-donor immune response. When *in vitro* studying the functional capacity of Treg at different time-points in a suppression assay, the variability in the Teff response is always left out of consideration. The Teff population may be affected by several factors as for example kidney failure, uremia, dialysis, the transplant, immunosuppressants and other various factors, as the proliferation in response to donor-antigens decreased after transplantation (Figure 2A). Given that the proliferation capacity of Teff is the actual read-out of Treg function, comparing Treg function of several time-points may lead to a misinterpretation of the results.

In the present study, we excluded the variability of Teff by performing a suppression assay in which the Teff population was kept constant and not hindered by immunosuppressive drugs. Our results demonstrated that after transplantation, lower numbers of $CD4^+CD25^{bright}$ T cells and a lower CD25 expression per cell are found. However, the donor-directed suppression by Treg improved after transplantation (Figures 3A and 4A). These donor-directed Treg might be involved in inducing a state of donor-specific hyporesponsiveness in the patient on the long term.

Many experimental animal models have shown that antigen specific $CD4^+CD25^{bright}$ regulatory T cells are involved in the induction and maintenance of tolerance after transplantation[24-26]. In human T cells, Treg function can be examined by studying the indirect and direct pathway. In a patient cohort of Salama et al. in 40% of stable renal transplant recipients, there was evidence of an antigen-specific regulatory $CD25^+$ cell population suppressing responsiveness towards alloantigens in the indirect pathway[15]. Indeed, there was also evidence that in a subset of CNI-free kidney allograft recipients long after transplantation, functional $CD4^+CD25^{bright}$ regulatory T cells are present in the peripheral blood that are -at least partially- responsible for the state of proliferative donor non-responsiveness[13, 16].

The post-transplant decrease in the percentage of $CD4^+$ T cells that express CD25, and the decrease in CD25 expression per cell can be explained by the interaction of cyclosporine, steroids and MMF with signaling of cytokines of the IL-2-family, necessary for their homeostasis[27-29]. In several studies it was found that CNIs reduce the number of circulating regulatory T cells in stable transplant recipients[30, 31]. In addition, Noris et al. clearly demonstrated that $CD4^+CD25^{bright}$ regulatory T cells do not develop during CsA treatment and they suggest that this is due to the immunosuppressive treatment[2]. Despite our and other *in vitro* data showing that CNIs negatively affect the fraction of Treg after transplantation[18, 30, 31], we found an improvement in Treg function. Therefore, to study the role of Treg in alloreactivity it is clear that functional analysis is critically important. Nowadays, the CD25 molecule is the most commonly marker to isolate T cells with regulatory capacities, but FoxP3 is regarded as a more specific marker to define regulatory T cells, therefore we characterized the $CD4^+CD25^{bright}$ T cells also for their FoxP3 expression[20-23]. In an additional new cohort, we stained PBMC of kidney transplant patients for FoxP3. We found that the $CD4^+CD25^{bright}$ T cells pre- and post-transplantation

predominantly expressed FoxP3. Moreover, our isolated $CD25^+$ T cells did not proliferate upon stimulation with antigen and were functional, suggesting that the majority of these cells consist of Treg. These data again emphasize the need of functional analysis of Treg in addition to flowcytometric analysis.

The improvement in Treg activity after transplantation might be due to an impaired function of the immune system of the patient before transplantation that might be influenced by several factors such as kidney failure, uremia and dialysis. Transplantation as treatment of end-stage renal disease results in a better kidney function and may even result in a general 'boost' of the immune system that *in vivo* is overruled by immunosuppressive drugs. After transplantation, the immune system regains the ability to mount immune responses to foreign pathogens. This recovered immune system triggers an anti-donor response and consequently may induce Treg activity that is directed against donor-antigens. *In vitro* this potent response can be measured, because the cells are not any more exposed to immunosuppressants.

It is unknown however, whether donor-specific Treg are induced *de novo* after transplantation or whether they act as expanded Treg that control alloactivated Teff cells after transplantation[32]. Accumulating evidence suggests that Treg are not only thymically-derived but can also be induced from naïve $CD25^-$ T cells (adaptive Treg) in the periphery after low-dose antigen stimulation[24, 33].

Considering the lower percentage of $CD4^+CD25^{bright}$ T cells after transplantation in our study, it is not very likely that there is induction of *de novo* (donor-specific) Treg by conversion or expansion of antigen-specific Treg. However, Treg activity could be improved, because the proportion of Treg with the memory phenotype is augmented due to induction of donor-specific Treg that corresponds to a smaller compartment with naïve Treg[34]. The proportion Treg with the effector memory or central memory phenotype can be measured by flow cytometric immunostaining for memory markers, such as CD27 and CD45RO.

To conclude, we report that although the frequency of Treg was even lower after than before transplantation, their suppressive capacity after transplantation improved in comparison with before transplantation. These Treg may be involved in the development of donor-specific hyporesponsiveness after kidney transplantation, although passive mechanisms as clonal deletion and anergy of Teff against donor-antigens still may also occur[4]. We, therefore hypothesize that to investigate changes in Treg function, the proliferation of the Teff population that is influenced by several factors, should remain constant.

Having these conclusions drawn, one should take into account that the small number of patients is a limitation of this study. Finally, since we show that donor-directed Treg are generated after transplantation, another interesting question to be investigated is whether the time-span after transplantation has an effect on the existence of donor-directed regulatory T cells after transplantation. Therefore, blood samples obtained at fixed time points after transplantation should be analyzed.

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# Chapter 3

## Generation of Donor-Specific Regulatory T-Cell Function in Kidney Transplant Patients

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## ABSTRACT

In the search for mechanisms that can induce and maintain transplant tolerance, donor-specific $CD4^{+}CD25^{bright}FoxP3^{+}$ regulatory T cells have been frequently mentioned. However, it remains to be demonstrated, whether these cells are generated after clinical transplantation. We prospectively analyzed the phenotype and function of peripheral regulatory $CD4^{+}CD25^{bright}$ T cells of 79 patients before, 3, 6, and 12 months after kidney transplantation. The immune regulatory capacities of $CD4^{+}CD25^{bright}$ T cells were assessed by their depletion from peripheral blood mononuclear cells (PBMC) and in co-culture with $CD25^{neg/dim}$ responder T-cells in mixed lymphocyte reactions (MLR). In the first year after transplantation, the number and proportion of $CD4^{+}CD25^{bright}$ T cells significantly decreased ($p<0.05$ and $p<0.001$, respectively). In the MLR, we observed donor-specific hyporesponsiveness in the presence of significantly increased proliferation to third and fourth party-antigens, ($p<0.001$ and $p<0.05$, respectively). Furthermore, functional analysis of $CD25^{bright}$ cells showed that the effect of depletion of these cells from PBMC, and their suppressive capacities in co-culture with donor-antigens stimulated $CD25^{neg/dim}$ responder T-cells (1:10 ratio) significantly improved ($p<0.01$ and $p<0.001$, respectively). Moreover, the difference between the stimulation with donor-antigens and third party-antigens became apparent at 6 months after transplantation. These findings demonstrate that donor-specific $CD4^{+}CD25^{bright}$ regulatory T-cell function is generated in fully immune suppressed renal recipients in the first year after transplantation.

## INTRODUCTION

The ultimate challenge in organ transplantation is to achieve transplant tolerance. Although studies describing this condition after clinical kidney transplantation (KTx) are already extremely rare[1-3], the development of tolerance in humans remains elusive. Nevertheless, the involvement of and even cell therapy with $CD4^+CD25^{bright}$ regulatory T cells (Treg) has been frequently suggested[3-8].

Associations between tolerance and Treg were found in immunosuppression-free liver transplant recipients from whom the proportion and number of Treg was elevated[5, 7]. Also, in a small group of operational tolerant renal recipients the level of peripheral Treg as well as the transcription factor for Treg, FoxP3, was higher when compared with patients with chronic rejection[9, 10].

Although these findings suggest an association between transplant tolerance and the presence of Treg, data providing evidence for the presence of functional donor-specific Treg after transplantation are only available from stable immune suppressed kidney transplant recipients[11-13]. However, it remains to be demonstrated that Treg also play a role in the induction of donor-specific hyporesponsiveness in patients after transplantation.

Unlike experimental animals, kidney transplant patients receive lifelong immunosuppression to prevent graft rejection. Because these regimens influence T cells[14], they may also affect the induction and function of Treg[15]. Particularly, because most of these drugs target the IL-2 pathway, which is crucial for the function, homeostasis and survival of $CD4^+CD25^+FoxP3^+$ T cells[16-19]. Thus, these immunosuppressive drugs may interfere with the development of donor-specific Treg thereby impairing a potential key player responsible for graft acceptance.

Therefore, we performed a prospective study on 79 fully immunosuppressed kidney transplant patients to determine whether donor-specific Treg are induced in the first year after transplantation. Understanding the dynamic features of antigen-specific regulatory T cells will contribute to our understanding of the role of these cells in antidonor reactivity.

## MATERIALS AND METHODS

### *Subjects*

The medical ethics committee of Erasmus Medical Centre approved the study protocol and all patients provided informed consent (medical ethics committee number 2004-264). As part of a multicentre trial[20], patients were enrolled from March 2004 until March 2006 and follow-up was performed for 1 year. We included 79 patients, who were equally randomized to treatment arm 1 with tacrolimus/rapamycin (n=39) or arm 2 with tacrolimus/mycophenolate mofetil (MMF, n=40, Table 1). There were no significant differences in patient characteristics between the two arms of treatment at baseline. In both arms of treatment, patients received prednisone for the first 4 to 6 weeks. The dosing and aimed whole blood trough levels of the study medication are summarized in Table 2. Peripheral blood samples were obtained within 24 hr before and 3, 6, and 12 months after KTx. Blood samples before KTx were obtained before patients received immunosuppressive medication. In addition, blood was obtained from 17 healthy controls (HC), consisting of 10 men and 7 women with a mean age of 52±8.6 years. These characteristics of our HC were comparable with our patient population.

**Table 1.** Patient characteristics at baseline

| Demographics | Arm 1 (n=39) | Arm 2 (n=40) |
|---|---|---|
| Gender (M/F) | 27 / 12 | 24 / 16 |
| Age (yr) | 51 ± 17 | 51 ± 16 |
| Dialysis type HD/ PD/ ND | 20/ 12/ 7 | 19/ 15/ 6 |
| Time on dialysis (mo) | 11 (0-75)* | 17 (0-280)* |
| Origin of donor kidney | | |
| Living related/ deceased | 25 / 14 | 27 / 13 |
| 1st KTx / >1st KTx | 34 / 5 | p35 / 5 |
| HLA A mismatch | 1.0 ± 0.7 | 0.9 ± 0.7 |
| HLA B mismatch | 1.1 ± 0.7 | 1.2 ± 0.7 |
| HLA DR mismatch | 1.1 ± 0.7 | 1.0 ± 0.7 |
| Primary kidney disease | | |
| Immunological disease | 8 | 9 |
| Hypertensive nephropathy | 8 | 8 |
| Diabetic nephropathy | 6 | 8 |
| Unknown | 7 | 7 |
| Polycystic kidney disease | 5 | 5 |
| Urological disease | 5 | 3 |

Mean ± SD, *Median (range), Arm 1; Tacrolimus/Rapamycin, Arm 2; Tacrolimus/MMF, HD=Hemodialysis, PD=Peritoneal Dialysis, ND=No Dialysis

### *Flow cytometric analysis*

Blood samples were collected in heparinized tubes and analyzed for the presence of T-cell subsets by four-color flow cytometry using mAbs directly conjugated to fluorescein isothiocyanate (FITC), phycoerythrin (PE), allophycocyanin (APC) or peridinin chlorophyll protein (PerCP). One hundred microliters of blood was incubated with 10 µL of the dual mAb combinations

**Table 2.** Dosing and trough levels of study medication

| | Pre-operative | Post-operative | Day 1-14 | Day 15-28 | Day 28-42 | Day 43-365 |
|---|---|---|---|---|---|---|
| Tacrolimus arm 1 | 0.2 mg/kg* | 0.2 mg/kg | 10-15 ng/mL** | 4-8 ng/mL | 4-8 ng/mL | 4-6 ng/mL |
| Sirolimus arm 1 | - | 6 mg | 2 mg | 2 mg | 1 mg | - |
| Tacrolimus arm 2 | 0.2 mg/kg | 0.2 mg/kg | 10-15 ng/mL | 8-12 ng/mL | 8-12 ng/mL | 5-10 ng/mL |
| MMF arm 2 | 1000 mg | - | 2 x 1000 mg/d | 2 x 500 mg/d | 2 x 500 mg/d | 2 x 500 mg/d |
| (Methyl)prednisone | 500 mg*** | 125 mg | 20 mg | 15 mg | 5 mg | - |

*The initial daily dose is 0.2 mg/kg p.o. given in two doses (pre/postoperatively), **and then adjusted by whole blood trough levels. *** intravenously bolus.

CD45-FITC/CD14-PE; $IgG_1$-FITC/$IgG_{2b}$-PE; $IgG_1$-PerCP/ $IgG_1$-APC as isotype control. Furthermore, we used the mAb CD3-FITC, CD4-PerCP, CD8-APC and CD25-PE. To further determine how Treg evolve, we added a combination of CD4-PerPC/CD25-PE/CD45RO-APC/CCR7-FITC to 100 µL whole blood. The antibodies were purchased from BD Biosciences (San Jose, CA) and R&D Systems (Abingdon, UK). After 30 min of incubation at room temperature, red blood cells were lysed with fluorescence-activated cell sorter solution (BD Biosciences) during 10 min. Cells were then washed twice, and analyzed on a flow cytometer (FACSCalibur, BD Biosciences) using SimulSet and CELL Quest Pro software (BD Biosciences). To establish an analysis gate that included at least 90% of the lymphocytes, the CD45/CD14 reagent was used. At least 20,000 gated lymphocyte events were acquired from each tube. Cells with a $CD45RO^-$ phenotype were considered to be naive cells and cells with a $CD45RO^+$ phenotype memory cells.

*Expression of FoxP3 and CD127*

FoxP3 is a transcription marker for Treg and in July 2006 it was shown that the expression of CD127 inversely correlates with FoxP3 expression and the suppressive function of Treg[21]. We began the experiments on our study cohort using fresh materials before the anti-FoxP3 antibody became available for analysis (eBioscience, San Diego, CA, USA) and before its correlation with CD127 was reported. Therefore, to gain insight into the expression profile of FoxP3 and CD127 in our patient materials, we stained peripheral blood samples of an additional cohort of patients (n=34). These samples were taken 24 hr pre KTx and stained with CD4-PerCP (BD Biosciences), CD25-PE (epitope B, BD PharMingen, San Diego, CA), CD127-FITC (eBioscience) and FoxP3-APC (clone PCH101, eBioscience). Patient characteristics from this additional cohort were comparable with our study population from Table 1.

*Isolation of peripheral blood lymphocytes*

Peripheral blood mononuclear cells (PBMC) were isolated from 49 mL heparinized peripheral blood by density gradient centrifugation using Ficoll-Paque (density 1.077 g/mL; Amersham, Uppsala, Sweden). PBMC were collected from the interphase, washed twice in Roswell Park

Memorial Institute 1640 (BioWhittaker, Verviers, Belgium) and resuspended in Human Culture Medium (HCM) consisting of Roswell Park Memorial Institute 1640-Dutch Modification (Gibco, BRL, Scotland, UK) supplemented with 10% heat inactivated pooled human serum, 4 mM L-Glutamine (Gibco BRL), 100 IU/mL penicillin (Gibco BRL) and 100 μg/mL streptomycin (Gibco BRL).

*Isolation of CD25$^{bright}$ cells*

After isolation, PBMC were washed once and resuspended in 45μL MACS-buffer/10x10$^6$ PBMC prepared according to the manufacturer's protocol (Miltenyi, Bergisch Gladbach, Germany). The CD25$^{bright}$ cells were depleted from PBMC by incubating PBMC with anti-CD25 microbeads (Epitope A, Miltenyi Biotec) followed by a positive selection (POSSELD-program) on the autoMACS (Miltenyi). Cells not selected by the microbeads were referred to as the CD25$^{neg/dim}$ fraction[11]. To control for the autoMACS procedure, 6x10$^6$ PBMC were treated by the same protocol in the absence of anti-CD25 microbeads.

Purity of the fractions was measured by flow cytometry using CD3-FITC, CD4-PerCP, CD8-APC (BD Bioscience) and CD25-PE (epitope B, BD PharMingen). Phenotypical analysis of both fractions demonstrated that the average proportion of CD4$^+$ cells in the CD25$^{bright}$ fraction was 95% and in the CD25$^{neg/dim}$ fraction 62% (Figures 1A and B). The proportion of CD4$^+$CD25$^{bright}$ cells in the CD25$^{bright}$ fraction was 72% (Figure 1C) and the proportion of CD4$^+$CD25$^{neg/dim}$ cells in the CD25$^{neg/dim}$ fraction was 96% (Figure 1D). These proportions were not different over time and comparable with proportions measured in samples from HC (Figure 1).

*Mixed Lymphocyte Reactions*

In the MLR, 5x10$^4$ freshly isolated patient-PBMC and CD25$^{neg/dim}$ cells were stimulated with 5x10$^4$ irradiated (40 Gy) donor PBMC (donor-antigens) and 5x10$^4$ (40 Gy) HLA A, B and DR fully mismatched third party PBMC. Because it has been described that improved histocompatibility between recipient and donor enhances immune regulation and graft survival after KTx[22], we also stimulated patient-PBMC and CD25$^{neg/dim}$ cells with 5x10$^4$ (40 Gy) fourth party PBMC. Fourth party PBMC have the same number of mismatches at the HLA A, B and DR level to the recipient as the donor to the recipient, but the mismatches are based on different antigens. The same third party and fourth party were used for an individual patient at all analysed time points. The MLR was performed in HCM, in triplicate, in a 96-wells round bottom plate for 7 days. At day 6, $^3$H-thymidine 0.5 μCi/well was added to the culture; and 16 hr later, samples were harvested and radioactivity was measured in counts per minute (CPM) using a β-counter (PerkinElmer, Oosterhout, the Netherlands).

*Regulation of alloantigens stimulated responder cells by CD25$^{bright}$ cells*

Regulation of proliferation by CD25$^{bright}$ cells was quantified both by their depletion from PBMC and in co-culture experiments with the CD25$^{neg/dim}$ responder cells. After depletion the increase

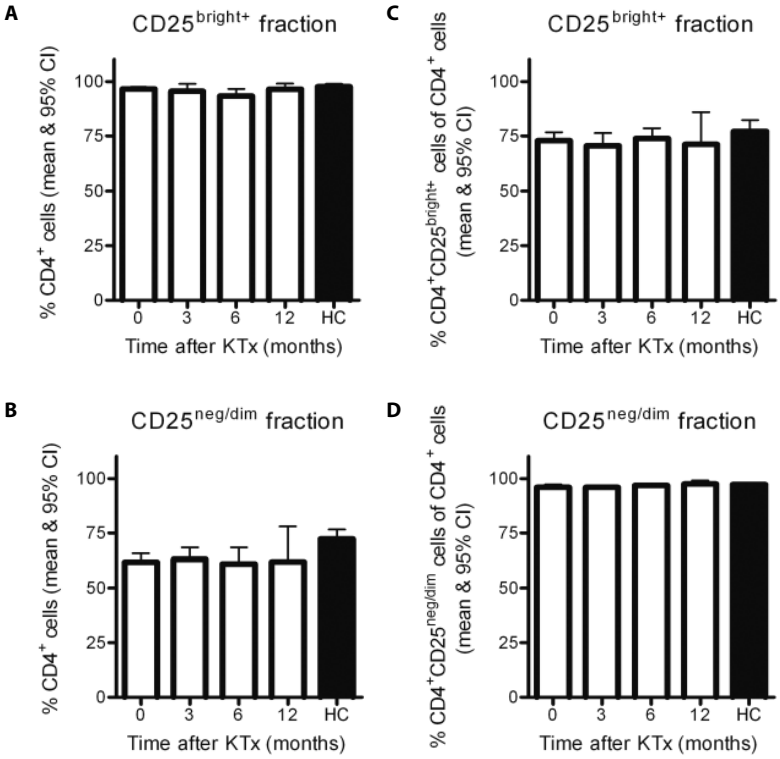


**Figure 1.** Isolation of $CD25^{bright}$ cells by autoMACS

**(A-B)** The average proportion of $CD4^+$ cells in the isolated $CD25^{bright}$ fraction was 95% and 62% in the $CD25^{neg/dim}$ fraction. **(C)** In the $CD25^{bright}$ fraction, the proportion of $CD4^+CD25^{bright}$ cells was 72%. **(D)** The proportion of $CD4^+CD25^{neg/dim}$ cells in the $CD25^{neg/dim}$ fraction was 96%. Proportions were not different over time (ANOVA) and comparable to proportions measured in samples from healthy controls (Unpaired t test). All data were normally distributed.

in proliferation reflects the regulatory capacity of the $CD25^{bright}$ cells. To compare the effect of depletion over time, we calculated the percentage of increase (% increase) in those cultures where the effect of depletion was positive.

$$\% \text{ Increase} = \frac{\text{CPM } CD25^{neg/dim} \text{ cells} - (\text{CPM PBMC})}{\text{CPM } CD25^{neg/dim} \text{ cells}} \times 100$$

In the MLR, isolated $CD25^{bright}$ cells were added to $CD25^{neg/dim}$ responder cells at a ratio of 1:5, 1:10, 1:20, and 1:40. The effect was calculated as the percentage of inhibition (% IH), when the proliferative response of alloactivated $CD25^{neg/dim}$ cells was more than 1000 CPM; and the effect of depletion was positive.

$$\% \text{ Inhibition} = \frac{\text{CPM } CD25^{neg/dim} \text{ cells} - (\text{CPM } CD25^{neg/dim} \text{ cells} + CD25^{bright} \text{ cells})}{\text{CPM } CD25^{neg/dim} \text{ cells}} \times 100$$

*Proliferation of mitogen stimulated cells*

We determined the capacity of PBMC and $CD25^{neg/dim}$ cells ($5x10^4$) to proliferate upon stimulation with 1 μg/mL Phytohemagglutinin (PHA; Murex Biotech LTd, Kent, UK). All cultures were performed in HCM, in triplicate in a 96-wells plate for 3 days. At day 2, $^3$H-thymidine 0.5 μCi/well was added to the culture; and 16 hr later, the samples were harvested and radioactivity was counted.

*Statistical Analysis*

All calculations were performed using GraphPad Prism 4.0 or SPSS 11.5. On the basis of the distribution of the data, we performed parametric or nonparametric testing. For paired analysis, the paired t test was performed; and to compare data from patients versus HC, we used the unpaired t test. For nonparametric testing, the Mann Whitney U test was performed. To determine if a certain parameter changed significantly over time, One-Way ANOVA or Kruskal Wallis test was used. To analyze several variables at a fixed time point, Cox or linear regression analysis was performed. A p value less than 0.05 is marked with *, p less than 0.01 with **, and p less than 0.001 with ***. For each analysis, statistics are described more specifically in the appropriate table and figure legends.

## RESULTS

Of 79 randomized patients receiving a kidney transplant, 62 (78%) completed the study and 17 (22%) were withdrawn due to adverse events. There was no difference between patients treated in arm 1 (tacrolimus/rapamycin) or arm 2 (tacrolimus/MMF) for adverse events (8 vs. 9), patient survival (97% vs. 98%), graft survival (97% vs. 93%), rejection incidence (13% vs. 10%), or renal function (serum creatinine 119 μmol/L vs. 130 μmol/L) at one year. Blood trough levels of the medication were within target range. The trough levels of tacrolimus were higher in arm 2 than in arm 1, which was consistent with the study protocol (Table 2). All the patients who had rejection were treated with antirejection therapy and are therefore described separately.

*Characterization of $CD4^+CD25^{bright}$ regulatory T cells*

Analysis of whole blood samples from patients and healthy controls (HC) was performed for lymphocyte subsets, including Treg defined as the $CD4^+CD25^{bright}$ T-cell population in combination with slightly less CD4 expression (Figure 2A). Flow cytometry showed that the absolute number of Treg and their proportion, decreased within the first year after KTx (Table 3 and Figure 2B, $p<0.05$ and $p<0.001$, respectively).

We also analyzed the expression of CD45RO, CCR7, FoxP3 and CD127 by Treg. These results revealed that the absolute number of $CD4^+CD25^{bright}CD45RO^+$ cells (Table 3) and their proportion decreased after KTx ($p<0.05$ and $p=0.06$, respectively). The absolute number of $CD4^+CD25^{bright}CCR7^+$ cells did not change in the first year after transplantation (Table 3), whereas their proportion increased ($p<0.01$).

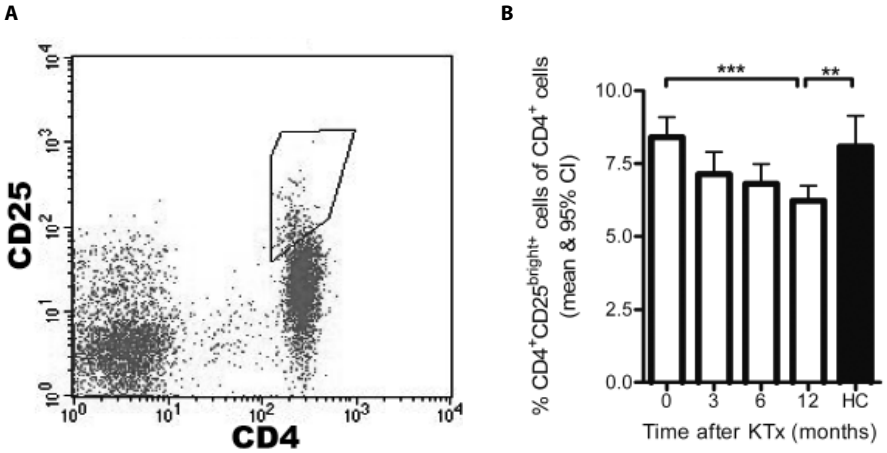


**Figure 2.** Phenotypic analysis of whole blood

**(A)** Dotplot of $CD3^+$ T cells stained for CD4 and CD25 gated for the $CD4^+CD25^{bright}$ cells. **(B)** The data were normally distributed and statistical analysis showed that the percentage of $CD4^+CD25^{bright}$ T cells of $CD4^+$ T cells from patients decreased in the first year after transplantation (ANOVA, $p<0.001$) and was lower than healthy controls (HC) at 12 mo (Unpaired t test, $p<0.001$).

**Table 3.** Flow cytometric results of patients without rejection

| Cell Subsets | Month 0 | Month 3 | Month 6 | Month 12 | HC |
|---|---|---|---|---|---|
| $CD3^+$ | 792** (695-890) | 859 (698-1025) | 872* (759-986) | 900 (774-1027) | 1150 (810-1490) |
| $CD8^+$ | 298 (250-346) | 348 (267-429) | 353 (291-415) | 357 (290-424) | 375 (213-536) |
| $CD4^+$ | 488*** (423-554) | 505* (409-601) | 509** (440-577) | 527** (455-600) | 772 (571-974) |
| $CD25^{bright}$ | 38** (33-43) | 34** (27-41) | 33*** (28-28) | 32*** (27-37) | 60 (43-77) |
| $CD25^{bright}CD45RO^+$ | 33** (29-37) | 26*** (22-31) | 26*** (22-30) | 26*** (22-29) | 48 (34-62) |
| $CD25^{bright}CCR7^+$ | 22*** (19-25) | 23** (17-27) | 22*** (18-26) | 22*** (18-26) | 41 (28-53) |

Absolute numbers in cell/µL (mean & 95% CI), HC = Healthy Controls. All data were normally distributed. An unpaired t test was performed for patients versus HC at all time points. One way ANOVA demonstrated that the number of $CD4^+CD25^{bright}$ cells and $CD4^+CD25^{bright}CD45RO^+$ cells significantly decreased over time (both $p<0.05$). Significance is presented as * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

As described in *Materials and Methods* we determined the expression of FoxP3 and CD127 by Treg on peripheral blood samples from an additional cohort of patients before KTx. Based on the gate in Figure 2A, the average percentage of Treg that expressed FoxP3 was 72%. This finding is in line with the results described in an article by Liu et al[21]. The average percentage of $FoxP3^+$ Treg with a $CD127^{neg/low}$ phenotype was 87%.

*Proliferation of PBMC*

The average proliferation of patient-PBMC to the mitogen PHA was more than 51000 CPM at all tested time points and comparable with the proliferation of PBMC from HC (57000 CPM). Before transplantation, proliferation of patient PBMC to donor-antigens, third and fourth party-antigens was significantly lower as compared with proliferation of PBMC from HC to alloantigens (Figure 3, all $p<0.001$). After transplantation, proliferation of PBMC to donor-antigens remained low whereas increasing proliferation to third and fourth party-antigens, was measured (Figure 3, $p<0.001$ and $p<0.05$, respectively). Thus, we observed a proportional hyporesponsiveness towards donor-antigens.

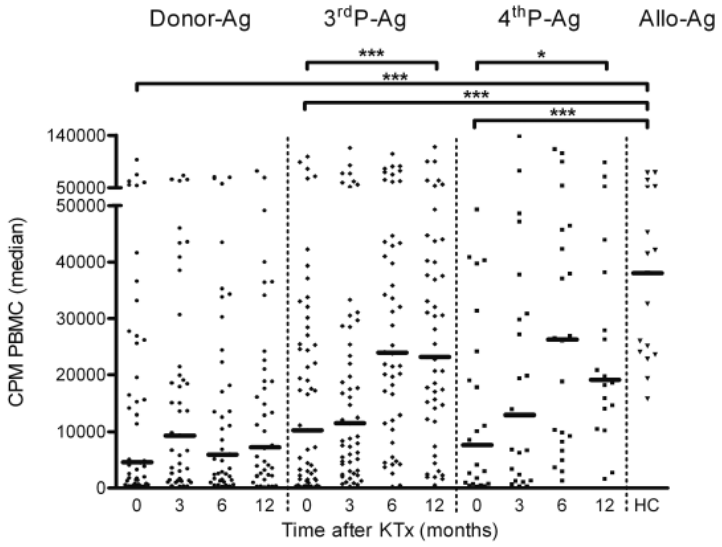


**Figure 3.** Proliferation of PBMC

The data were not always normally distributed and therefore statistical analysis was based on a nonparametric distribution. Before transplantation, proliferation of patient-PBMC to donor-antigens, third party antigens and fourth party antigens was significantly lower as compared to proliferation of PBMC from healthy controls to alloantigens (Mann Whitney U test, all $p<0.001$). After transplantation, proliferation to donor-antigens remained low, while the response to third party antigens and fourth party antigens increased (Kruskal-Wallis Test, $p<0.001$ and $p<0.05$ respectively).

*The suppressive function of CD25$^{bright}$ cells*

The effect of depletion of CD25$^{bright}$ cells from PBMC on direct alloresponses was determined in MLR. Because of the limited amount of peripheral blood available from our patients, we could not analyze the effect of depletion of CD25$^{bright}$ cells in cultures stimulated with fourth party-antigens. After depletion of the CD25$^{bright}$ fraction, we observed an overall increase of the proliferative response in cultures stimulated with donor-antigens and third party-antigens before and after transplantation (Figure 4A). Before transplantation, the average effect of depletion on proliferative responses of alloreactive cells was 51% on stimulation with donor-antigens and

57% upon stimulation with third party-antigens (Figure 4B). After transplantation, the effect of depletion increased significantly in co-cultures stimulated with donor-antigens and less so with third party-antigens (Figure 4B, $p<0.01$ and $p=0.07$, respectively). Furthermore, at 6 and 12 months after transplantation the % increase was higher in cultures stimulated with donor-antigens than with third party-antigens (Figure 4B, $p<0.05$ and $p=0.09$, respectively).

The suppressive capacity of the isolated $CD25^{bright}$ cells on a per cell basis was determined in co-culture experiments with $CD25^{neg/dim}$ responder cells. The isolated $CD25^{bright}$ cells did not proliferate upon stimulation with allo-antigens. Co-culture experiments proved that the suppressive effect of $CD25^{bright}$ cells on $CD25^{neg/dim}$ responder cells is a dose-dependent phenomenon (Figures 4C and D). Before transplantation, the capacity of $CD25^{bright}$ cells to suppress donor-antigens or third party-antigens stimulated $CD25^{neg/dim}$ responder cells (ratio 1:10) was significantly lower when compared with HC, (both $p<0.05$, Figure 4E). After transplantation, the average suppressive capacity of $CD25^{bright}$ T cells improved significantly in cultures stimulated with donor-antigens (Figure 4E, 51%-75%, $p<0.001$), but not with third party-antigens (50%-57%, $p=0.49$). Furthermore, at 6 and 12 months after transplantation, the capacity of $CD25^{bright}$ cells to suppress donor-antigens stimulated $CD25^{neg/dim}$ cells was significantly higher than on stimulation with third party-antigens (Figure 4E, $p<0.01$ and $p<0.001$ respectively). The results on the % inhibition at a 1:10 ratio were comparable with the ratio of 1:5, 1:20 and 1:40, but significance was lost at 1:20 and 1:40.

*Rejectors versus non-rejectors*

In this study, 9 out of 79 patients (11%) had a rejection episode. All rejections occurred in the first month after transplantation (median 12 days; range 3-28). Antirejection therapy consisted of high dose solumedrol. At baseline, no differences were found between rejectors and non-rejectors for clinical characteristics, flow cytometric results, proliferation of PBMC and the suppressive function of $CD25^{bright}$ cells. Therefore, we found none of these factors are a predictor for rejection before transplantation.

We analyzed the suppressive capacity of $CD25^{bright}$ cells from rejectors at 12 months after transplantation. Our results show that this suppressive capacity in co-cultures of $CD25^{neg/dim}$ responder cells stimulated with donor-antigens or third party-antigens was not different from nonrejectors at month 12. This is in line with a study from Demirkiran et al.[23] on liver transplant recipients.

*Immunosuppressive drugs: tacrolimus/MMF versus tacrolimus/rapamycin*

We compared the two arms of treatment to determine whether therapy with tacrolimus/MMF versus tacrolimus/rapamycin affected Treg differently. No difference was observed for any of the phenotypical or functional Treg-characteristics analyzed in this study between these arms of treatment.

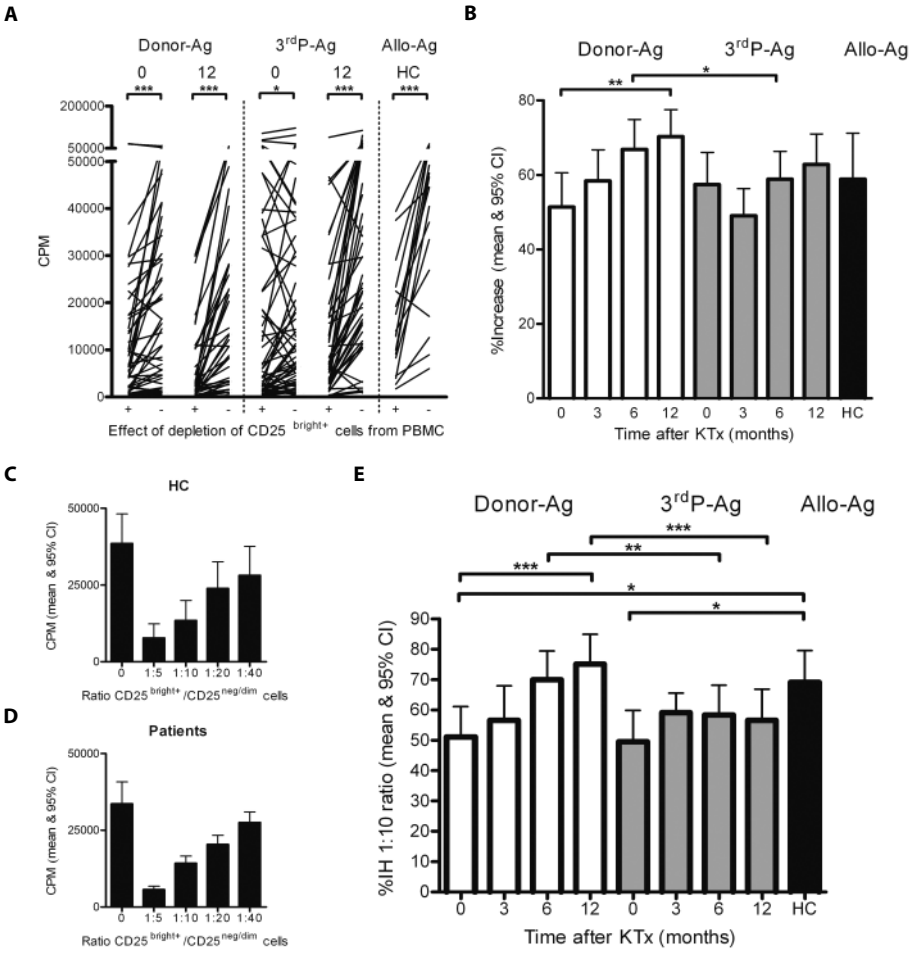


**Figure 4.** Functional analysis of CD25$^{bright}$ cells

Except for the data in **(A)**, all data were normally distributed. **(A)** PBMC (+) and CD25$^{neg/dim}$ cells (-). Depletion of CD25$^{bright}$ cells from PBMC resulted in improved proliferation in donor-antigens and third party-antigens stimulated co-cultures from patients before (0) and 12 mo after transplantation (Mann Whitney U test). **(B)** The effect of depletion was calculated as the percentage increase. After transplantation, the effect of depletion increased significantly in co-cultures stimulated with donor-antigens and less vigorously with third party antigens (ANOVA, $p<0.01$ and $p=0.07$, respectively). At 6 and 12 mo, the percentage increase was higher in co-cultures stimulated with donor-antigens than with third party antigens (paired t-test, $p<0.05$ and $p=0.09$, respectively). **(C-D)** In HC and patients (12 mo, third party antigens stimulated co-cultures), inhibition of alloantigens stimulated CD25$^{neg/dim}$ cells by CD25$^{bright}$ cells proved to be dose dependent. **(E)** The suppressive capacity of CD25$^{bright}$ cells was calculated as the percentage inhibition. Before transplantation, the percentage inhibition was significantly lower in co-cultures stimulated with donor-antigens and third party antigens when compared to HC (unpaired t test, both $p<0.05$). After transplantation, the percentage inhibition increased in co-cultures stimulated with donor-antigens (ANOVA, $p<0.001$), but not significantly with third party antigens ($p=0.49$). At 6 and 12 mo, the percentage inhibition was significantly higher in co-cultures stimulated with donor-antigens than with third party antigens (paired t test, $p<0.01$ and $p<0.001$, respectively).

*Multivariate analysis*

In a multivariate analysis the factors like gender, recipient age, dialysis type, time on dialysis, origin of donor kidney, first KTx/more than first, number of HLA mismatches, primary kidney disease, blood group, cytomegalovirus status, and the level of panel reactive antibodies before KTx were not associated with the number, proportion, or function of $CD25^{bright}$ T cells on a fixed time point or over time.

## DISCUSSION

To investigate whether donor-specific $CD4^{+}CD25^{bright}$ regulatory T-cell function is generated in *de novo* kidney transplant recipients; we prospectively analyzed their suppressive capacity in the first year after transplantation. In the MLR, depletion of $CD25^{bright}$ cells from PBMC, and their capacity to suppress the proliferation of $CD25^{neg/dim}$ cells, demonstrated improved Treg function in the first year. We also found donor-specific hyporesponsiveness, whereas Treg activity was significantly more donor-directed compared with third party-antigens.

Data from *in vitro* and animal studies indicated that immunosuppressive drugs have detrimental effects on Treg[15, 17, 24]. However, the development of donor-specific Treg in the present study shows that the immune system can bypass these unfavorable effects *in vivo* to a certain extent. Apart from the restored kidney function[25], an explanation might be that immunosuppressive drugs like cyclosporine and tacrolimus do not inhibit the transcription of IL-2[14], an important cytokine for the function and survival of $CD4^{+}CD25^{bright}FoxP3^{+}$ T cells[16-19]. However, the pivotal role for this cytokine was not always observed[26], as other members of the IL-2 family may compensate for the absence of IL-2[18, 27]. This probably results from their shared signalling through the common gamma chain (i.e. CD132), which activates the signal transducer and activator of transcription factor 5 (STAT5), and therefore induces the expression of the transcription factor for regulatory cells FoxP3[8, 28, 29].

The observed donor-specific hyporesponsiveness as compared with the reactivity to third and fourth party-antigens did not result from better histocompatibility between donor and recipient[22]. Regulation could be another explanation. Indeed, the suppressive function of Treg from our patients became increasingly potent to donor-antigens stimulated cultures after transplantation. It has been reported that Treg respond dynamically to their antigenic environment in a transgenic mouse model, which showed that these regulatory T cells proliferated in response to T-cell receptor engagement[30]. In the transplantation setting, the continuous presence of donor-antigens could therefore stimulate the peripheral proliferation and accumulation of Treg. Moreover, it has been reported that operationally tolerant patients have an unexpected strongly altered T-cell receptor Vβ usage and high TCR transcript accumulation in selected T cells[31]. This may explain why we found generation of donor-specific regulatory T cell function and not higher Treg numbers. Also, development of potent Treg might be favoured

by the lymphopaenic state of transplant patients (Table 3), because stimuli that originate from lymphopaenia favor their homeostatic proliferation and enhance their suppressor function[32].

In the present study, we measured donor-specific hyporesponsiveness in the direct pathway of allorecognition, which was mediated by $CD4^+CD25^{bright}$ Treg. In contrast, a cross-sectional study from Game et al.[33] stated that Treg do not contribute to the direct pathway of hyporesponsiveness in stable transplant patients. The difference between their findings and the present study may be explained by differences in immunosuppressive strategies, the lower number of patients studied (n=12 vs. n=79) and the time after transplantation (2-20 years vs. ≤1 year, respectively). Especially the latter may be essential, because the indirect pathway becomes more important in the long term[4]. In addition, other mechanisms could be envisioned that contribute to the measured donor-specific hyporesponsiveness, including anergy, ignorance and clonal deletion of donor-specific effector T cells.

The generation of donor-specific Treg function occurred in the presence of immunosuppressive agents that have the potential to hamper their development and suppressive function[15, 24, 27]. Therefore, the individual effect of these drugs or their combinations may still have influenced the dynamics by which donor-specific Treg function is generated. Several studies indicated that rapamycin does not interfere with the suppressive activity of $CD4^+CD25^{bright}FoxP3^+$ T cells and favors their expansion *in vivo*[34-36], whereas MMF and calcineurin inhibitors for example, tacrolimus prevent the expansion of these cells[14, 34]. Here, we did not observe a difference in the effect of treatment with tacrolimus/rapamycin or tacrolimus/MMF on the number and function of Treg. These findings can be explained by the dominant effect of tacrolimus in both arms of treatment. Especially, because calcineurin inhibitor-based treatment is associated with decreased numbers of Treg and possibly impairs their functional development[15, 24, 34, 37].

Another explanation for the observed changes in the peripheral compartment may be an increased recruitment of Treg to secondary lymphoid tissues and the transplanted organ[26, 30, 35, 38-40]. CCR7 is a homing marker for the lymphoid tissues and in this study we demonstrated that the proportion of Treg that expressed CCR7 significantly increased. This suggests an increased potential of the peripheral Treg compartment to home to lymphoid tissues[41, 42]. Also CCR7 is expressed by naive T cells[41, 42] and indeed flow cytometric analysis revealed a decreased proportion of Treg with a memory phenotype, indicating an increased proportion of naive Treg. Because it has been demonstrated that especially naive Treg give rise to potent antigen-specific Treg[43], their strong proportional increase may have favored the development of the observed donor-specific Treg function.

In summary, we prospectively analyzed the development of peripheral $CD4^+CD25^{bright}$ T cells from kidney transplant recipients in the first year after transplantation. Our results demonstrated that even in the presence of full immunosuppression potent donor-specific $CD4^+CD25^{bright}$ regulatory T-cell function is generated in these patients.

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# Chapter 4

## The Effect of Rabbit Anti-Thymocyte Globulin Induction Therapy on Regulatory T cells in Kidney Transplant Patients

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## ABSTRACT

Prevention of alloreactivity by rabbit Anti-Thymocyte Globulins (rATG) may not only result from immunodepletion, but also from the induction of T cells that control allogeneic immune responses. In the present prospective and controlled study, we investigated the effect of rATG on the frequency, function and phenotype of peripheral immunoregulatory $CD4^+$ T cells in kidney transplant patients. After transplantation, 16 patients received ATG-induction therapy and triple therapy consisting of tacrolimus, mycophenolate mofetil (MMF) and steroids. The control group (n=18) received triple therapy only. By flow cytometry, T cells were analyzed for CD25, FoxP3, CD127, CD45RO and CCR7. To study their suppressive capacities, $CD25^{bright}$ T cells were co-cultured with $CD25^{-/dim}$ T cells (Teff) in mixed lymphocyte reactions, stimulated with donor and third party (3P)-antigens. Pre-transplant levels of $FoxP3^+CD127^{-/low}$ T cells were 6% of $CD4^+$ T cells. One wk post-ATG treatment, no measurable numbers of regulatory T cells were present ($p<0.01$). After 4 weeks, the cell numbers of $CD4^+FoxP3^+CD127^{-/low}$ T cells slowly reappeared and thereafter remained low ($p<0.01$). At 14 weeks, a significant shift towards the $CD45RO^+CCR7^+$ (central memory) phenotype within $CD4^+FoxP3^+$ T cells was observed ($p<0.01$). At 26 weeks, the proliferative alloresponses of the peripheral blood mononuclear cells (PBMC) and $CD25^{-/dim}$ Teff profoundly decreased compared with pre-transplant ($p=0.01$ and $p=0.02$ respectively), while the regulatory capacity of the $CD25^{bright}$ T cells, of which 90% consisted of $FoxP3^+CD127^{-/low}$ T cells, remained unaffected. The $CD25^{bright}$ T cells suppressed the anti-donor (94%) and 3P responses (93%). Our findings show that rATG therapy does not spare peripheral immunoregulatory T cells *in vivo*, but after regeneration preserves their suppressive activity.

## INTRODUCTION

Thymus-derived natural immunoregulatory $CD4^+$ T cells do not only have important activities in the prevention of autoimmunity[1, 2], but also control immune responses towards transplanted organs and tissues[2, 3]. They require IL-2 for their homeostasis, function and maintenance and therefore highly express the IL-2 receptor α-chain, CD25[4]. IL-2 and other members of the IL-2 family that signal via the common γ-chain ($\gamma_c$; CD132) are important for the induction of immunoregulatory $CD4^+$ T cells[5, 6]. The markers most generally used to define human immunoregulatory $CD4^+$ T cells are that they constitutively express the forkhead/winged helix transcription factor FoxP3[7, 8], while they do not constitutively express the IL-7 receptor α-chain and are therefore $CD127^{-/low}$[9]. The majority of the immunoregulatory $CD4^+$ T cells express the CD45RO memory marker, GITR, CTLA-4[10] and the homing markers CCR7 and CD62L, both necessary for their migration[11]. The proliferation of $CD25^{-/dim}$ Teff can be inhibited by anergic peripheral $CD4^+CD25^{bright}$ T cells either directly in a cell-cell contact-dependent manner[12, 13] or via the suppression of the IL-2 and IFN-γ production[14-16]. Other molecular mechanisms of immunoregulatory $CD4^+$ T cell-mediated suppression are through the secretion of IL-10 and TGF-β and via killing of the Teff directly by cytolysis[17, 18].

It has been shown that immunosuppressive drugs, such as the calcineurin inhibitors cyclosporine and tacrolimus negatively affect the frequency and function of $CD4^+CD25^{bright}$ T cells[19-21]. However, other agents, for instance the mTOR inhibitors and rabbit anti-thymocyte globulins, may favour the expansion of the $CD4^+CD25^{bright}$ T cells[22-24]. Induction therapy with anti T-cell depletion strategies as rATG is used to avoid rejection or to minimize the nephrotoxic effect of CNI on immediate graft function[25-27]. Known mechanisms of action by rATG are depletion of immunocompetent cells through complement-dependent lysis or activation-associated apoptosis[28, 29], and modulation of several molecules on residual circulating leucocytes that are involved in regulating leucocyte-endothelium adhesion and leucocyte migration, e.g. the chemokine receptors CXCR4, CCR5 and CCR7[28,30]. Experimental studies suggested that the immunosuppressive activity of rATG may also result from its effect on $CD4^+CD25^{bright}FoxP3^+$ T cells by either the selective sparing of immunoregulatory $CD4^+$ T cells or by the induction and expansion of regulatory T cells[24, 31, 32]. Therefore, rATG-treatment in patients may modulate the immune system and enhance the process leading to hyporesponsiveness towards the allograft.

Here, we characterized the frequency, function and phenotypic characteristics (e.g. FoxP3, CD127, CD45RO and CCR7) of peripheral blood $CD4^+CD25^{bright}$ immunoregulatory T cells of kidney-transplant patients that received rATG induction therapy combined with triple therapy consisting of tacrolimus, mycophenolate mofetil and steroids, prior to transplantation and at 4, 14 and 26 weeks after transplantation. Patients without rATG therapy and only triple therapy served as controls.

## MATERIALS & METHODS

*Patients and study design*

Kidney transplant patients (n=34) were enrolled in this study. The patients (n=16) were given one infusion of 2 mg/kg Anti-thymocyte Globulin (Rabbit) (Thymoglobulin®, Genzyme Corporation, Cambridge, MA) each day at Day 1, 2 and 3 after transplantation followed by a triple therapy maintenance regimen consisting of tacrolimus, MMF and prednisone. MMF and prednisone were given from Day 1 and tacrolimus was given from Day 2 after transplantation. A control non-rATG patient group (n=18) was given triple therapy alone. Patient characteristics are depicted in Table 1. The patients were part of a feasibility study on a pilot for a future randomized controlled trial and were included between April and August 2007. After informed consent, peripheral blood was drawn pre-transplant and at 4, 14 and 26 weeks after transplantation. The Medical Ethical Commission of the Erasmus Medical Center approved the protocol.



**Table 1.** Demographics of Kidney Transplant Patients

| | rATG - group | Non - rATG group |
|---|---|---|
| Patients (n) | 16 | 18 |
| Recipient gender (M/F) | 7/9 | 8/10 |
| Recipient age at KTx | 53 ± 17* | 53 ± 13* |
| HLA Incompatibilities | | |
| HLA-A MM (number) | 0.8 ± 0.8* | 1.0 ± 0.8* |
| HLA-B MM (number) | 1.2 ± 0.7* | 1.3 ± 0.8* |
| HLA-DR MM (number) | 0.9 ± 0.7* | 1.1 ± 0.8* |
| Acute Rejection (n) | 1 (6%) | 3 (17%) |
| Infections (n) | 4 (25%) | 2 (11%) |

*Mean ± SD

*Flow cytometry*

EDTA-blood was analyzed for the presence of T-cell subsets by four-color flow cytometry using mAbs directly conjugated to fluorescein isothiocyanate (FITC), phycoerythrin (PE), allophycocyanin (APC) or peridinin chlorophyll protein (PerCP). Blood (100 µL) was incubated with CD45-FITC/CD14-PE, IgG1-FITC/IgG2b-PE and CD19-APC. Furthermore, CD3-FITC, CD4-PerCP, CD8-APC, and CD3/16/56-FITC/PE were also used (BD Biosciences, San Jose, CA and R&D Systems, Abingdon, UK). After 30-min incubation at RT, red blood cells were lysed. White blood cells were washed twice, and analyzed on a flow cytometer (FACSCalibur) using SimulSet and CELL Quest Pro software (BD Biosciences). The number of leucocytes was determined by the cell counter CASY® model TT (Schärfe System GmbH, Reutlingen, Germany).

FoxP3 intracellular staining was performed according to the manufacturer's instructions (FoxP3-APC, clone PCH101, eBiosciences, San Diego, CA). Extracellular staining was conducted prior to intracellular staining with FITC-conjugated CD127, IgG1, or CCR7, PERCP-conjugated CD4 and PE-conjugated CD25 or CD45RO at 4°C for 30 min (BD Biosciences. To discriminate between effector memory ($CD45RO^+CCR7^-$), central memory ($CD45RO^+CCR7^+$) and naïve

($CD45RO^{-}CCR7^{+}$) T cells, CCR7 was combined with $CD45RO^{33}$. Flow cytometric analysis was performed with at least 100 events in the gate.

*Isolation of Peripheral Blood Lymphocytes*

Peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation over Ficoll-paque, frozen in 10% DMSO enriched RPMI 1640 medium (BioWhittaker, Verviers, Belgium) and stored at -140˚C.

*Isolation of human $CD4^{+}CD25^{bright}$ T cells*

PBMC were thawed and resuspended in 10% human culture medium (HCM), that consisted of RPMI 1640 medium with L-glutamine (Bio Whittaker) supplemented with 10% pooled human serum, 100 IU/mL penicillin and 100 µg/mL streptomycin (Gibco BRL). The $CD25^{bright}$ T cells were isolated from PBMC after incubation with anti-CD25 microbeads, (Miltenyi Biotech, Bergisch Gladbach, Germany) followed by positive selection (POSSELD-program) on the autoMACS (Miltenyi Biotech). The untouched residual fraction consisted of $\geq$ 98% of $CD25^{-/dim}$ Teff and was used as responder population. Both fractions were stained with CD4-PERCP (BD Biosciences) and CD25-PE epitope B (clone M-A251, BD Biosciences). The purity of $CD25^{bright}$ was $\geq$ 90%.

*Suppression Assay*

The function of $CD4^{+}CD25^{bright}$ T cells was determined by MLR in which the suppressive capacity of $CD25^{bright}$ T cells was measured twice; firstly by their depletion from PBMC, and secondly by their ability to inhibit the proliferative responses to donor and 3P antigens of the $CD25^{-/dim}$ Teff. The $CD25^{-/dim}$ Teff were co-cultured in triplicate with and without $CD25^{bright}$ T cells. Irradiated (40 Gy) donor spleen cells and HLA mismatched (2-2-2) 3P spleen-cells were used as stimulator cells ($1x10^{5}$ cells/100 µL) and co-cultured with $5x10^{4}$ cells/100 µL of a mixture of $CD25^{bright}$ : $CD25^{-/dim}$ at 1:10, 1:20, 1:40 and 1:80 ratios in round-bottom 96-well plates (Nunc, Roskilde, Denmark). Moreover, the $CD25^{bright}$ T cells were co-cultured with irradiated $CD25^{-/dim}$ Teff in the presence of donor- and 3P antigens to confirm their anergic state. After 7 days' incubation at 37°C in a humidified atmosphere of 5% $CO_2$, the proliferation was measured after $^{3}$H-thymidine (0.5 µCi/well: Amersham Pharmacia Biotech) incubation during the last 16 hr before harvesting. The median counts per minute (cpm) for each triplicate was determined and the level of suppression of the $CD25^{-/dim}$ Teff by $CD25^{bright}$ T cells was calculated and expressed as the percentage inhibition of the Teff.

*Statistical analyses*

Statistical analyses were performed using Graphpad Prism (v.4.03). Based on the distribution of the data we performed non-parametric testing. For determination of the levels of statistical significance, the two-sided probability values according to the Kruskal-Wallis test, the Wilcoxon matched pairs test and the Mann Whitney U Test was used. For comparisons within the rATG-group

over time, the non-parametric Kruskal-Wallis test was used and the results were validated with the Wilcoxon matched pairs test. To test differences between groups the Mann Whitney U test (unpaired measurements) was used. Post-hoc analyses were performed using Bonferroni's test for multiple comparisons. P values $\leq 0.05$ were considered statistically significant.

## RESULTS

*Patients*

We observed acute rejections in three patients of the non-rATG group and in one patient of the rATG-group within 26 weeks post-transplantation (Table 1). The number of infections was not significantly different between both groups. There were no differences in renal function or blood trough levels of tacrolimus between both groups in the first 26 weeks. However, in the non-rATG group, the MMF trough level was significantly higher at 14 wks after transplantation compared with the rATG-group, probably due to adjustments according to leucocyte levels.



*Flow cytometry of lymphocyte subsets*

After rATG-treatment we observed a significant decrease in $CD3^+$ T cells, $CD3^-CD16/56^+$ NK cells, $CD8^+$ T cells, $CD4^+$ T cells, $CD4^+CD25^{bright}$ T cells and not in $CD19^+$ B-cells compared to pre-transplant levels in the rATG-group ($p<0.01$, Figure 1). The magnitude of the decrease in T cells, NK-cells, $CD4^+$ T cells, $CD4^+CD25^{bright}$ T cells, $CD4^+CD25^{bright}FoxP3^+$ T cells and $CD4^+CD25^{bright}FoxP3^+CD127^{-/low}$ T cells was more outspoken compared to the non-rATG group ($p<0.01$).

After 4 weeks, the number of all cell types in both groups increased although this recovery occurred more gradually in the rATG-group. The number of $CD3^+$ T cells in this group remained significantly lower compared to pre-transplant levels and partial recovery was only 50% of baseline at 26 weeks (Figure 1A). Along with the recovery of the $CD4^+$ T cells, the $CD4^+CD25^{bright}$ T cells recovered only to ~30% of baseline at 26 weeks ($p<0.01$, Figures 1E and F, respectively).

To determine the percentage of *bona fide* regulatory T cells, we further phenotyped $CD4^+CD25^{bright}$ T cells for the regulatory T-cell marker FoxP3. Pre-transplant, the percentage of $CD4^+CD25^{bright}$ T cells that expressed FoxP3 fluctuated between individuals, though the majority of the $CD4^+CD25^{bright}$ T cells expressed FoxP3 in both groups, which is in line with the results of Liu *et al.* (top 10% of $CD4^+CD25^{bright}$ in the gate; 72% ± 2, mean ± SEM, top 5%: ≥ 80% and top 2% ≥ 90%)[9]. Therefore, the pattern of the absolute cell number of $CD4^+CD25^{bright}FoxP3^+$ T cells (Figure 1G) is comparable to the $CD4^+CD25^{bright}$ T cell counts. Almost all $CD4^+CD25^{bright}FoxP3^+$ T cells were $CD127^{-/low}$ (90% ± 1, mean ± SEM, Figure 1H).

At all time-points after transplantation, the cell-numbers of the $CD4^+$, $CD4^+CD25^{bright}$, $CD4^+CD25^{bright}FoxP3^+$ and $CD4^+CD25^{bright}FoxP3^+CD127^{-/low}$ T cells were significantly lower in the rATG group than in the non-rATG-group. In the non-rATG group, at 26 weeks, all cell numbers returned to baseline levels (Figure 1).

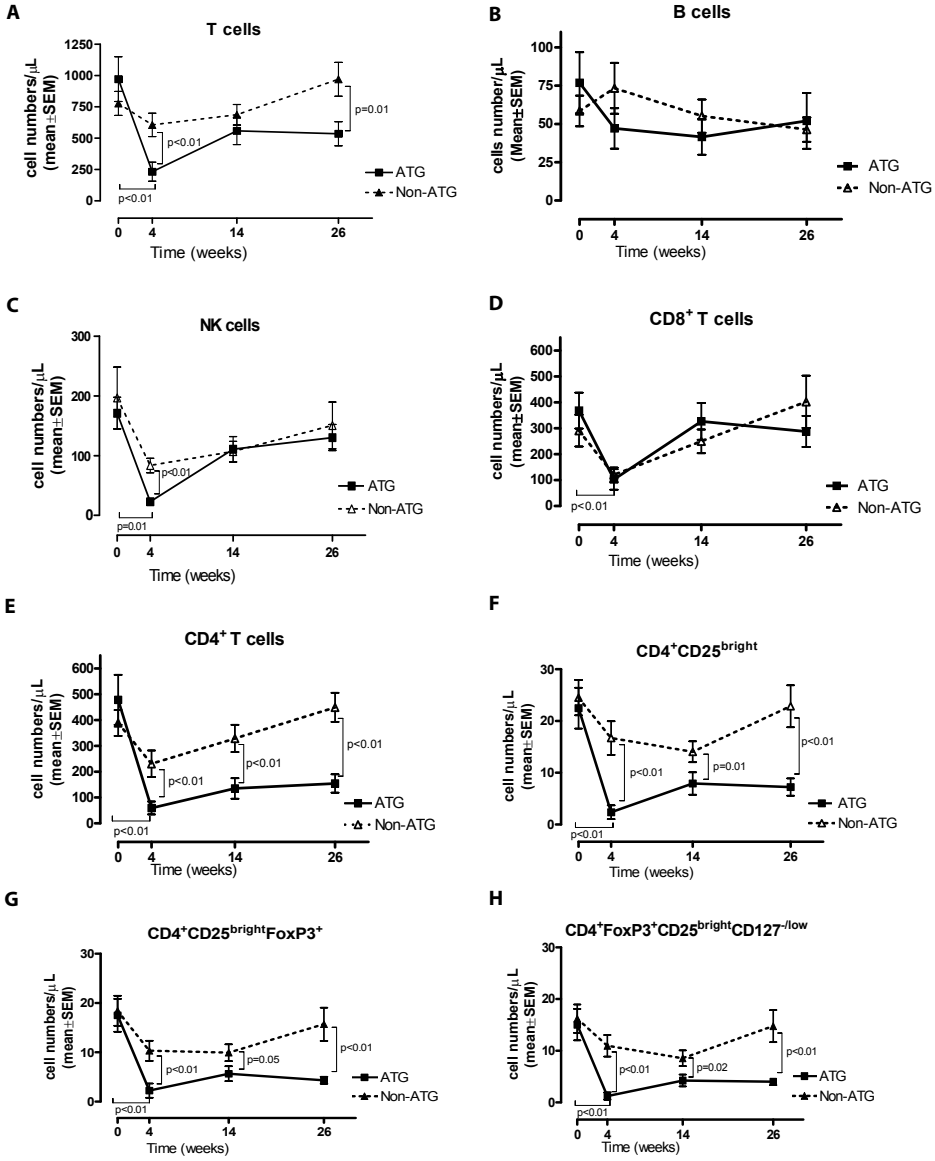


**Figure 1.** Prospective analysis of PBMC and T-cell subpopulations by flow cytometry

Kinetics of different cell types and cell subsets in the rATG treated patients (n=16, solid line) and non-rATG treated patients (n=18, dotted line). Data are depicted as mean ± SEM. **(A)** T cells **(B)** B cells **(C)** NK cells **(D)** $CD8^+$ T cells **(E)** $CD4^+$ T cells **(F)** $CD4^+CD25^{bright}$ T cells of $CD4^+$ T cells **(G)** $CD4^+CD25^{bright}FoxP3^+$ T cells of $CD4^+$ T cells and **(H)** $CD4^+CD25^{bright}FoxP3^+CD127^{-/low}$ of $CD4^+$ T cells. Statistically significant differences within the rATG group over-time were tested by Kruskal-Wallis Test and validated by the Wilcoxon matched pairs test and differences between the rATG- and non-rATG groups were tested with the Mann-Whitney U Test.

*Suppressive capacity of CD4$^{+}$CD25$^{bright}$FoxP3$^{+}$CD127$^{-/low}$ T cells*

The proliferative capacity of the PBMC and the CD25$^{-/dim}$ Teff was analyzed in the MLR. At 14 and 26 weeks, the proliferative responses of the allo-activated PBMC population were reduced compared to pre-transplant (Figures 2A and B). This phenomenon was not observed in the non-rATG group. After depletion of the CD25$^{bright}$ T cells, the proliferation of the CD25$^{-/dim}$ Teff in response to alloantigens showed the same pattern as the PBMC, as their proliferative capacity is affected by rATG (Figures 2C and D). These findings suggest that the anti-donor hyporesponsiveness can be the result of impaired responses by effector T cells and suppressive actions by regulatory T cells.

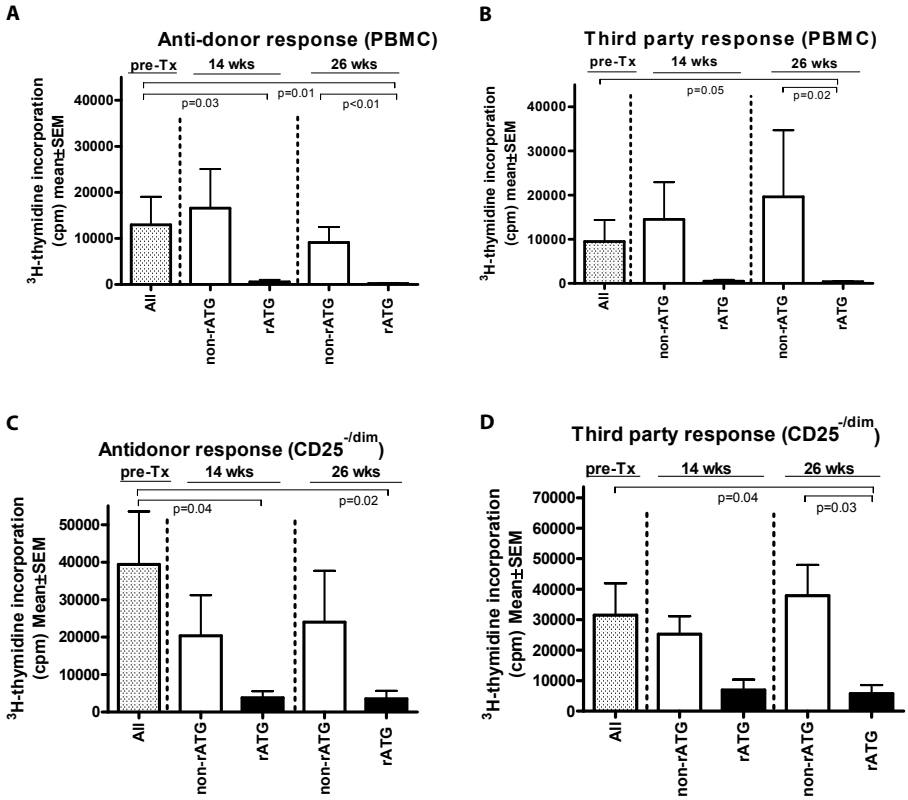


**Figure 2.** Mixed lymphocyte reactions with PBMC and CD25$^{-/dim}$ Teff

PBMC of rATG treated patients (n=6) and non-rATG treated patients (n=6) were isolated pre-transplant (dotted bars) and at 14 and 26 weeks and were stimulated *in vitro* with donor **(A)** and 3P spleen cells **(B)** in the MLR. **(C)** CD25$^{-/dim}$ T cells were stimulated in vitro with donor and **(D)** 3P antigens. Proliferation is depicted as counts per minute (cpm) after $^{3}$H-Thymidine incorporation. Error bars represent mean ± SEM.

Therefore, we studied the suppressive capabilities of the CD4$^{+}$CD25$^{bright}$FoxP3$^{+}$CD127$^{-/low}$ T cells at 26 weeks after rATG treatment. Due to the low number of T cells, it was not possible to study the regulatory activities of CD25$^{bright}$ T cells at 4 and 14 weeks after rATG-treatment. At 26 weeks, the number of PBMC was sufficient and the function of the CD25$^{bright}$ T cells was

measured. After depletion of the $CD25^{bright}$ T cells, the anti-donor and 3P proliferative responses of the $CD25^{-/dim}$ Teff increased, pre- and post transplant (Figure 3). The $CD25^{bright}$ T cells of post-transplant were anergic in response to donor- and 3P antigens and in the presence of irradiated $CD25^{-/dim}$ Teff. Pre- and post-transplant, co-culture of $CD25^{bright}$ T cells and the $CD25^{-/dim}$ Teff resulted in the inhibition of the anti-donor and 3P proliferative responses of the $CD25^{-/dim}$ Teff in a dose-dependent manner (Figure 3). When the percentage of inhibition of the $CD25^{-/dim}$ Teff response to 3P antigens was calculated at different $CD25^{bright}$ : $CD25^{-/dim}$ ratios, this remained proportionally unaltered (Figure 3). Post-transplant, the percentage of inhibition of the anti-donor response at a 1:10 ratio was similar to the non-rATG-group (proliferation [cpm] 0:1 vs. 1:10 ; 20684 ± 11107 vs. 3142 ± 2326, mean ± SEM, percentage inhibition: 85%). These results show that though diminished in number, the $CD25^{bright}$ T cells have proportionally adequate suppressor activity after rATG-treatment, whereas the proliferative capacity of the $CD25^{-/dim}$ Teff is significantly affected by rATG.

*Phenotypical characterization of $CD4^+FoxP3^+$ T cells*

We subsequently quantified the $CD4^+$ regulatory T cells using an approach independent of CD25 expression (Figure 4A). At 1 week after rATG-treatment, no measurable numbers of $CD4^+FoxP3^+CD127^{-/low}$ T cells were present in the circulation. At 4 weeks, 5 out of 16 patients (31%) had a sufficient number of T cells to perform a FoxP3 staining. In the rATG-group, the percentage of $CD4^+FoxP3^+$ T cells that had the $CD127^{-/low}$ phenotype did not change (89% ± 1.0, mean ± SEM) as depicted in Figure 4B, indicating that the recovered $CD4^+FoxP3^+$ T cells harbored the phenotype of genuine regulatory T cells. After 4 weeks, along with the depletion of $CD4^+$ T cells in the rATG-group, the number of patients that had detectable cell numbers of $CD4^+FoxP3^+CD127^{-/low}$ T cells increased (88%), but in each patient, the homeostatic reconstitution occurred slowly (Figure 4C).

*Naïve, central memory and effector memory regulatory T cells*

In the literature it has been described that T cells with the memory phenotype are resistant of immunodepletion by rATG[34] but in our patient cohort we did not measure any T cells immediately after rATG-treatment. Hence, the percentage of $CD4^+CD45RO^+$ profoundly rose above baseline and was higher than non-rATG group (Figure 5A). The percentage of $CD4^+CCR7^+$ massively decreased after depletion and was lower than the non-rATG group at 14 and 26 weeks (Figure 5B).

Subsequently, we analyzed whether the recovered $CD4^+FoxP3^+$ T cells after rATG induction were predominantly of the memory phenotype as homeostasis-driven proliferation after immunodepletion may account for an increase in memory $CD4^+FoxP3^+$ T cells[35]. Therefore, we phenotyped the $CD4^+FoxP3^+$ T cells for CD45RO and CCR7. The combination of both markers allows a distinction between the central memory and effector memory T cells[33, 36, 37].

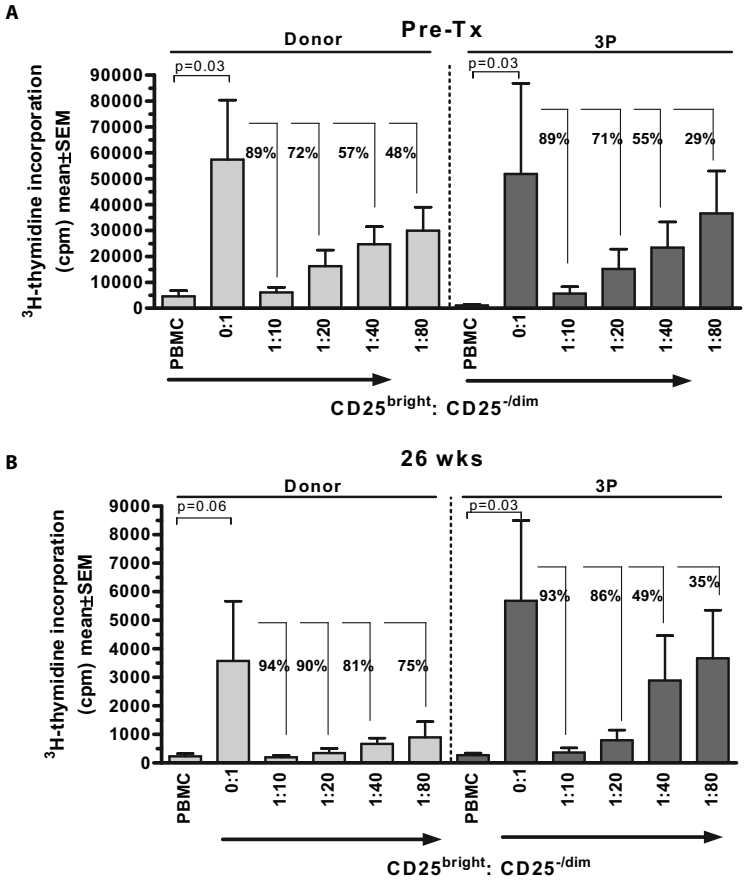


**Figure 3.** Suppressive capacity of regulatory T cells before and after rATG treatment in suppression assays
The ability of $CD25^{bright}$ T cells to suppress the anti-donor (grey bars) and 3P responses (dark gray bars) of the $CD25^{-/dim}$ Teff was analyzed before and after rATG-treatment. Proliferation is depicted as counts per minute (cpm) after $^3$H-Thymidine incorporation and the percentage of inhibition of the $CD25^{-/dim}$ effector T cell response (mean) is shown. **(A)** Suppression assay with PBMC before rATG-treatment. The anti-donor and 3P response of the PBMC and the $CD25^{-/dim}$ Teff is given. After co-culture of $CD25^{-/dim}$ Teff and $CD25^{bright}$ T cells, the anti-donor and 3P responses of the $CD25^{-/dim}$ Teff were inhibited in a dose-dependent manner. **(B)** At 26 weeks after rATG-treatment, $CD25^{-/dim}$ Teff were also inhibited in a dose-dependent manner. Note that the y-axis is only 10% of the pre-transplant values. Error bars represent mean ± SEM of n=6.

Within the rATG-treated patients in whom we measured $CD4^+FoxP3^+$ T cells, a shift in the proportion of $FoxP3^+$ cells expressing CD45RO was observed. Along with the $CD4^+$ T cells, the proportion of $CD4^+FoxP3^+$ T cells that expressed $CD45RO^+$ was higher at 14 and 26 weeks compared to baseline levels and higher than the non-rATG group (Figure 5C). rATG also influenced the $CD4^+FoxP3^+$ T cells expressing CCR7, as the proportion of $CD4^+FoxP3^+CCR7^+$ was lower than in the non-rATG group at 14 and 26 weeks (Figure 5D).

After rATG-treatment, significant differences were observed in the composition of the naïve ($CD45RO^-CCR7^+$), central memory ($CD45RO^+CCR7^+$) and effector memory ($CD45RO^+CCR7^-$) $CD4^+FoxP3^+$ T cell populations. At 14 and 26 weeks, the percentage of naïve T cells within the

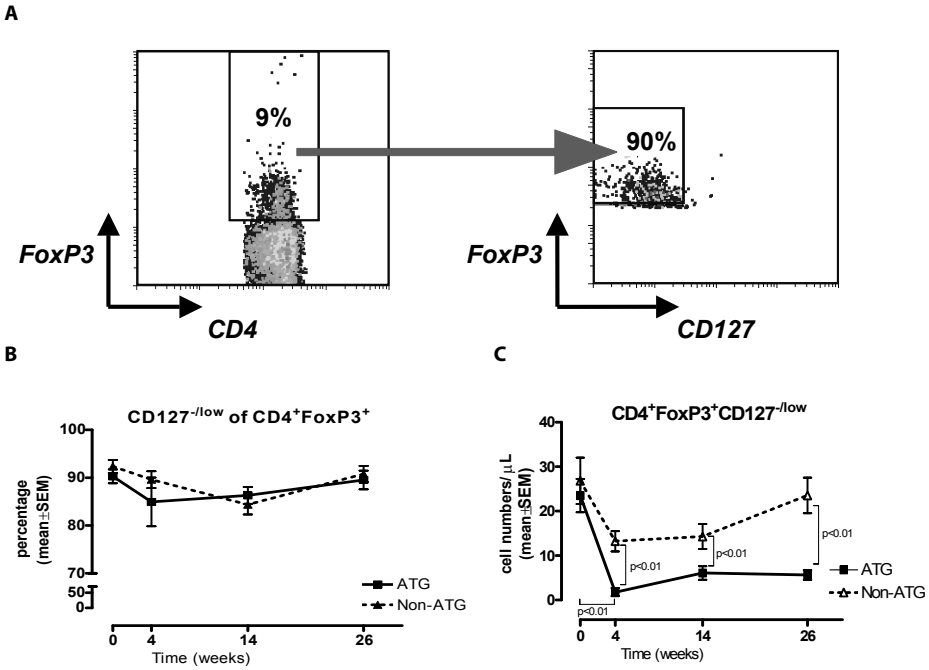


**Figure 4.** Characterization of regulatory T cells that are recovered after rATG treatment

**(A)** Representative flow cytometric results of $CD127^{-/low}$ expression within $CD4^+FoxP3^+$ T cells. About 90% of the $CD4^+FoxP3^+$ T cells have the $CD127^{-/low}$ phenotype. **(B)** The percentage of $CD127^{-/low}$ of $CD4^+FoxP3^+$ T cells remained constant both in the rATG and non-rATG group. **(C)** Evaluation of the kinetics of $CD4^+FoxP3^+CD127^{-/low}$ T cells before and after transplantation. Absolute cell numbers are shown. $CD4^+FoxP3^+CD127^{-/low}$ T cells were depleted after rATG-treatment ($p<0.01$) and showed similar kinetics and pattern as the $CD4^+CD25^{bright}$ T cells. Data are depicted as mean ± SEM.

rATG-group was lower than pre-transplant and lower compared to the non-rATG group (Figure 5E). The fall in the proportion of naïve T cells after rATG-treatment was associated with an increase in the proportion of the $CD45RO^+$ memory pool that was due to an increase of the central memory $CD4^+FoxP3^+$ T cells (Figure 5F). The percentage of effector memory $CD4^+FoxP3^+$ T cells remained stable over time, but was higher than in the non-rATG group at 14 and 26 weeks (Figure 5G).

## DISCUSSION

In the present prospective controlled study, we investigated the effect of rATG induction therapy on the function and phenotype of peripheral $CD4^+CD25^{bright}FoxP3^+CD127^{-/low}$ regulatory T cells in KTx patients. Our findings demonstrate that the $CD3^+$ T cells, $CD3^-CD16/56^+$ NK cells, and $CD4^+$ T cells, but not $CD19^+$ B cells are depleted after rATG-treatment (Figure 1). The number of T cells steadily recovered to 50% of baseline at 26 weeks. Interestingly, the recovery the number of $CD4^+$T cells remained at 30% of baseline. This may be attributed by a slow regeneration and thymic output of $CD4^+$ T cells, which occurs more gradually in the elderly[38].

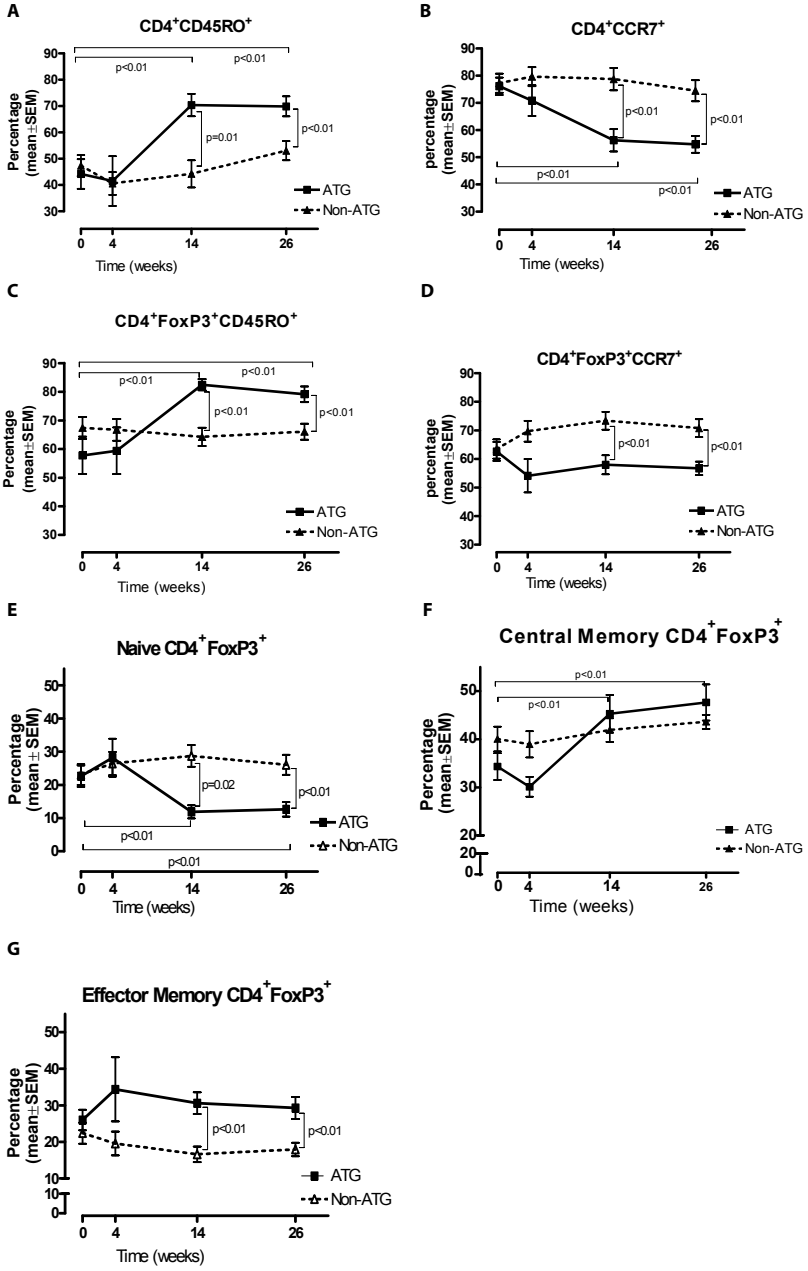


**Figure 5.** Phenotyping of the naïve, effector memory and central memory regulatory T cells

Longitudinal and detailed analysis of $CD4^+$ T cells and $CD4^+FoxP3^+$ T cells in the rATG-group (solid line) and the non-rATG group (dotted line). Within the $CD4^+FoxP3^+$ T cells the **(A)** $CD4^+CD45RO^+$ T cells as $CD45RO^+$ of $CD4^+$ **(B)** $CD4^+CCR7^+$ T cells as $CCR7^+$ of $CD4^+$ **(C)** $CD4^+FoxP3^+CD45RO^+$ T cells as $CD45RO^+$ of $CD4^+FoxP3^+$ **(D)** $CD4^+FoxP3^+CCR7^+$ T cells as $CCR7^+$ of $CD4^+FoxP3^+$ **(E)** Naïve ($CD4^+FoxP3^+CD45RO^-CCR7^+$) T cells as $CD45RO^-CCR7^+$ of $CD4^+FoxP3^+$ **(F)** Central memory ($CD4^+FoxP3^+CD45RO^+CCR7^+$) T cells as $CD45RO^+CCR7^+$ of $CD4^+FoxP3^+$ and **(G)** Effector memory ($CD4^+FoxP3^+CD45RO^+CCR7^-$) T cells as $CD45RO^+CCR7^-$ of $CD4^+FoxP3^+$ were characterized. Results are expressed as the percentage of positive cells. Error bars represent mean ± SEM.

We found that the regulatory T cells whether defined as $CD4^+CD25^{bright}$, $CD4^+CD25^{bright}FoxP3^+$, $CD4^+CD25^{bright}FoxP3^+CD127^{-/low}$ or $CD4^+FoxP3^+CD127^{-/low}$ T cells, were totally depleted from the peripheral blood after rATG-treatment (Figures 1 and 4). Our results are in accordance with Louis *et al.* who reported that ATG did not specifically spare the $CD4^+CD25^{bright}$ T cells[29].

In concert with the slow repopulation of the $CD4^+$ T cells, the $CD4^+CD25^{bright}$ and $CD4^+FoxP3^+CD127^{-/low}$ T cells did not fully recover and showed an impaired homeostasis in the first 26 weeks after rATG-treatment. We found that the FoxP3 expression did not change at the protein level after rATG treatment within 26 weeks after transplantation. Again in line with this, it was described that the FoxP3 mRNA transcripts were not upregulated within 2 years after transplantation[29]. From these data it can be concluded that regulatory and non-regulatory $CD4^+$ T cells show the same behavior after rATG induction therapy. Several studies with other immunodepleting agents as e.g. Campath-1H (anti-CD52) support our and above mentioned data by showing that the number of $CD4^+CD25^{bright}FoxP3^+$ regulatory T cells significantly decreased after treatment and remained very low thereafter, suggesting that this effect is driven by immunodepletion in general[39-41].

After rATG-treatment, the proliferative capacity of the PBMC and $CD25^{-/dim}$ Teff markedly decreased as shown in Figures 2 and 3. Besides general phenomena that account for the hyporesponsiveness as anergy, clonal deletion and ignorance of donor-directed effector T cells, we have several rATG-specific explanations for the observed hyporesponsiveness. Firstly, the low proliferation can be attributed to a low percentage of T cells among the lymphocyte population and the low number of $CD4^+$ T cells within the T cell population as this resulted in an inverse $CD4^+$ to $CD8^+$ ratio (Figure 1). The low number of $CD4^+$ T cells is associated with an inferior T-helper cell response to $CD8^+$ T cells in comparison with before transplantation. A second explanation is given by Preville *et al.* who demonstrated in non-human primates that non-depleted $CD3^+$ and $CD4^+$ T cells in lymph nodes that were coated by rATG, down regulated CD2, CD3, CD4 and CD8 molecules and had impaired responsiveness in the MLR[42]. Thirdly, other studies reported that T-helper cell function is affected by rATG by impaired co-stimulatory signals delivered by monocytes[43] or by decreased expression of the co-stimulatory molecule CD28 on T cells[44, 45] and fourthly, by a post-transcriptional defect of CD25 expression resulting in a reduced IL-2 response, whereas a normal IL-2 secretion is preserved[43]. Fifthly, as demonstrated by Haidinger *et al.*, the low proliferative capacities of the Teff population could also be due to disruption of the T-cell/Antigen Presenting Cell (APC) interface by rATG[46]. Sixthly, rATG triggers lysis of dendritic cells[47, 48] and impairs their maturation[49] and therefore DCs will not play an active role in the antigen presentation to T cells. Here, we report another explanation for the hyporesponsiveness of the PBMC, i.e. the partial involvement of functional regulatory T cells. Depletion of the $CD25^{bright}$ population from the PBMC resulted in an increased proliferation and in co-culture experiments these $CD25^{bright}$ T cells profoundly suppressed the anti-donor and 3P responses after rATG-treatment (Figure 3). Therefore, our main finding is that rATG affected the function of recovered Teff whereas the suppressive activity of the

newly generated regulatory T cells remained proportionally unaltered. The $CD4^+FoxP3^+$ T cells measured in the rATG-group are probably not regenerated by the thymus as the percentage of naïve T cells profoundly decreased as shown in Figure 5. This suggests that there could be a transient effect on the regenerative capacity of thymus. As we observed a shift in the frequency of $CD4^+FoxP3^+CD127^{-/low}$ T cells towards the central memory phenotype (Figure 5), it is likely that the naïve $CD4^+FoxP3^+$ T cells differentiated into memory $CD4^+FoxP3^+$ T cells. Factors such as donor-antigens or rabbit immunoglobulins that act as foreign antigens might contribute to this differentiation. Since the increase in the proportion of $CD4^+FoxP3^+$ memory T cells was not observed in the control group, particularly rabbit immunoglobulins must play an important role. To our knowledge, we provide the first evidence that rATG therapy in KTx patients affects the function of $CD25^{-/dim}$ Teff and preserves $CD4^+CD25^{bright}$ regulatory T-cell function. As one might expect from experimental studies, the therapeutic effect of rATG *in vivo* neither arose from the expansion of residual regulatory T cells nor from *de novo* generation that resulted in an increase above baseline. Most importantly, the regulatory T cells that recovered after rATG-treatment were able to effectively govern allogeneic immune responses by effector T cells as before rATG-treatment.



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# Chapter 5

## Characterization of Rabbit Anti-Thymocyte Globulins-Induced CD25$^+$ Regulatory T Cells from Cells of Patients with End Stage Renal Disease

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## ABSTRACT

Rabbit Anti-Thymocyte Globulins (rATG) are known to convert $CD4^+CD25^{neg}FoxP3^{neg}$ T cells from healthy individuals into $CD4^+CD25^+FoxP3^+$ T cells. In this study, we investigated the effect of rATG on the induction of regulatory T cells (Treg) from blood cells of patients with end-stage renal disease who are candidates for transplantation and rATG-induction therapy. The induced Treg were analyzed and compared with naturally occurring $CD4^+CD25^+FoxP3^+$ T cells. The $CD25^{neg}$ T cells of pre-transplant patients (n=7) and healthy controls (n=4) were stimulated with rATG or control rabbit immunoglobulins for 24 hr. The phenotype of induced regulatory T cells was examined by flow cytometry and their function was studied in the conventional suppression assay. Further characterization was performed by mRNA-analyses. After 24 hr, the percentage of $CD4^+CD25^+FoxP3^+CD127^{-/low}$ T cells and $CD8^+CD25^+FoxP3^{neg}CD127^+$ T cells became higher in the rATG-treated samples compared with the rIgG-treated samples ($p<0.01$). The rATG-induced $CD25^+$ T cells, whether $CD4^+$ or $CD8^+$ inhibited the allogeneic responses of $CD25^{-/dim}$ effector T cells as vigorously as natural $CD25^+$ T cells. However, the proportion of $FoxP3^+$ within the top 2% rATG-induced $CD4^+CD25^+$ T cells was lower than within the natural $CD4^+CD25^+$ T cells (11± 2% vs. 95± 5%, $p<0.01$). The mRNA-expression levels of IL-27, IL-10, IFN-γ, perforin and granzyme B were markedly higher compared with natural $CD25^+$ T cells (all $p=0.03$), whereas CTLA4 ($p=0.03$), TGF-β ($p=0.02$) and RORγt ($p=0.04$) were lower. rATG allows the induction of regulatory T cells from patient peripheral blood mononuclear cells (PBMC) *in vitro*. In comparison with natural Treg, the rATG-induced Treg are phenotypically distinct, but have similar regulatory activities. rATG may beneficially contribute to the mechanisms that control alloreactivity.

## INTRODUCTION

Immunoregulatory T cells may play an important role in the suppression of both autoreactivity[1] and alloreactivity[2,3]. Apart from the naturally occurring $CD4^+CD25^+FoxP3^+CD127^{-/low}$ T cells that comprise 2% to 7% of the peripheral $CD4^+$ T cells, the adaptive regulatory T cells (Treg) are a field of interest, for example, the $FoxP3^+$TGF-β-producing T helper (Th) 3 cells[4] and the $FoxP3^{neg}$IL-10-producing Tr1 cells that upregulate FoxP3 after antigen stimulation[5].

The suppressive nature of Treg can be exploited to develop a cell-based therapy to acquire tolerance in the setting of organ transplantation. In the past, much effort has been taken to expand peripheral Treg *in vitro* with the ultimate goal to reinfuse them into the patient and induce graft acceptance[6, 7]. However, the *in vitro* expansion procedure encounters major hurdles with the specificity and purity of the expanded cells and their migratory capacity *in vivo*.

A more promising way of skewing the immune system towards Treg has been shown *in vitro* and in experimental animal models with immunosuppressive drugs such as the mTOR-inhibitors, sirolimus and everolimus[8, 9]; co-stimulatory inhibitors, CTLA4Ig[10] and anti-CD40L[11]; the inosine monophosphate inhibitor mycophenolate mofetil (MMF)[12]; and T-cell depletion agents such as anti-CD3 antibody[13], Campath-1H[14], and the polyclonal rabbit anti-thymocyte globulins (rATG)[15-17]. rATG is generally used as induction therapy for organ transplant patients to prevent rejection[18, 19]. However, the *in vitro* induction assays to generate Treg were performed with peripheral blood cells of healthy individuals, whereas patients that receive rATG-induction therapy post-transplantation had end-stage renal disease (ESRD). The peripheral blood mononuclear cells (PBMC) of these patients are affected by the toxic effects of, for example, uremia that is further intensified by treatment with dialysis, which may result in impaired T-cell function, despite evidence of activation markers of the immune system[20-23]. Therefore, these PBMC may, thus, respond in a differential manner to rATG than the PBMC of healthy individuals.

In this report, we studied whether functional Treg can be induced *in vitro* with rATG in PBMC of ESRD patients. Furthermore, we examined the characteristics of rATG-induced Treg and compared these with the $CD4^+CD25^+FoxP3^+CD127^{-/low}$ naturally occurring Treg. Our study does not only provide a better perceptive of the phenotype and suppressive activities of induced Treg in patients after rATG-therapy but also may have therapeutic implications for the drug-driven induction and expansion of Treg in these patients.

## MATERIALS & METHODS

*Regulatory T-cell induction experiments*

PBMC were isolated by density gradient centrifugation over Ficoll-paque (Amersham Pharmacia Biotech, Uppsala, Sweden) from the peripheral blood or buffy coats from 11 subjects (n=7 patients with ESRD or n=4 healthy blood bank donors, Sanquin Blood Bank, Rotterdam, the Netherlands). PBMC were washed twice and were then resuspended in 10% Human Culture Medium (HCM), which consisted of Roswell Park Memorial Institute 1640 medium with L-glutamine (Bio Whittaker, Verviers, Belgium) supplemented with 10% pooled human serum and 100 IU/mL penicillin and 100 µg/mL streptomycin (Gibco BRL, Scotland, UK). The $CD25^+$ T cells were depleted from the PBMC by incubation with anti-CD25 microbeads, (Miltenyi Biotech, Bergisch Gladbach, Germany) followed by negative selection on the autoMACS (Miltenyi Biotech, DEPLETE-S program). The untouched residual fraction consisted of $CD25^{neg}$ cells (98%).

To induce Treg with rATG, the residual $CD25^{neg}$ fraction was washed and resuspended in HCM to a final concentration of $5x10^5$/mL. rATG (Thymoglobulin, Genzyme Corporation, Cambridge, MA) or a control polyclonal rabbit IgG antibody (rIgG, Sigma-Aldrich, St.Louis, MO) in a final concentration of 10 µg/mL was added to 12-wells plates (Greiner, Alphen a/d Rijn, the Netherlands) for 24 and 72 hr.

Binding of rATG to lymphocytes was determined by incubation of $CD25^{neg}$ T cells in the presence of 10 µg/mL rATG or rIgG for 0.5 hr and 24 hr. Before flow cytometric analysis, cells were washed twice with Roswell Park Memorial Institute 1640 medium.

To check the function of induced $CD4^+CD25^+$ and $CD8^+CD25^+$ T cells after 24 hr, $CD3^+CD4^+$ and $CD3^+CD8^+$ T cells were sorted with the FACSAria (BD Biosciences, San Jose, CA) on day 0 and then depleted for CD25 by autoMACS. To examine whether the induction of $CD25^+$ Treg is rATG-specific, we also stimulated $CD25^{neg}$ cells with CD3/CD28 Dynabeads (Dynal, Invitrogen, Breda, the Netherlands).

*Flow cytometry*

Flow cytometry was performed using antibodies directly conjugated to fluorescein isothiocyanate (FITC), phycoerythrin (PE), allophycocyanin (APC) or peridinin chlorophyll protein (PerCP) and Amcyan. After incubation with rATG or rIgG, PBMC were harvested and FoxP3-intracellular staining was performed in round-bottom 96-wells plates (Nunc, Roskilde, Denmark, 200 µL/well) according to the manufacturer's instructions (FoxP3-APC, clone PCH101, e-Bioscience, San Diego, CA). Extracellular staining was conducted before intracellular staining with FITC-conjugated CD127 (eBioscience), IgG1, PERCP-conjugated CD4, PE-conjugated CD25 epitope B (clone M-A251, BD Biosciences, San Jose, CA), PE-conjugated CD25 and Amcyan-conjugated CD3 at 4°C for 30 min (BD Biosciences).

For *in vitro* detection of rATG antibodies PE-conjugated sheep-anti-rabbit (Serotec, Oxford, UK) was used. Isotype controls IgG1-FITC and IgG1-PE for CD25 and CD127 and IgG2a-APC were included at each staining as controls and the Fluorescence Minus One controls[24].

Intracellular staining for PE conjugated granzyme B (Sanquin, CLB) and APC-conjugated FoxP3 was performed after extracellular staining with CD3-Amcyan, CD4-PERCP, CD25 epitope B PE-Cy7 (clone M-A251) and CD127-FITC.

Intracellular cytokine staining was performed as described previously[25, 26]. In brief, during the last 5 hr of incubation with rATG, the Protein Transport Inhibitor Monensin (BD Biosciences) was added to the cell cultures. After extracellular staining, cells were permeabilized and then stained with either PE-labeled IL-10 (BD Biosciences). Flow cytometry was performed on an eight-color FACS Canto II supplemented with DIVA software (BD Biosciences).

To determine the effect of the depletive mechanism of rATG *in vitro*, an apoptosis assay was performed on PBMC from ESRD patients. PBMC were stained for the apoptotic cell marker Annexin V-PE according to the protocol of the manufacturer (BD Biosciences) and measured on the FACS Canto II (BD Biosciences). The late apoptosis and necrosis marker 7-aminoactinomycin D (7-AAD) was added to each sample 15 min before measurement. PBMC before and after rATG treatment were also microscopically analyzed to determine the cell counts on day 0 and after 24 hr to calculate the percentage of cells in culture after 24 hr. Trypan blue staining was performed to determine the percentage of dead cells.

For the sorting experiments, PBMC were labeled with CD3-Amcyan, CD8-FITC and CD4-APC (BD Biosciences). The purity of $CD3^+CD4^+$ and $CD3^+CD8^+$ T cells on day 0 after sorting on the FACS Aria II was more than or equal to 98%.

*Isolation of human natural and induced regulatory T cells and suppression assays*

To test their function, $CD25^+$ T cells were isolated from the PBMC after incubation with anti-CD25 microbeads, (Miltenyi Biotech) followed by a positive selection (POSSELD-program) on the autoMACS (Miltenyi Biotech). The isolated and the residual fractions were washed and resuspended in HCM for functional analysis. The untouched residual fraction consisted for more than or equal to 98% of $CD25^{-/dim}$ cells and 2% $CD25^{bright}$ T cells. The purity of CD25 in the $CD25^+$ isolated fraction was more than or equal to 90%.

The function of regulatory T cells was determined by mixed lymphocyte reactions (MLR) in which the suppressive capacity of $CD25^+$ T cells was measured by their ability to inhibit the proliferative response of the $CD25^{-/dim}$ effector T cells ($CD25^{-/dim}$ Teff). $CD25^{-/dim}$ Teff were co-cultured in triplicate with natural $CD25^+$ T cells or with induced $CD25^+$ T cells. Control experiments were performed in which $CD25^{-/dim}$ Teff were co-cultured with and without the residual fraction after isolation of the induced $CD25^+$ T cells.

To determine the potency of the induced Treg, $CD25^{-/dim}$ cells of a allogeneic HLA-mismatched individual or of the patient were co-cultured with induced $CD25^+$ T cells. Irradiated (40 Gy) 2-2-2 HLA mismatched allogeneic PBMC or spleen cells were used as stimulator cells

(1x$10^5$ cells/100 µL) and co-cultured with 5x$10^4$ cells/100 µL of a $CD25^+$ : $CD25^{-/dim}$ mixture at 1:10, 1:20 and 1:40 in triplicate wells in round-bottom 96-well plates (Nunc, Roskilde, Denmark). Moreover, the natural and rATG-induced $CD25^+$ T cells were co-cultured with irradiated $CD25^{-/dim}$ Teff in the presence of alloantigens to confirm their anergic state.

After 7 days of incubation at 37°C in a humidified atmosphere of 5% $CO_2$, proliferation was measured after $^3$H-thymidine (0.5 µCi/well: Amersham Pharmacia Biotech, Buckinghamshire, UK) incubation for the last 16 hr before harvesting. $^3$H-thymidine incorporation into DNA was assessed using a Betaplate counter (LKB-Wallac, Turku, Finland).

Before adding $^3$H-thymidine to the 96-wells plate in the MLR, supernatant was collected to determine the IL-2 and IFN-γ levels. Cytokine levels (picograms per millilitre) were assessed in duplicate by ELISA (IL-2; e-Bioscience and IFN-γ; U-CyTech Bioscience Utrecht, the Netherlands). To investigate the role of IL-10 in the mechanism of suppression of rATG-induced $CD25^+$ T cells, suppression assays were performed in the absence and presence of 5 µg/mL anti-IL10 receptor antibody (purified anti-human CD210 [IL-10R] BioLegend, San Diego, CA).



*Quantitative (Q) PCR*

To analyze gene expression patterns, total RNA was isolated from the various cell population using the High Pure RNA Isolation kit (Roche Applied Science, Penzberg, Germany) as described previously[27]. In brief, RNA concentrations were measured with the NanoDrop (ThermoScientific, Wilmington, Delaware) and cDNA was synthesized from mRNA. The mRNA expression levels of IL-2, IFN-γ, TGF-β, IL-4, IL-10 and IL-35 [EBI3 and IL-12A], IL-27 [EBI3 and IL-27p28], GATA3, FOXP3, RORγt, CTLA-4, GITR and the cytotoxic molecules granzyme B and perforin were analyzed. The choice of primers and probes for GATA3, IL-4, perforin and granzyme B was defined using the primer express software (Applied Biosystems, Forster City, CA). Assay-on-demand products for the measurement of 18S (Hs99999901_s1), FOXP3 (Hs00203958_m1), RORγt (Hs00172858_m1), CTLA-4 (Hs00175480), CD25 (Hs00166229_m1), IL-2 (Hs00174114_m1), IL-10 (Hs00174086), EBI-3 (Hs01057148.m1), IL-27p28 (Hs00377366.m1) and IL-12A (Hs00168405.m1), IFN-γ (Hs00174143.m1) and TGF-β (Hs00171257_m1) mRNA were designed by Applied Biosystems (Forster City, CA). After 2 min at 50°C and 10 min 95°C, the polymerase chaine reaction was performed by 40 cycles of 15 sec at 95°C and 1 min at 58°C (GATA-3, IL-4, Granzyme B), 59°C (perforin 1) or 60°C (18S, FoxP3, RORγt, CTLA-4, CD25, IL-2, IL-10, EBI-3, IL-27p28, IL-12A, IFN-γ and TGF-β) using a StepOnePlus Real-Time PCR System (Applied Biosystems). The amount of each target molecule was quantified by measuring the cycle threshold (Ct) values, which were transformed to the number of cDNA copies ($2^{[40-Ct]}$). Each run contained several negative controls (no template) and the same two positive reference samples to check intra- and inter-assay variations. The Ct values within and between experiments were all less than 0.25. The relative concentrations of the analyzed markers were normalized to the relative concentration of the housekeeping gene 18S that was present in each sample and multiplied by $10^6$ because of the lower concentration of the target gene compared with the concentration of 18S[28].

*Cytotoxic T lymphocyte Mediated Lysis assay*

Activated $CD25^{-/dim}$ T cells (n=6 blood bank donors) that served as target cells were isolated by autoMACS as described above and were stimulated with 200 IU/mL recombinant IL-2 (proleukin; Chiron BV, Amsterdam, the Netherlands), 2 µg/mL of the mitogen phytohemagglutinin (PHA) and 100 ng/mL IFN-γ (to upregulate HLA-class II, U-Cytech, the Netherlands) in 24-wells plates (Greiner) for 7 days at 37°C. On day 7, these autologous or allogeneic target blasts (Target [T]) were labeled with europium-diethylenetriamene pentaacetate (DTPA). HLA-class II expression by the target cells was analyzed after staining with HLA-DR FITC and CD4-PERCP by flow cytometry (BD Biosciences).

For effector cells we used PBMC that were stimulated with allogeneic irradiated PBMC and 200 IU/mL IL-2 for 7 days at 37°C. At day 7, these activated cells (Effector [E]) were cocultured in 96-wells plates for 4 hr with autologous or allogeneic target blasts and served as the negative and positive control for cytotoxicity, respectively. Natural $CD25^{+}$ T cells and rATG-induced $CD25^{+}$ T cells were also used as effector cells and cocultured with autologous and allogeneic target blasts at different E:T ratios (40:1, 20:1, 10:1 and 5:1). After 4 hr of incubation, the plates were centrifuged, and 20 µL of the supernatant was harvested. Enhancement solution (100 µL, PerkinElmer, Groningen, the Netherlands) was added the wells. Fluorescence of the released Europium was measured and expressed in counts per second. The percentage lysis was calculated as follows: (measured E:T europium release – background europium release (0:1) / (max. Europium release – background europium release) x100%). Maximum europium release was measured by incubation of target cells with 1% Triton (Sigma-Aldrich, Zwijndrecht, the Netherlands).

*Statistical analyses*

Statistical analysis of the flow cytometric and MLR data was performed using Graphpad Prism (version 4.03). To test whether the values have normal distribution, the Kolmogorov-Smirnov test was used. For the determination of levels of statistical significance, the two-sided probability values according to the Wilcoxon matched pairs test, Mann Whitney U test or Student's t test were used. P values less than or equal to 0.05 were considered statistically significant.

## RESULTS

*Binding assay of rATG on PBMC*

To evaluate whether rATG changes the phenotype and function of $CD25^{neg}$ T cells, we first detected the binding of rATG to these T cells. To elucidate this, $CD25^{neg}$ T cells were incubated for 0.5 hr and 24 hr with 1, 10 and 100 µg/mL rATG or with a rabbit IgG control antibody. In the presence of 1 µg/mL rATG, 50% of the cells were bound to rATG already after 0.5 hr. In the presence of 10 µg/mL, rATG was attached to all $CD25^{neg}$ T cells and saturation was maximal and

persisted for at least 24 hr (Figure 1). These results were not found during incubation without any antibody or with the control rIgG antibody that is not directed against epitopes on human thymocytes (Figure 1).

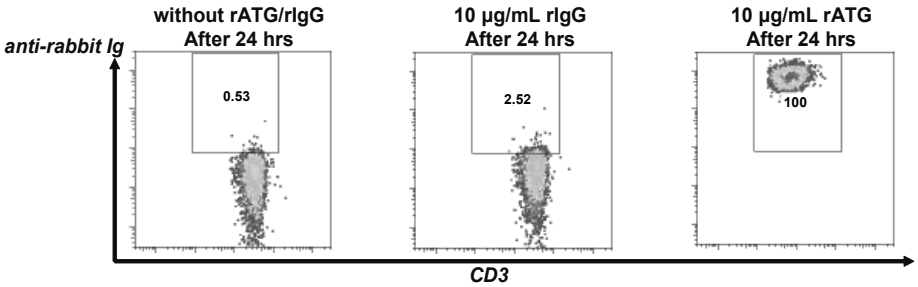


**Figure 1.** Flow cytometric analysis of the binding of rATG to $CD3^{+}CD25^{neg}$ T cells
After 24 hrs of incubation with rATG (10 µg/mL), rATG is attached to all T cells.



*Induction of regulatory T cells by rATG*

Next, different doses of rATG were incubated with $CD25^{neg}$ T cells of healthy individuals for 24 hr to test the most optimal dose to accomplish the induction of regulatory T cells. This resulted in a substantial dose-dependent increase in the percentage of $CD25^{+}$ T cells (Figure 2A). However, in the presence of 10 and 100 µg/mL rATG, the percentages of cells in culture after 24 hr were substantially lower (33% and 20% respectively, mean n=3) than in the absence of rATG (65%). Indeed, the highest rATG-dose induced the largest percentage of dead lymphocytes after 24 hr (Figure 2B). Therefore, the induction experiments with patient PBMC were performed with 10 µg/mL rATG. A representative example of rATG-induced CD25-expression in patient $CD25^{neg}$ T cells at this particular dose is given in Figure 2C. rATG induces an increase in the proportion of $CD25^{+}$ T cells up to 40%.

In all $CD25^{neg}$ samples of patients, the percentage of $CD4^{+}CD25^{+}$ T cells increased to 38 ± 5.5% (mean ± SEM) with rATG, but not rIgG stimulation after 24 and 72 hr (Figure 2D). As within $CD4^{+}$ T cells, $CD25^{+}$ T cells were also induced within $CD8^{+}$ T cells after 24 hr (26 ± 2.4%, mean ± SEM, Figure 2E).

When combining CD25 with the expression of the transcription factor FoxP3, that is involved with the development and function of $CD4^{+}CD25^{+}$ regulatory T cells,[29, 30] we observed that of the top 2% rATG-induced patient $CD4^{+}CD25^{+}$ T cells (Figure 2C), almost 10% harbored the $FoxP3^{+}CD127^{-/low}$ phenotype, which is significantly higher than the rIgG-treated cells (Figure 2F). The percentage of $FoxP3^{+}CD127^{-/low}$ within the induced $CD4^{+}CD25^{+}$ T cells is significantly lower than within the natural $CD25^{+}$ T cells (Figure 2G, 9.4 ± 1.3% vs. 95 ± 5%, $p<0.01$). These low proportions of $FoxP3^{+}$ T cells were also found in samples of healthy individuals. In contrast to the rATG-induced $CD4^{+}CD25^{+}$ T cells, the rATG-induced $CD8^{+}CD25^{+}$ T cells were all $FoxP3^{neg}CD127^{+}$ (data not shown).

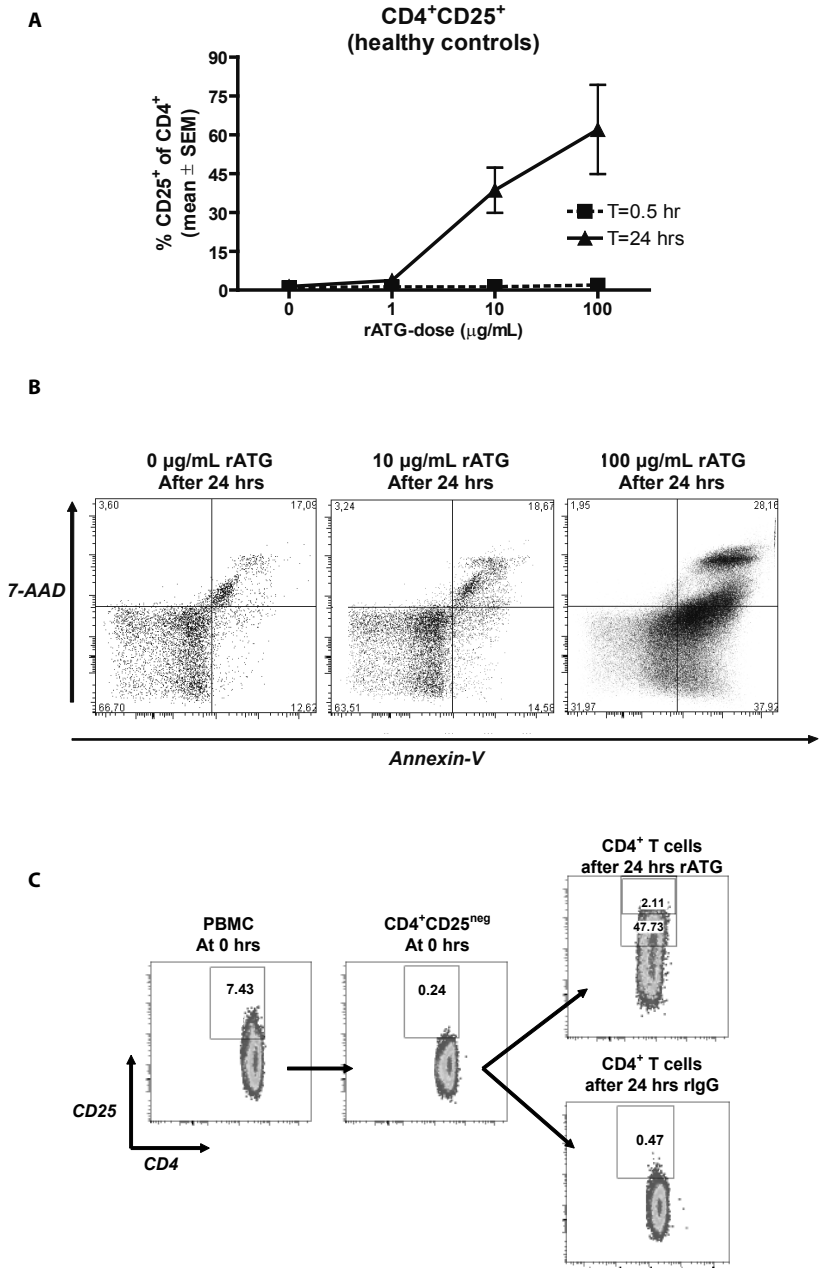


**Figure 2.** Induction of regulatory T cells

**(A)** rATG dose-response curve for the induction of $CD25^+$ T cells in healthy controls, n=3. **(B)** Representative example of rATG-induced cell-death. $CD25^{neg}$ T cells were stimulated with 10 and 100 µg/mL rATG for 24 hrs. After 24 hrs, the percentage of 7-AAD/Annexin-V double-positive cells was largest in the presence of 100 µg/mL. **(C)** One representative example of rATG-driven induction of $CD25^+$ T cells from $CD25^{neg}$ T cells that were isolated from PBMC of an ESRD-patient.

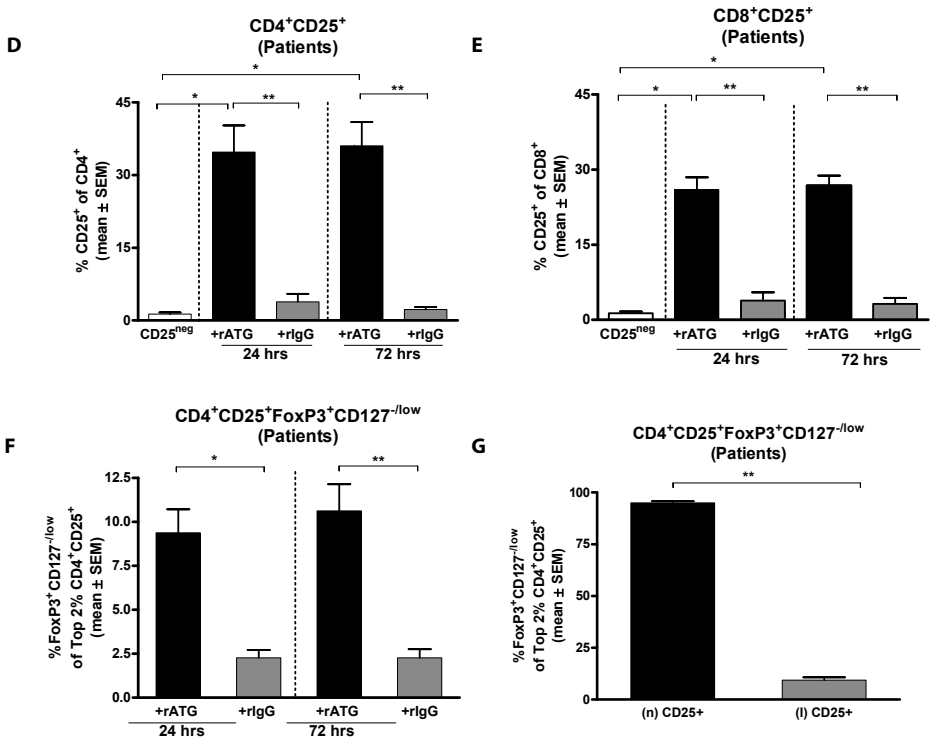


**Figure 2.** Induction of regulatory T cells (continued)

The percentage of $CD25^+$ T cells within the **(D)** $CD4^+$ T cells and **(E)** $CD8^+$ T cells of patients increased in the presence of rATG (black bars) after 24 hrs of incubation and was significantly higher compared to the rIgG-treated samples (gray bars) and remained stable for 72 hrs. **(F)** $FoxP3^+CD127^{-/low}$ T cells were induced within the rATG-induced $CD4^+CD25^+$ T cells. Data are depicted as mean ± SEM of N=7 ESRD patients. **(G)** $FoxP3^+CD127^{-/low}$expression in natural (n) and induced (I) $CD4^+CD25^+$ T cells from N=7 ESRD patients. Differences were statistically tested by the Wilcoxon matched paired test. *p<0.05, **p<0.01.

### *Functional analysis of induced $CD25^+$ T cells*

To test the functional capacities of the rATG-induced $CD25^+$ T cells, we isolated these cells and co-cultured them with autologous $CD25^{-/dim}$ Teff of the same ESRD patient in a suppression assay. The function of the rATG-induced $CD25^+$ T cells was assessed by their potency to inhibit the proliferation of $CD25^{-/dim}$ T cells in the MLR and to inhibit cytokine secretion by $CD25^{-/dim}$ T cells when stimulated with allo-antigen.

The allogeneic proliferative responses of the $CD25^{-/dim}$ Teff were lower in the presence of natural and rATG-induced $CD25^+$ T cells from patient PBMC samples; a representative example of is depicted in Figure 3A. Moreover, similar to the natural $CD25^+$ T cells, the rATG-induced $CD25^+$ T cells were also able to inhibit the allogeneic immune responses of autologous $CD25^{-/dim}$ Teff cells (Figure 3A). Subsequently, the level of suppression by the natural and rATG-induced $CD25^+$ T cells was calculated and expressed as the percentage of inhibition. The percentage inhibition by natural $CD25^+$ T cells from ESRD patients at a $CD25^{-/dim}$ : $CD25^+$ ratio of 10 to 1 was

90% (mean) and at a 20 to 1 ratio 70% (mean). These percentages did not significantly differ from the suppression by their corresponding rATG-induced $CD25^+$ T cells (Figure 3B). The data obtained from PBMC of healthy controls are in line with that of ESRD patients (Figure 3C). As an important function of natural $CD25^+$ T cells is to inhibit the cytokine production of activated $CD25^{-/dim}$ T cells in the MLR, we also examined whether the rATG-induced $CD25^+$ T cells have this capability. After co-culture of $CD25^{-/dim}$ T cells and rATG-induced $CD25^+$ T cells, both the IL-2 and IFN-γ production by $CD25^{-/dim}$ T cells in response to stimulation with allo-antigen were indeed lower, (Figures 3D and E, respectively). Another key characteristic of natural $CD25^+$ suppressor T cells is that they do not proliferate upon stimulation with alloantigen. We found that the rATG-induced $CD25^+$ T cells also did not show a proliferative response upon stimulation with alloantigen for 7 days, suggesting that the cells are anergic (487 ± 153, mean ± SEM).

Next, we questioned whether the $CD4^+CD25^+$ or $CD8^+CD25^+$ T cells account for the inhibition that was observed by all rATG-induced $CD25^+$ T cells. Therefore, we first isolated $CD4^+CD25^{neg}$ and $CD8^+CD25^{neg}$ T cells from PBMC of healthy individuals on day 0 and incubated both populations for 24 hr with rATG or rIgG. The rATG-induced $CD4^+CD25^+$ and $CD8^+CD25^+$ T cells inhibited the proliferative responses of autologous $CD25^{-/dim}$ T cells at the same magnitude compared with the total $CD25^+$ T cells (Figure 3F).

We further examined whether the suppressive properties of the rATG-induced $CD25^+$ T cells were HLA-restricted. Therefore, the inhibition of the proliferative responses on a HLA-A, -B, and -DR 2-2-2 mismatched $CD25^{-/dim}$ cell population was analyzed. Interestingly, the rATG-induced $CD25^+$ T cells showed equal suppressive properties in the inhibition of allogeneic $CD25^{-/dim}$ cells of a 2-2-2 HLA mismatched individual compared with autologous $CD25^{-/dim}$ cells (Figure 3G).

To address whether the induction of suppressive $CD25^+$ T cells is rATG-specific, we also stimulated $CD25^{neg}$ T cells with anti-CD3/CD28 for 24 hr. The anti-CD3/CD28-induced $CD25^+$ T cells were not able to suppress allogeneic immune responses by autologous $CD25^{-/dim}$ cells, showing that the induction of regulatory function is a rATG-specific property (Figure 3H).

*The effect of rATG on the function of natural $CD25^+$ T cells in mixed lymphocyte reactions*

To determine the direct effect of rATG on the function of natural rATG-treated $CD25^+$ T cells, $CD25^{-/dim}$ cells were co-cultured with natural $CD25^+$ T cells in a suppression assay in the absence and presence of 10 μg/mL rATG at a $CD25^+$ : $CD25^{-/dim}$ ratio of 0:1, 1:10, and 1:20. When the $CD25^{-/dim}$ cells were co-cultured with the $CD25^+$ T cells in the presence of rATG, the allogeneic response (counts per minute: 4924 ± 1128, mean ± SEM) was proportionally inhibited in a similar manner as in the absence of rATG (percentage of inhibition: +rATG: 58 ± 18; -rATG: 43 ± 9%). This suggests that in the presence of rATG, immunoregulatory function of the $CD25^+$ T cells remains proportionally intact.

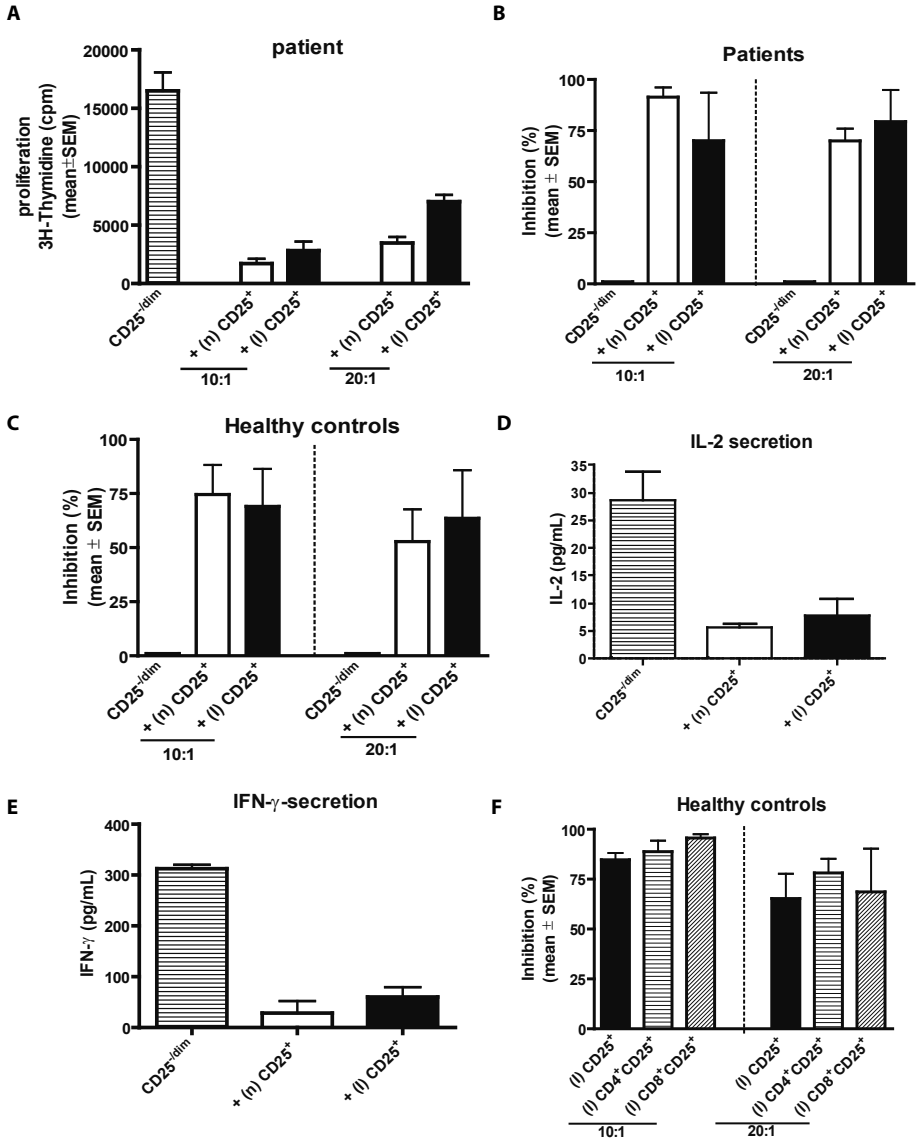


**Figure 3.** Suppression assays with natural (n) and rATG-induced (l) $CD25^+$ T cells

$CD25^+$ T cells were isolated and co-cultured with autologous $CD25^{-/dim}$ cells. **(A)** One representative example of proliferative responses after $^3$H-Thymidine incorporation in counts per minute (cpm) of one ESRD patient. Proliferative responses by $CD25^{-/dim}$ cells after stimulation with alloantigen (horizontally striped bars) are lower in the presence of natural (white bars) and rATG-induced (black bars) $CD25^+$ T cells. Error bars are mean ± SEM of each triplicate. **(B)** Suppressive capacities of (n) and (l) $CD25^+$ T cells from N=5 patients, expressed as percentage of inhibition at a 10:1 and 20:1 $CD25^{-/dim}$ :$CD25^+$ ratio. **(C)** Percentage inhibition of (n) and (l) $CD25^+$ T cells of N=3 healthy controls, N=3. **(D)** IL-2 and **(E)** IFN-γ production in the MLR at a [$CD25^+$ : $CD25^{-/dim}$] ratio of 0:1 and 1:10, N=3 healthy controls. In the presence of (l) $CD25^+$ T cells, the secretion of these cytokines is partially inhibited. **(F)** rATG-induced $CD4^+CD25^+$ and $CD8^+CD25^+$ T cells from healthy controls, N=3 were isolated and tested for their regulatory activities. Percentage of inhibition is shown.

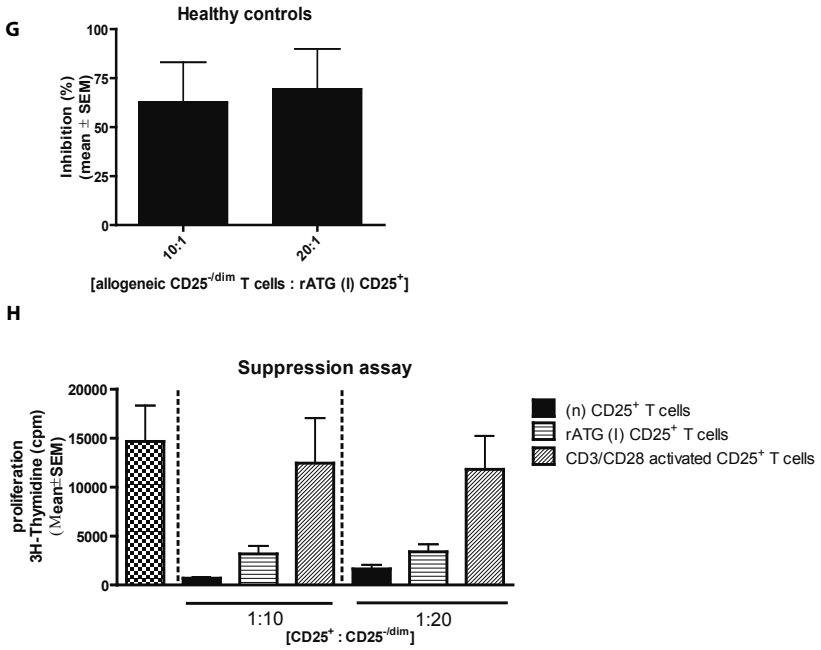


**Figure 3.** Suppression assays with natural (n) and rATG-induced (I) $CD25^+$ T cells (continued)
**(G)** Suppression assay in which rATG-induced $CD25^+$ T cells were co-cultured with allogeneic $CD25^{-/dim}$ cells. **(H)** Representative example of suppression assay with CD3/CD28 induced $CD25^+$ T cells. The proliferation in the absence [$CD25^+$ : $CD25^{-/dim}$] of 0:1 and in the presence of $CD25^+$ T cells is shown. Data are shown as mean ± SEM of each triplicate

### *Gene expression analysis of induced $CD25^+$ T cells*

First, we analyzed the gene-expression patterns of the rATG-induced $CD25^+$ T cells in healthy individuals and compared these with the natural $CD25^+$ T cells. The rATG-induced $CD25^+$ T cells are characterized by low levels of FOXP3, GATA3, CTLA-4, TGF-β and IL-12A (Figure 4). High mRNA expression levels were found for GITR, IL-10, EBI3, the heterodimeric cytokine IL-27 that is comprised of EBI3 and a unique IL-12p35-like protein IL-27p28, IFN-γ, perforin, and granzyme B. The latter cytotoxic molecule was abundantly expressed in the rATG-induced $CD25^+$ T cells compared with the natural $CD25^+$ T cells (Figure 4A). IL-2, IL-4 and the transcription factor for Th17-cells RORγt were undetectable. To assess whether the FAS/FAS ligand (FASL) apoptotic pathway might be involved in the function of rATG-induced regulatory T cells, we also assessed the mRNA expression levels of FAS and FASL in the rATG-induced $CD25^+$ T cells; however the mRNA-levels of both genes were not significantly higher compared with the natural $CD25^+$ T cells and unstimulated $CD25^{neg}$ T cells of day 0, thereby not attributing an important role to cell death, mediated by this pathway (Figure 4A).

Second, we assessed the mRNA expression patterns of natural $CD25^+$ T cells in the absence and presence of rATG. Even in the rATG-treated natural $CD25^+$ T cells, FOXP3 and GITR were upregulated 2-fold and IL-10, EBI3, IL-27p28, perforin and granzyme B were upregulated 10-fold compared with the untreated natural $CD25^+$ T cells (Figure 4B).

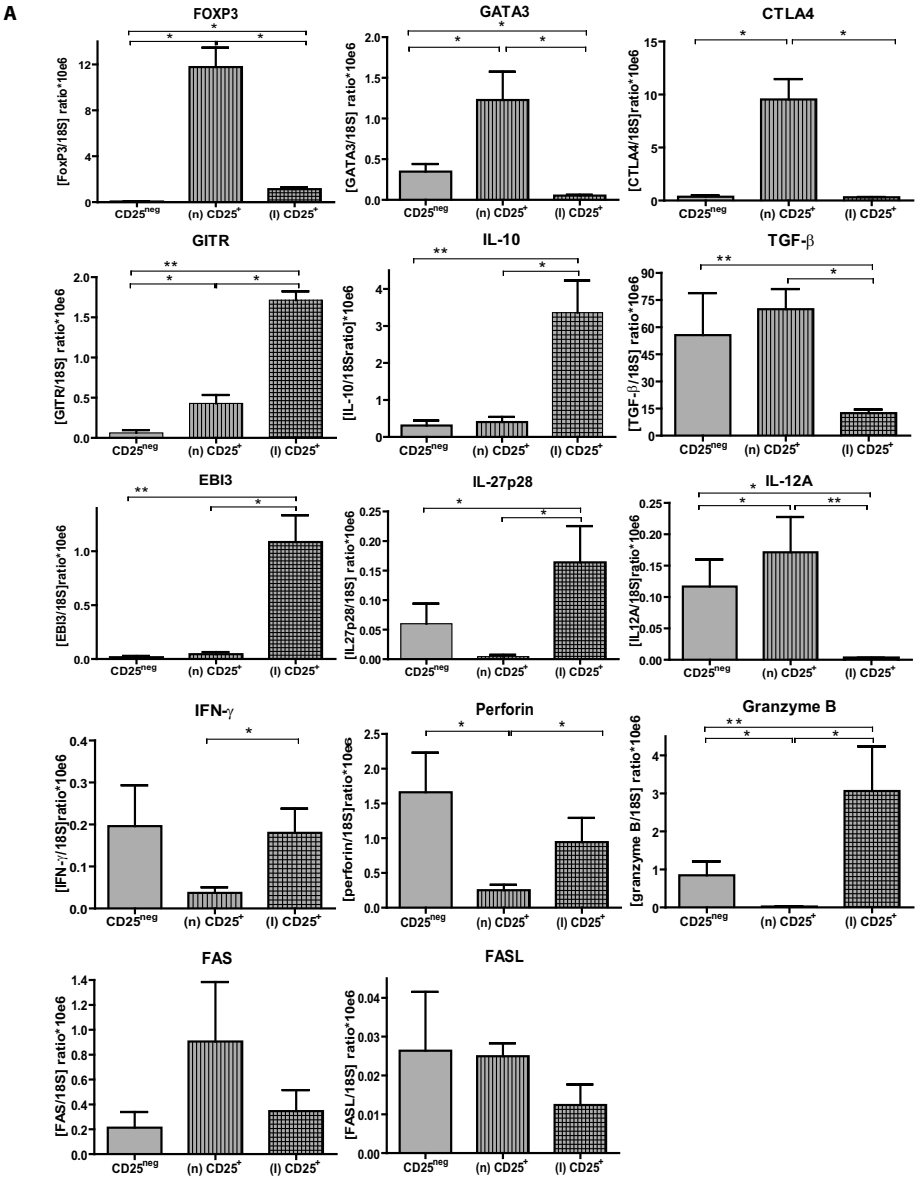


**Figure 4.** mRNA-profiles of regulatory T cells

**(A)** mRNA expression patterns of $CD25^{neg}$ T cells, natural (n) $CD25^{+}$ T cells and rATG-induced (l) $CD25^{+}$ T cells that were isolated from PBMC of N=4 healthy controls after 24 hrs of stimulation with rATG. Gray bars represent $CD25^{neg}$ cells; vertically striped bars are (n) $CD25^{+}$ T cells and squared bars the (l) $CD25^{+}$ T cells. Relative [target gene/18S] ratios are shown for FOXP3, GATA3, CTLA4, GITR, IL-10, TGF-β, EBI3, IL-27p28, IL-12A, IFN-γ, perforin, granzyme B, FAS and FASL.

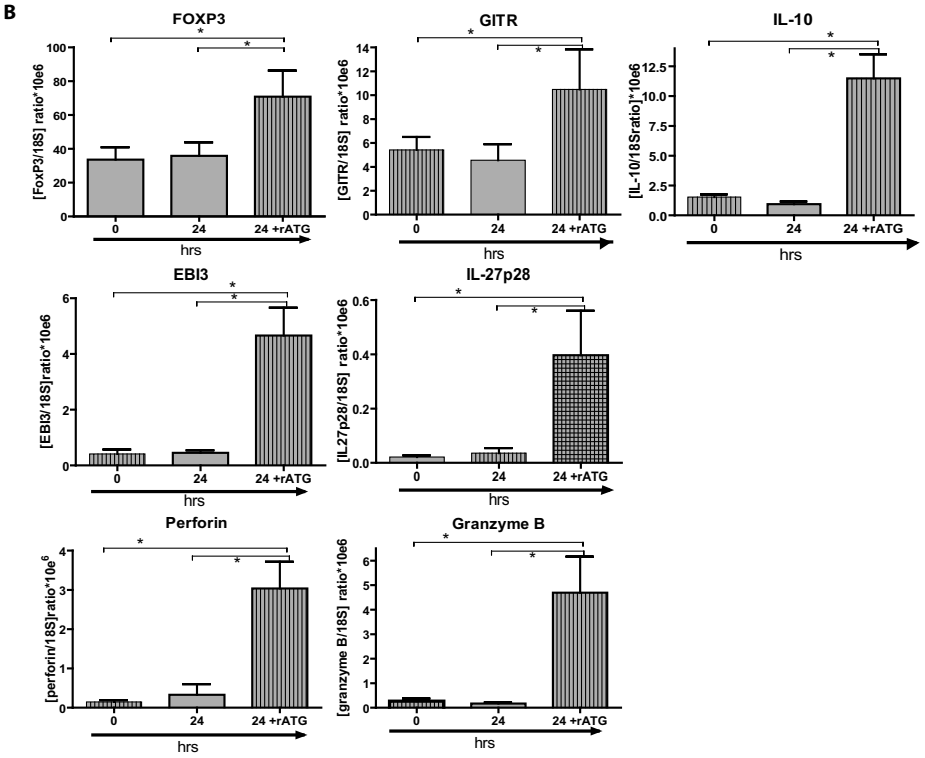


**Figure 4.** mRNA-profiles of regulatory T cells (continued)

**(B)** mRNA expression patterns of (n) $CD25^+$ T cells isolated from PBMC on day 0 and (n) $CD25^+$ in the absence and presence of rATG-incubation. Relative [target gene/18S] ratios are shown for FOXP3, GITR, IL-10, EBI3, IL-27p28, perforin and granzyme B. Error bars represent mean ± SEM. Significance was tested by the Mann-Whitney U Test, *$p<0.05$.

### *Flow cytometric analyses of Granzyme B and IL-10 expression and their functional significance*

To confirm that granzyme B is one of the potential effector molecules by which the rATG-induced $CD25^+$ T cells regulate immune responses, we determined granzyme B expression at the protein level on $CD25^+$ T cells by flow cytometry. Again, we compared the rATG-induced $CD25^+$ T cells with the natural $CD25^+$ T cells. Furthermore, the rATG-induced $CD4^+CD25^+$ T cells were double stained for FoxP3 to examine whether rATG-induced $CD4^+CD25^+FoxP3^+CD127^{-/low}$ T cells coexpressed granzyme B. As shown in Figure 5, the proportion of rATG-induced $CD4^+CD25^+$ (Figures 5A and B) and rATG-induced $CD4^+CD25^+FoxP3^+CD127^{-/low}$ T cells (Figures 5C and D) that expressed granzyme B was significantly higher compared with the natural $CD25^+$ T cells. Within the rATG-induced $CD8^+CD25^+$ T cells, the majority (59 ± 4.4%, mean ± SEM) expressed granzyme B.

To check whether the rATG-induced $CD25^+$ T cells have cytotoxic activities, we performed a cytotoxic T lymphocyte mediated lysis assay. Natural and rATG-induced $CD25^+$ T cells were used

as effector cells (E). Target (T) blasts were generated from autologous and allogeneic PBMC. Activated target blasts were incubated in the presence of IFN-γ to upregulate HLA-class II expression. As shown in Figure 5E, T-cell blasts abundantly expressed HLA-class II in the presence of IFN-γ. Alloantigen-stimulated PBMC lysed allogeneic and not the negative control; the autologous target blasts. The natural and induced $CD25^+$ cells showed no cytotoxicity on allogeneic blasts (Figure 5F).

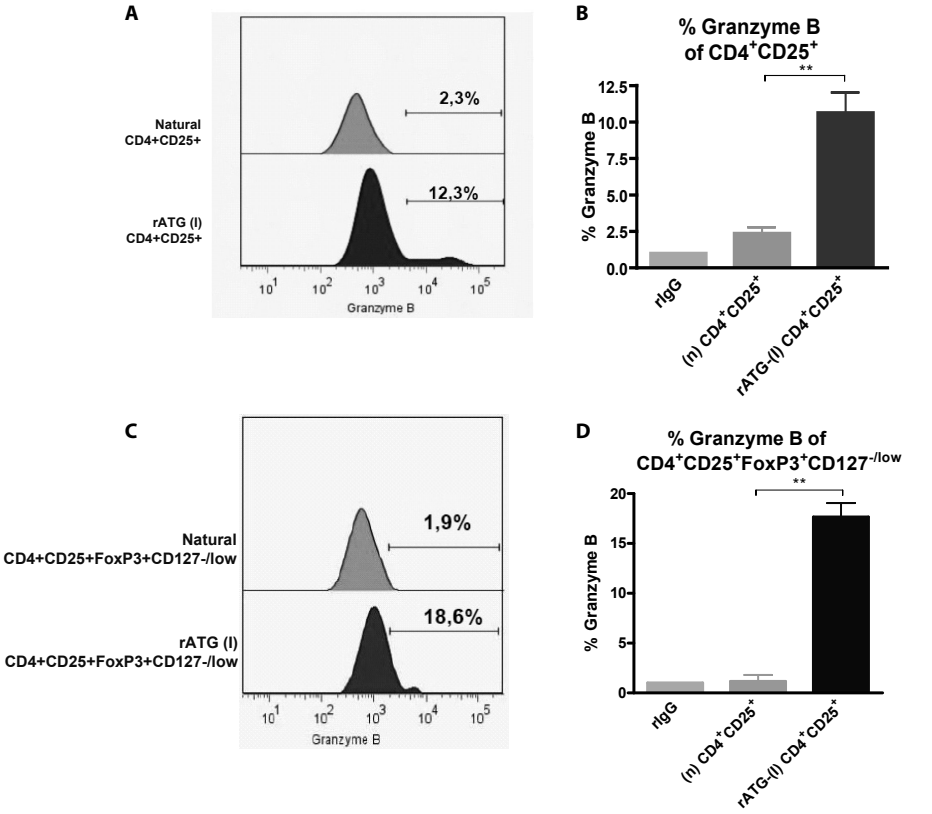


**Figure 5.** Validation of mRNA-expression levels by flow cytometry

Flow cytometric analyses of granzyme B expression within natural (n) and rATG-induced (I) $CD4^+CD25^+$ and $CD4^+CD25^+FoxP3^+CD127^{-/low}$ T cells. **(A)** Representative example of relative granzyme B expression within (n) and (I) $CD4^+CD25^+$ T cells. Percentage of positive cells is shown by the marker. **(B)** Within the rATG-induced $CD4^+CD25^+$ T cells of healthy controls, the proportion of granzyme B was significantly higher compared to the natural (n) $CD25^+$ T cells. Mean ± SEM are shown of N=3 healthy controls. **(C)** Representative example of relative granzyme B expression within natural and rATG-induced $CD4^+CD25^+FoxP3^+CD127^{-/low}$ T cells. **(D)** The frequency of granzyme B expression is significantly higher within the $CD4^+CD25^+FoxP3^+CD127^{-/low}$ T cells compared to the natural $CD4^+CD25^+FoxP3^+CD127^{-/low}$ T cells. Mean ± SEM are shown of N=3 healthy controls.

Furthermore, IL-10-expression was also induced by rATG not only within the induced $CD25^+$ T cells but also within the (n) $CD25^+$ T cells that were stimulated with rATG and therefore, we also verified in the rATG-induced $CD25^+$ T cells whether the IL-10 mRNA is translated into the protein. In contrast to the mRNA, the IL-10 protein was found at low levels (<5%) in the induced Treg compared with the natural Treg. To confirm whether these low IL-10 protein levels have

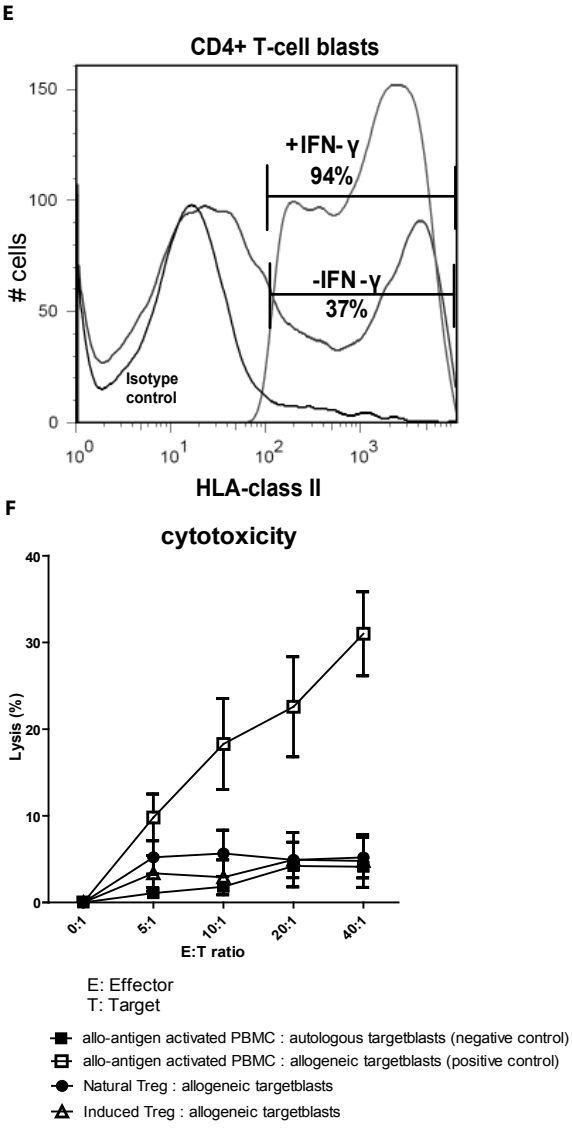


**Figure 5.** Validation of mRNA-expression levels by flow cytometry (continued)
**(E)** One representative example of upregulation of HLA-class II on $CD4^+$ T cell blasts (gated on $CD3^+CD4^+$ T cells) in the presence of IFN-γ. **(F)** Cytotoxicity assay with natural and induced Treg as effector cells (E) and europium labeled target blasts (T). Natural and induced Treg are cocultured with allogeneic target blasts. Mean ± SEM are shown for N=6 healthy controls. Differences were statistically tested by the Students t Test. **p<0.01.

biological activity, the role of IL-10 in the mechanism of suppression by rATG-induced $CD25^+$ T cells was studied in suppression assays that were performed in the absence and presence of anti-IL-10 receptor antibody. In the presence of the anti-IL-10 receptor antibody, the suppressive capacity by rATG-induced $CD25^+$ T cells was not abolished, data not shown.

## DISCUSSION

In this study, we examined whether rATG is able to induce functional Treg in PBMC of patients with ESRD, who are candidates for rATG-induction therapy. The characteristics of these rATG-induced $CD25^+$ T cells were compared with natural $CD25^+$ T cells.

We show that at a rATG dose of 10 µg/mL converts T cells from patients into suppressive $CD4^+CD25^+FoxP3^+CD127^{-/low}$ T cells at the same magnitude as T cells from healthy controls (Figures 2 and 3). This indicates that the induction of $CD25^+$ T cells is able to occur in kidney transplant patients during and after rATG-induction therapy. Although immune cells from ESRD patients cells are extensively exposed to the toxic effects of uremia and stressed by dialysis and, therefore, do not function properly, as shown in several publications[20, 21, 31], our findings demonstrate that the T cells of these patients are apparently not defective in their ability to induce regulatory T cells after rATG-stimulation *in vitro*.

The top 2% rATG-induced $CD4^+CD25^+$ T cells only consisted of 10% $FoxP3^+CD127^{-/low}$ T cells (Figure 2), whereas others and we have reported that the top 2% of natural $CD4^+CD25^+$ T cells consisted for more than or equal to 90% $FoxP3^+CD127^{-/low}$ T cells[32, 33]. The induced $CD4^+CD25^+$ T-cell population is thus heterogeneous and comprises both $FoxP3^+CD127^{-/low}$ T cells with the regulatory phenotype and other T cells with the $FoxP3^{neg}CD127^+$ phenotype. In contrast, the rATG-induced $CD8^+CD25^+$ T cells did not express FoxP3 and were $CD127^+$. However, the total induced $CD25^+$ T-cell population had profound regulatory properties that were comparable with natural $CD25^+$ T cells and were not markedly different in their suppressive activities between patients and healthy individuals (Figure 3). Moreover, the rATG-induced $CD4^+CD25^+$ and $CD8^+CD25^+$ T cells were able to effectively suppress the allogeneic immune responses (Figure 3). The function of natural $CD25^+$ T cells treated with rATG remained proportionally unaltered.

Gene expression analyses revealed that the rATG-induced $CD25^+$ T cells and natural $CD25^+$ T cells both highly express GITR. However, there were also marked differences. The rATG-induced $CD25^+$ T cells expressed EBI3, IL-10, IL-27, IFN-γ, perforin, and granzyme B compared with the natural $CD25^+$ T cells. The latter suggests that their mechanism of suppression may differ from that of natural $CD25^+$ T cells (Figure 4). EBI3 was upregulated in the induced $CD25^+$ T cells, whereas the other transcript that forms the heterodimeric cytokine IL-35[34], IL-12A, was not upregulated. Natural $CD25^+$ T cells highly expressed IL-12A but not EBI3 compared with the $CD25^{neg}$ T cells; therefore IL-35 is not supposed to be a reliable marker for $CD4^+CD25^+$ Treg[35]. IL-27 is encoded by IL-27p28 and EBI3[36]. It promotes Th1-cell differentiation of naïve $CD4^+$ T cells and induces the expression of the IL-12Rβ2 gene by naïve $CD4^+$ T cells to make these cells responsive to IL-12[34, 37]. In contrast, as an attenuator of immune responses, it suppresses the differentiation into Th17-cells. In its latter role, IL-27 also induces IL-10 expression[38]. Thus in our study, the high IL-10 levels might be due to IL-27 production. In T cells, IL-27 induces IFN-γ, perforin and granzyme B production and enhances CTL-activity[37, 39, 40]. The mRNA expression

levels of granzyme B and IL-10 were also upregulated when natural $CD25^+$ T cells were treated with rATG, showing that the expression of the mRNA and protein is driven by rATG. It has been demonstrated that IL-10 was required for inducing perforin and granzyme B in human IFN-γ/IL-10 producing $CD4^+$ T cells that showed cytolytic activity[41] and these cells could enhance cytolytic function in activated $CD8^+$ T cells[42, 43]. Cell death mediated by the FAS-FASL pathway does not seem to play an important role according to their lower mRNA expression levels compared with the (n) $CD25^+$ T cells. Brown et al.[44] who reported that the perforin pathway and the granule exocytosis and not the FASL pathway are predominant in $CD4^+$ T cells pathway support these data. Our data show that the rATG-induced $CD25^+$ T cells are anergic and inhibit the proliferation and cytokine secretion of T cells and express IL-10 mRNA, but that the IL-10 pathway does not contribute to the suppressive mechanism of rATG-induced Treg. Furthermore, the rATG-induced Treg do not operate by cytotoxicity as shown in Figure 5F. There could be two explanations for the absence of cytotoxic activity by the rATG-induced T cells. First, the frequency of granzyme B expressing cells is lower in the rATG-induced $CD4^+CD25^+$ T cells (11% mean, Figure 5B) than in cytotoxic $CD8^+$ T cells or NK cells (40% of $CD8^+$ T cells and 50% of NK cells after stimulation)[45]. The frequency of granzyme B expressing cells is thus expected to be higher to accomplish cytotoxic activity. Second, granzyme B expression in the induced regulatory T cells is merely a phenotypic marker of T-cell activation and is not linked to cytotoxic activities (cell granule exocytosis). The lack of correlation between granzyme B expressing PBMC in ELIspot and cytotoxic activities has also been found earlier by our group[46] and has been shown in other human non-cytotoxic granzyme B-expressing cells types[47]. In these cells and the rATG-induced Treg, granzyme B may have other nonapoptotic functions[47].

The question remains, what the driving force is behind the induction of functional suppressor T cells that seems to be a unique characteristic for rATG. Nonspecific activation by CD3/CD28 although perfectly able to induce FoxP3, does not lead to the generation of functional regulatory T cells as shown by our findings (Figure 3) and by others[48, 49]. In this study, we found that rATG not only induces FoxP3 but also induces regulatory function independent of FoxP3 as the rATG-induced $CD8^+CD25^+FoxP3^{neg}CD127^+$ T cells. However, the rATG-induced $CD8^+CD25^+$ T cells might regulate through cytotoxic mechanisms in an irreversible manner, instead of reversible suppression via cell-cell contact or cytokines.

It is well known that TGF-β promotes and maintains FoxP3-expression in expanded $CD4^+CD25^+$ T cells and is required for their immunosuppressive capacity[50]. Addition of TGF-β into $CD4^+CD25^{neg}$ cells induced $FoxP3^+$ T cells with regulatory capacities[51]. In our rATG-treated cultures, activated non-Treg within the $CD25^{neg}$ cells on day 0 might operate as a source of TGF-β. An additional explanation for the induction of regulatory function by rATG might be the direct effect of an unspecified rabbit protein present in the rATG-preparation that skews $CD25^{neg}$ T cells into a regulatory phenotype[52]. Otherwise, a component within the rATG-mixture could promote acetylation or inhibition of histone deacetylation through histone deacetylases (HDACs), leading to FOXP3 transcription that may result in the generation of Treg[53].

Recently, we reported the phenotypical and functional analyses of $CD4^+CD25^+FoxP3^+$ T cells in the peripheral blood of rATG-treated patients[32]. The results obtained from the rATG-group were compared with that of a non-rATG control group. The most important conclusions were that the $CD25^+$ T cells slowly recover after rATG-treatment until approximately 30% of baseline at 6 months posttransplantation. After rATG-treatment, the function of the isolated $CD25^+$ T cells was proportionally comparable with before, showing that rATG treatment has an effect on their numbers but does not affect their function. After transplantation patients receive combination therapy consisting of rATG induction therapy, tacrolimus, MMF and steroids. Tacrolimus, MMF or steroids may influence the induction of Treg by rATG. Therefore, in this study, we determined to what extent rATG can convert T cells from ESRD patients into cells that may control the allogeneic immune response in a clean study set up.

Taken together, we here demonstrate that cells of ESRD patients are not defective in their ability to induce Treg after rATG-stimulation as rATG induces $CD4^+CD25^+FoxP3^+CD127^{-/low}$ T cells in PBMC of ESRD patients to the same extent as in healthy controls. This indicates that $CD25^+$ T cells can be induced in patients who are candidates for transplantation followed by rATG-induction therapy. Furthermore, rATG also induces Treg within the $CD8^+$ T-cell population that are $CD25^+$, but effectively suppress allogeneic immune responses, independently from FoxP3. Although, different in gene expression characteristics, the induced $CD25^+$ T cells exhibit equal suppressive potency compared with natural $CD25^+$ T cells. Therefore, rATG may beneficially contribute to the mechanisms that control alloreactivity. These findings may provide more insight in the characterization of induced regulatory T cells after rATG induction therapy in kidney transplant patients. More research has to be done to reveal the exact mechanism of suppression by the rATG-induced Treg.



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# Chapter 6

## The Calcineurin Inhibitor Tacrolimus Allows the Induction of Functional $CD4^+CD25^+$ Regulatory T Cells by Rabbit Anti-Thymocyte Globulins

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## ABSTRACT

Rabbit Anti-Thymocyte Globulins (rATG) induce $CD4^+CD25^+FoxP3^+$ regulatory T cells that control alloreactivity. In the present study, we investigated whether rATG convert T cells into functional $CD4^+CD25^+FoxP3^+CD127^{-/low}$ regulatory T cells in the presence of drugs that may hamper their induction and function, i.e. calcineurin inhibitors. $CD25^{neg}$ T cells were stimulated with rATG or control rIgG in the absence and presence of tacrolimus for 24 hr. Flow cytometry was performed for CD4, CD25, FoxP3 and CD127 and the function of $CD25^+$ T cells was examined in suppression assays. mRNA-expression profiles were composed to study the underlying mechanisms. After stimulation, the percentage $CD4^+CD25^+FoxP3^+CD127^{-/low}$ increased (from 2 to 30%, mean, $p<0.01$) and was higher in the rATG-samples than in control rIgG-samples (2%, $p<0.01$). Interestingly, $FoxP3^+$ T cells were also induced when tacrolimus was present in the rATG-cultures. Blockade of the IL-2 pathway did not affect the frequency of rATG-induced $FoxP3^+$ T cells. The rATG-tacrolimus induced $CD25^+$ T cells inhibited proliferative responses of alloantigen-stimulated effector T cells as vigorously as rATG-induced and natural $CD4^+CD25^+FoxP3^+CD127^{-/low}$ T cells (67 ± 18% vs. 69 ± 16% vs. 45 ± 20%, mean ± SEM, respectively). At the mRNA-expression level, rATG-induced $CD25^+$ T cells abundantly expressed IL-10, IL-27, IFN-γ, perforin and granzyme B in contrast to natural $CD25^+$ T cells (all $p=0.03$), whereas FoxP3 was expressed at a lower level ($p=0.03$). These mRNA data were confirmed in regulatory T cells from kidney transplant patients. Our findings demonstrate that tacrolimus does not negatively affect the induction, phenotype and function of $CD4^+CD25^+$ T cells, suggesting that rATG may induce regulatory T cells in patients who receive tacrolimus maintenance therapy.

## INTRODUCTION

The major goal of transplantation immunobiology is to prevent alloreactivity by inducing a state of donor-specific hyporesponsiveness in order to acquire graft acceptance. There are several protocols to establish this. First, a straightforward way of inhibiting alloreactivity can be accomplished by immunosuppressive therapy. However, a major limitation of the most common immunosuppressive regimens is that they lack specificity as they do not only dampen the immune responses against the allograft. Secondly, apart from mechanisms like clonal deletion, anergy, or activation induced cell death (AICD), the *in vivo* skewing of the immune system towards the regulatory T cells that control alloreactivity seems to be promising in obtaining donor-specific hyporesponsiveness as demonstrated in experimental transplantation models[1, 2].

It is tempting to speculate that immunosuppressive drugs may also contribute to the development of donor-specific hyporesponsiveness via the active induction of regulatory T cells. Indeed, experimental studies analyzing the effects of various immunosuppressive agents suggest that these drugs beneficially contribute to immunoregulatory mechanisms[3-5]. For instance, rabbit Anti-Thymocyte Globulins (rATG), which are given as induction therapy after transplantation, convert human $CD25^{neg}$ T cells into functional suppressive $CD4^+CD25^+FoxP3^+$ T cells *in vitro*[5, 6].

Previously, we reported that the number of $CD4^+CD25^{bright}$ regulatory T cells slowly and incompletely recovered in kidney transplant recipients within 6 months after rATG-induction therapy when given in combination with a calcineurin inhibitor and MMF[7]. Nevertheless, the donor-specific suppressive properties of these peripheral $CD4^+CD25^{bright}$ T cells were equivalent to that of the $CD4^+CD25^{bright}$ T cells before transplantation. In line with these results, it has been shown that steroids do not hamper the recovery of $CD4^+CD25^+$ regulatory T cells after treatment of kidney transplant patients with a non-depletive rATG-solution[8]. Moreover, the patient group that received rATG-treatment without steroids did not show enhanced levels of regulatory T cells after treatment. Our study and the latter implicate that CNIs might be responsible for the lack of enhanced regulatory T cell numbers after rATG therapy compared with pre-treatment.

As $CD4^+CD25^+$ regulatory T cells require IL-2 and other members of the IL-2 cytokine family for their development, homeostasis and function [9-12], their frequency or function might be affected by CNIs that inhibit the transcription factor NFAT required for IL-2 transcription[13-15] or by anti-IL-2 receptor antibodies (daclizumab/basiliximab) that block IL-2 signaling[15]. This may imply that in patients, these agents may negatively influence the beneficial effects of rATG on the induction of regulatory T cells.

To understand the factors that enhance or harm the development of functional rATG-induced regulatory T cells, we investigated the induction of rATG induced-regulatory T cells in the presence and absence of a CNI (tacrolimus), antibodies that abolish IL-2 (anti-IL-2) and that block IL-2R signaling (daclizumab) on human peripheral blood cells. Furthermore, the rATG-treated

cells were functionally analyzed and characterized according to their gene-expression patterns to reveal their underlying mechanisms. The gene-expression profiles obtained *in vitro* were verified in kidney transplant patients who received rATG induction therapy and CNI maintenance therapy and were compared with a non-rATG group. These findings may have important implications for understanding one of the mechanisms of action of rATG in transplanted patients after rATG induction therapy which is followed by concomitant immunosuppression.

## MATERIALS AND METHODS

*Induction of regulatory T cells*

PBMC were isolated by density gradient centrifugation over Ficoll-Paque (Amersham Pharmacia Biotech, Uppsala, Sweden) from buffy coats of 5 blood bank donors (Sanquin Blood Bank, Rotterdam). PBMC were washed twice and resuspended in 10% Human Culture Medium (HCM), which consisted of RPMI 1640 medium with L-glutamine (Bio Whittaker, Verviers, Belgium) supplemented with 10% pooled human serum and 100 IU/mL penicillin and 100 µg/mL streptomycin (Gibco BRL). The $CD25^+$ T cells were depleted of the PBMC by incubation with anti-CD25 microbeads, (Miltenyi Biotech, Bergisch Gladbach, Germany) followed by negative selection on the autoMACS (Miltenyi Biotech, DEPLETE-S program). The untouched residual fraction consisted of $CD25^{neg}$ cells (>95%, Figure 1A). To induce regulatory T cells with rATG, the residual ($CD25^{neg}$) fraction was washed and resuspended in HCM to a final concentration of $5x10^5$/mL. RATG (10 µg/mL, Thymoglobulin, Genzyme Corporation, Cambridge, MA), or a control polyclonal rabbit IgG antibody (rIgG, 10 µg/mL, Sigma-Aldrich, St. Louis, MO) was added for 24 and 72 hr. Tacrolimus (10 ng/mL, Astellas, Tokyo, Japan), monoclonal anti-human IL-2Rα antibody (1 µg/mL, R&D Systems, Minneapolis, MN) or anti-human IL-2 antibody (1 µg/mL, R&D Systems) was added to the rATG-treated cultures for 24 hr.

*Flow cytometry*

Flow cytometry was performed using antibodies directly conjugated to fluorescein isothiocyanate (FITC), phycoerythrin (PE), allophycocyanin (APC) or peridinin chlorophyll protein (PerCP) and Amcyan. After incubation with rATG or rIgG, PBMC were harvested and FoxP3-intracellular staining was performed according to the manufacturer's instructions (FoxP3-APC, clone PCH101, eBioscience, San Diego, CA). Extracellular staining was conducted prior to intracellular staining with FITC-conjugated CD127 (eBioscience), IgG1, PERCP-conjugated CD4, PE-conjugated CD25 epitope B (clone M-A251, BD Biosciences, San Jose, CA), PE-conjugated CD25 and Amcyan-conjugated CD3 at 4°C for 30 min. (BD Biosciences). In a separate plate, PE-conjugated granzyme B (Pelicluster, clone CLB-GB11, CLB, Amsterdam) was added simultaneously during incubation with the FoxP3-antibody. Prior to this intracellular staining, extracellular staining with PE-Cy7-conjugated CD25 epitope B (clone M-A251, BD Biosciences) and PERCP-conjugated CD4 was

performed. Isotype controls IgG1-FITC and IgG1-PE for CD25 and granzyme B and CD127 and IgG2a-APC were included at each staining as controls as well as the Fluorescence Minus One (FMO) controls[16].

Phosphospecific flow cytometry was performed according to the manufacturer's specifications (BD Biosciences) on $CD25^{neg}$ cells (buffy coats blood bank donors, Sanquin, n=3) before (unstimulated) and after 24 hr of rATG (10 µg/mL) and rATG-tacrolimus (10 ng/mL) stimulation. PBMC and $CD25^{neg}$ cells ($1x10^6$) that were stimulated with PMA-ionomycine 10 min at 37°C served as the positive controls. Phosphorylation of P38 in $CD3^+CD4^+$, $CD4^+CD25^+$ and $CD4^+CD25^{neg}$ gated cells was determined by five color flow cytometry using the following antibodies: MAPK-P38-PE (clone 36/p38 pT180/pY182, BD Biosciences), CD3-PERCP, CD4-PB, CD25-PE-Cy7 epitope B (clone M-A251, BD Biosciences). Cells were analyzed on a FACSCanto II flow cytometer (BD Biosciences) for data analysis. Twenty thousand gated lymphocyte events were acquired from each tube. Median fluorescent intensity values (MFI) were generated by analyzing the data with Diva 6.0 software (BD Biosciences). FMO controls and unstained control tubes were also included[16].

*Isolation of human natural and induced regulatory T cells and suppression assays*

To test their function, natural and induced $CD25^+$ T cells were isolated from the PBMC after incubation with anti-CD25 microbeads, Miltenyi Biotech) followed by a positive selection (POSSELD-program) on the autoMACS (Miltenyi Biotech) as described before[17]. The isolated and the residual fractions were washed and resuspended in HCM for functional analysis. The untouched residual fraction consisted for > 98% of $CD25^{-/dim}$ effector cells. The purity of the $CD25^+$ isolated fraction was > 90%[17].

The function of $CD25^+$ T cells was determined by mixed lymphocyte reactions in which their suppressive capacities were measured by their ability to inhibit the proliferative response of autologous $CD25^{neg}$ effector T cells ($CD25^{neg}$ Teff). $CD25^{neg}$ Teff were co-cultured in triplicate with natural $CD25^+$ T cells or with induced $CD25^+$ T cells. Irradiated (40 Gy) 2-2-2 HLA mismatched allogeneic PBMC were used as stimulator cells ($10^5$ cells/100 µL) and co-cultured with $5x10^4$ cells/100 µL of a $CD25^+$ : $CD25^{neg}$ mixture at 1:10 in triplicate wells in round-bottom 96-well plates (Nunc, Roskilde, Denmark). Moreover, the natural and rATG-induced $CD25^+$ T cells were co-cultured with irradiated $CD25^{-/dim}$ Teff in the presence of alloantigens to confirm their anergic state.

After 7 days of incubation at 37°C in a humidified atmosphere of 5% $CO_2$, proliferation was measured after $^3$H-thymidine (0.5 µCi/well: Amersham Pharmacia Biotech, Buckinghamshire, UK) incubation for the last 16 hr before harvesting. $^3$H-Thymidine incorporation into DNA was assessed using a Betaplate counter (LKB-Wallac, Turku, Finland).

*Patient samples*

Kidney transplant patients (n=6) were enrolled in this study and were part of a feasibility study. Patients (n=3) were given one infusion of 1.5 mg/kg Anti-thymocyte Globulin (Rabbit) (Thymoglobulin®, Genzyme Corporation, Cambridge, MA) each day at day 1, 2 and 3 after transplantation followed by a triple therapy maintenance regimen consisting of tacrolimus, MMF and prednisone. MMF and prednisone were given from day 1 and tacrolimus was given from day 2 after transplantation. A control non-rATG group (n=3) was treated with tacrolimus, MMF and prednisone. Heparinized blood was drawn at 12 weeks (median) post-transplant, when median T-cell numbers were 400 T cells/μL (N=3 rATG patients). PBMC were isolated as described above by density gradient centrifugation over Ficoll-Paque. Subsequently, $CD4^+CD25^+CD127^{-/low}$ and $CD4^+CD25^{neg}CD127^+$ T cells were sorted with the FACSAria II (BD Biosciences) on PERCP-conjugated 7-AAD, Pacific Blue-conjugated CD4, PE-Cy7-conjugated CD25 and PE-conjugated CD127 (BD Biosciences). The purity of $CD25^+$ within sorted cells was ≥ 98%. After isolation, T-cell subsets were snap-frozen for PCR analyses.

*Quantitative (Q) PCR*

To analyze the gene expression patterns of the samples obtained from healthy individuals and patients, the $CD25^{neg}$ T cells on day 0, rATG-induced and rATG-tacrolimus induced $CD25^+$ and $CD25^{neg}$ T cells after 24 hr, and natural $CD25^+$ T cells were harvested and total RNA was isolated using the High Pure RNA Isolation kit (Roche Applied Science, Penzberg, Germany), according to the manufacturer's instructions. RNA-isolation from the patient samples was performed by extraction with phenol. In brief, cDNA was synthesized from total RNA with random primers as described before[15]. Q-PCR was applied to quantify the mRNA expression levels of IL-4, RORγt, perforin and granzyme B using the primer express software (Applied Biosystems, Forster City, CA). Assay-on-demand products for the detection and quantification of 18S (Hs99999901_s1), CD25 (Hs00166229.m1), FOXP3 (Hs00203958_m1), IL-2 (Hs00174114.m1), IL-10 (Hs00174086.m1), EBI-3 (Hs01057148.m1), IL27p28 (Hs00377366.m1) and IFN-γ (Hs00174143.m1) mRNA was designed by Applied Biosystems (Forster City, CA). The PCR-reactions were performed using a StepOnePlus Real-Time PCR System (Applied Biosystems). The amount of each target molecule was quantified by measuring the cycle threshold (Ct) values, which were transformed to the number of cDNA copies $[2(40\text{-}Ct)]$[18]. Each run contained several negative controls (no template) and two positive reference samples to check intra- and inter-assay variations. The same reference samples were used in all experiments. There were no significant differences in Ct values of reference samples within and between the experiments (all <0.25 Ct). The relative concentrations of the analyzed markers were normalized to the relative concentration of the housekeeping gene 18S that was present in each sample and multiplied by $10^6$ due to the lower concentration of the target gene compared with the concentration of 18S[18].

*Cytotoxic T lymphocyte Mediated Lysis (CML) assay*

Activated $CD25^{-/dim}$ T cells (n=6 blood bank donors) that served as target cells were isolated by autoMACS as described above and were stimulated with 200 IU/mL recombinant IL-2 (proleukin; Chiron BV, Amsterdam, the Netherlands), 2 μg/mL of the mitogen phytohemagglutinin (PHA) and 100 ng/mL IFN-γ (to upregulate HLA-class II, U-Cytech, the Netherlands, Figure 5D) in 24-wells plates (Greiner) for 7 days at 37°C. On day 7, these autologous or allogeneic target blasts (Target; T) were labeled with Europium-DTPA. HLA-class II expression by the target cells was analyzed after staining with HLA-DR FITC and CD4-PERCP by flow cytometry (BD Biosciences).

For effector cells we used PBMC that were stimulated with allogeneic irradiated PBMC and 200 IU/mL IL-2 for 7 days at 37°C. At day 7, these activated cells (Effector; E) were cocultured in 96-wells plates for 4 hr with autologous or allogeneic target blasts and served as the negative and positive control for cytotoxicity, respectively. Natural $CD25^{+}$ T cells, rATG-induced and rATG-tacrolimus induced $CD25^{+}$ T cells were also used as effector cells and cocultured with autologous and allogeneic target blasts at different E:T ratios (40:1, 20:1, 10:1, 5:1). After 4 hr of incubation, the plates were centrifuged and 20 μL of the supernatant was harvested. Enhancement solution (100 μL, PerkinElmer, Groningen, the Netherlands) was added the wells. Fluorescence of the released Europium was measured and expressed in counts per second (cps). The percentage lysis was calculated by [(measured E:T Europium release – background Europium release (0:1) / (max. Europium release – background Europium release) (x100)]. Max. Europium release was measured by incubation of target cells with 1% Triton (Sigma-Aldrich, Zwijndrecht, the Netherlands).

*Calculations and statistical analyses*

Statistical analysis of flow cytometry was performed using Graphpad Prism (version 5). To test if the values have normal distribution, the Kolmogorov-Smirnov test was used. For the determination of levels of statistical significance, the two-sided probability values according to the Mann Whitney U Test. The One way ANOVA was performed for multiple testing and post hoc analyses were performed using Bonferroni's test for multiple comparisons. P values $<0.05$ were considered statistically significant.

## RESULTS

*Induction of human $CD4^+CD25^+FoxP3^+CD127^{-/low}$ regulatory T cells with rATG*

We performed induction experiments with rATG by the incubation of human $CD25^{neg}$ T cells that were depleted from PBMC (Figure 1A). A typical example of induced CD25-expression after stimulation with rATG is shown in Figure 1B. $CD25^+$ T cells were induced in the rATG-treated samples (27% ± 1.7, mean ± SEM, n=5, Figure 1C) and the percentage $CD25^+$ was significantly higher than in the rIgG-treated samples (2.6% ± 0.5, Figure 1C). Also, when incubated with rATG in the presence of tacrolimus, the proportion of $CD25^+$ T cells was significantly higher than the rIgG-treated samples (41% ± 1.6, Figure 1C), whereas tacrolimus alone had no effect on the percentage of $CD25^+$ T cells compared with the rIgG-treated samples.

To evaluate the effect of IL-2, we subsequently inhibited the IL-2 pathway. Incubation of $CD25^{neg}$ T cell cultures in the presence of an IL-2Rα inhibitor and rATG for 24 hr also resulted in a significantly higher percentage of CD25-expressing $CD4^+$ T cells than in the rIgG treated samples (IL-2Rα: 21% ± 3.9 and anti-IL-2: 34% ± 8.1, Figure 1C). Next, the percentage of $FoxP3^+CD127^{-/low}$ T cells was assessed within the $CD4^+CD25^+$ T cells. $FoxP3^+CD127^{-/low}$ T cells were induced within the $CD25^+$ T cells in all cultures with rATG (Figure 1D). The percentage of $FoxP3^+CD127^{-/low}$ of $CD4^+CD25^+$ T cells for the rIgG, rATG, rATG-tacrolimus, IL-2Rα and anti-IL-2 antibody were 2% ± 0.6, 30% ± 3.9, 19% ± 4.4, 27% ± 2.2 and 30% ± 3.5 respectively (Figure 1E).

It has been reported that phosphorylation P38, an important component of the Mitogen-activated Protein Kinase (MAPK) pathway that is linked to NFAT, can be inhibited by CNI[19-21]. Interestingly, P38 phophorylation is required for TGF-β induced conversion of $CD4^+CD25^{neg}$ cells into $CD4^+CD25^+FoxP3^+$ regulatory T cells[22]. Therefore, we questioned whether P38 phosphorylation was also induced after stimulation of $CD25^{neg}$ cells with rATG to induce FoxP3-expression. We found that phosphorylation was present at low level in unstimulated $CD25^{neg}$ cells (Figure 2A) and increased after 10 min. of stimulation with PMA-ionomycine.

Subsequently, the phoshphorylation level of P38 was measured in the total $CD4^+$ population after 24 hr of incubation of $CD25^{neg}$ cells with rATG and rATG-tacrolimus. The phosphorylation of P38 was increased in the rATG-treated samples and in the rATG-tacrolimus incubated samples. We did not find a difference in P38 phosphorylation level the between rATG- and the rATG-tacrolimus treated samples (Figure 2B). When gated on the rATG-induced $CD25^+$ T cells, the level of phosphorylated P38 was comparable with that in natural $CD25^+$ (Figure 2C). Moreover, the level of phosphorylated P38 was higher in natural and rATG-induced $CD25^+$ than in $CD25^{neg}$ (Figure 2C).

*Functional analysis of regulatory T cells*

Subsequently, we studied the suppressive capacity of rATG- and rATG-tacrolimus induced $CD4^+CD25^+$ T cells. Therefore, these induced $CD4^+CD25^+$ T cells were isolated from the total PBMC population after 24 hr and cocultured in a suppression assay with autologous $CD25^{neg}$ T cells during stimulation with alloantigen.

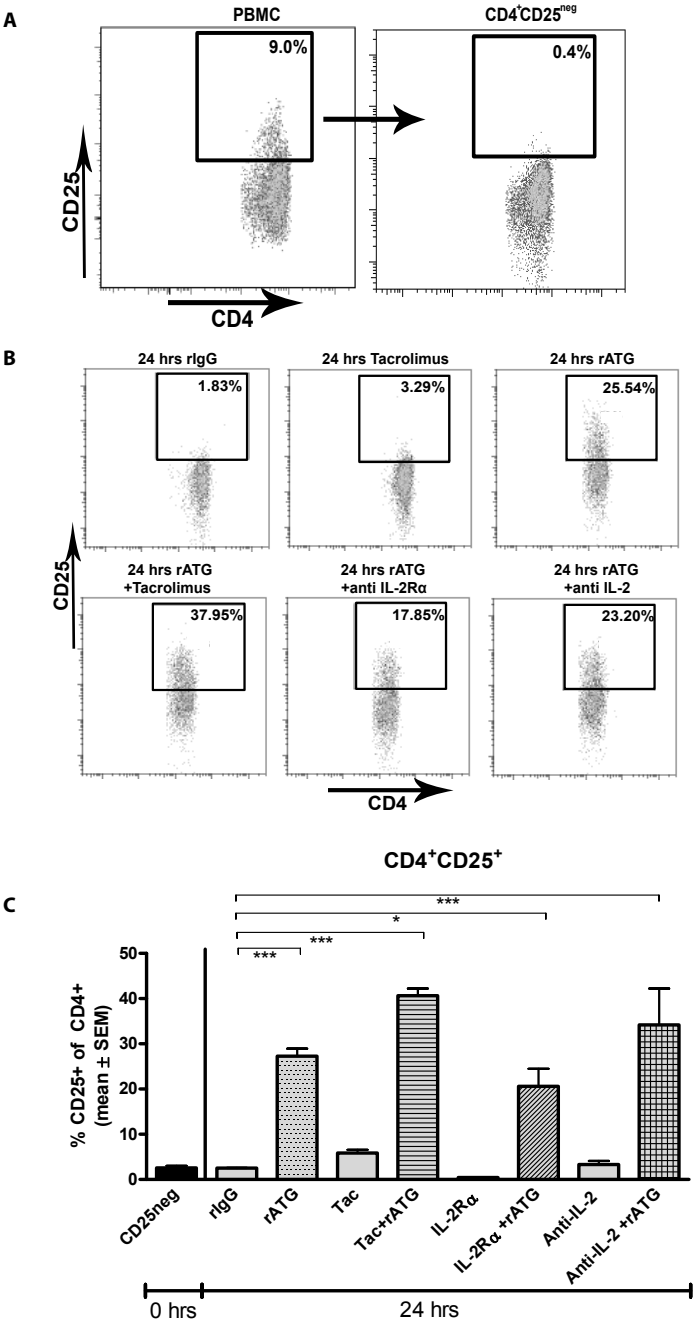



**Figure 1.** Induction of regulatory T cells

**(A)** Depletion of $CD25^+$ T cells from PBMC. **(B)** Incubation of $CD25^{neg}$ T cells with rIgG, Tacrolimus, rATG, rATG+Tacrolimus, rATG+IL-2 receptor (R) α and rATG+anti-IL-2. Gate shows the percentage of $CD25^+$ of $CD4^+$ T cells. **(C)** Percentage of $CD25^+$ T cells of $CD4^+$ of n=3. Differences were statistically tested by ANOVA, $p<0.0001$.

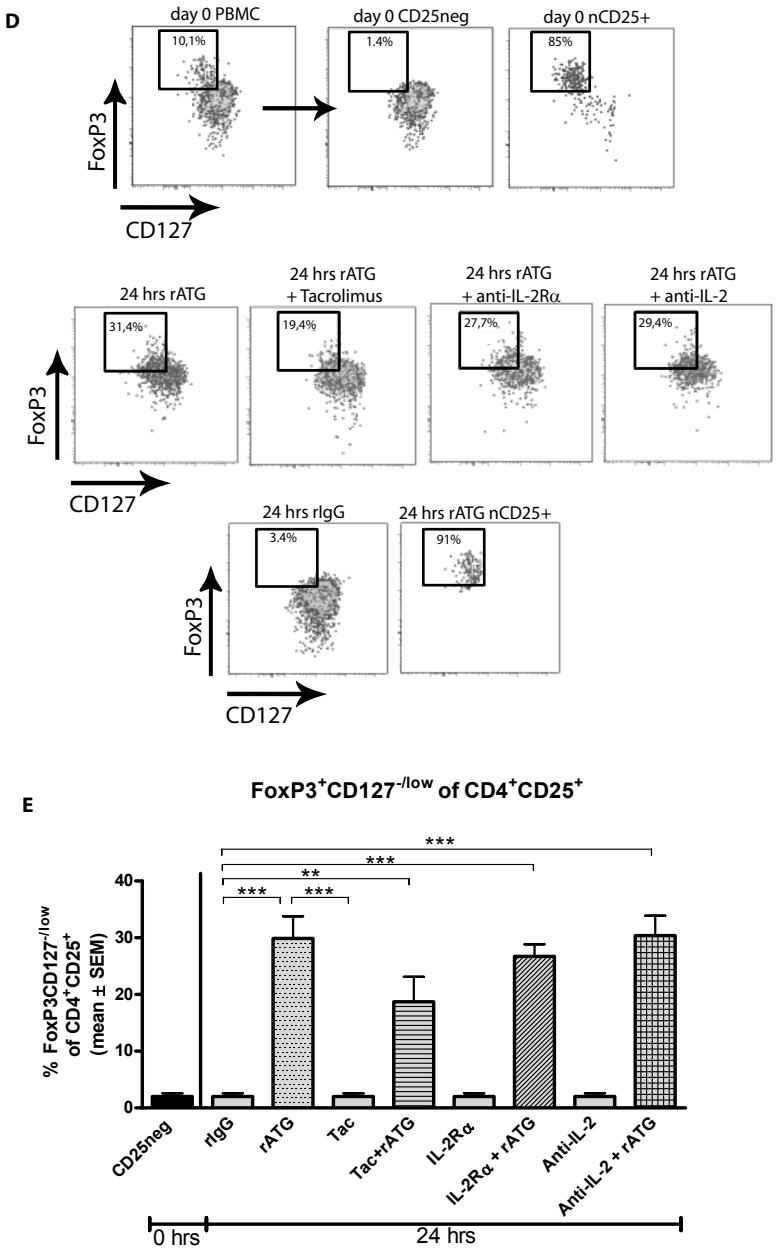



**Figure 1.** Induction of regulatory T cells (continued)

**(D)** Representative example of percentage FoxP3$^{+}$CD127$^{-/low}$ within the induced CD25$^{+}$ T cells of all cultures in the presence of rATG. CD25$^{neg}$ cells from PBMC (top panels) were stimulated for 24 hrs with rATG, rATG + Tacrolimus, rATG + anti-IL2Rα and rATG + anti-IL-2. After 24 hrs, induced CD25$^{+}$ T cells were gated and analysed for their FoxP3$^{+}$CD127$^{-/low}$ expression (middle panels). Gates for positivity were set on FoxP3$^{+}$CD127$^{-/low}$ cells within natural (nCD25$^{+}$) T cells after 24 hrs of incubation with rATG. FoxP3$^{+}$CD127$^{-/low}$ cells were absent within CD25$^{neg}$ cells incubated with control rIgG (lower panels). **(E)** Percentage of FoxP3$^{+}$CD127$^{-/low}$ of induced CD25$^{+}$ T cells, n=3 healthy individuals. Error bars represent mean ± SEM. Differences were statistically tested by ANOVA, $p<0.0001$, *$p<0.05$, ***$p<0.001$.

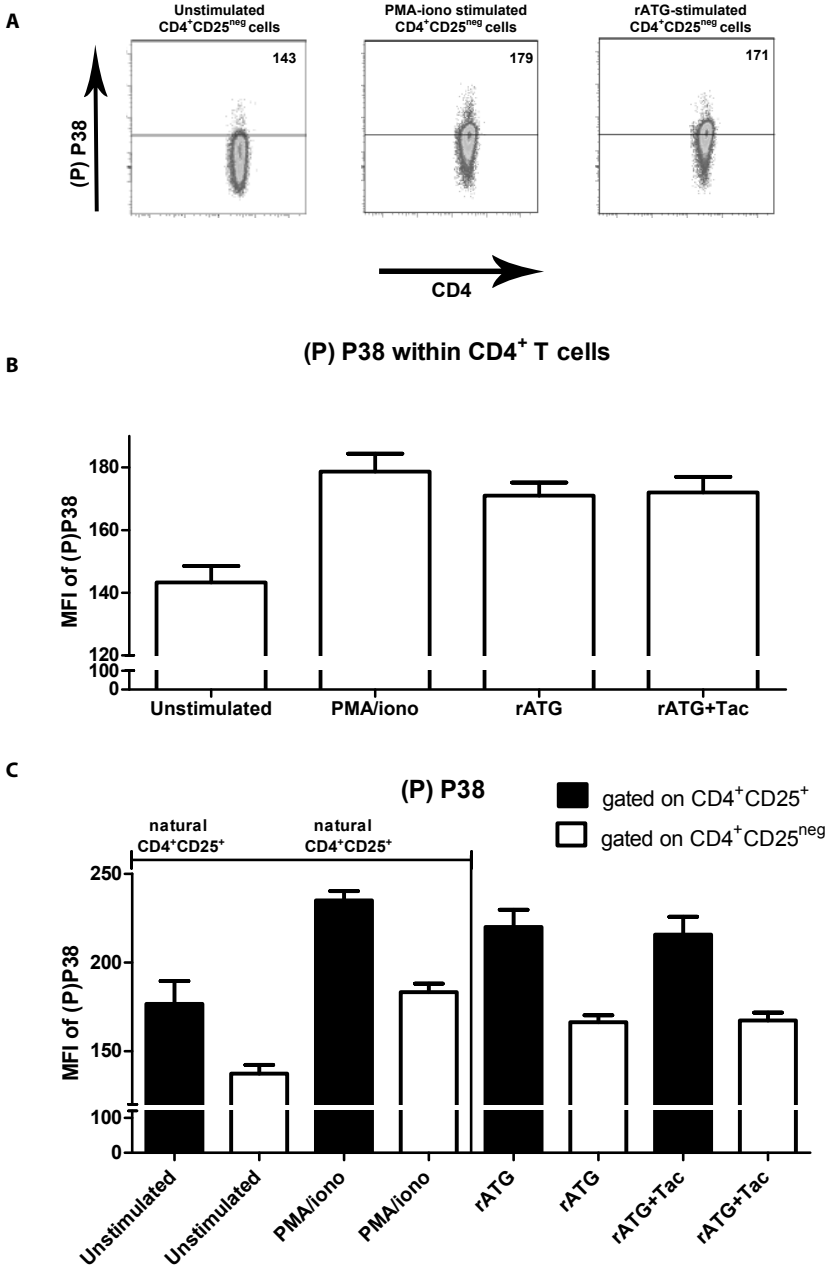



**Figure 2.** P38 phospho-specific flow cytometry

**(A)** Representative example of P38 phosphorylation levels in unstimulated, PMA-ionomycine stimulated, rATG-stimulated $CD4^+CD25^{neg}$ cells. Median Fluorescence intensities are depicted of phosphorylated (P) P38 in $CD4^+$ T cells after 24 hrs. of culture **(B)** P38 phosphorylation in unstimulated $CD4^+CD25^{neg}$ cells, PMA-ionomycine stimulated $CD4^+CD25^{neg}$ cells, rATG stimulated $CD4^+CD25^{neg}$ cells and rATG-tacrolimus stimulated $CD4^+CD25^{neg}$ cells, n=3 **(C)** Phosphorylation of P38 in unstimulated and PMA-ionomycine stimulated natural $CD4^+CD25^+$ ($nCD4^+CD25^+$) and $CD4^+CD25^{neg}$ cells from PBMC and rATG-induced $CD4^+CD25^+$ and $CD25^{neg}$ cells.

After coculture with natural CD25$^+$, rATG-induced CD25$^+$ or rATG-tacrolimus induced CD25$^+$ T cells, the proliferative responses by CD25$^{neg}$ T cells were lower (Figure 3A). The rATG-induced CD25$^+$ T cells that were induced in the presence or absence of tacrolimus inhibited the response at a comparable level to natural CD25$^+$ T cells (mean ± SEM) at a 1:10 ratio (rATG-induced CD25$^+$: 69% ± 16, rATG-tacrolimus-induced CD25$^+$: 67% ± 18 vs. natural CD25$^+$: 45% ± 20, Figure 3B). The analogous suppressive capacities of the rATG-induced and the rATG-tacrolimus-induced CD25$^+$ T cells suggest that rATG promotes *bona fide* regulatory T cells even in the presence of tacrolimus.

A key characteristic of natural CD25$^+$ suppressor T cells is that they do not proliferate upon stimulation with alloantigen. We found that the rATG-induced and rATG-Tacro induced CD25$^+$ T cells also did not show a proliferative response upon stimulation with alloantigen for 7 days, suggesting that the cells are anergic (rATG: 487 ± 153, rATG-Tacro: 369 ± 186, mean ± SEM).

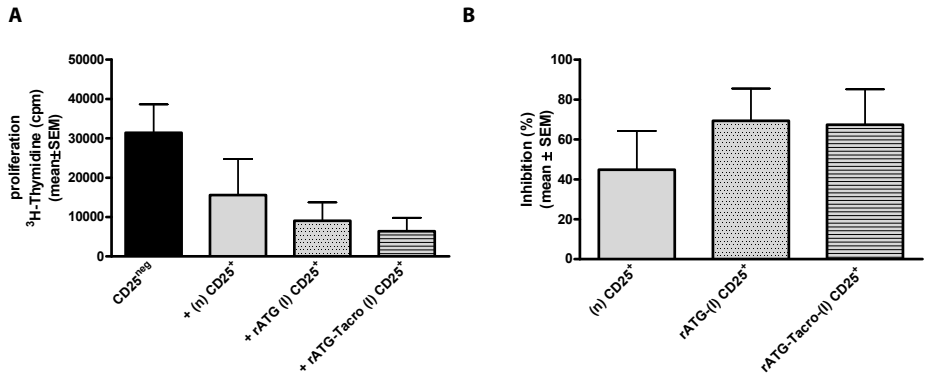



**Figure 3.** Suppression assays with natural (n) and rATG-Induced (I) and rATG-Tacrolimus (Tacro) Induced CD25$^+$ T cells
**(A)** Proliferative responses after $^3$H-Thymidine incorporation are shown in counts per minute (cpm) of CD25$^{neg}$ T cells in response to allo-antigen (black bar) and of CD25$^{neg}$ T cells in the presence of (n) CD25$^+$ T cells (gray bar), rATG (I) (dotted bar) and rATG-Tacrolimus induced (striped bar) CD25$^+$ T cells. **(B)** Percentage inhibition of the proliferation of CD25$^{neg}$ T cells by (n) (gray bar), rATG-(I) (dotted bar) and rATG-Tacrolimus induced (striped bar) CD25$^+$ T cells. Mean ± SEM are shown for n=5 healthy individuals.

### *Gene expression profile of rATG-induced regulatory T cells in the absence and presence of tacrolimus*

To reveal the key molecules involved in the mechanism of action of rATG tacrolimus induced CD25$^+$ T cells, we investigated their gene-expression profiles on these T cells and compared them with rATG-induced and natural CD25$^+$ T cells. The purity of CD25$^+$ within sorted cells was ≥ 98%.

The mRNA expression of FOXP3 was significantly lower in the rATG-induced and rATG-tacrolimus induced CD25$^+$ T cells compared with the natural CD25$^+$ T cells (Figure 4). IL-10, EBI3, IL-27p28, IFN-γ, perforin and Granzyme B were all abundantly expressed in the rATG- and rATG-tacrolimus induced CD25$^+$ T cells compared with the natural CD25$^+$ T cells (Figure 4).

Next, we compared the profiles of the rATG-induced CD25$^+$ T cells in the presence and absence of tacrolimus and it appeared that the mRNA-expression of all tested genes, with the exclusion of FOXP3 and IL-27p28, were significantly higher in the rATG-(I) CD25$^+$ T cells in the

presence of tacrolimus (Figure 4). Overall, the gene expression profile of the rATG-tacrolimus induced CD25$^+$ T cells was comparable with the rATG-induced CD25$^+$ T cells whereas distinct from that of the natural CD25$^+$ T cells (Figure 4). IL-2, IL-4 and the transcription factor for Th17-cells RORγt were undetectable.

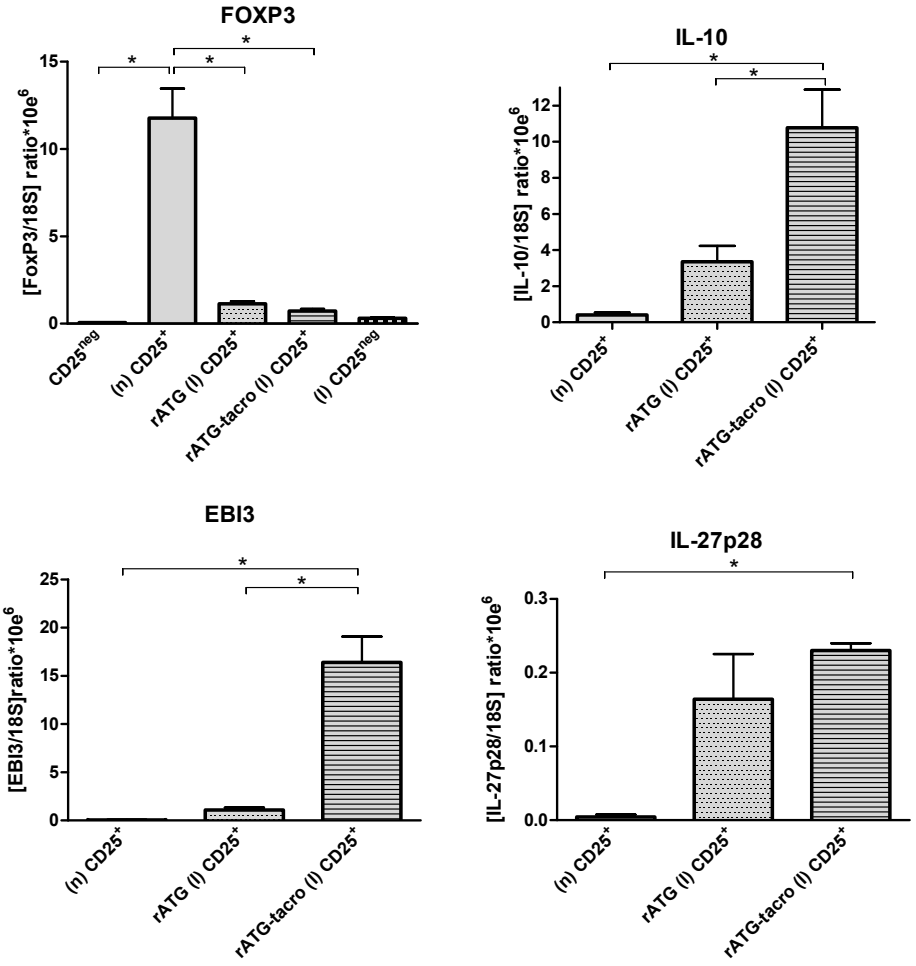



**Figure 4.** Relative mRNA expression patterns of (n) (gray bar), rATG (I) (dotted bar) and rATG-tacrolimus (I) (striped bar) CD25$^+$ T cells for FOXP3, IL-10, EBI3, IL27p28, IFN-γ, perforin and granzyme B for n=4 healthy individuals. Error bars represent mean ± SEM. Significance was tested by ANOVA; FOXP3; $p<0.0001$, IL-10; $p=0.0007$, EBI3; $p<0.0001$, IL-27p28; $p=0.01$, IFN-γ; $p=0.0008$, perforin; $p<0.0001$, Granzyme B; $p=0.0014$, *$p<0.05$.

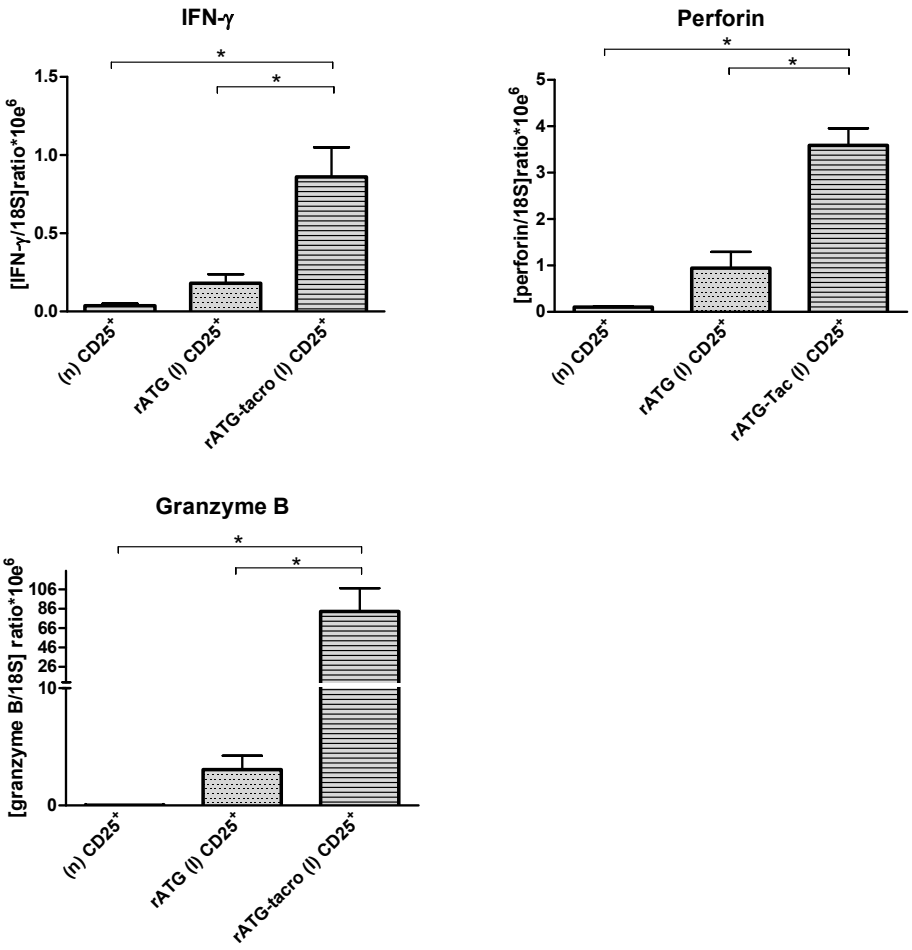



**Figure 4.** (continued) Relative mRNA expression patterns of (n) (gray bar), rATG (I) (dotted bar) and rATG-tacrolimus (I) (striped bar) $CD25^+$ T cells for FOXP3, IL-10, EBI3, IL27p28, IFN-γ, perforin and granzyme B for n=4 healthy individuals. Error bars represent mean ± SEM. Significance was tested by ANOVA; FOXP3; $p<0.0001$, IL-10; $p=0.0007$, EBI3; $p<0.0001$, IL-27p28; $p=0.01$, IFN-γ; $p=0.0008$, perforin; $p<0.0001$, Granzyme B; $p=0.0014$, *$p<0.05$.

*Flow cytometric analyses of Granzyme B and cytotoxic activities by induced regulatory T cells*

The finding that granzyme B mRNA levels were upregulated in the rATG-induced and >80-fold in rATG-tacrolimus induced $CD25^+$ T cells compared with natural $CD25^+$ T cells, prompted us to link the results on transcriptional level with the translational level. Therefore, we investigated the expression of this protein in these T cells as well. The percentages and fluorescence intensities of granzyme $B^+$ T cells within the $CD4^+CD25^+FoxP3^+$ T cells were measured to determine co-expression of granzyme B and FoxP3. A representative example of granzyme $B^+$ T cells within $CD4^+CD25^+FoxP3^+$ T cells is shown in Figure 5A. A considerably higher percentage was granzyme $B^+$ of the rATG-tacrolimus-induced $CD4^+CD25^+FoxP3^+$ T cells, compared with natural

$CD4^+CD25^+FoxP3^+$ T cells (Figures 5A and B). Also the fluorescence intensities of granzyme B were higher in the rATG-tacrolimus and rATG-induced $CD25^+FoxP3^+$ T cells compared with the natural $CD25^+FoxP3^+$ T cells, suggesting a higher per-cell expression (Figure 5A). The percentage of granzyme $B^+$ T cells was the same between rATG and rATG-tacrolimus induced $CD25^+$ T cell populations, whether defined as $CD4^+CD25^+FoxP3^+$ (Figure 5B) or $CD4^+CD25^+$ T cells (Figure 5C).

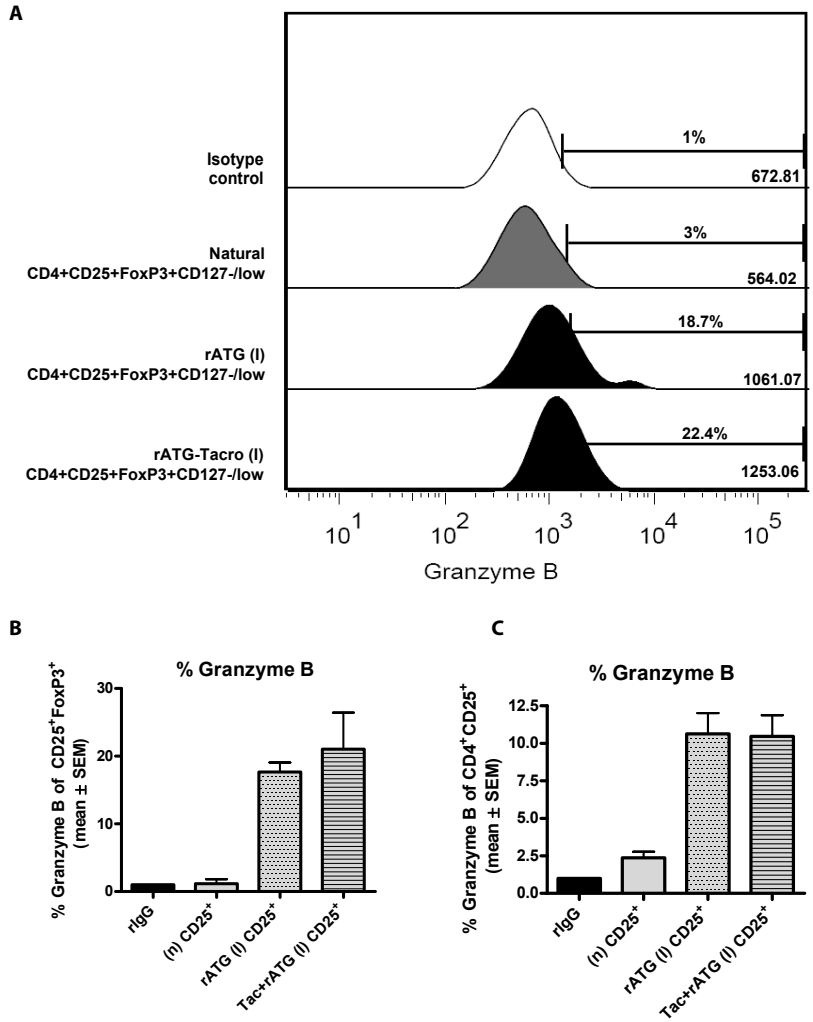
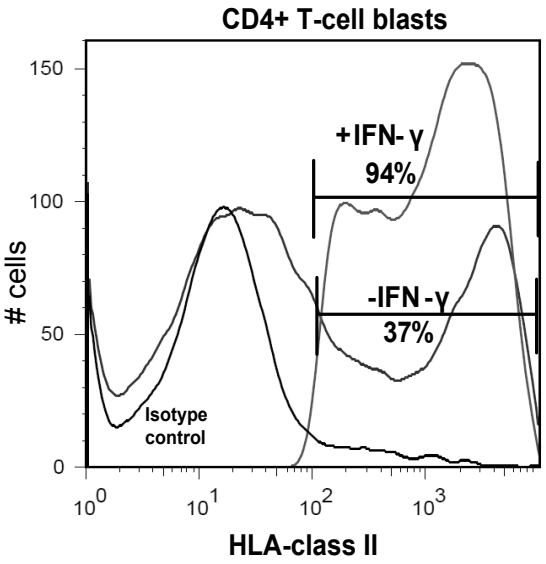



**Figure 5.** Flow cytometric analyses of granzyme B expression **(A)** within natural, rATG (I) and rATG-Tacro (I) $CD25^+FoxP3^+CD127^{low}$ T cells. The marker represents percentage of Granzyme B positive cells. Median fluorescence intensities of granzyme B are also depicted. **(B)** Percentage granzyme B positive cells within $CCD25^+FoxP3^+$ T cells for n=3 healthy individuals and **(C)** percentage granzyme B positive cells within $CD4^+CD25^+$ T cells of n=3 healthy individuals. Mean ± SEM are shown.

D

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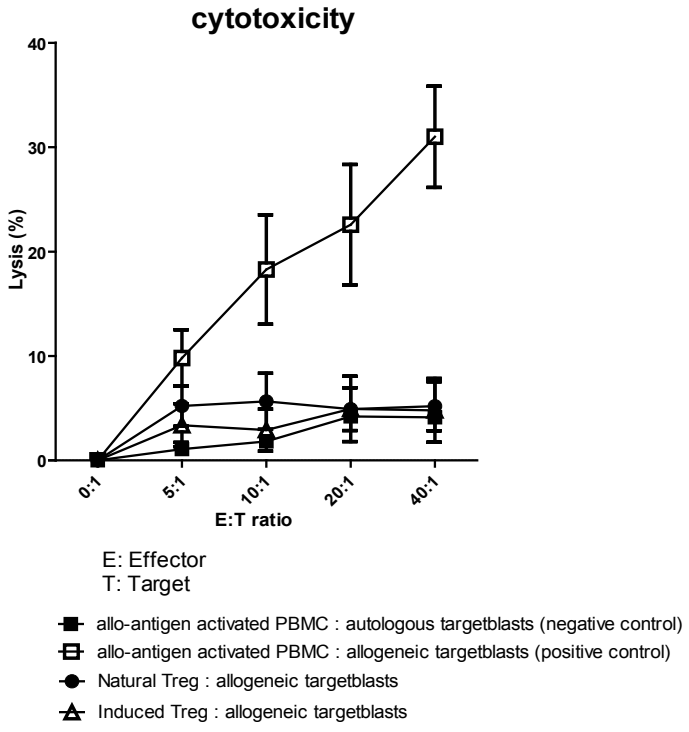


**Figure 5.** (continued) **(D)** One representative example of upregulation of HLA-class II on $CD4^+$ T cell blasts (gated on $CD3^+CD4^+$ T cells) in the presence of IFN-γ. **(E)** Cytotoxicity assay with natural, rATG and rATG-tacro Induced Treg as effector cells (E) and europium labeled target blasts (T). Natural and Induced Treg are cocultured with allogeneic target blasts. Mean ± SEM are shown for N=6 healthy controls.

To check whether the rATG-induced and rATG-Tacro induced $CD25^+$ T cells have cytotoxic activities, we performed a cytotoxic T lymphocyte mediated lysis (CML) assay. Natural, rATG-induced and rATG-Tacro induced $CD25^+$ T cells were used as effector cells (E) and Target (T) blasts were generated in the presence of IL-2 and IFN-γ from autologous and allogeneic PBMC and labeled with Europium. First, we checked the HLA-class II expression on T cell blasts. In the presence of IFN-γ, T cell blasts abundantly upregulated HLA-class II (Figure 5D). Allogeneic stimulated PBMC were able to lyse allogeneic but not autologous target blasts. The percentage lysis of allogeneic target blasts by natural, rATG and rATG-tacro-induced $CD25^+$ was comparable with the autologous control, showing that they do not lyse allogeneic target blasts (Figure 5E).

*Gene expression profile of regulatory T cells in kidney transplant patients after rATG induction therapy*

To confirm expression levels found *in vitro* in cells from healthy individuals, the mRNA-expression patterns of the same set of genes on $CD25^+$ T cells from kidney transplant patients that received rATG-induction therapy followed by maintenance therapy that consisted of tacrolimus. A non-rATG group that received maintenance therapy alone served as control because their PBMC are composed of only natural regulatory T cells and are not heterogeneous as expected for the rATG-treated patients. The FACS-sorted $CD4^+CD25^+CD127^{-/low}$ T cells of patients within the rATG-group showed significantly higher expression of CD25 and FoxP3 than the same T-cell subset from patients in the non-rATG group (Figure 6). Moreover, the mRNA-expression levels of IL-10, IL-2, IFN-γ, perforin and Granzyme B were also highly expressed in the $CD4^+CD25^+CD127^{-/low}$ from rATG-treated patients compared with the $CD4^+CD25^+CD127^{-/low}$ T cells from the control group (Figure 6). The mRNA levels of IL-27p28 and EBI3 were not detectable in the patient samples.

## DISCUSSION

The main mechanism of action of most immunosuppressive drugs is to downregulate immune responses against allo-antigen by effector T cells. However, it became apparent that immunoglobulins can serve as the trigger for cytokines as i.e. IL-10 or TGF-β in the microenvironment of T cells to induce or expand regulatory T cells.

In the present study, we explored the effect of immunosuppressants that hamper the IL-2 pathway, on inducible regulatory T cells by rATG. We show that rATG induce CD25 and FoxP3-expression both in the presence and absence of tacrolimus and Daclizumab (Figure 1). These rATG-tacrolimus $CD25^+$ T cells have the same phenotype (Figure 1) and P38-phosphorylation levels (Figure 2), exhibit equal suppressive activities (Figure 3) and have similar gene-expression patterns (Figure 4) compared with rATG-induced $CD25^+$ T cells, showing that tacrolimus does not negatively affect Treg induction by rATG. The gene-expression levels of rATG-induced $CD25^+$ T cells in the presence of tacrolimus even seem to be enhanced. When the gene-expression

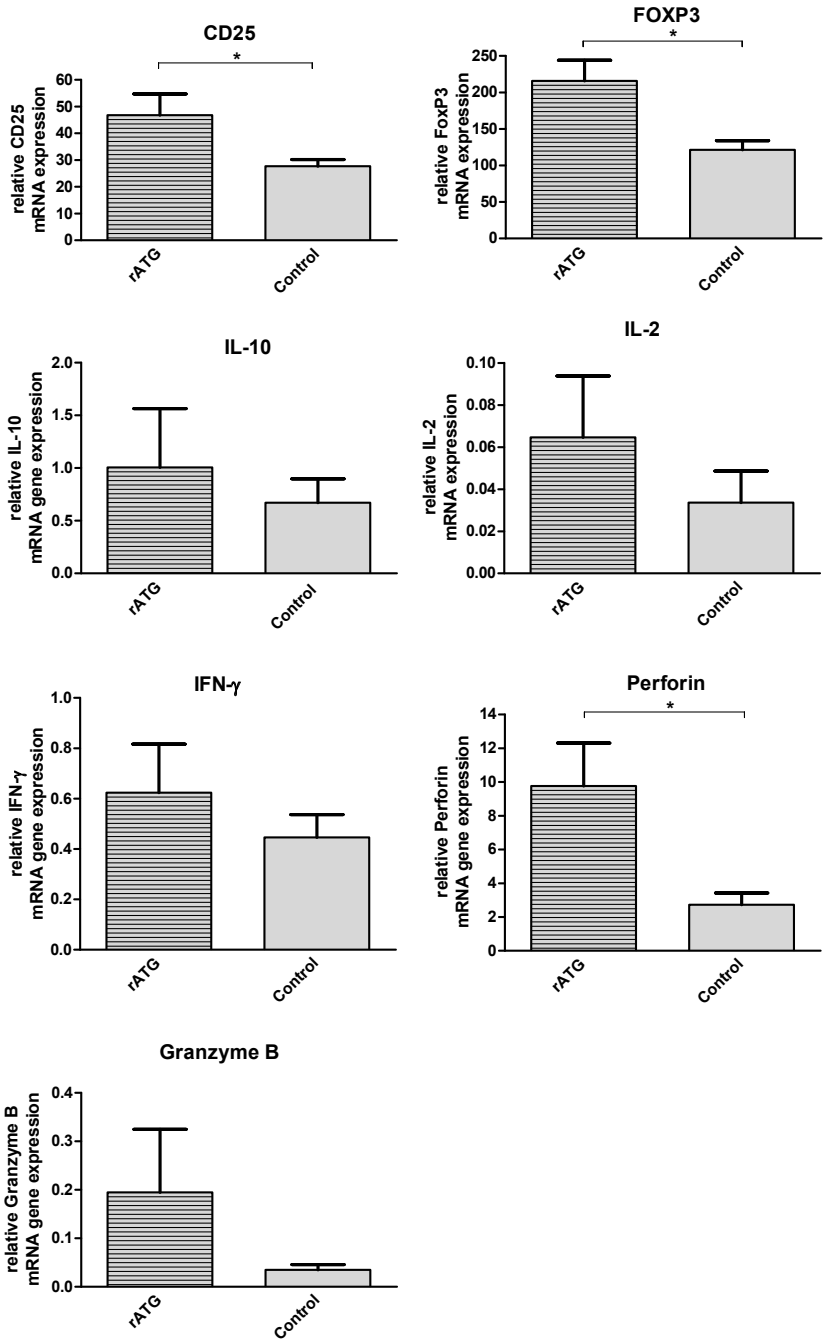


**Figure 6.** Relative mRNA expression patterns of CD25, FOXP3, IL-10, IL-2, IFN-γ, perforin and granzyme B within $CD25^+$ T cells that were isolated from PBMC of rATG (striped bars, n=3) and control patients (gray bars, n=3). Data are shown as mean ± SEM. Statistical significance was tested by Mann-Whitney U Test, *$p<0.05$.

levels were verified in kidney transplant patients that received rATG-induction therapy, the *in vitro* data were confirmed (Figure 6).

As it is well known that IL-2 expands $CD4^+CD25^{bright}FoxP3^+$ T cells *in vitro*[23-25], the role of IL-2 in the development and homeostasis of $FoxP3^+$ T cells with suppressive function is inconclusive. Incubation of human $CD4^+CD25^{neg}$ T cells with IL-2 does not necessarily result in induction of CD25 or FoxP3[26, 27]. After activation with anti-CD3/CD28 and IL-2, the $FoxP3^{neg}$ T cells only transiently express FoxP3 but do not exhibit suppressive properties, showing that FoxP3 has to be constitutively expressed to induce regulatory activity[26, 28, 29]. In contrary to these results, it has been reported that FoxP3 is present in IL-$2^{-/-}$, IL-$2R\alpha^{-/-}$ and IL-$2R\beta^{-/-}$ Treg that have regulatory function, whereas T cells from an IL-$2R\gamma^{-/-}$ mice do not express FoxP3[9]. Thus in line with our results using human cells, the direct (anti-IL-2) or indirect blockade of IL-2 (calcineurin inhibitor) still permits T cell signaling by other cytokines of the common γ-chain (CD132) that may compensate for the absence of IL-2 and may provide signals involved in the development, homeostasis and function of regulatory T cells[10]. As it has been reported that blockade of the IL-2 pathway or the expression of Th17-inducing cytokines (IL-6 and IL-23)[30] may lead to the induction of Th17 cells and not of regulatory T cells, we now show that $FoxP3^+$regulatory T cells are induced even in the presence of IL-2 pathway inhibitors.

Antigenic stimulation alone is sufficient to induce conversion of conventional T cells into regulatory T cells. This conversion even takes place at a higher rate when IL-2 is blocked, resulting in reduced proliferation of naïve T cells that will differentiate into $FoxP3^+$ regulatory T cells[31]. Regulatory T cells that can be induced in the periphery upon encounter with antigen have been reported earlier, e.g. the $FoxP3^+$TGF-β-producing Th3-cells and the $FoxP3^{neg}$IL-10-producing Tr1 cells that upregulate FoxP3 after antigen stimulation[4]. In our rATG-tacrolimus model, foreign rabbit antigens may serve as TCR trigger. However, although this resulted in an increased proportion of CD25, this was not associated with a higher proportion of FoxP3 positivity when the IL-2 pathway was blocked. Thus, for the induction and development of FoxP3-expression, both $\gamma_c$-cytokines and TCR stimulation are required.

Interestingly, the protein levels of other cytokines of the IL-2 family, IL-7 and IL-15 are increased in the serum of renal allografts recipients during the early post-transplant period as demonstrated by Simon T et al.[32]. These other cytokines of the IL-2 family may thus bypass the function of IL-2 itself to facilitate a certain FoxP3 expression and may allow the induction of regulatory T cells in the presence of a calcineurin inhibitor *in vitro* and *in vivo* as shown by our findings. IFN-γ is highly expressed by rATG-induced Treg. It has been described earlier that this cytokine may also play a crucial role in the function of induced regulatory T cells *in vivo*[33, 34]. Furthermore, a convincing role has been attributed to perforin and granzyme B in regulatory T cells to mediate suppression and induce tolerance[35, 36]. However, the rATG- and rATG-tacro induced Treg do not operate by cytotoxicity as shown in Figure 5E. There could be two explanations for the absence of cytotoxic activity by the rATG-induced T cells. First, the frequency of granzyme B expressing cells is lower in the rATG-induced $CD4^+CD25^+$ T cells (11% mean, Figure

5C) than in cytotoxic $CD8^+$ T cells or NK cells (40% of $CD8^+$ T cells and 50% of NK cells after stimulation)[37]. Thus, to accomplish lysis of target blasts, the frequency of granzyme B expressing cells should be higher. Secondly, granzyme B expression in the induced regulatory T cells is merely a phenotypic marker of T cell activation and is not linked to cytotoxic activities (cell granule exocytosis). The lack of correlation between granzyme B expressing PBMC in ELISPOT and cytotoxic activities has also been found earlier by our group[38] and has been shown in other human non-cytotoxic granzyme B-expressing cells types[39]. In these cells and the rATG-induced regulatory T cells, granzyme B may have other non-apoptotic functions[39].

RATG-induced $CD4^+$ and $CD4^+CD25^+$ T cells are characterized by high NFAT1-expression as has been described by Feng et al.[6]. This observation clarifies the induction of FoxP3 in these cells, thereby conferring FOXP3-expression and regulatory activity. A disruption of the interaction between FOXP3 with NFAT resulted in a graded manner with the ability of FOXP3 to repress the expression of IL-2 and to upregulate CTLA-4 and CD25[40, 41]. Thus, one may speculate that by the addition of a calcineurin inhibitor, NFAT cannot be phosphorylated and is not able to form a complex with FoxP3[41], resulting in a downregulation of FOXP3. Phosphorylation of MAPK-P38, that regulates the transcription and activation NFAT[19, 20] has been shown to be necessary for the *in vitro* induction of $FoxP3^+$ regulatory T cells with TGF-β[22]. Our results show that the phosphorylation of P38 was induced in rATG-incubated cells and reached almost the same level as after PMA-ionomycine stimulation. Considering that the NFAT and MAPK pathways interact with each other and both pathways can be inhibited by a calcineurin inhibitor[21], one would expect that rATG-induced P38 phosphorylation is inhibited in the rATG-tacrolimus-induced Treg.

Our findings show that the rATG-induced P38 phosphorylation was not inhibited in the presence of tacrolimus and FoxP3-expression was also not impaired (Figure 2B). Apparently, rATG is such a potent stimulus of this pathway and dominates the inhibitory effect of tacrolimus. Interestingly, the P38 phosphorylation level was higher within the rATG-induced $CD25^+$ cells than within $CD25^{neg}$ cells and comparable with the natural $CD25^+$ level, showing that they are *bona fide* regulatory T cells (Figure 2C). Moreover, the suppressive capacities of rATG-tacrolimus-induced $CD25^+$ T cells were the same as rATG- and natural $CD25^+$ T cells. There are three possible explanations for this phenomenon. Firstly, it could be that there is $Ca^{2+}$-independent transcription of IL-2 as a calcineurin inhibitor only inhibits IL-2 production for 70-80% and thus is not the only pathway leading to proliferation[42, 43], providing enough IL-2 for Treg homeostasis and function. Secondly, it has been demonstrated that therapeutic levels of cyclosporine A, do not inhibit all calcineurin that is present in the cell (only about 50%), thereby allowing strong signals to trigger cytokine expression[44-46]. Thirdly, in a study with atopic dermatitis patients, the authors reasoned that partial T cell activation to induce activation-induced cell death makes Teff more sensitive to cyclosporine A than Treg[47]. This notion is supported by our findings, as tacrolimus apparently only affects the signaling of effector T cells by inhibiting their activation and their IL-2 production. In addition, an important function of calcineurin is to prevent deletion of activated T cells through the inhibition of activation-induced cell death. A CNI alleviates

this inhibition by transducing a 'stress' activation signal into the cell that subsequently triggers apoptosis. The stress activation signal may account for the 'activated' T-cell gene-expression pattern of rATG-induced Treg in the presence of tacrolimus.

In kidney transplant patients with rATG induction therapy and CNI maintenance therapy, regulatory T cells with the memory phenotype slowly recover after rATG-induction therapy as reported previously[7]. This recovery can be attributed to homeostatic proliferation in the presence of low-dose (rabbit) antigen that triggers the output of naïve T cells by the thymus and conversion into memory (regulatory) T cells in the periphery. The naïve and memory (regulatory) T cells will proliferate to refill the empty space. The latter phenomenon does not only result in a recovery of regulatory T cells but may also lead to a higher regulatory T cell numbers or FoxP3-expression compared with baseline. Indeed, in our patients the gene expression of FoxP3 was higher than in the non-rATG control group (Figure 6).

One has to take into account that the induced FoxP3-expression in our model may not only represent a marker for regulatory T cells, but also for T-cell activation. Our rATG-induced $CD25^+$ cells expressed FoxP3 at lower level (19% to 31%) than natural regulatory T cells (85%). Furthermore, according to their mRNA-pattern they show a rather 'activated T-cell' phenotype. These data emphasize that their phenotype does not fully resemble natural regulatory T cells. However, the suppression assays show that the rATG-induced $CD25^+$ cells and natural regulatory T cells have comparable regulatory activities. It is therefore essential to address whether the induced $FoxP3^+CD25^+$ T cells within the heterogeneous $CD25^+$ T-cell population or $CD25^+FoxP3^{neg}$ cells exhibit suppressive activities. To date, it is not possible to study the function of human $FoxP3^+$ T cells, because it is an intracellular marker. Thus, phenotypical analysis or mRNA expression analysis alone is not sufficient to classify cells into a particular T-cell subset. It is essential to examine the function of T cells and combine these data with the phenotype analysis as we did in the present study.

Taken together, in the present report, we show that in the presence of immunosuppressants that indirectly inhibit IL-2 (tacrolimus) or IL-2 pathway inhibitors (anti-IL-2, anti-IL-2Rα), it is still possible to induce regulatory T cells by rATG that are phenotypically, functionally and at the gene-expression level identical as regulatory T cells that are induced by rATG in the absence of these immunosuppressants. Furthermore, it remains unclear whether natural or induced regulatory T cells are more important in their contribution to donor-specific hyporesponsiveness after transplantation. More research has to be done to characterize these rATG-induced T cells and to fully utilize their immunosuppressive properties to promote graft-acceptance.

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# Chapter 7

## The Jak Inhibitor CP-690,550 Preserves the Function of $CD4^{+}CD25^{bright}FoxP3^{+}$ Regulatory T Cells and Inhibits Effector T Cells

*Manuscript submitted*

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## ABSTRACT

The Janus kinase (jak) inhibitor CP-690,550 inhibits alloreactivity and is currently being investigated for prevention of allograft rejection after transplantation. In this study, we examined the effect of CP-690,550 on IL-2-mediated Jak/STAT5 phosphorylation by $CD4^+CD25^{bright}FoxP3^+CD127^{-/low}$ T cells (Treg) and $CD4^+CD25^{neg}$ effector T cells (Teff) in kidney transplant patients. Phospho-specific flow cytometry was used to study the effect of CP-690,550 on IL-2-induced intracellular Signal Transducer and Activator of Transcription (STAT) 5-phosphorylation. IL-2 induced phosphorylation of STAT5 (P-STAT5) in both Treg and Teff, which was significantly higher for $CD4^+CD25^{bright}$ Treg (increased by 71%, mean) than for $CD4^+CD25^{neg}$ Teff (increased by 42%). In the presence of 100 ng/mL CP-690,550, a clinically relevant exposure, IL-2 induced P-STAT5 was partially inhibited in $CD4^+CD25^{bright}$ Treg (% inhibition; 51%), while almost blocked in the Teff (% inhibition; 84%, p=0.03). The $IC_{50}$ was 2-3 times higher for Treg (104 ng/mL) than for Teff (40 ng/mL, p=0.02). In the presence of CP-690,550, Treg exhibited additional suppressive activities on the alloactivated proliferation of Teff (% inhibition; 56%, mean). In addition, $CD4^+CD25^{bright}$ Treg from kidney transplant patients receiving CP-690,550 vigorously suppressed the proliferation of Teff (% inhibition; 87%). Our findings show that the Jak inhibitor CP-690,550 effectively inhibits Teff function but preserves the suppressive activity of $CD4^+CD25^{bright}$ regulatory T cells.

## INTRODUCTION

After organ transplantation, alloreactivity is triggered by antigen-presenting cells that activate T cells via three signals: I. via the T-cell receptor, II. via co-stimulation and III. via the cytokine signal[1]. The cytokine signal is important in the mediation of growth signals to the cell and activates a signaling cascade that is transduced via the Janus family of kinases (Jaks). Cytokines of the IL-2 family exert their biological functions through Jak molecules 1 and 3 and via STAT (signal transducer and activator of transcription) transcription factors. Phosphorylation of Jaks results in recruitment of STAT molecules to the receptor. Subsequently, STAT molecules dimerize and then translocate into the nucleus, leading to gene transcription. These molecules regulate multiple aspects of T-cell differentiation and function. Therefore, Jaks have emerged as targets for drug development to block T-cell growth, differentiation, activation, and to suppress alloreactivity[2-4].

T-cell receptor triggering and IL-2/IL-15 stimulation induce FoxP3-expression in both antigen-specific adaptive $CD4^+CD25^+$ regulatory T cells (Treg) and in $CD4^+CD25^{neg}$ effector T cells that requires STAT5-signaling[5, 6]. However, $FoxP3^+$ regulatory T cells are distinct from activated $FoxP3^+$ effector T cells by their stable and constitutive expression of FoxP3 and by their suppressive capacities. It has been reported that $STAT5^{-/-}$ mice have a dramatic reduction in Treg[7] and blockade of STAT5b in mice or a mutation in the STAT5b gene in humans results in decreased accumulation and regulatory function of $CD4^+CD25^{bright}$ T cells[8]. The IL-2Rβ-chain, Jak3 and STAT5 are also essential for Treg development and maintenance[9, 10].

The Jak inhibitor CP-690,550 is being developed for clinical organ transplantation in a calcineurin (CNI)-free protocol. CP-690,550 has been shown to be a potent immunosuppressive agent in inhibiting alloreactivity in various (animal) experimental models[11-13]. In a prospective Phase I study, we demonstrated that the number of peripheral blood $CD4^+CD25^+$ Treg decreased in kidney transplant patients after CP-690,550 treatment[14]. The regulatory capacities of the residual Treg remained unchanged during treatment.

The immunomodulatory effect of CP-690,550 on T cells can be directly measured by the detection and quantification of cytokine-induced STAT5 phosphorylation in a flow cytometric assay[15]. In this study, we investigated whether there is a differential inhibitory effect of CP-690,550 on IL-2-activated STAT5 phosphorylation in Teff and Treg. Moreover, we analyzed the proliferative function of Teff and the suppressive activity of $CD25^{bright}$ T cells in kidney transplant patients who were part of a Phase II trial and received CP-690,550 therapy in combination with basiliximab induction therapy, mycophenolate mofetil (MMF) and prednisolone. These results were compared with a control group consisting of kidney transplant patients who received cyclosporine instead of CP-690,550.

## MATERIALS & METHODS

*Whole blood staining for P-STAT5 in the presence of IL-2*

Whole blood samples (200 µL) were freshly obtained from healthy individuals (n=4) and stimulated with culture medium (RPMI-1640) containing IL-2 at a final concentration of 2000 IU/mL (130 ng/mL) and incubated for 30 min at 37 °C. Cells were lysed and fixed by adding Lyse/Fix Buffer (BD Biosciences, San Jose, CA) to the tubes at a final concentration of 2% and incubated for 10 min at 37°C. Next, cells were washed in staining buffer (flow buffer with 0.5% bovine serum albumin (BSA) and permeabilised with cold 70% methanol for 30 min on ice. Cells were washed twice in staining buffer and resuspended in 25 µL staining-buffer.

IL-2 induced phosphorylation of STAT5 in different T-cell subpopulations was determined by five color flow cytometry using antibodies directly conjugated to phycoerythrin (PE), phycoerythrin-Cy7 (PE-Cy7) Peridinin chlorophyll protein (PerPC), allophycocyanin (APC) and Pacific Blue (PB). Cells were simultaneously incubated for 30 min with the following antibodies according to manufacturer's specifications: P-STAT5 (Y694)-PE, FoxP3-APC (clone PCH101, eBiosciences, San Diego, CA), CD3-PERCP, CD4-PB, CD25-PE-Cy7 epitope B (clone M-A251, BD Biosciences, San Jose, CA) and CD127-FITC. Cells were then washed in flow buffer and analyzed on a FACSCanto II flow cytometer (BD Biosciences, San Jose, CA) for data analysis. Twenty thousand gated lymphocyte events were acquired from each tube. Median fluorescent intensity values (MFI) were generated by analyzing the data with Diva 6.0 software (BD Biosciences, San Jose, CA). The effect of IL-2 on STAT5 phosphorylation was calculated by the percentage P-STAT5-PE positive cells or the median fluorescence intensity of P-STAT5-PE of the IL-2 stimulated sample minus the unstimulated sample (background). Fluorescence Minus One (FMO) controls and unstained control tubes were also included.

*Whole blood staining for P-STAT5 after pre-incubation with CP-690,550*

Whole blood samples (200 µL) were pre-incubated for 60 min with culture medium containing CP-690,550 at a final concentration of 1, 2.5, 10, 25, 50, 100, 250 or 1000 ng/mL (provided by Pfizer Inc, New London, CT). Next, cells were incubated in culture medium containing IL-2 (130 ng/mL) and then the phosphoflow cytometry protocol as described above was performed. The percentage of inhibition of STAT5 phosphorylation was calculated at each concentration of CP-690,550 and expressed as a percentage of the IL-2-induced STAT5 phosphorylation. Sigmoid curves were drawn using Graphpad Prism (v.5.02) and $IC_{50}$ was calculated.

*Patients*

Kidney transplant patients who were part of a Phase II trial (A3921030) were included in this study. Patients (n=4) in the study group received 15 mg CP-690,550 twice daily (B.I.D) for at least 90 days after transplantation and received maintenance therapy consisting of mycophenolate mofetil (MMF, Cellcept, Roche Laboratories, New Jersey) 500 to 1000 mg B.I.D and prednisolone

5 to 7.5 mg daily. Patients in the comparative group (n=3) received cyclosporine (Neoral, Novartis Pharma BV, Basel), MMF and prednisolone. Patients in both groups received 20 mg of basiliximab anti-CD25 monoclonal antibody induction therapy on the day of transplantation and on day 4 after transplantation. After informed consent was obtained from the participating patient, heparin tubes with blood were obtained 1 day before and 56 days after transplantation. The medical ethical committee of the Erasmus MC, Rotterdam approved the study.

*Isolation of Peripheral Blood Lymphocytes for functional studies*

Peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation over Ficoll-paque from buffy coats of healthy blood bank donors (n=6, Sanquin Blood Bank, Rotterdam, the Netherlands) and from heparinized blood of kidney transplant patients (n=7). PBMC were then frozen in 10% DMSO enriched RPMI 1640 medium (BioWhittaker, Verviers, Belgium) and stored at -140˚C.

*Isolation of human CD25$^{bright}$ T cells from peripheral blood of healthy individuals*

PBMC were thawed and resuspended in 10% Human Culture Medium (HCM), which consisted of RPMI 1640 medium with L-glutamine (Bio Whittaker) supplemented with 10% pooled human serum, 100 IU/mL penicillin and 100 µg/mL streptomycin (Gibco BRL). CD25$^{bright}$ T cells were isolated from PBMC after incubation with anti-CD25 microbeads, (Miltenyi Biotech, Bergisch Gladbach, Germany) followed by positive selection (POSSELD-program) on the autoMACS (Miltenyi Biotech). The untouched residual fraction consisted of $\geq$ 98% CD25$^{-/dim}$ Teff and was used as responder population in the suppression assays. Both fractions were stained with CD4-PERCP (BD Biosciences) and CD25-PE epitope B (clone M-A251, BD Biosciences) and the purity of CD25$^{bright}$ was determined by flow cytometry ($\geq$ 90%).

*Isolation of human CD25$^{bright}$ T cells from peripheral blood of kidney transplant patients*

All patients in the CP-690,550 group (n=4) and in the cyclosporine group (n=3) were treated with basiliximab anti-CD25 induction therapy, MMF and prednisone maintenance therapy. Anti-CD25 induction therapy caused shedding of the IL-2Rα-complex. Therefore, it was not possible to isolate the CD25$^{bright}$ T cells on epitope A. To recover the CD25-expression on T cells, PBMC were rested overnight in HCM with low dose IL-2 (5 ng/mL). After 24 hr, the CD25-expression recovered to normal levels (Figure 4) and the CD25$^{bright}$ T cells, consisting of 85% FoxP3$^{+}$ T cells, could be isolated according to the protocol described above.

*Mixed lymphocyte reactions*

The effect of CP-690,550 on the proliferative capacity of CD25$^{-/dim}$ Teff from healthy individuals was studied by mixed lymphocyte reaction. The CD25$^{bright}$ T cells were depleted from PBMC and the residual CD25$^{-/dim}$ T cells were activated with irradiated (40 Gy) HLA mismatched

(2-2-2) PBMC as stimulator cells (1x$10^5$ cells/100 µL) in the presence of various CP-690,550 concentrations (10, 25, 50, 100, 250, 500 and 1000 ng/mL).

The function of $CD25^{bright}$ cells of healthy individuals was analyzed by their ability to inhibit the proliferative response of alloantigen-activated $CD25^{-/dim}$ Teff. The $CD25^{-/dim}$ Teff were co-cultured in triplicate with and without $CD25^{bright}$ T cells. Irradiated (40 Gy) HLA mismatched (2-2-2) PBMC were used as stimulator cells (1x$10^5$ cells/100 µL) and co-cultured with 5x$10^4$ cells/100 µL of a mixture of $CD25^{bright}$ : $CD25^{-/dim}$ at an 1:5 ratio in round-bottom 96-well plates (Nunc, Roskilde, Denmark). Suppression assays with PBMC of healthy individuals were performed in the presence of 50 and 100 ng/mL CP-690,550 to study the effect of CP-690,550 on Treg function.

Suppression assays with PBMC of kidney transplant patients (n=7) were performed in the same manner as described above but now only in the presence of 100 ng/mL CP-690,550 or 100 ng/mL cyclosporine. There were two reasons for choosing these concentrations. First, the CP-690,550 Phase I study showed that trough plasma levels after CP-690,550 oral administration stabilized at 50 to 100 ng/mL in kidney transplant patients who received CP-690,550 30 mg B.I.D. Secondly, from the MLRs of healthy individuals, it became apparent that the proliferative responses of Teff exposed to CP-690,550 concentrations above 100 ng/mL were not utilizable as a read-out for Treg function in the suppression assay (mean cpm<1000).

After incubation for 7 days at 37°C in a humidified atmosphere of 5% $CO_2$, the proliferation was measured after $^3$H-thymidine (0.5 µCi/well: Amersham Pharmacia Biotech) incubation during the last 16 hr before harvesting. The median counts per minute (cpm) for each triplicate was determined and the level of suppression of the $CD25^{-/dim}$ Teff by $CD25^{bright}$ T cells was calculated and expressed as the percentage inhibition of the Teff.

*Statistical analysis*

Statistical analysis was performed using Graphpad Prism (v.5.02). To test whether there is a normal distribution, the Kolmogorov-Smirnov Test was used. For determination of the levels of statistical significance, the two-sided probability values according to the Wilcoxon matched pairs test or Mann Whitney U Test were used. P values $\leq$ 0.05 were considered statistically significant, without correction for multiple comparisons.



## RESULTS

*IL-2 induced STAT5 phosphorylation in $CD4^+CD25^{neg}$, $CD4^+CD25^{dim}$ and $CD4^+CD25^{bright}$ T cells in the absence and presence of CP-690,550*

Activation of the Jak-STAT5 signaling pathway after IL-2 stimulation was examined in the $CD4^+CD25^{neg}$, $CD4^+CD25^{dim}$ and $CD4^+CD25^{bright}$ T-cell subsets in freshly obtained peripheral whole blood samples from healthy individuals. Approximately 57% (mean) of $CD4^+$ T cells

were CD25$^{neg}$ T cells (of which 0.9% was FoxP3$^{+}$), 38% were CD25$^{dim}$ T cells (8% FoxP3$^{+}$) and 5% were CD25$^{bright}$ T cells (84% FoxP3$^{+}$, Figure 1A). Of all CD4$^{+}$CD25$^{bright}$FoxP3$^{+}$ T cells, 90% were CD127$^{-/low}$. After 30 min of IL-2 stimulation, the percentage of P-STAT5 markedly increased in the CD25-positive subsets, a typical example of STAT5 phosphorylation is shown in Figure 1B. The highest proportions of P-STAT5 were found within the CD25$^{bright}$ T cells (mean % P-STAT5 within CD25$^{dim}$: from 4 to 71%, and within CD25$^{bright}$: from 12 to 83%, Figure 1C). STAT5 molecules were also phosphorylated in the CD25$^{neg}$ T cells (from 1 to 43%). Analogous data were obtained when the median fluorescence intensities (MFI) of P-STAT5 were analyzed in all CD4$^{+}$ T-cell subsets (MFI of P-STAT5 within CD25$^{neg}$: from 213 to 655, CD25$^{dim}$: from 225 to 1108 and CD25$^{bright}$: from 200 to 1270, Figure 1D).

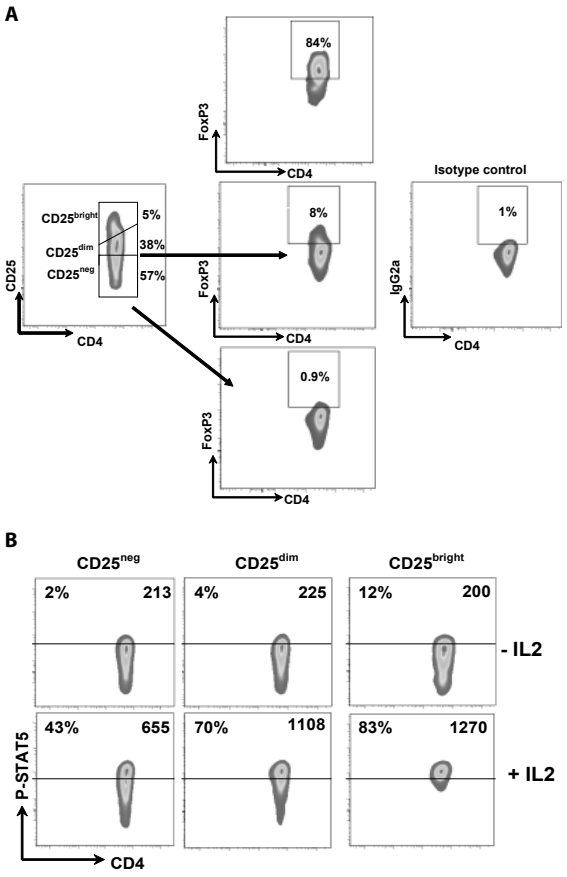


**Figure 1.** IL-2 induced STAT5 phosphorylation

**(A)** Representative example of CD25-expression within CD4$^{+}$ T cells and FoxP3-expression within the CD4$^{+}$CD25$^{neg}$, CD4$^{+}$CD25$^{dim}$ and CD4$^{+}$CD25$^{bright}$ T cells. Gates for FoxP3-positivity are set on the isotype control (IgG2a) control. Percentages positive cells are shown within the gates. **(B)** The percentage P-STAT5 within CD4$^{+}$CD25$^{neg}$, CD4$^{+}$CD25$^{dim}$ and CD4$^{+}$CD25$^{bright}$ T cells is analyzed after phospho-specific flow cytometry on whole blood samples. Percentage positive cells are shown in the left corner of the FACS plots and median fluorescence intensity (MFI) is shown in the right corner.

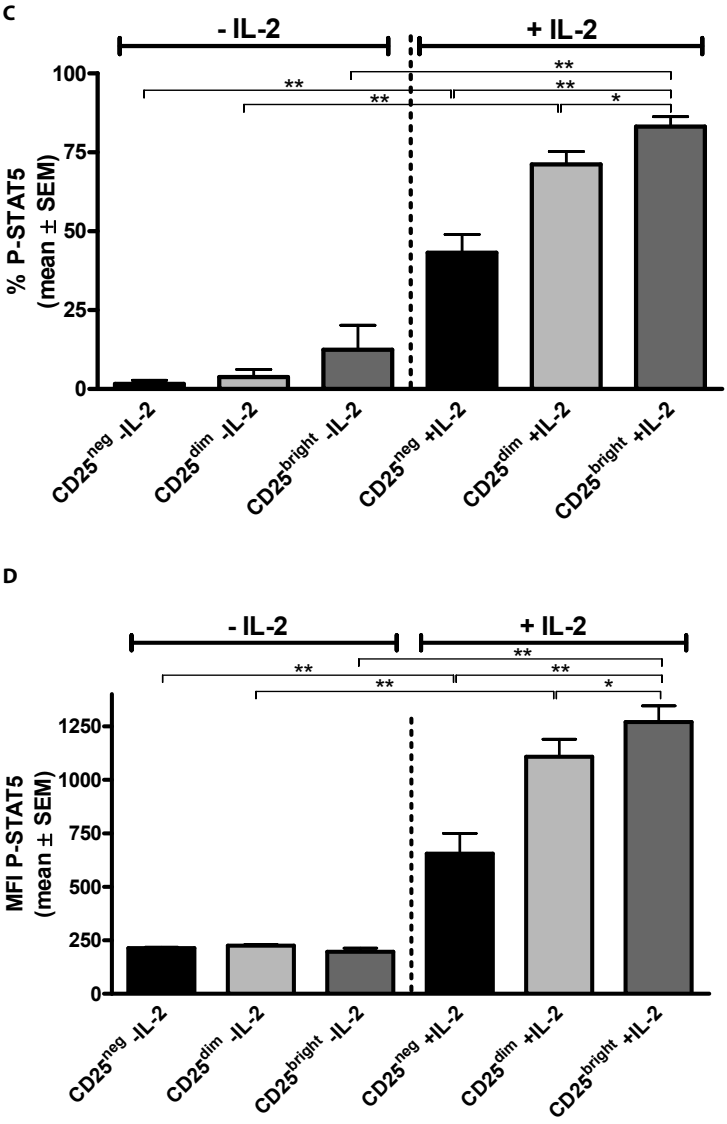


**Figure 1.** IL-2 induced STAT5 phosphorylation (continued)

**(C)** Percentages P-STAT5 positive cells and **(D)** median fluorescence intensities of P-STAT5 are shown within $CD4^+CD25^{neg}$, $CD4^+CD25^{dim}$ and $CD4^+CD25^{bright}$ T cells of n=4 patients. Mean ± SEM are depicted. Differences are statistically tested by Mann-Whitney U Test or Wilcoxon matched pairs Test. *p<0.05, **p<0.01.

To study the effect of CP-690,550 on STAT5-phosphorylation and the activation status of the $CD4^+CD25^{neg}$, $CD4^+CD25^{dim}$ and $CD4^+CD25^{bright}$ T cells, whole blood was preincubated with increasing concentrations of CP-690,550 followed by IL-2 stimulation. A representative example of the phosphoflow cytometry of the IL-2 induced P-STAT5 and the inhibition of CP-690,550 is depicted in Figure 2A. The IL-2 induced P-STAT5 was partially inhibited in the presence of 100 ng/mL CP-690,550

(Figure 2B, mean %P-STAT5: $CD25^{neg}$; from 37 to 6%, $CD25^{dim}$: from 70 to 16% and $CD25^{bright}$: from 81% to 40%) and almost fully inhibited (>95%) in the presence of 1000 ng/mL. Inhibition of P-STAT5 levels in unstimulated cells was not observed, showing that there is no basal level of P-STAT5 in fresh whole blood cells (Figure 2B). CP-690,550 was able to inhibit the P-STAT5 levels in the three subsets of all blood samples in a dose-dependent manner (Figure 2B). However, $IC_{50}$ was significantly higher in $CD25^{bright}$ T cells (104 ng/mL, mean), compared with $CD25^{dim}$ (45 ng/mL, $p<0.05$) and $CD25^{neg}$ T cells (40 ng/mL, $p<0.05$, Figure 2C). The same results were found with the MFI of P-STAT5 ($CD25^{neg}$: from 614 to 262, $CD25^{dim}$: from 1044 to 369 and $CD25^{bright}$: from 1212 to 677, Figure 2D and mean $IC_{50}$ $CD25^{bright}$: 90 ng/mL vs. $CD25^{dim}$: 37 ng/mL, $p<0.05$, vs. $CD25^{neg}$: 35 ng/mL, $p<0.05$, Figure 2E).

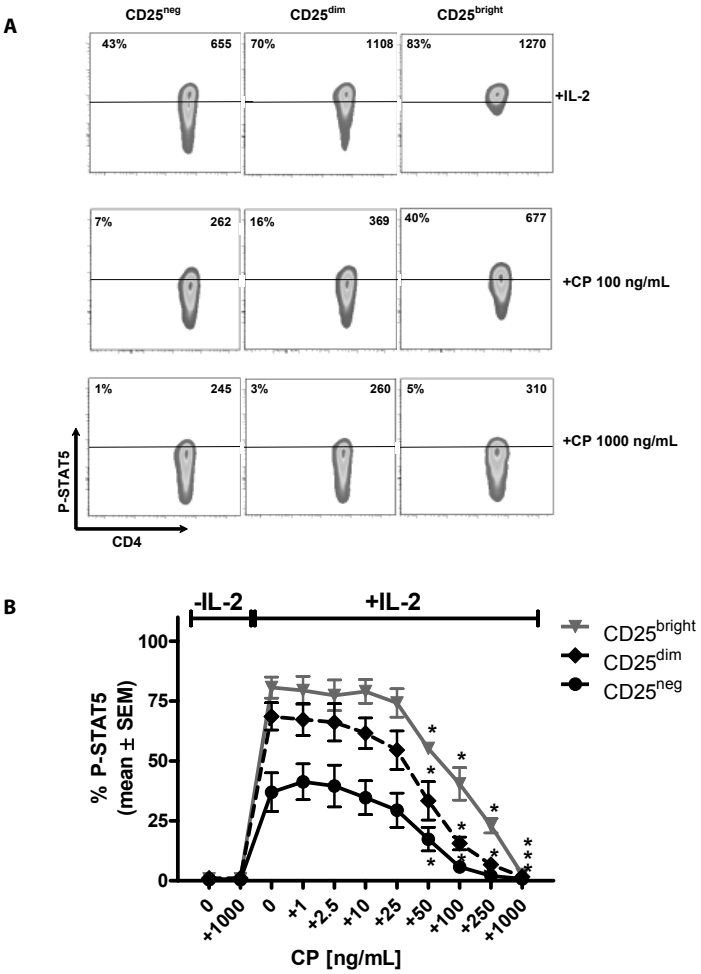


**Figure 2.** The effect of CP-690,550 on IL-2 induced STAT5 phosphorylation

**(A)** Flow cytometric example of IL-2-induced STAT5 phosphorylation in $CD4^+CD25^{neg}$, $CD4^+CD25^{dim}$ and $CD4^+CD25^{bright}$ in the absence and presence of 100 ng/mL and 1000 ng/mL CP-690,550. **(B)** Percentage positive cells of P-STAT5 at increasing doses of CP-690,550 are shown within $CD4^+CD25^{neg}$, $CD4^+CD25^{dim}$ and $CD4^+CD25^{bright}$ for n=4 patients. Mean ± SEM are shown at each dose.

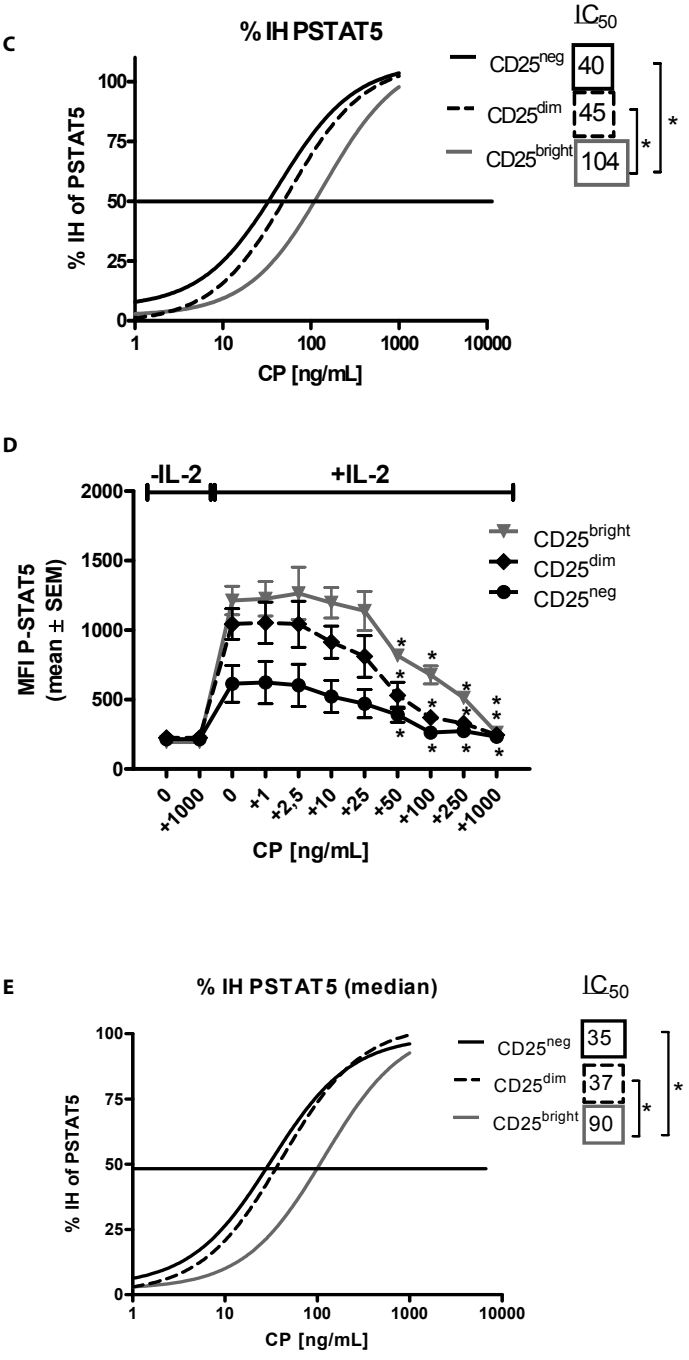


**Figure 2.** The effect of CP-690,550 on IL-2 induced STAT5 phosphorylation (continued)

**(C)** Sigmoid curves with percentage inhibition of P-STAT5 by CP-690,550 calculated on the percentage STAT5 positive cells. The $IC_{50}$ is shown for the $CD25^{neg}$, $CD25^{dim}$ and $CD25^{bright}$ T cells within $CD4^{+}$ T cells. **(D)** Median fluorescence intensities of P-STAT5 at increasing doses of CP-690,550. **(E)** Sigmoid curve with percentage inhibition of P-STAT5 by CP-690,550 calculated on the median fluorescence intensities. *$p<0.05$.

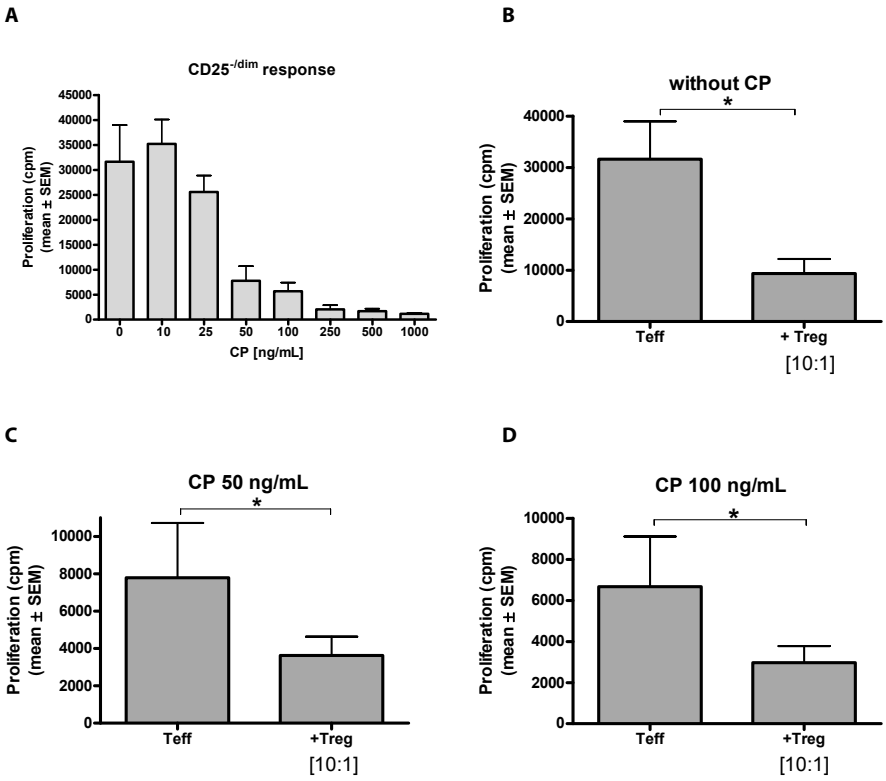


**Figure 3.** Functional assays with cells of healthy individuals

**(A)** Dose-response curve for the inhibition of proliferation of alloactivated CD25$^{-/dim}$ T cells by CP-690,550 in the MLR. The proliferation of CD25$^{-/dim}$ T cells is shown in counts per minute (cpm) **(B)** Suppression assay in which alloactivated CD25$^{-/dim}$ T cells are cocultured without and with CD25$^{bright}$ T cells. The proliferation is shown at a [CD25$^{-/dim}$ : CD25$^{bright}$] ratio of 10:1. **(C)** Suppression assay in the presence of 50 ng/mL CP-690,550 and **(D)** 100 ng/mL CP-690,550. Data are shown for n=6 healthy individuals. Error bars represent mean ± SEM. Differences are statistically tested by the Mann-Whitney U Test, *p<0.05.

*The effect of CP-690,550 on the function of Teff and Treg from healthy individuals*

To study the effect of CP-690,550 on the proliferation of alloactivated CD25$^{-/dim}$ Teff from healthy individuals, MLRs were performed. Teff were stimulated with allo-antigen for 7 days in the presence of increasing concentrations of CP-690,550, ranging from 1 to 1000 ng/mL. Starting at 50 ng/mL, CP-690,550 inhibited alloreactivity by Teff in a dose-dependent manner (mean percentage inhibition at 50 ng/mL: 77%, 100 ng/mL: 87%, 250 ng/mL: 94%, 500 ng/mL: 95% and 1000 ng/mL: 96%, Figure 3A). The regulatory activities of CD25$^{bright}$ T cells in the presence of CP-690,550 were analyzed by suppression assays in which CD25$^{bright}$ T cells were co-cultured with the Teff at a ratio of 1 to 10, respectively. The suppression assays were performed in the presence of 50 or 100 ng/mL CP-690,550. These concentrations were most appropriate because the maximal drug effect on Treg could be achieved as the Teff proliferation was not completely inhibited. In the absence of CP-690,550, the proliferative response of allo-antigen stimulated Teff was significantly lower when cocultured with CD25$^{bright}$ T cells (cpm: [CD25$^{-/dim}$] 31627

and [$CD25^{-/dim}$ + $CD25^{bright}$] 9361, p<0.05, Figure 3B). In the presence of the aforementioned CP-690,550 concentrations, Treg exerted suppression of the proliferation by Teff in response to alloantigens (cpm at 50 ng/mL: [$CD25^{-/dim}$] 7783 and [$CD25^{-/dim}$ + $CD25^{bright}$] 3625, p<0.05 and at 100 ng/mL [$CD25^{-/dim}$] 6674 and [$CD25^{-/dim}$ + $CD25^{bright}$] 2969, p<0.05, Figure 3C and D). These results suggest that Teff (77-87%) are more sensitive to the effect of clinically relevant CP-690,550 levels than Treg (23-13% decrease in function).

*The function of Teff and Treg from kidney transplant patients treated with CP-690,550*

As CP-690,550 preserves Treg suppressive function *in vitro*, we next questioned what the effect of CP-690,550 would be on PBMC of kidney transplant patients who were treated with this agent. Pre- and post-transplant (56 days) PBMC were collected from 7 kidney transplant patients who were part of a controlled Phase II study, with cyclosporine as comparator. All patients in the CP-690,550 group (n=4) and in the cyclosporine group (n=3) were treated with basiliximab anti-CD25 induction therapy, MMF and prednisone maintenance therapy. This anti-CD25 induction therapy post-transplant caused shedding of the IL-2Rα-complex, which makes isolation of $CD25^{bright}$ T cells impossible. To recover the CD25-expression of the basiliximab-treated patients for isolation, PBMC were rested overnight in culture medium containing 5 ng/mL IL-2. After 24 hr, the CD25-expression recovered to common levels (Figure 4) and the $CD25^{bright}$ T cells (consisting of 85% $FoxP3^+$ T cells) could then be isolated. Previously, we have shown that anti-CD25 mAb therapy does not negatively affect FoxP3 protein expression and $CD25^{bright}$ T-cell function [16].

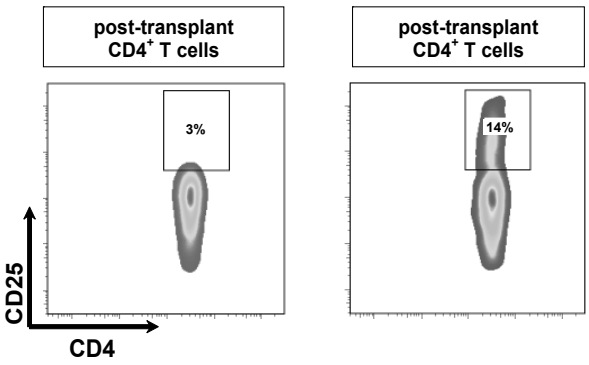


**Figure 4.** CD25 expression on $CD4^+$ T cells of kidney transplant patients treated with basiliximab
CD25-expression disappeared after treatment (left plot). CD25-expression recovered after overnight incubation of PBMC in culture medium with 5 ng/mL IL-2 (right plot).

The proliferative capacity of Teff was studied by depletion of $CD25^{bright}$ T cells from the PBMC. The proliferation in response to alloantigens was significantly lower post-transplantation in both groups, (cpm: 13073 pre-transplant to 4057 post-transplant within the CP-690,550 group, and to 3219 post-transplant within the cyclosporine treated group, Figure 5A). The functionality of

the suppressor cells was studied in conventional suppression assays. Pre-transplant CD25$^{bright}$ T cells were able to nearly completely suppress the proliferation of pre-transplant Teff at a Treg-to-Teff ratio of 1:5 (cpm: [CD25$^{-/dim}$] 13073 and [CD25$^{-/dim}$ + CD25$^{bright}$] 500, percentage inhibition: 96%, Figure 5B).

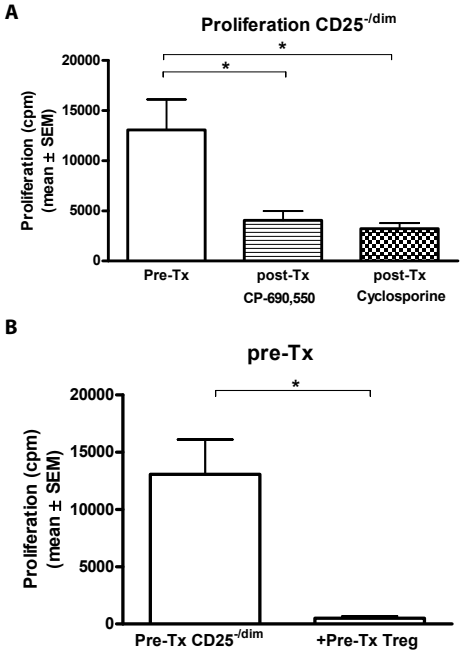


**Figure 5.** Functional assays with cells of kidney transplant patients

**(A)** Proliferation of alloactivated CD25$^{-/dim}$ T cells of *pre-Tx* of all patients (white bar) and *post-Tx* from patients of the CP-690,550 group (striped bar) and of the cyclosporine group (squared bar). **(B)** Suppression assay in which alloactivated CD25$^{-/dim}$ T cells of *pre-Tx* are cocultured with Treg (CD25$^{bright}$ T cells) of *pre-Tx* at a 5 to 1 ratio.

We studied the effect of CP-690,550 on Treg function by adding this compound at various concentrations to our system. This was done to be absolutely certain of the maximal drug effect and to minimize the influence of other drugs on the PBMC of the patients *in vivo*. Clinically relevant levels of CP-690,550 or cyclosporine were added to the suppression assays on day 0. Considering that the post-transplant Teff showed a low proliferative response (Figure 5A), we chose to use the pre-transplant Teff as a read-out for post-transplant Treg function in the suppression assay. The proliferation of pre-transplant Teff decreased when CP-690,550 was added to the MLR (Figure 5C). When post-transplant CD25$^{bright}$ T cells isolated from CP-690,550-treated patients were co-cultured with pre-transplant Teff exposed to CP-690,550, the proliferation was further inhibited (percentage inhibition; 85%, Figure 5C). The same set of experiments was carried out with PBMC from patients of the cyclosporine group. As shown in Figures 5A and D, there was a decrease in the proliferation of the pre-transplant Teff in the presence of

cyclosporine. Post-transplant CD25$^{bright}$ T cells from cyclosporine-treated patients were able to further inhibit these responses (percentage inhibition; 89%).

When the percentage of inhibition was calculated, there was neither a difference between the suppressive capabilities post-transplant CD25$^{bright}$ T cells and pre-transplant CD25$^{bright}$ T cells nor did we find significant differences between the groups (percentage inhibition pre-transplant vs. post-transplant CP-690,550 group: 94% vs. 85% and cyclosporine group: 97% vs. 89%, Figure 5E).

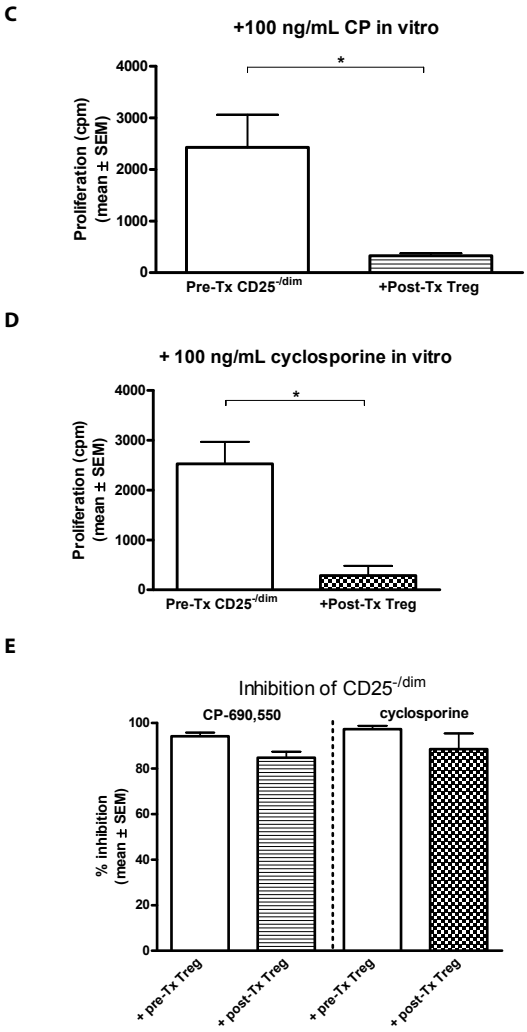


**Figure 5.** (continued) **(C)** Suppression assay of PBMC from patients in the CP-690,550 group in which CD25$^{-/dim}$ T cells of *pre-Tx* are cocultured with Treg (CD25$^{bright}$ T cells) of *post-Tx* at a 5 to 1 ratio in the presence of 100 ng/mL CP-690,550. **(D)** Suppression assay of PBMC from patients in the cyclosporine group in which CD25$^{-/dim}$ T cells of *pre-Tx* are cocultured with Treg (CD25$^{bright}$ T cells) of *post-Tx* at a 5 to 1 ratio in the presence of 100 ng/mL cyclosporine. **(E)** Percentage inhibition of the proliferative response of *pre-Tx* CD25$^{-/dim}$ by *pre-Tx* and *post-Tx Treg* of both groups.

## DISCUSSION

Cytokines of the IL-2 family that signal via the $\gamma_c$ are important in lymphoid development, homeostasis and differentiation as well as in immune regulation and inflammation[3, 17]. Therefore, interference with this pathway has been considered a suitable target for the suppression of alloreactivity after clinical organ transplantation. As part of a Phase I study, the effect of the Jak-inhibitor CP-690,550 has been tested on the number of circulating lymphocyte subsets and the function of PBMC of kidney transplant patients who received therapy consisting of the immunosuppressive drug CP-690,550[14]. In a previous study, we showed that the number of Treg decreased after CP-690,550 treatment but the remaining cells still exerted their function, whereas the IFN-γ secretion by Th1 cells was inhibited. These data suggest that the function of Teff cells is more sensitive to the effects of CP-690,550 than the regulatory capacity of Treg[14]. In the present study we investigated whether there is a differential effect of CP-690,550 on Treg and Teff. Therefore, we examined the effect of CP-690,550 on the IL-2 induced STAT5-phosphorylation of $CD25^{neg}$, $CD25^{dim}$ and $CD25^{bright}FoxP3^{+}$ T cells within $CD4^{+}$ T cells in whole blood samples from healthy individuals. Furthermore, the proliferative and suppressive functions of the $CD25^{-/dim}$ and $CD25^{bright}$ T cells isolated from kidney transplant patients who received CP-690,550 therapy were examined pre- and post treatment. These results were compared with a control group, consisting of kidney transplant patients who received cyclosporine therapy.

By applying phospho-specific flow cytometry on whole blood samples of healthy individuals, we showed that Teff are more susceptible to blockade of Jak by CP-690,550 compared with Treg (Figure 2). The functional studies with PBMC of healthy individuals and kidney transplant patients confirmed the difference in sensitivity to CP-690,550 between Treg and Teff (Figures 3 and 5). These findings may favor the onset of hyporesponsiveness, particularly because Teff cells are the major culprits in causing alloreactivity and rejection of the allograft after organ transplantation.

An intriguing observation was that T cells that lacked CD25-expression also showed enhanced levels of phosphorylated STAT5 after IL-2 stimulation. This phenomenon can be explained by the binding of IL-2 via the dimeric low affinity receptor complex IL-2Rβγ that is mostly found on naïve and memory T cells[18]. Pre-B cells, thymocytes and NK cells have low levels of CD25, whereas activated T cells and Treg have a trimeric IL-2Rαβγ-receptor complex and have a high affinity binding by cooperative interactions between the α and β chains and a low activation threshold[19-21]. Therefore, the highest levels of P-STAT5 can be found in the two latter T-cell subsets. Nevertheless, the β and γ-chains remain the most crucial elements for IL-2 signaling.

After binding of the cytokines of the IL-2 family, heterodimerization of the receptor subunits occurs and Jak3 associates with the β and γ-receptor chains and Jak1 associates with the β-chain. The Jaks auto-phosphorylate and cause STAT5 dimerization/trimerization after activation[22]. There are several explanations for the different sensitivity for CP-690,550. Firstly,

by a different distribution of the IL-2 α, β, and γ chains or a variation in the recruitment of Jak1 and Jak3 molecules between Treg and Teff. Treg might have more Jak3 and/or Jak1 molecules and therefore need more CP-690,550 to inhibit all molecules[23]. Furthermore, it is also unclear how many configurations of STAT homo- and heterodimeric complexes are present in the Treg and Teff cells before, during and after cytokine stimulations. Secondly, it has been shown that different regions of the cytoplasmic domain of the IL-2Rβ-chain interact and couple with distinct signaling pathways and cellular responses in Treg and Teff[24]. In Teff, IL-2 stimulation predominantly results in IFN-γ production and proliferation, whereas in Treg IL-2 is required to induce and maintain FoxP3-expression and suppressive function. IL-2 induced STAT5 phosphorylation might not be important for Treg function, but apparently is important for Teff function, because inhibition of the STAT5 phosphorylation by CP-690,550 results in an almost 90% decrease in proliferation of Teff, whereas Treg function is still present when only 40% of the cells have P-STAT5. FOXP3-expression is induced by STAT5 as STAT5-tetramers are able to bind a highly conserved binding site on the promoter region of the FOXP3-gene[25]. Accordingly, Treg in our study already express FoxP3, and therefore cannot be affected, so only the induction of *de novo* FoxP3-expression and the development of Treg are inhibited. On the other hand, FoxP3-expression alone is not sufficient for the acquisition of suppressive capacity, as it has been shown that activated Teff cells can also induce FoxP3, in a STAT5-dependent manner[5]. To maintain FoxP3, more pathways are necessary and apparently these pathways operate separately. This may explain the presence of functional Treg.

Collectively, our data demonstrate that although STAT5-phosphorylation is partially inhibited in Treg in the presence of the Jak-inhibitor CP-690,550, they still exhibited suppressive activities. Apparently, in $CD25^{bright}$ T cells, STAT5 phosphorylation is not (or partly) coupled with their function, in contrast to the $CD25^{neg}$ and $CD25^{dim}$ T cells that require STAT5 phosphorylation for their function (Figures 2, 3 and 5). It already has been reported that there is a fundamental difference between allo-responsive Treg and Teff in the susceptibility to a diverse set of immunosuppressive agents such as anti-CD4 mAb, rATG, anti-CD40L and the IL-2 signal inhibitor rapamycin. These agents commonly lead to the selective expansion of antigen-specific natural Treg or higher resistance of Treg than Teff to an anti-proliferative or apoptosis-inducing effect of these agents[26-28]. According to our findings, this list can be expanded with CP-690,550 and cyclosporine. In summary, we have shown that the function of Treg is largely independent of IL-2/Jak3/STAT5 signaling as the indirect inhibitor of the functions of IL-2, CP-690,550 and the CNI cyclosporine both spare Treg function, whereas they effectively inhibit Teff STAT5 phosphorylation and function. Our findings may have important implications in the usage of these immunosuppressive drugs and their mechanisms in the modulation of anti-donor responses after clinical organ transplantation.

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# Chapter 8

## Conversion from Calcineurin Inhibitor to Mycophenolate Mofetil-Based Immunosuppression Changes the Frequency and Phenotype of $CD4^+FoxP3^+$ Regulatory T Cells

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## ABSTRACT

$CD4^+Foxp3^+$ regulatory T cells (Treg) depend on IL-2 for their function and survival. By interfering with the IL-2 production, calcineurin inhibitors (CNI) may negatively affect Treg. Here, we describe the effects of conversion from CNI to mycophenolate mofetil (MMF) monotherapy on renal function, and on Treg frequency and phenotype in liver transplant recipients. Patients (n=16) with renal impairment on CNI were converted to MMF and received a single dose of the IL-2-receptor blocking antibody (Daclizumab). Control patients (n=8) continued CNI treatment. Renal function rapidly and significantly improved after conversion. Daclizumab treatment resulted in a 75% blocking of CD25 at one month causing a significant reduction in the percentage of $CD4^+CD25^+$ cells but not affecting the percentage of $CD4^+CD25^+Foxp3^+$ cells. Six months after conversion to MMF, the percentage of $CD4^+CD25^+Foxp3^+$ cells increased significantly by 125%. FOXP3 mRNA analysis of mononuclear cells confirmed the enrichment of Foxp3 in peripheral blood. Interestingly, the CD25 expression level on $CD4^+Foxp3^+$, but not $CD4^+Foxp3^-$ cells significantly increased compared with preconversion. Conversion to MMF increases the percentage and CD25 expression of $CD4^+Foxp3^+$ cells indicating that MMF therapy can overturn the repressive effect of CNI on circulating Treg levels and therefore may promote Treg-mediated suppression of alloreactivity.

## INTRODUCTION

The checks and balances of immune reactive and immune suppressive cells likely determine whether there is immune reactivity to donor antigens or non-responsiveness (tolerance). Tolerance to self and foreign antigens involves the action of regulatory T cells (Treg)[1, 2]. These T cells with suppressive capacities were first identified for their ability to prevent organ specific autoimmune diseases in mice[1]. In experimental transplant models these cells can transfer tolerance to donor antigens as well[2]. Recent studies suggest that calcineurin inhibitors (CNI, cyclosporine A or tacrolimus), belonging to the current standard immunosuppressive therapy after organ transplantation (Tx), interfere with the expansion, survival and possibly the function of Treg *in vitro*[3, 4] and *in vivo*[5, 6]. CNI are known as potent blockers of IL-2 production and it is established that IL-2 is critically important for the survival of Treg[7]. This could explain why CNI have been reported to be detrimental to both spontaneous experimental transplant tolerance *in vivo* and tolerance induced by co-stimulatory blockade[8, 9]. The clinical use of CNI is associated with considerable non-immunological side effects, most importantly renal dysfunction due to nephrotoxicity[10] but also diabetes mellitus, hyperlipidemia and hypertension.

Clinical studies have shown that conversion of CNI to immunosuppressants like rapamycin or mycophenolate mofetil can halt or even reverse CNI associated side effects. In liver Tx, CNI conversion to MMF based immune suppression improves renal function but is also associated with a considerable risk of acute rejection[11, 12]. Recent studies suggest that rapamycin, unlike CNI, does not interfere with the survival and alloantigen-driven expansion of Treg *in vitro*[3, 4] and *in vivo*[6]. There is now accumulating evidence that the inosine monophosphate inhibitor MMF, an IL-2 independent immunosuppressant, has similar effects on Treg as rapamycin. It was shown in different experimental transplant models that MMF does not interfere with Treg function and positively affects tolerance induction[5, 9, 13].

In the present study stable liver transplantation patients were converted from CNI to MMF monotherapy. To minimize the risk of conversion-associated acute rejection, recipients were treated with additional induction IL-2 receptor blockade. The aim of this study was to observe the effects of conversion on the frequency and phenotype of circulating $CD4^+Foxp3^+$ Treg. After conversion to MMF, rapid effects on renal function as well as changes in the frequency and CD25 expression of circulating $CD4^+Foxp3^+$ Treg were observed. Possible implications of these changes with respect to transplant tolerance will be discussed.

## PATIENTS AND METHODS

*Patients and study design*

Liver transplant recipients transplanted at the Erasmus MC (University Medical Center Rotterdam) on CNI maintenance monotherapy and at least 12 months after Tx, were screened for renal dysfunction. Renal dysfunction was defined based on increased serum creatinin and blood urea nitrogen (BUN) levels and a calculated creatinin clearance of less than 60 mL/min. To exclude other causes of renal dysfunction, ultrasound of kidneys and aorta were performed. Only patients older than 18 years were consecutively enrolled. Exclusion criteria included abnormal graft function (increased serum levels of aminotransferase, alkaline phosphatase, gamma-glutamyl transferase and bilirubin, without histological signs of acute rejection), active infection, malignancy, and a history of severe (steroid-resistant or repeated) rejection and other causes of renal dysfunction. This study was approved by the Medical Ethical Committee of the Erasmus MC and an appropriate informed consent was obtained from all patients.

Initially this was an open, randomized study comparing MMF-Daclizumab based regimen with standard CNI based drug regimen. At time-point zero one infusion of 2 mg/kg bodyweight of Dacluzimab was given intravenously and oral MMF was started at a dose of 2 g a day. CNI was stopped at the same day. MMF dose was not adapted to trough levels. The composite primary endpoint of this study was the improvement of renal function: decrease in serum creatinin, BUN and increase in creatinin clearance during the first 6 months after randomization. Creatinin clearance was calculated with the Cockroft-Gault equation and in a subset of patients glomerular filtration rate (GFR) was measured. Secondary endpoints included biopsy proven rejection, allograft dysfunction, patient death at 6 months and graft loss at 6 months. During the follow up, liver and renal functions were assessed on regular basis in the outpatient clinic. When acute rejection was diagnosed intravenous methylprednisolon (Solumedrol, 3x1000mg) was administered together with re-introduction of CNI and discontinuation of MMF. Acute rejection was confirmed by histological examination of liver biopsies using the Banff classification. General characteristics of the study group are summarized in Table 1. For flow cytometric and reverse-transcriptase polymerase chain (RT-PCR) analysis five heparinized peripheral blood samples (in total 30 mL) were obtained from all patients before and at 1, 3, and 6 months after randomization.

*Flow cytometric analysis*

Peripheral Blood Mononuclear Cells were obtained by density gradient centrifugation over Ficoll-Paque plus (Amersham Biosciences, Buckinghamshire, UK) and stored frozen at –135°C. After thawing, PBMC were washed twice with phosphate-buffered saline containing 0.3% bovine serum albumin followed by staining with primary monoclonal antibodies in phosphate-buffered saline/0.3% bovine serum albumin (30 min at 4 °C). The following fluorochrome-conjugated monoclonal antibodies were used: CD25 (clone 2A3)-fluorescein isothiocyanate

**Table 1.** Baseline patient characteristics

| | Study patients (n=16) | Controls (n=8) | p-value |
|---|---|---|---|
| Mean age ± SD (yr) | 60±11 | 66±5 | NS |
| Gender (M/F) | 8/8 | 3/5 | NS |
| Mean time after Tx (yr) | 9±4 | 7±2 | NS |
| Indication LTx | | | |
| Sclerosing Cholangitis | 3 | 2 | NS |
| Alcoholic Cirrhosis | 2 | 2 | NS |
| Cryptogeneic Cirrhosis | 3 | 1 | NS |
| Biliary Cirrhosis | 3 | 1 | NS |
| Viral Hepatitis | 2 | 0 | NS |
| Acute Liver Failure | 3 | 1 | NS |
| Haemangioendothelioma | 0 | 1 | NS |
| Maintenance Immunosuppression | | | |
| Cyclosporine A monotherapy | 11 | 5 | NS |
| Tacrolimus monotherapy | 5 | 3 | NS |

Tx, Transplantation; LTx, liver transplantation

(FITC), CD25 (clone M-A251)-phycoerythrin (PE), and CD4-PerCP-Cy5.5 from Becton Dickinson (San Jose, USA); CTLA-4-APC, CD3-fluorecein isothiocyanate, and IgG2a-PE from Immunotech, (Marseille, France); Foxp3-APC and isotype IgG2a-APC from eBiosciences (San Diego, USA). To determine the level of CD25 blockade by Daclizumab, we used two different clones of anti-CD25 antibody, which bind different epitopes. Antibody concentrations were titrated to obtain the same mean fluorescence intensity for both CD25 antibodies. In this manner, the extent of CD25 blocking by cell-bound Daclizumab was calculated. To define maximal receptor blocking as control, $5x10^6$ PBMC were incubated with 25 µg/mL Daclizumab (10 min at 4 °C) *in vitro* before CD25 staining.

After primary incubation cells were washed, and for staining of intracellular FoxP3, the cells were fixed and permeabilized using fixation/permeabilization supplied by eBiosciences. Analysis by flow cytometry was performed using FACS Calibur and CELLQuest Pro software (Becton Dickinson, San Jose, CA). The percentages of Treg were calculated as a percentage of total $CD3^+CD4^+$ T cells. The relative CD25 expression within the $FoxP3^+CD4^+$ and $FoxP3^{neg}CD4^+$ cell populations was calculated based on the geometric mean fluorescence intensity for both populations at baseline (t=0), which were considered 100%.

*Quantitative RT-PCR*

From PBMC ($1x10^6$ cells), erythrocytes were lysed with a buffer containing ammoniumchloride, sodium bicarbonate and EDTA by incubation for 7 min on ice. The remaining leukocyte fraction was pelleted and resuspended in 0.5 mL TRIzol reagent (Life Technologies, GmbH, Karlsruhe, Germany) at 4°C. After homogenization, 20 µg poly A (Boehringer, Mannheim, Germany) was added and the TRIzol lysates were directly stored at -80°C. Total RNA was extracted with 160 µL of cold chloroform-isoamylalcohol and subsequently precipitated with ice-cold 80% ethanol (350 µL). The precipitated solution was then loaded on an RNA-isolation column from the

Qiagen RNeasy isolation kit (Qiagen, Venlo, the Netherlands) and RNA was isolated according to the manufacturer's instructions. Total RNA was denaturated for 10 min at 80°C and then chilled to 4°C. First-strand cDNA synthesis was performed as previously described[14]. Real-time RT-PCR was used to quantify FOXP3 mRNA. The constant region of the T cell receptor α chain (TCR-Cα) RNA was quantified to use as reference gene. The primers and probes for FOXP3 were obtained from Assays-on-Demand Gene Expression Product and pre-developed Taqman PDAR assays (Applied Biosystems, Forster City, CA). Each Taqman probe was labeled at the 5' end with the reporter dye molecule 6-carboxyfluorescein. Five microliters cDNA was added to 20 µL PCR mixture containing 12.5 µL Universal PCR Master Mix (Applied Biosystems), 0.625 µL primer/probe mix and 6.875 µL $H_2O$. The choice of primer and probe for the measurement of TCR-Cα transcripts was as previously described[14].

Amplifications were performed using the ABI 7700 sequence detector system (Applied Biosystems) under the following conditions: a first step of 2 min 50°C and 10 min 95°C followed by 40 cycles of 15 sec at 95°C and 1 min at 60°C (for TCR-Cα 1 min at 58°C). The target message was quantified by measuring threshold cycle ($C_t$). Two negative controls (no template), and two positive reference samples were included for each determination. Samples negative for the TCR-Cα genes were excluded from further analysis. The relative FOXP3 expression based on TCR-Cα was calculated using the comparative $\Delta\Delta C_t$ formula: $\Delta\Delta CT$ ($C_t$ Target – $C_t$ TCR-Cα) $_{sample}$ - ($C_t$ Target – $C_t$ TCR-Cα) $_{control}$. The fold change was calculated using $2^{-\Delta\Delta Ct}$.

*Statistical Analysis*

Statistical analysis was performed using SPSS Inc. software version 11.0 (Chicago, IL, USA). For the clinical data the intention-to-treat analyses was used. Comparisons were performed with Wilcoxon matched paired test or the Mann-Whitney U test. P-values less than 0.05 were considered to be significant.



## RESULTS

*Clinical Outcomes*

Sixteen liver transplantation recipients with renal impairment on CNI were converted to MMF immunosuppression and received a single dose of IL-2-receptor blocking antibody (Daclizumab). Control group (n=8) continued CNI treatment. Two cases of acute rejection occurred in the conversion group: one at 1 month and the other at 6 months after conversion. Rejection was confirmed with histologically and successfully treated with intravenous methylprednisolon and re-introduction of CNI. Improvement of renal function was observed as early as 1 month after conversion to MMF. Relative to baseline values, serum creatinin decreased with a mean of 16% ± 9% (SD) at 6 months after conversion (Table 2). BUN levels also significantly decreased. In the control group, serum creatinin and BUN remained unchanged. The calculated creatinin

clearance increased significantly in the conversion group (p=0.001), whereas remaining stable in the control group (Table 2). In seven patients in the conversion group, the GFR was measured and showed a significant increase at 6 months confirming the improvement of renal function (p=0.018), whereas no changes were seen in the control group (n=4). Conversion patients at 6 months (n=13) had a significant lower serum bilirubin level (p=0.034), whereas this remained unchanged in control patients (n=7; Table 2).

**Table 2.** Improved renal function after conversion from CNI to MMF monotherapy

| Patients | Baseline | 1 mo | 3 mo | 6 mo | N |
|---|---|---|---|---|---|
| Mean serum creatinin (µmol/L) | | | | | |
| Control | 154±23 | 150±19 | 150±25 | 152±32 | 7 |
| Conversion | 158±35 | 141±27* | 143±31* | 132±30* | 13 |
| Mean serum BUN (mmol/L) | | | | | |
| Control | 12±2 | 12±2 | 12±2 | 11±3 | 7 |
| Conversion | 13±4 | 10±3* | 10±3* | 9±2* | 13 |
| Creatinin Clearence (mL/min) | | | | | |
| Control | 38±16 | ND | ND | 42±14 | 7 |
| Conversion | 45±14 | ND | ND | 56±19* | 13 |
| GFR (mL/min) | | | | | |
| Control | 44±13 | ND | ND | 44±12 | 4 |
| Conversion | 39±7 | ND | ND | 46±11* | 7 |
| Serum Bilirubin | | | | | |
| Control | 10±2 | ND | ND | 9±2 | 7 |
| Conversion | 10±3 | ND | ND | 8±2* | 13 |

Shown are the means ± SD *p< 0.05 statistical significantly different from baseline values.

### *Changes in $CD4^+CD25^{bright}$ T cells after conversion to MMF*

To assess the effect of immunosuppression conversion on Treg, flowcytometric analysis of CD4 and CD25 in PBMC was performed. A representative dot plot of CD4 and CD25 double staining within the $CD3^+$ T cells is shown in Figure 1A. For the detection of CD25, an antibody (clone M-A251) was used that can bind independently from blocking by Daclizumab. One month after conversion to MMF, CD25 expression on $CD4^+$ T cells was significantly decreased by a mean of 64% ± 4% SEM ($p \leq 0.001$; Figure 1B). In order to determine whether this down-regulation is associated with CD25 blocking by Daclizumab, double immunostainings were performed using dependent (i.e. does not bind in the presence of Daclizumab) and independent CD25 detection antibodies. As shown in Figure 1C, maximal CD25 blocking was observed at 1 month after conversion (75%) and this coincided with the reduction of $CD4^+CD25^{bright}$ T cells. Blocking by Daclizumab was reduced to baseline levels by 6 months (Figure 1C). At 6 months, when CD25 blocking by Daclizumab was alleviated, all conversion patients showed a more than 25% increase in $CD4^+CD25^{bright}$ cells. The relative levels of $CD4^+CD25^{bright}$ cells, as a percentage of baseline, was significantly increased and also statistically significant different from the levels in the control group (p=0.003; Figure 1B). The mean percentage of $CD4^+CD25^+$CTLA-$4^+$ within total $CD3^+$ T cells was increased at 6 months after conversion from 2.0 ± 0.8 SEM at baseline

to 4.3 ± 2.4 SEM ($p<0.05$, data not shown). None of these changes were seen in the control group. In sum, these data indicate that Daclizumab reduces the percentage of $CD4^+CD25^{bright}$ T cells whereas after 6 months conversion from CNI to MMF a significant increased proportion of circulating $CD4^+CD25^{bright}$ cells was observed.

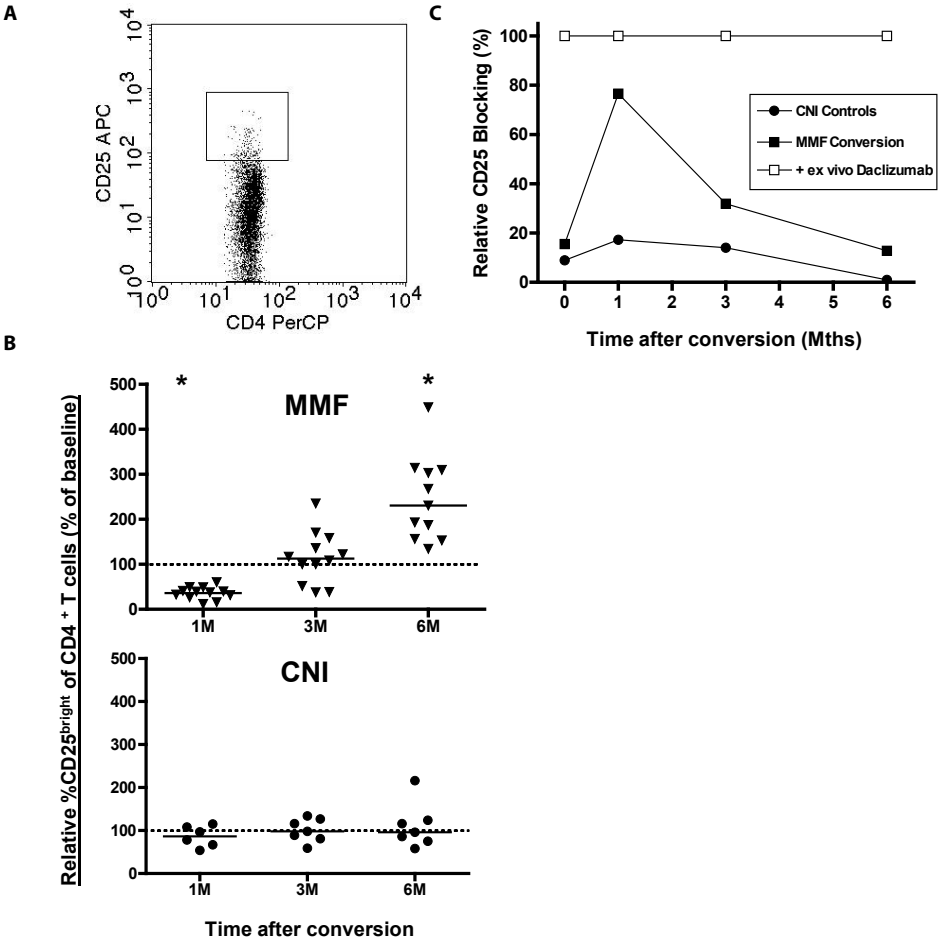


**Figure 1.** Changes in percentage of $CD4^+CD25^{bright}$ T cells after conversion to MMF monotherapy

**(A)** Representative dot plots of $CD4^+CD25^{bright}$ cells within the $CD3^+$ T-cell population. **(B)** Shown are the relative percentage of $CD4^+CD25^{bright}$ cells, as a percentage of baseline, in conversion (MMF, n=11) and control patients (CNI, n=7). At 1 mo after conversion, a median decrease of 62% ± 4 SEM in the relative percentage of $CD4^+CD25^{bright}$ cells was observed (*$p \leq 0.001$). At 6 mo, all conversion patients showed at least a 25% increase in $CD4^+CD25^{bright}$ cells over baseline values. The median increase was 130% (*$p \leq 0.001$) and was also significantly different from the control group at 6 mo ($p=0.003$). Bar represents median and dashed lines indicate baseline levels. **(C)** Level of CD25 blocking by Daclizumab on $CD4^+$ T cells. Blocking was calculated based on differential staining of two CD25 detecting antibodies, one binding to the same epitope as Daclizumab and one binding a distinct epitope. Maximum level of CD25 blocking (>75%) was observed at 1 mo and coincided with the down regulation of CD25 expression. Blocking values returned to baseline at 6 mo after conversion (n=9). In the control group (n=5) approximately 15% CD25 blocking was observed which was unrelated to Daclizumab, but possibly related to bound IL-2.

*Increase of CD25$^+$Foxp3$^+$T cells after conversion to MMF*

To further confirm the effect of immunosuppression conversion on Treg, additional flow cytometric analyses were performed for Foxp3. A representative dot plot of CD25 and Foxp3 expression within the CD4$^+$ T cells is shown in Figure 2A. The total proportion of CD25$^+$Foxp3$^+$ cells within the CD4$^+$ T cells significantly increased after conversion (Figure 2B). Ten out of 11 patients showed a more then 20% increase in the percentage of CD25$^+$Foxp3$^+$ cells at 6 months. After conversion, the mean increase over baseline levels at 6 months was 125% ± 43% SD (p=0.004; Figure 2B). Also a significant difference was observed (p=0.007) when comparing the relative percentage CD25$^+$Foxp3 of conversion and control groups. No significant changes from baseline were seen in the control CNI group. RT-PCR analysis confirmed the increase of Foxp3$^+$ cells at transcript level at 6 months after conversion (60% increased FOXP3 mRNA over baseline levels, p=0.002), whereas no changes were observed in the control group. In two patients who were withdrawn from MMF and reconverted to CNI monotherapy (at 3 and 6 months after conversion), the proportion of Foxp3$^+$ cells and FOXP3 mRNA expression decreased after reintroduction of CNI (Table 3). No association was observed between the percentage of CD25$^+$Foxp3$^+$ cells and the development of acute rejection. Overall, these findings suggest that removal of CNI and introduction of MMF positively affect the percentage of circulating CD4$^+$CD25$^+$Foxp3$^+$ Treg.

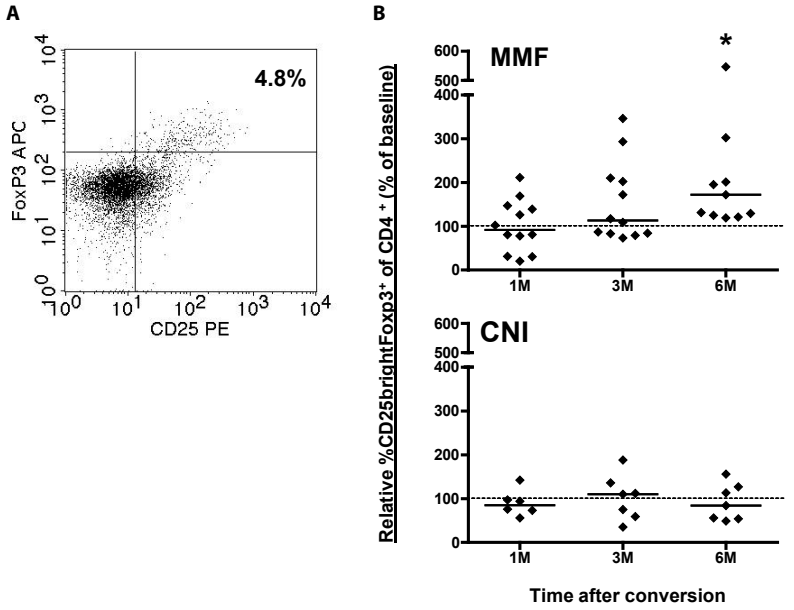


**Figure 2.** Increase in proportion of CD25$^+$Foxp3$^+$ T cells after conversion to MMF

**(A)** Representative dot plot showing CD25$^+$Foxp3$^+$ cells within the CD4$^+$ T-cell population. **(B)** The relative percentage of CD25$^+$Foxp3$^+$ cells within the CD4$^+$ population, as a percentage of baseline values, are shown from conversion (MMF, n=12) and control patients (CNI, n=7). No decrease was observed at 1 mo, but the relative levels of CD25$^+$Foxp3$^+$ cells increased significantly at 6 mo after conversion (*p=0.004) and was also different from the control group at 6 mo (p=0.008). Quantitative RT-PCR analysis of PBMC confirmed the enrichment of Foxp3 mRNA at 6 mo after conversion (p=0.002 compared to baseline; data not shown). No significant changes were observed within the control patients who continued on CNI. Bar represents median and dashed lines indicate baseline levels.

**Table 3.** Changes in Foxp3$^+$ cells after re-conversion to CNI in two patients

| | % Foxp3$^+$ of CD4$^+$ T cells | | | Relative FOXP3 mRNA expression | | |
|---|---|---|---|---|---|---|
| | Pre | MMF | After CNI restart | Pre | MMF | After CNI restart |
| Patient 1 | 2.7 | 6.3 | 3.2 | 67.4 | 208.6 | 103.6 |
| Patient 2 | 5.2 | 7.7 | 5.0 | 33.7 | 68.3 | 31.0 |

Reconversion (patient 1 at 3 mo, patient 2 at 6 mo) from MMF to CNI decreased the percentage of CD4$^+$Foxp3$^+$ T cells and FOXP3 mRNA levels

*Changes in CD25 expression on Foxp3$^+$ cells after conversion to MMF*

We examined the differential expression of CD25 on CD4$^+$Foxp3$^+$ and CD4$^+$Foxp3$^-$ cells after conversion. The increased CD25 expression on CD4$^+$ T cells observed at 6 months after conversion was predominantly confined to CD4$^+$Foxp3$^+$ cells (Figure 3). The CD25 expression of CD4$^+$Foxp3$^+$ cells was increased at 6 months by 140%±56 SEM ($p<0.05$) over baseline levels. An increase of 56%±51 SEM in CD25 expression was observed on CD4$^+$Foxp3$^-$ cells, but this did not reach statistical significance.

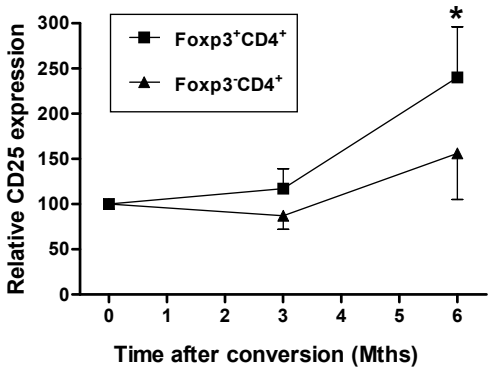


**Figure 3.** Changes in CD25 expression on Foxp3$^+$ cells after conversion

The relative CD25 expression was calculated based on the geometric mean fluorescence intensity at baseline (100%). At 6 mo after conversion, CD25 expression significantly increased on Foxp3$^+$ cells (*$p<0.05$) but not on Foxp3$^-$ cells. Shown is mean ± SEM of 11 conversion patients.



## DISCUSSION

The success of solid-organ transplantation depends on the continuous administration of toxic and antigen non-specific immunosuppressive drugs to prevent rejection. Currently, maintenance immunosuppressive therapy in most liver transplant recipients includes a CNI. This study shows that the percentage of CD25$^+$Foxp3$^+$ cells within the CD4$^+$ T cells significantly increased after conversion from CNI to MMF (Figure 2) and in two patients it seemed to decrease again after reconversion from MMF to CNI (Table 3). Conversion to MMF also resulted in phenotypical changes within the Treg population. After conversion, an increase of CD25 expression level (i.e.,

mean fluorescence intensity) on Foxp3$^+$ cells was observed (Figure 3) suggesting that these cells may have become more susceptible to IL-2 signals.

IL-2 has been shown to be of critical importance for the function and survival of Treg[7,15]. Immunosuppression interfering with the IL-2 pathway may therefore affect the suppressive capacity and homeostasis of Treg. CNI interfere with the nuclear factor of activated T-cells (NFAT) signaling pathway that is an important regulator of IL-2 production. Recently, a close relationship between NFAT and FOXP3 was shown to be critical for Treg function[16]. By interfering with this interaction, CNI are believed to inhibit Treg[17]. In experimental bone marrow transplantation CsA administration inhibited Treg function *in vivo* by reducing IL-2 production[5]. Exogenous IL-2 was shown to overturn the suppressed FoxP3 expression and the suppressive function of Treg induced by cyclosporine A, underlining the importance of IL-2 for Treg. In this same study it was shown that rapamycin and MMF had no detrimental effect on the function of Treg, both *in vitro* and *in vivo*[5]. There is now accumulating evidence that MMF like rapamycin spares Treg in their survival and function[18]. It was shown in different experimental transplant models that MMF does not interfere with Treg function and positively affect tolerance induction[5, 9, 13]. In the clinical setting, Segundo et al.[19] recently demonstrated that the use of CNI, and not rapamycin, is associated with a reduced percentage of circulating Treg in renal transplant recipients. To our knowledge for the first time our study shows that conversion to MMF therapy can actually reverse the suppressive effect of CNI on the percentage of Treg in circulation.

In our study, we showed that the percentage of CD4$^+$CD25$^+$Foxp3$^+$ Treg increased after conversion from CNI to MMF. Interestingly, this increase was accompanied by a significant increase of CD25 expression on CD4$^+$Foxp3$^+$ Treg (Figure 3). However, 1 month after conversion, when over 75% of CD25 is effectively blocked by Daclizumab (Figure 1C), CD25 expression was significantly decreased (Figure 1B), both on CD4$^+$Foxp3$^+$ and CD4$^+$Foxp3$^-$ cells. The CD25 downregulation was not due to epitope blocking because the antibody used (clone M-A251) for detection recognizes a different (independent) epitope than Daclizumab. Contrarily, loss of CD25 expression may be due to Daclizumab-associated receptor internalization or shedding. Kreijveld et al.[20] reported that during Daclizumab treatment in kidney transplant recipients levels of Foxp3$^+$ Treg decreased. In contrast, Kohm et al.[21] showed a functional inactivation, rather than depletion, of Treg by CD25 antibodies. These contradicting findings may be due to the intensive immunosuppressive therapy following kidney transplantation, which may act synergistically on Treg. In our study, monotherapy with MMF in combination with one gift of Daclizumab did not result in decreased levels of CD4$^+$Foxp3$^+$ Treg 1 or 3 months after conversion, but in an increase at 6 months and beyond (data not shown) and was supported by analysis of CD4$^+$CD25$^+$CTLA-4$^+$ T cells.

The possible negative effect of CNI on tolerance by affecting Treg is accompanied with non-immunological side effects, in particular nephrotoxicity, causing significant morbidity. This initially randomized study shows that conversion from a CNI based immunosuppressive regimen to MMF monotherapy improves renal function with a low risk of acute rejection, supporting

previous conversion studies in liver transplant recipients[11, 12]. Two patients developed an episode of acute rejection, one at 1 month and the other at 6 months after conversion. The patient who developed acute rejection at 6 months after conversion had an MMF blood trough level of 1.8 mg/L at the time of rejection, whereas being approximately 7 mg/L in the months before. After initial improvement with steroids, biliary obstruction was diagnosed, which may have contributed to the portal cellular infiltrate seen in the liver biopsy. No differences in MMF trough levels were seen in the other patient, who developed acute rejection, and graft function normalized after treatment.

In conclusion, we show that CNI withdrawal in liver transplant recipients increases the proportions of circulating $CD4^+CD25^+Foxp3^+$ Treg. Whether this has important implications on the balance between alloreactivity and tolerance remains to be determined. Clinically, conversion from CNI to MMF clearly results in an improvement of renal function and a decrease in blood pressure.

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# Chapter 9

## Summary

## General Discussion

## Samenvatting en Conclusie

## SUMMARY

For the last decades, solid organ transplantation is the most appropriate treatment for patients with organ failure in terms of improving their quality of life and survival. Without any therapeutic interference, the immune system of the patient will reject the new organ, because it is regarded as foreign. Acute cellular rejection can occur within several days after transplantation and is mediated by Teff cells that will proliferate in response to antigens of the allograft. To prevent allograft rejection, organ transplant patients need immunosuppression consisting of a combination of immunosuppressive drugs (a calcineurin inhibitor, a proliferation inhibitor and steroids). However, these drugs do not only suppress immune responses triggered by donor antigens (anti-donor reactivity). They generally inhibit immune responses including those to pathogens, leading to an increased number of infections. In particular CNIs and mTOR-inhibitors have toxic side-effects to the kidneys and some classes of these immunosuppressive drugs can cause cancer which can also be a major drawback for their long-term use. This a-specific mode of action during the life long administration of immunosuppressive drugs demands a more specific treatment after transplantation. However, for graft acceptance, suppression of ongoing immune responses by immunosuppressive drugs is essential. Preferably, these drugs must specifically target the donor-reactive Teff cells, which is rather complicated to establish.

The $CD4^+CD25^{bright}FoxP3^+CD127^{-/low}$ T cells comprise an endogenous specialized subset of T cells that govern immune responses against self-antigens and are therefore assigned as regulatory T cells (Treg)[1]. These 'suppressor' cells may control proliferative immune responses by effector T cells (Teff) that are directed against the allograft. Next to the $CD4^+CD25^{bright}FoxP3^+CD127^{-/low}$ T cells, there is a diversity of Treg subsets with immunosuppressive activities: $CD4^{neg}CD8^{neg}$ T cells[2], γδ-cells, natural killer T cells[3], IL-10-producing Tr1 T cells[4], TGF-ß-producing Th3 T cells[5] and $CD8^+CD25^+FoxP3^+$ T cells. This dissertation focuses on the role of $CD4^+FoxP3^+CD25^{bright}CD127^{neg/low}$ T cells in transplantation patients. In addition, the effect of immunosuppressive medication on their generation, characteristics and function is studied. As an endogenous source, these Treg have a lot of potential to induce tolerance as they can suppress the proliferation of donor-reactive Teff cells, which are held responsible for the onset of cellular rejection of the graft after transplantation.

The main mechanism of action of the most often used immunosuppressive drugs for transplant patients is that they directly and indirectly inhibit the proliferation of activated T cells. These agents have different targets on the T cell as they can block signal 1; T-cell receptor triggering (CNIs) or signal 3; the cytokine signal (anti-CD25, Jak-inhibitor and mTor-inhibitor). These immunosuppressive agents will not only exert their effect on activated T cells as the may influence the frequency, phenotype and function of $CD4^+CD25^{bright}FoxP3^+CD127^{-/low}$ T cells. Recent experimental studies showed that these agents do not only hamper the function of Treg but may also contribute to the mechanisms that control alloreactivity. Therefore it is tempting

to speculate that immunosuppressive drugs may even be used to manipulate the immune system in favor of these T cells.

Little is known about the specific effects of immunosuppressive drugs on immune regulation by Treg in humans. The **central aim of this thesis** is to investigate the effect of immunosuppressive agents on immune regulation by $CD4^+CD25^{bright}FoxP3^+CD127^{-/low}$ T cells in the peripheral blood of organ transplant patients. The results in this dissertation may contribute to a better understanding and may allow skewing of the immune system towards immune regulation by a fine-tuned immunosuppressive therapy that is beneficial for the patients' Treg. An overview of the diverse Treg subsets, the characteristics of $CD4^+CD25^{bright}FoxP3^+CD127^{-/low}$ Treg and the different types of immunosuppressive drugs with their side-effects and mechanisms of action at the cellular immunological level are described in **chapter 1**.

In **chapter 2** we report that antigen-specific Treg are induced in kidney transplant patients who received triple therapy consisting of cyclosporine, MMF and prednisone. The frequency and function of $CD25^{bright}$ T cells of nine stable kidney-transplant patients before and 0.5 - 2 years after clinical kidney transplantation were measured. Within this relatively short period after transplantation, patients can be considered as fully immune suppressed. To more accurately study the function of $CD25^{bright}$ T cells post-transplant, we modified the 'conventional' suppression assay in which pre-transplantation Treg function is determined by coculture with allo-activated pre-transplantation Teff and post-transplantation Treg function by coculture with allo-activated post-transplantation Teff. To exclude the influence of other confounding factors after transplantation on the proliferation of Teff cells, which is the read-out of the suppression assay, the suppressive capacities of Treg pre- and post transplantation were compared, whereas the Teff population was kept constant (only of pre-transplantation). After transplantation, the percentage of $CD4^+CD25^{bright}$ T cells significantly decreased. However, the lower percentage of post-transplant $CD4^+CD25^{bright}$ T cells was not associated with reduced, but rather improved suppressor function of these cells. The proliferative response of pre-transplant Teff to donor-antigens was more profoundly suppressed by post-transplant Treg than by pre-transplant Treg and was comparable with third party antigens. In immune suppressed kidney transplant patients, the donor-directed suppressive capacity of $CD4^+CD25^{bright}$ Treg improved, which may contribute to the development of donor-specific hyporesponsiveness against the graft.

In **chapter 3,** we questioned whether donor-specific $CD4^+CD25^{bright}FoxP3^+$ cells are generated after clinical kidney transplantation. Therefore, in a prospective study, the phenotype and function of peripheral $CD4^+CD25^{bright}$ T cells of patients before and 3, 6 and 12 months after kidney transplantation were analyzed. Patients were equally randomized to treatment arm 1 with tacrolimus/rapamycin or arm 2 with tacrolimus/MMF. The immune regulatory capacities of $CD4^+CD25^{bright}$ T cells were assessed by their depletion from PBMC and in co-culture

with Teff cells in the MLR. In the first year after transplantation, the number and proportion of $CD4^+CD25^{bright}$ T cells significantly decreased. In the MLR, we observed donor-specific hyporesponsiveness in the presence of significantly increased proliferation to third- and fourth party antigens. Functional analysis of $CD25^{bright}$ cells showed that the effect of depletion of these cells from PBMC, as well as their suppressive capacities in co-culture with donor antigens stimulated Teff cells significantly improved. Moreover, the difference in proliferation by Teff cells between anti-donor and anti-third party stimulation became apparent at 6 months after transplantation. Although the combined use of tacrolimus and rapamycin results in a synergistic inhibitory effect, there was no difference in the number and function of Treg between the patients in the two arms of therapy. Thus, donor-specific $CD4^+CD25^{bright}$ Treg are induced even under effective immunosuppression in renal recipients within the first year after clinical kidney transplantation.

In **chapter 4, 5 and 6,** rabbit anti-thymocyte globulins (rATG) are the major topics. One of the mechanisms of action of rATG is that they can convert $CD4^+CD25^{neg}FoxP3^{neg}$ T cells from healthy individuals into $CD4^+CD25^+FoxP3^+$ T cells as has been described in the literature. We investigated the effect of rATG-induction therapy on Treg from kidney transplant patients. Moreover, two *in vitro* studies describe the *in vitro* effect of rATG on PBMC from patients with end stage renal diseases who are candidates for rATG-induction therapy. The induction of Treg by rATG and the effect of rATG on the function of natural occurring Treg were examined on PBMC of candidates for kidney transplant patients and of healthy individuals.

In **chapter 4,** the effect of rATG on the phenotype, frequency and function and of peripheral immunoregulatory $CD4^+$ T cells in kidney transplant patients was established in a prospective and controlled study. Patients received ATG-induction therapy and triple therapy consisting of tacrolimus, MMF and steroids. The control group received triple therapy only. Pre-transplant levels of $FoxP3^+CD127^{-/low}$ T cells were 6% of $CD4^+$ T cells. One week post-ATG treatment, no measurable numbers of Treg were present. After 4 weeks, the cell numbers of $CD4^+FoxP3^+CD127^{-/low}$ T cells slowly reappeared and thereafter remained low. At 14 weeks, a significant shift towards the $CD45RO^+CCR7^+$ (central memory) phenotype within $CD4^+FoxP3^+$ T cells was observed. At 26 weeks, the proliferative alloresponses of the PBMC and $CD25^{-/dim}$ Teff profoundly decreased compared with pre-transplant, whereas the regulatory capacity of the $CD25^{bright}$ T cells, of which 90% consisted of $FoxP3^+CD127^{-/low}$ T cells, remained unaffected. The $CD25^{bright}$ T cells suppressed the anti-donor and third party responses. These findings show that rATG therapy does not spare peripheral Treg *in vivo*, but after regeneration preserves their suppressive activity.

**Chapter 5** describes the *in vitro* effect of rATG on the induction of Treg from blood cells of patients with end-stage renal disease who are candidates for transplantation and rATG-induction therapy. The rATG-induced Treg were in depth characterized and compared with naturally occurring $CD4^+CD25^+FoxP3^+$ T cells. The $CD25^{neg}$ T cells of pre-transplant patients

and healthy controls were stimulated with rATG or control rabbit immunoglobulins (rIgG) for 24 hr. After 24 hr of culture, the percentage of $CD4^+CD25^+FoxP3^+CD127^{neg/low}$ T cells and $CD8^+CD25^+FoxP3^{neg}CD127^+$ T cells became higher in the rATG-treated samples compared with the rIgG-treated samples. There was no difference between patients and healthy individuals. The rATG-induced $CD4^+CD25^+$ and $CD8^+CD25^+$ T cells inhibited the allogeneic responses of $CD25^{neg/dim}$ Teff as vigorously as natural $CD4^+CD25^+$ T cells. However, the proportion of $FoxP3^+$ within the top 2% rATG-induced $CD4^+CD25^+$ T cells was lower than within the natural $CD4^+CD25^+$ T cells. The mRNA levels of IL-27, IL-10, IFN-γ, perforin and granzyme B were markedly higher than in the natural $CD25^+$ T cells, whereas CTLA4, TGF-β and RORγt were lower. RATG allows the induction of cells with suppressive activities from patient PBMC *in vitro*. In comparison with natural Treg the rATG-induced Treg are distinct on their phenotype and gene-expression profile, but have similar regulatory activities. Thus, rATG may beneficially contribute to the mechanisms that control alloreactivity.

In **chapter 6,** we elaborated on the *in vitro* Treg-induction capacity by rATG and addressed whether rATG convert T cells into functional $CD4^+CD25^+FoxP3^+CD127^{neg/low}$ T cells in the presence of drugs that may hamper their induction and function, i.e. CNIs. $CD25^{neg}$ T cells from healthy individuals were stimulated with rATG or control rIgG in the absence and presence of tacrolimus for 24 hr in culture. Interestingly, $FoxP3^+$ T cells were also induced when tacrolimus was present in the rATG-cultures. Blockade of the IL-2 pathway did not affect the frequency of rATG-induced $FoxP3^+$ T cells. The rATG-tacrolimus induced $CD25^+$ T cells inhibited proliferative responses of alloantigen-stimulated Teff cells as vigorously as rATG-induced and natural $CD4^+CD25^+FoxP3^+CD127^{neg/low}$ T cells. In contrast to natural $CD25^+$ T cells, rATG-induced $CD25^+$ T cells abundantly expressed IL-10, IL-27, IFN-γ, perforin and granzyme B mRNA, whereas FoxP3 mRNA was expressed at a lower level. These mRNA data were confirmed in Treg from kidney transplant patients who received rATG induction therapy. The findings of this study indicate that tacrolimus does not affect the induction and function of $CD4^+CD25^+$ T cells, suggesting that rATG may induce Treg in patients who receive tacrolimus maintenance therapy.

In **chapter 7,** we focused on the third signal that is required for T-cell growth and activation; the cytokine signal. Cytokines of the IL-2 family that signal via the common γ chain activate the Jak-STAT pathway. These cytokines are critical factors for the growth, differentiation and function of $CD4^+CD25^{neg/dim}$ Teff cells and $CD4^+CD25^{bright}$ Treg. In this study, we examined whether CP-690,550 has an effect on the regulatory activity of $CD4^+CD25^{bright}FoxP3^+CD127^{-/low}$ T cells. The effect of CP-690,550 on the IL-2-induced intracellular STAT5-phosphorylation of Teff and Treg was investigated in peripheral blood samples from healthy controls by phospho-specific flow cytometry. The function of Teff and Treg from healthy individuals and kidney transplant patients was examined by MLRs and suppression assays in the presence and absence of CP-690,550. IL-2 induced phosphorylation of STAT5 was significantly higher for $CD4^+CD25^{bright}$

Treg than for $CD4^+CD25^{neg}$ Teff. In the presence of 100 ng/mL CP-690,550, a clinically relevant dose, the IL-2 induced P-STAT5 was partially inhibited in $CD4^+CD25^{bright}$ Treg, whereas almost completely blocked in the Teff. The $IC_{50}$ was 2-3 times higher for Treg than for Teff cells. In the presence of CP-690,550, the Treg exhibited additional suppressive activities on the alloactivated proliferation of Teff. Also the $CD4^+CD25^{bright}$ Treg from kidney transplant patients receiving CP-690,550 therapy vigorously suppressed the proliferation of Teff. These findings show that the Jak-inhibitor CP-690,550 effectively inhibits Teff function but preserves the suppressive activity of $CD4^+CD25^{bright}$ Treg.

In **chapter 8,** we investigated the effects of conversion from CNI to IMPDH inhibitor (MMF) monotherapy on renal function and on Treg frequency and phenotype in liver transplant recipients. Patients with renal impairment on CNI were converted to MMF and received a single dose of IL-2-receptor blocking antibody (Daclizumab). Control patients continued CNI treatment. Renal function rapidly and significantly improved after conversion. Daclizumab treatment resulted in blocking of CD25 at month 1 and a significant reduction in the percentage of $CD4^+CD25^+$ cells but did not affect the percentage of $CD4^+CD25^+Foxp3^+$ cells. Six months after conversion to MMF, the percentage of $CD4^+CD25^+Foxp3^+$ cells increased by 125%. FOXP3 mRNA analysis of mononuclear cells confirmed the enrichment of Foxp3 in peripheral blood. Interestingly, the CD25 expression level on $CD4^+Foxp3^+$, but not $CD4^+Foxp3^{neg}$ cells significantly increased compared with pre-conversion. Thus, conversion to MMF increases the percentage and CD25 expression of $CD4^+Foxp3^+$ cells indicating that MMF therapy can overturn the repressive effect of CNI on circulating Treg levels and therefore may promote Treg-mediated suppression of alloreactivity.

## GENERAL DISCUSSION

Hyporesponsiveness to donor-antigens may occur through different mechanisms including T-cell and B-cell deletion, anergy, and immune regulation. Transplanted patients would benefit from immune-specific therapy that governs immune reactions which are specifically directed against the allograft. The aspecific mechanisms of actions of the currently used immunosuppressive drugs lead to adverse reactions. Therefore, a cell based therapy specifically directed against the donor graft may lead to an early onset of donor specific hyporesponsiveness as compared with treatment with immunosuppressive drugs. Trials with Treg are indeed ongoing as Tr1 cells are already infused into diabetes patients[6] and used in hematopoietic stem cell transplantation[7, 8]. *In vitro* expanded $CD4^+CD25^+CD127^-$ Treg have been transferred to patients with acute or chronic graft versus host disease (GvHD) and allowed for significant alleviation of the symptoms[9].

However, there are several major drawbacks with the *in vitro* expansion and reinfusion of $CD4^+CD25^{bright}CD127^{-/low}FoxP3^+$ T cells. First, isolation of a 100% pure $FoxP3^+$ T cell population is challenging due to the intracellular localization of FoxP3. Thus, surrogate surface markers (CD25, CD127) can be used for isolation to enrich for FoxP3. Isolation of $CD25^{bright}CD127^{-/low}$ T cells seems to increase the purity for FoxP3 compared with isolation of T cells solely based on $CD25^{bright}$ [10]. Secondly, the culture conditions (medium, growth factors, time-span) to obtain a high yield of donor-specific $CD25^{bright}CD127^{-/low}$ T cells are not fully optimized yet. Thirdly, once injected, the FoxP3-expression of the expanded cells might be unstable[11] and can be lost within an inflammatory and activated microenvironment which may lead to the generation of IL-17 producing cells[12] or pathogenic memory T cells *in vivo*[13].

Hence, it seems more attractive to manipulate the immune system with immunosuppressive drugs by skewing the Teff-Treg balance towards the Treg. This can be done by *in vivo* generation of Treg with current therapeutic drug regimens. Such a drug-therapy should stimulate immune regulation by its beneficial effects on Treg and would be more easily and rapidly applicable into the clinic. It can be just a matter of reducing, adjusting and fine-tuning the doses of immunosuppressive drugs to beneficially influence the Teff to Treg ratio in order to induce tolerance. With this thesis, we aimed to reveal the effect of immunosuppressive agents on immune regulation in order to find the most optimal combination for a positive skewing of Treg by their induction, expansion or sparing in organ transplant patients.

### *The influence of calcineurin inhibitors on regulatory T cells*

In line with others[14, 15], we show in **chapter two** that cyclosporine, as part of the immunosuppressive drug regimen of kidney transplant patients, contributes to a decrease in the frequency of $CD4^+CD25^{bright}$ T cells. This decline in the frequency is not only due to the CNI, but can also be attributed to steroids. There is evidence showing that cyclosporine accounts for this decrease in the frequency. Cyclosporine prevents the induction of $FoxP3^+$ in the MLR[16] and that it affects

the frequency of $CD4^{+}CD25^{bright}$ T cells in liver transplant patients [14, 17]. Moreover, it has been reported that cyclosporine affects another subset of Treg, the $CD8^{+}CD28^{neg}$ T cells[18]. It is obvious that Treg need IL-2 to maintain their homeostasis and when the IL-2 production is blocked, they immediately go into apoptosis.

In contrast to their frequency, we demonstrate that the function of the remaining $CD25^{bright}$ T cells when analyzed ex vivo was not affected by the immunosuppressive therapy that included cyclosporine, which is in line with another group[18]. TCR stimulation and cytokine signaling are both required for Treg to properly exert their function. Hence, Treg function remains possible because not all Teff cells are affected in their IL-2 production and there is enough IL-2 left for Treg to exert their suppressive activities. Secondly, functional Treg are present because they consume other cytokines of the IL-2 family, e.g. IL-15, of whom the production is calcineurin-independent. Third, one has to take into account that there is also a small amount of calcineurin-independent secretion of IL-2 itself that could explain the IL-2 that is present[19].

Because of the inhibitory effect of immunosuppressive drugs and other factors on Teff cell proliferation which is the read-out of Treg function post-transplant, we adjusted the 'conventional' suppression assay to determine Treg function post-transplant, **chapter two**. The Teff of pre-transplant was used as the read-out for Treg function post-transplant instead of the Teff post-transplant, thereby excluding any intrinsic defect in the proliferative capacity caused by immunosuppressive drugs. By using this different suppression assay, we concluded that cyclosporine does not affect Treg function.

An interesting future experiment could be to culture Treg in the presence of cyclosporine for 24 hr and to subsequently analyze their suppressive capacity in a suppression assay. However, Treg probably will not survive the 24 hr of culture without TCR stimulation and may die; therefore this can only be done in the presence of TCR-stimulation or IL-2 stimulation. **Chapter seven**, the Jak-inhibitor study, also describes the effect of cyclosporine on the function of Treg from kidney transplant patients treated with cyclosporine (the control group). In the presence of clinically relevant levels of cyclosporine in the suppression assays, Treg from the cyclosporine-treated patients were still able to additionally suppress the proliferation of the Teff cells that survived. In this study, the results that were found in **chapter two** were confirmed, as we show again that Treg can exert their function during exposure to cyclosporine.

After analyzing the effect of CNI's on the suppressive activity of Treg, we subsequently examined their effect on the induction of Treg *in vitro*. In the literature, it has been shown that rATG induces Treg in $CD25^{neg}$ cells from healthy individuals[20]. However, in **chapter four** we stated that Treg levels did not increase in kidney patients after rATG-treatment but declined and slowly recovered during standard immunosuppression consisting of tacrolimus, MMF and prednisone. Considering that IL-2 is an essential growth factor that is required for the generation of Treg[21, 22] we speculated that the replenishment of Treg into the peripheral compartment of these patients might be delayed by tacrolimus that prevents Treg induction. This hypothesis was studied *in vitro* by Treg-induction experiments in the presence of tacrolimus. Interestingly,

Treg were generated by rATG even in the presence of tacrolimus, anti-IL-2R antibody or anti-IL-2 antibody. The stimulatory effect of rATG, which includes enhanced NFAT1-expression[20] is dominantly present and can overturn the repressive effect of tacrolimus. Therefore, rATG-induction therapy may allow the replenishment of functional Treg under standard therapy.

Furthermore, an important finding was that CNI therapy does not changes the phenotype and composition of naïve/memory Treg post transplant as we observed in **chapter four** in the control group for the rATG-group that received tacrolimus, MMF and prednisolone. According to our findings, the CNI-dose that is used for organ transplant patients is deleterious for Teff cells and does not affect Treg. The CNI dose can be reduced, to find a proper balance that preserves Treg numbers; however, it is questionable whether this will not lead to an increased number of (acute) rejections. Most importantly, CNIs allow immune regulation of the remaining Teff by Treg that are spared.

*Immunosuppressive therapy and the induction of donor-specific regulatory T cells*

In **chapters two, three, and four** we show that the proliferation of donor-directed immune responses by Teff cells is significantly lower than the third party immune responses after coculture with Treg post-transplant in the suppression assay. This observation implies that donor-specific Treg are generated within 1 year after transplantation under the influence of maintenance therapy (consisting of a CNI or rapamycin, MMF and prednisolone). Donor-specific Treg are generated in the periphery and can be considered as adaptive Treg. The immune system adapts to donor antigens by the generation of these donor-specific Treg. Whereas the third party response of PBMC improves as can be seen in **chapter three**, the responses against the donor are lower and remain at a stable level. Inversely, the donor-specific regulation improves after transplantation, whereas the regulation of third party responses remains stable. It is unknown whether these Treg are generated as a result of adaptation; as they may also be characterized as natural occurring Treg that respond by cross reactivity and recognize part of the donor-antigens as their own. For instance, Treg may suppress responses against broad HLA-antigens (e.g. HLA-Bw4 and HLA-Bw6). On the other hand, Treg suppression might be HLA-restricted in a way that Treg only inhibit responses that have a certain HLA- A, B or DR subtype (e.g. if the patient has a HLA-B51-specificity, Treg of the patient may recognize HLA-B51 that may be present on donor (stimulator cells in the MLR). The Treg of the patient will suppress the immune responses that are considered to be directed against these 'self' antigens.

In **chapter four**, we determined the function of the Treg present in the peripheral blood at 26 weeks after transplantation when the number of Treg in the peripheral blood was sufficient to isolate the induced Treg and to study their function. The Treg from patients in the rATG-group seem to govern the donor-directed immune responses by Teff cells more vigorously than the third party immune responses. The Treg from kidney transplant patients in the control group (thus without rATG induction therapy) did not show this trend, indicating that rATG-induction therapy may trigger the generation of donor-specific Treg.

A condition for the induction of Treg is T cell receptor signaling which can be triggered by the presence of suboptimal, low dose antigen. Furthermore, cytokines and growth factors are also required. Thus, donor antigens that are present after transplantation and rabbit proteins from the rATG mixture both may serve as the trigger for activation of the immune system and the following induction of donor-specific adaptive Treg. Another phenomenon that may explain the induction of donor-specific Treg is the homeostatic proliferation after rATG-induced T-cell depletion in the presence of donor-antigens. This homeostatic proliferation accounted for the massive increase in the proportion of memory FoxP3$^{+}$CD45RO$^{+}$ T cells that may have generated donor-directed specificity. Taken together, donor-specific regulatory T cells can be generated during standard maintenance immunotherapy by their regeneration in the thymus or may be induced in the presence of donor antigens in the periphery. RATG-induction therapy may accelerate the induction of donor-specific Treg process by homeostatic proliferation after immune depletion and by the induction of T cells with suppressive function.

*In vitro induction of regulatory T cells by rATG*

Apart from depletion of lymphocytes *in vivo*, rATG induces Treg *in vitro*[20]. One can speculate about the active component in the rATG mixture that *in vitro* triggers the induction of CD25 and FoxP3-expression. Although this thesis is predominantly focused on FoxP3$^{+}$CD25$^{+}$ T cells, interestingly, rATG also induced FoxP3$^{neg}$CD25$^{neg/dim}$ T cells with suppressive activities after 24 hr of culture (30% inhibition at a [Treg: Teff] ratio of 1:10). Apparently, rATG confers regulatory activity to cells in a FoxP3-independent manner.

As we demonstrate Treg induction in CD25$^{neg}$ cells the absence of allo-antigen stimulation, it remains to be investigated whether there is conversion of allo-activated CD25$^{neg}$ Teff cells to Treg during the 7-day MLR in the presence of rATG. If this phenomenon occurs in the MLR, it will have a positive effect in the 7-day suppression assay, in which alloactivated Teff cells were cocultured with natural Treg in the presence of rATG. Potential rATG-induced Treg may synergise with the existing natural Treg in the downregulation of alloactivated Teff cells. We show in **chapter five** that the rATG affected alloactivated Teff response is additionally inhibited by Treg.

It is interesting to dissect the components of the rATG-immunoglobulins mixture. The human thymocyte suspensions are derived from thymus fragments that were surgical waste during cardiac surgery in children. Thymus donors must be less than 14 years old and fit the selection criteria. They must also be at low risk for Creutzveldt-Jacob disease and other transmissible subacute spongiform encephalopathies. Blood samples from these donors are screened for a number of viruses. After sensitization of specific pathogen-free rabbits with human thymus fragments, γ immune globulins are purified and pasteurized. The immunosuppressive product contains cytotoxic antibodies directed against a broad array of surface antigens expressed on T cells and adhesion molecules including CD2, CD3/TCR, CD4, CD8, CD11a, CD25, CD28, CD45, HLA-Class I and II.

Factors that influence the *in vivo* induction of Treg include the strength of the co-stimulatory molecules and TCR-mediated signals, microenvironmental factors and the cytokine milieu[23-25]. Rabbit proteins could be the potential TCR trigger for *in vitro* Treg induction. Taking this into account, we incubated human $CD25^{neg}$ T cells with control polyclonal rabbit immunoglobulins. After 24 hr there was no induction of CD25 or FoxP3 expression. Moreover, Feng et al.[20] reported that horse-ATG does not have the ability to induce or expand Treg, showing that the part of the immunoglobulins that is arranged in a different species does not stimulate the TCR. Apparently, the variable part of the immunoglobulins that is directed against epitopes on human T cells is responsible for TCR stimulation. For future research, it would therefore be interesting to separate the variable from the constant part of the antibodies after purification of the antibodies from rabbits and incubate these parts separately with human $CD25^{neg}$ T cells *in vitro*. Another idea for future research is to investigate whether a non-depleting rATG mixture that is directed against the lymphoblastic Jurkat T-cell line is also able to induce Treg *in vitro*. It has been reported that Fresenius ATG did not enhance Treg levels in kidney transplant patients after transplantation[26].

The development of immune regulation and immunoregulatory mechanisms is often elicited as a feedback mechanism during alloactivation to prevent the outgrowth of aggressive Teff cells. Thus, rATG induces CD25-expression on $CD25^{neg}$ cells and when they become activated, Treg arise to downregulate this activation status. As it is well known that FoxP3 is an activation marker, one could speculate that the FoxP3-expression induced in T cells induced by rATG functions as a T-cell activation marker. Other evidence for this statement was derived from the mRNA expression profiles that showed that granzyme B also served as an activation marker as the granzyme B-expression by rATG-induced $CD25^+$ T cells did not result in cytotoxicity. In our hands, high mRNA- and protein levels did not automatically correlate with functional activity. To prove that Treg induction was rATG-specific, and not the result of a-specific T-cell activation, we included an essential control experiment. We showed that CD3/CD28 activated $CD25^+$ cells did not inhibit allogeneic immune responses and that our rATG-induced $CD4^+CD25^+$ and $CD8^+CD25^+$ T cells were able to suppress allogeneic immune responses, by not only autologous Teff cells but even allogeneic Teff cells. The latter finding shows that they can suppress the proliferation of each activated T cell, independent of the HLA-specificity. Moreover, they suppress their cytokine production and their proliferation, which is a characteristic of natural $CD4^+CD25^{bright}$ Treg, indicating that there is no 'sopping up' or scavenging of growth factors which is a characteristic of activated T cells.

If granzyme B is not responsible for the suppressive capacity of the rATG-induced Treg, which molecule ìs? Besides IL-10 and granzyme B, we found that IL-27 was significantly upregulated. However, one of the functions of IL-27 is that it upregulates granzymes and perforins and stimulates cytotoxicity via $CD8^+$ T cells. Cytotoxicity may play an important role in elimination of T cells *in vivo*.

Future research may focus on the stability of the rATG-induced Treg that can be tested according to the methylation-sites of FOXP3. These methylation sites are found to be a novel target to explore within the experimental field of Treg. True *'bona fide'* Treg can be identified in a heterogeneous population including activated T cells that transiently express FoxP3 and Treg that constitutively express FoxP3[27]. It would therefore be interesting to unravel the methylation-status of FoxP3 Treg-specific demethylated regions (TSDR) to determine whether the expression of FoxP3 is stable or not; this could reveal something about the stability and presence of Treg. To become Treg, T cells can be incubated with a DNA demethylation agent. Demethylation of FOXP3 gene has been shown to increase the number of Treg in experimental models[28]. Wieczorek et al. analyzed FOXP3 demethylation prior to and after rATG treatment. According to a decline of demethylated sites in the FOXP3 gene on the DNA after rATG-treatment, they found that Treg levels decreased[27]. They concluded that DNA Methylation Analysis of FOXP3 can be used to quantify Treg because demethylation of the FOXP3 TSDR is a feature of stable '*bona fide*' Treg[27].

*rATG induction therapy depletes regulatory T cells*

The clinical relevance of the rATG-induced T cells with suppressive function in the presence of high dose rATG that is given in the clinic is hard to deduce. In **chapter four** we reported that the $CD4^{+}CD25^{bright}FoxP3^{+}CD127^{-/low}$ T cells are completely depleted after three doses of 2 mg/kg rATG and slowly reappear slowly at 4 weeks after rATG-induction therapy. Their level does not reach baseline in the first 26 weeks after treatment. Other groups have demonstrated that the recovery of $CD4^{+}$ T cells can take for more than five years and the levels still do not reach baseline levels[29]. The origin of Treg that reappear in the peripheral blood at 14 and 26 weeks after rATG-treatment is unknown. They might be replenished by the lymphoid organs or they might be newly thymus-derived emigrants. The idea of thymus-derived generation is supported by a study with non-human primates, in whom rATG-mediated T-cell depletion occurs in all compartments of the body. Regeneration is only possible by the thymus which is the only source of Treg present in the periphery.

The phenotype of the $FoxP3^{+}$ T cells present in the blood after rATG-treatment changes, as the number of naïve $FoxP3^{+}$ T cells decreased after rATG-treatment and memory $FoxP3^{+}$ T cells increased. This change is due to the homeostatic proliferation of memory T cells that are replenished. Otherwise, they could be converted from naïve T cells into memory T cells in the periphery in the presence of suboptimal doses of antigens (induction). Whether there is induction *in vivo* (thus conversion from $CD25^{neg}FoxP3^{neg}$ T cells to $CD25^{+}FoxP3^{+}$ T cells) is complicated to investigate in patients. We did not find an increase in the proportion or absolute numbers. Publications with enhanced Treg frequencies after rATG-induction therapy or that demonstrate the suppressive capabilities of Treg immediately after rATG-induction therapy are lacking. However, Treg induction is still an option when rATG is present in the body. Thus, in the first weeks after rATG-treatment, rATG may induce T cells, until it is broken down. In contrast, we

found that in between rATG-treatment and 4 weeks after treatment, T-cell depletion prevailed and no T cells were present in the circulation, **chapter four**. After rigid depletion of T cells, they start to slowly recover so conversion of Teff into Treg may occur. Although patients receive maintenance therapy consisting of a CNI, this should not be a restriction for Treg-induction, as clearly demonstrated in **chapter five**. The only explanation for the absence of enhanced Treg numbers could be the absence of components of the complement-system *in vitro*. RATG eliminates peripheral blood cells by inducing cell-death via complement-dependent lysis. In **chapter five**, we demonstrate *in vitro* apoptosis of PBMC at high concentrations of rATG that resulted in an even higher proportion of Treg among the cells that survived. In contrast, *in vivo*, all T cells die after rATG-induction therapy and there is no sparing or survival (of a selective subset) of cells. Therefore, the confounding factor is to be identified.

The findings described in this thesis can therefore contribute to the determination of the right choice and dosage adjustment of immunosuppressive drugs. This dissertation may contribute in making the right combination of 'pro-tolerogenic' drugs. According to the findings of this thesis, some recommendations for treatment of transplant patients with rATG induction therapy can be made. First, rATG induction therapy should be given to candidates for kidney transplantation prior to transplantation; to deplete Teff cells and to create the most optimal circumstances for antigen-specific induction of Treg before inflammation is elicited, instead of during inflammation. A study in which $CD4^+FoxP3^+$ regulatory T cells were quantified in kidney allograft infiltrates showed that their proportion of $CD4^+$ T cell infiltrates was higher in the borderline change and subclinical cellular rejection biopsies than in acute cellular rejection biopsies. In the acute phase of the allogenic response, these regulatory T cells could act to diminish the interstitial inflammation and its associated lesions[30]. Secondly, if potent enough to prevent graft rejection, patients should receive a rATG dose of <2 mg/kg (at this particular dose, total depletion of Treg occurs), to allow Treg induction. Partial depletion might trigger immune activation, induction of regulatory T cells and expansion of the remaining natural Treg that survive. In this manner, Treg numbers will recover earlier because there is no harsh immunodepletion. In particular the recovery of Treg by the thymic output and their induction in the periphery in the presence of donor-antigens may contribute to donor-specific hyporesponsiveness, which is favorable for the patient. Moreover, in the presence of a low rATG dose, the immune system will not be prone to (viral) infections, as with high doses but is able to cope with these pathogens by triggering a proper immune response.

*Calcineurin-free protocols and regulatory T cells*

As shown by our data, Treg do not require STAT5 signaling to exert their function. Inhibition of Jaks which results in the suppression of STAT5 phosphorylation in the presence of clinically relevant doses of CP-690,550 does not influence their suppressive activities. We therefore suggest that STAT5 signaling is only considered necessary for the induction of *de novo* FoxP3-expression and thus for the generation of *de novo* Treg. The clinical significance of CP-690,550 in

the prevention of graft rejection is currently still investigated in a clinical trial phase. The results of a randomized pilot trial with CP-690,550 (part of the phase II study) showed that treatment of kidney transplant patients with CP-690,550 did result in clinically relevant immunosuppression comparable with the tacrolimus control group[31]. If CP-690,550 prevents graft rejection and improves graft function by the end of the trial phases, it can be considered as a drug therapy that is 'harmless' to Treg function, whereas Treg frequency is affected. A previous paper from our group show that Treg numbers recovered quickly after CP-690,550 therapy[32]. Donor-specific immune regulation by the immune system is not affected by all immunosuppressive drugs that are investigated in the present thesis.

To examine the impact of the Jak-inhibitor CP-690,550 on Teff and Treg subsets, we used phospho-specific flow cytometry, **chapter seven**. This technique allowed us to characterize the IL-2 induced STAT5 phosphorylation downstream of Jak in Treg and Teff cells. Furthermore, by using this technique, we revealed differential levels of STAT5 phosphorylation in Treg versus Teff in the presence of the Jak-inhibitor CP-690,550. The observed difference in sensitivity for CP-690,550 in Treg versus Teff was confirmed in functional assays.

It can be interesting to study the influence of an immunosuppressive drug in patients by obtaining blood and then *in vitro* perform functional tests. However, one has to take into account that the immunosuppressive drug has already exerted its effect on the blood cells. Therefore, functional experiments should be performed in the presence of therapeutic concentrations of the drugs, or after incubation of the Treg separately for 24 hr and then tested for their function in a suppression assay which may reveal the effect of these drugs on the function of directly. In this way we are able to demonstrate that the immunosuppressive drugs can target T cells in the concentrations that correlate with the trough levels as measured in the patients.

In **chapter eight** we demonstrate that the level of $FoxP3^+$ cells increased after conversion from a CNI to MMF. Conversion to MMF therapy can actually reverse the suppressive effect of CNI on the percentage of Treg in circulation. Whether the $FoxP3^+$ Treg in the presence of MMF modulates anti-donor reactivity after liver transplantation is to be tested. However, in kidney transplant patients that were converted to MMF monotherapy, the suppressive capacity of $CD4^+CD25^{bright}FoxP3^+$ T cells was preserved[33]. Rapamycin has been shown to be a beneficial agent for Treg because it renders Treg less prone to apoptosis than activated Teff cells[16, 34]. Conversion of triple therapy consisting of tacrolimus, MMF and prednisone to rapamycin monotherapy has been shown to increase Treg numbers in kidney transplant patients[33]. The suppressive activities on the anti-donor proliferation by Treg in the presence of rapamycin monotherapy were comparable with before conversion to rapamycin[33]. For rapamycin, the preservation of Treg suppressive capacity is due to the maintenance of their FoxP3 mRNA expression[16]. These results were also supported by an animal experimental model of graft versus host disease that showed that rapamycin and MMF preserve the suppressive function of Treg[35].

*Conclusion: Immunosuppressive drug therapy that promotes regulatory T cells*

CNIs do not affect Treg function, which is most important, but do affect the frequency of Treg. Yet, CNI-free protocols (CP-690,550, MMF or rapamycin) are 'Treg-sparing' because these protocols induce a rapid recovery of Treg, which can even result in enhanced levels compared to baseline. This rapid recovery of functional Treg allows immune regulation when it is needed most; early after transplantation during inflammation and an elevated activation state of the immune system. The combination of a T-cell depleting agent with rapamycin increase *de novo* Treg, as has been reported before[36]. RATG induction therapy and MMF or rapamycin maintenance therapy would be a Treg-promoting regimen. We show that conversion from CNI to MMF clinically clearly results in an improvement of renal function and a decrease of blood pressure in liver transplant patients. Conversion from a CNI to rapamycin has been reported to improve renal function, with acceptable rates of adverse events[37].

The world-wide transplantation program 'Kidney Disease Improving Global Outcomes' (KDIGO) and the Transplantation Society recommend -at the time of writing- anti-IL-2R induction therapy for all kidney transplantation patients in combination with maintenance therapy consisting of a CNI and MMF[38]. These recommendations by the KDIGO were made from a clinical point of view as they prevent graft rejection by the inhibition of alloreactivity. A (possible) favorable effect of immunosuppressive drugs on immune regulation by the immune systems' endogenous source of regulatory T cells was not explored.

The present dissertation provides **two key messages**. First, we show that Treg are generated during immunosuppressive therapy which allows us to move the Teff-Treg balance from Teff cells towards Treg with the right choice and combination of immunosuppressive agents. Second, the research described in this thesis may also contribute in fine-tuning the immunosuppressive regimen after organ transplantation in favor of regulatory T cells in the periphery, that are involved in donor-specific hyporesponsiveness. Fine-tuning implies that the dosage given to the patient affects or eliminates Teff cells, whereas at the same time maintaining Treg levels and their function. For all drugs used in this thesis, mixed lymphocyte reactions and suppression assays in the presence of the immunosuppressive drug of relevance, were performed to mimic the *in vivo* immune responses. A summary of the impact of the immunosuppressive drugs on immune regulation as described in this thesis is depicted in Table 1.

**Table 1.** Effects of immunosuppressive drugs on effector T cells (Teff) and regulatory T cells (Treg) as described in this thesis

| Immunosuppressive agent / T-cell population | Teff Frequency *(In vivo)* | Teff Function *(Ex vivo and In vitro)* | Treg Frequency (In vivo) | Treg Induction *(In vitro)* | Treg Function *(antigen-specific)* |
|---|---|---|---|---|---|
| **Cyclosporine** | ↓ | ↓ | ↓ | ↓ | ↑ |
| **Tacrolimus** | ↓ | ↓ | ↓ | ↓ | ↑ |
| **rATG** | ↓ | ↓ | ↓ | ↑ | ↑ |
| **rATG + Tacrolimus** | ↓ | ↓ | ↓ | ↑ | ↑ |
| **Mycophenolate Mofetil** | ↓ | ↓ | ↑[33] | ↓ | Unaffected[33,35] |
| **Prednisolone** | ↓ | ↓ | ↓ | ↑[39,40] | ↑[40] |
| **CP-690,550** | ↓ | ↓ | ↓[32] | ↓ | Unaffected |
| **Rapamycin** | ↓ | ↓ | ↑ | ↓ | Unaffected/↑[41] |

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## SAMENVATTING

Orgaantransplantatie is de ultieme behandelingsmogelijkheid voor patiënten met een falend orgaan. Het probleem dat zich echter voordoet na orgaantransplantatie is dat het immuunsysteem van de patiënt het nieuwe orgaan echter als lichaamsvreemd ziet en het transplantaat daarom kan afstoten. Sir Peter Medawar heeft in 1944 in een huidtransplantatiemodel in het konijn ontdekt dat transplantaatafstoting een verworven activiteit is van het immuunsysteem[1]. In 1945, leidde een transplantatie bij tweelingkalveren met een gemeenschappelijke placenta en daardoor dezelfde weefselkenmerken, tot het besef dat orgaantransplantatie alleen kan slagen, indien de immuunreactie van de ontvanger tegen het transplantaat kan worden voorkomen of onderdrukt[2]. Dit werd bevestigd door de eerste succesvolle niertransplantatie bij de mens, bij een eeneiige tweeling, waar donor en ontvanger ook identiek waren voor hun weefselkenmerken[3]. Tussen niet-identieke personen is naast een zo groot mogelijke overeenkomst tussen weefselkenmerken van donor en ontvanger, onderdrukking van de immuunreactie bij de ontvanger noodzakelijk.

De ontdekking van de eerste geneesmiddelen die immuunreacties tegen het transplantaat onderdrukken (ook wel immunosuppressiva genoemd), zorgden ervoor dat goede transplantaatoverleving niet alleen voorkwam bij transplantaties tussen familieleden. Door betere weefseltyperingen en het voortgaande onderzoek naar nieuwe en effectievere immunosuppressieve geneesmiddelen, werd het ook mogelijk om transplantaties tussen niet-verwante personen te verrichten[4].

Immuunreacties worden gemedieerd door T cellen van het immuunsysteem, een gespecialiseerde groep cellen die in de thymus wordt gegenereerd (Figuur 1 introduction) en zich via de bloedbaan verplaatst via de lymfebanen naar de lymfeklieren, of naar het bolwerk van T cellen in de milt. In de lymfeklieren komen T cellen in aanraking met partikels (antigenen) die afkomstig zijn van bacteriën en virussen. Omdat deze antigenen lichaamsvreemd zijn, zullen T cellen hierop reageren door een immuunreactie op gang te brengen. Zo zorgen zij ervoor dat alles wat lichaamsvreemd is, verwijderd wordt uit het lichaam. In een immuunreactie krijgen T cellen door middel van antigenen het signaal om in actie te komen (raken geactiveerd) en gaan vervolgens interleukine-2 (IL-2) produceren; een groeifactor dat zij nodig hebben voor hun groei (proliferatie) en rijping (differentiatie). Door de massale productie en consumptie van IL-2 worden T cellen gestimuleerd om zich te vermenigvuldigen (expanderen) en om uit te rijpen tot zogenaamde 'effector' T cellen (T-eff). Deze T-eff cellen vallen vervolgens bacteriën en virussen aan, waardoor infecties voorkomen worden. De natuurlijke functie van T cellen van het immuunsysteem is dus een eerstegraads verdediging tegen schadelijke bacteriën en virussen[4,5].

Na orgaantransplantatie worden antigenen die afkomstig zijn van het donororgaan gepresenteerd aan T cellen. Door de immuunreactie die optreedt tegen deze donorantigenen worden de donorcellen afkomstig van het donororgaan opgeruimd waardoor het transplantaat wordt afgestoten. Het optreden van acute transplantaatafstoting door T cellen (cellulaire

afstoting) binnen de eerste weken tot maanden na transplantatie is onder andere afhankelijk van de intensiteit van de immuunreacties die afhangt van de mate van overeenkomst van de weefselkenmerken tussen donor en ontvanger[4]. Het optreden van acute cellulaire transplantaatafstoting wordt eveneens sterk bepaald door de immunosuppressieve behandeling van de ontvanger na transplantatie. Om te onderzoeken wat het effect van een bepaald geneesmiddel op het immuunsysteem is, bootsen we in het laboratorium immuunreacties na met 'mixed lymfocyten reacties' (MLR). Perifere bloed cellen (PBMC), waar zich T cellen in bevinden, worden uit het bloed van de patiënt geïsoleerd en gestimuleerd met PBMC uit het bloed van een levende donor of T cellen uit de milt van een overleden donor. In een MLR wordt alleen naar de reactiviteit van patiënt op donor gekeken en niet andersom. Dit wordt bereikt door de PBMC van de donor te bestralen zodat ze het vermogen om te delen kwijtraken en alleen de T cellen van de patiënt kunnen delen. De mate van reactiviteit van patiënt op donor wordt bepaald aan de hand van de T-cel groei van de patiënt.

De ontwikkeling van de calcineurine remmer cyclosporine A, dat de IL-2 productie remt, en door Sir Roy Calne in 1980 als eerste getest werd, was een ware doorbraak in het transplantatieveld, aangezien dit de patiënt en transplantaatoverleving enorm heeft verbeterd[6,7]. Orgaantransplantatiepatiënten worden sindsdien vaak behandeld met standaardtherapie; een combinatie van de meest effectieve geneesmiddelen die transplantaatafstoting kunnen voorkomen. Deze combinatietherapie bestaat tegenwoordig uit een calcineurine remmer (cyclosporine A of het nog nieuwere tacrolimus), T-cel groei remmer (mycofenolaat mofetil; MMF of sirolimus/rapamycin of everolimus) en steroide; (prednisolon). Steroïden zijn erg krachtig doordat zij aangrijpen op diverse processen, waaronder de T-cel groei, activatie en de productie van groei- en ontstekingsfactoren (cytokines)[8]. Ook veroorzaken zij celdood en scheiden ze bepaalde factoren uit die de ontstekingsreactie dempen[8]. Standaardtherapie wordt bij niertransplantatiepatiënten met een verhoogd risico op transplantaatafstoting (patiënten die getransplanteerd worden met een nier van een overleden donor) vooraf gegaan door inductie therapie, bestaande uit anti-thymocyten immunoglobulines (ATG). Deze immunoglobulines worden opwekt in konijnen waarbij immuuncellen afkomstig van thymi van kinderen zijn ingespoten. Afhankelijk van de dosis- zorgt ATG ervoor dat (alle) T cellen in het lichaam vernietigd worden zodat zij geen immuunreacties op gang brengen. Om zijn effectieve werkingsmechanisme wordt ATG ook gegeven als afstotingstherapie.

Na orgaantransplantatie worden patiënten vele jaren tot decennia behandeld met standaardtherapie. Helaas remmen immunosuppressiva ook de natuurlijke functies van het immuunsysteem waaronder de afweer tegen bacteriën en virussen, waardoor patiënten een verhoogd risico op infecties oplopen. Naast deze bijwerkingen zijn er ook allerlei andere schadelijke neveneffecten en op de lange termijn is er een verhoogd risico op tumoren. Ook zijn in het bijzonder de calcineurine remmers schadelijk voor de nieren. Door de ernst van de vele bijwerkingen, de lange termijn symptomen en de nierschade is gezocht naar een vervangend immunosuppressivum dat wel net zo effectief is in het voorkomen van transplantaatafstoting,

maar minder bijwerkingen heeft. Het geneesmiddel CP-690,550 is een dergelijk immunosuppressivum waarvan onderzocht wordt of ter vervanging van calcineurine remmers kan worden gebruikt. CP-690,550 onderdrukt de gevoeligheid van T cellen voor IL-2, zodat dit niet 'geconsumeerd' kan worden en T cellen dus geremd worden in de groei en rijping[9].

Door de talrijke en ernstige bijwerkingen van de huidige immunosuppressiva, wordt ook veel onderzoek verricht naar alternatieve behandelmethodes die heel gericht T cellen aanpakken die specifiek op het transplantaat reageren en betrokken zijn bij afstoting. Een van die alternatieve behandelmethodes om acceptatie van het orgaan te bereiken zou kunnen liggen in de functie van een gespecialiseerde groep T cellen van het immuunsysteem; de regulatoire T cellen (T-reg). Er zijn verschillende groepen van T-reg. Echter, de $CD4^+CD25^{bright}$ T cellen hebben een hoge behoefte aan IL-2 en laten zich daarom op basis van hun uiterlijke kenmerken typeren door een verhoogde (bright) aanwezigheid van de IL-2α receptor (CD25) op het celoppervlak[10]. Een receptor is te vergelijken met een sleutelgat waar maar een specifieke sleutel in past. IL-2 functioneert als sleutel voor CD25 en door binding van IL-2 aan het CD25 eiwit kan de T cel externe signalen die het via IL-2 ontvangt doorgeven aan de binnenzijde van de cel. Bovendien maakt ruim 90% van de $CD4^+CD25^{bright}$ T cellen grote hoeveelheden Foxp3[11-13] en CTLA4[14] aan; twee belangrijke eiwitten die zich in de celkern bevinden en $CD4^+CD25^{bright}$ T cellen nodig hebben om hun functie uit te oefenen. Ook kenmerken deze cellen zich door afwezigheid van de receptor (CD127) voor de groeifactor interleukine-7, dat zich wel bevindt op alle andere T cellen[15]. In verschillende dierexperimentele modellen is aangetoond dat $CD4^+CD25^{bright}Foxp3^+CD127^{-/low}$ T cellen zich onderscheiden van de overige T cellen doordat zij immuunresponsen die gericht zijn tegen lichaamseigen weefsel onderdrukken[16].

In de mens is aangetoond dat deze immuunonderdrukkende regulatoire eigenschappen een belangrijke rol spelen in het voorkomen van auto-immuunziekten waarbij het immuunapparaat verstoord is en zich richt tegen lichaamseigen weefsel. In het laboratorium (*in vitro*) is door middel van suppressie assays (Figuur 1) aangetoond dat deze $CD4^+CD25^{bright}Foxp3^+CD127^{-/low}$ T cellen immuunresponsen (celdeling en o.a. de IL-2 productie van T-eff cellen[17]) onderdrukken door middel van cel-cel contact met T-eff cellen. Andere subgroepen van T-reg onderdrukken immuunreacties van T-eff cellen doordat zij veel van een bepaald anti-ontstekingseiwit produceren dat de T-eff groei remt. Zo produceren de Th3-cellen TGF-β[18] en de Tr1 cellen IL-10[19]. Mogelijk kunnen de $CD4^+CD25^{bright}Foxp3^+CD127^{-/low}$ T cellen ook na orgaantransplantatie op een specifieke manier immuunreacties onderdrukken die gericht zijn tegen het transplantaat. Aangezien de afweeronderdrukkende medicijnen de groei en rijping van T cellen belemmeren, is onze hypothese dat deze geneesmiddelen mogelijk ook het aantal en immuunregulatoire functie van $CD4^+CD25^{bright}FoxP3^+CD127^{-/low}$T cellen beïnvloeden.

Onderzoek naar T-reg vindt vooral plaats in het laboratorium en met proefdieren. Er is maar zeer weinig bekend over de specifieke effecten van immunosuppressiva op de immuunregulatie in patiënten die een orgaantransplantatie ondergaan. Dierexperimentele modellen hebben aangetoond dat ATG het ontstaan van T-reg juist gunstig beïnvloeden. Inductie therapie met

ATG kan mede hierdoor acceptatie van het donororgaan bij patiënten bevorderen. CP-690,550 grijpt aan op de signaaltransductie route die belangrijk is voor de groei van T cellen. Door op moleculair niveau de werking van dit geneesmiddel te bestuderen, zouden we misschien een verschil in gevoeligheid voor dit geneesmiddel tussen T-reg en T-eff kunnen aantonen.

In dit promotieonderzoek hebben wij het effect van verschillende immunosuppressiva op de uiterlijke kenmerken (het fenotype) en de functie van T-reg in nier- en levertransplantatiepatiënten bestudeerd. Dit hebben wij enerzijds onderzocht in prospectieve studies waarbij de $CD4^{+}CD25^{bright}FoxP3^{+}CD127^{-/low}$ T cellen aan de hand van hun uiterlijke kenmerken en functie in kaart zijn gebracht. Anderzijds hebben wij ook *in vitro* onderzoek gedaan naar de werkingsmechanismen van de immunosuppressiva op cellulair en moleculair niveau. De doelstelling van deze dissertatie is om te streven naar een therapie voor transplantatiepatiënten waarbij het aantal en de functie van T-reg niet worden aangetast of zelfs toeneemt en de functie wordt versterkt. T-reg onderdrukken dan de immuunreactiviteit door T-eff cellen die gericht is tegen het transplantaat, terwijl de afweer tegen infectieuze bacteriën en virussen behouden blijft.

In **hoofdstuk 2** onderzoeken wij of donorspecifieke T-reg geïnduceerd worden in patiënten met standaardtherapie bestaande uit cyclosporine, MMF en prednison. Om dit te onderzoeken hebben wij het percentage $CD25^{bright}$ T cellen van het totale aantal $CD4^{+}$ T cellen gemeten in het bloed van stabiele niertransplantatiepatiënten op 0.5-2 jaar na niertransplantatie. In deze periode na transplantatie kregen deze patiënten de volledige standaardtherapie in dezelfde doses als waarmee begonnen is, wat betekent dat de mate van immunosuppressie gelijk is aan kort na transplantatie. Naast het fenotype hebben we ook de functie van T-reg bestudeerd door middel van suppressie assays. In een zogenaamde 'conventionele' suppressie assay wordt de functie van pretransplantatie T-reg bepaald door deze in een kweek te brengen met de T-eff cellen van pretransplantatie en de functie van posttransplantatie T-reg door deze in kweek te brengen met de T-eff cellen van posttransplantatie. De groei van T-eff cellen die gericht is tegen donorcellen of tegen $3^{e}$ partij cellen wordt in af- en aanwezigheid van T-reg bepaald. Derde partij cellen zijn afkomstig van een individu die volledig verschilt in zijn weefseltypering van de patiënt. In aanwezigheid van T-reg verwachten we dat de groei van T-eff cellen onderdrukt wordt. De T-eff cellen van post transplantatie staan echter onder invloed van verschillende 'stress' factoren waaronder donorantigenen, immunosuppressiva etc., die de groei en vitaliteit van deze T-eff cellen belemmeren. Daarom hebben we in **hoofdstuk 2** om de functie van T-reg posttransplantatie te bepalen de conventionele suppressie assay aangepast. De functie van T-reg *posttransplantatie* is nu bepaald door de remming op de groei van T-eff cellen van *pretransplantatie* te meten, waarvan we weten dat deze beter groeien dan posttransplantatie T-eff cellen op donor- en $3^{e}$ partij cellen. Na transplantatie hebben we een daling in het aantal $CD4^{+}CD25^{bright}$ T cellen gevonden. Echter, functioneel gezien waren de T-reg van posttransplantatie in staat om de immuunresponsen tegen de donor nog sterker te onderdrukken dan de T-reg van pretransplantatie. Er was geen verschil in de remming door

T-reg van posttransplantatie op de T-eff groei tegen donorcellen of 3$^{e}$ partijcellen. Uit deze resultaten concluderen wij dat T-reg posttransplantatie in het lichaam betrokken zijn bij het onderdrukken van donorspecifieke immuunreacties tegen het donororgaan.

In **hoofdstuk 3** is getracht uit te zoeken of na klinische niertransplantatie donorspecifieke CD4$^{+}$CD25$^{bright}$FoxP3$^{+}$ T cellen ontstaan die de specifieke immuun reacties tegen het donororgaan kunnen onderdrukken. In een prospectieve studie is het fenotype en de functie van CD4$^{+}$CD25$^{bright}$ T cellen geanalyseerd op 3, 6 en 12 maanden na niertransplantatie. De patiënten zijn gerandomiseerd in verschillende behandelgroepen; arm 1 staat op tacrolimus en rapamycin therapie en arm 2 op tacrolimus en MMF therapie. De immuunregulatoire capaciteit van CD4$^{+}$CD25$^{bright}$ T cellen is op twee manieren bepaald. Ten eerste, door ze te isoleren uit PBMC en dan de respons tegen donor- en 3$^{e}$ partij cellen vóór en na isolatie te meten. Ten tweede door de geïsoleerde CD25$^{bright}$ T cellen in verschillende radio's met T-eff cellen in cocultuur te brengen zoals in een conventionele suppressie assay gedaan wordt. In het eerste jaar na transplantatie was er een significante daling in het absolute aantal en het percentage CD4$^{+}$CD25$^{bright}$ T cellen. In de MLR was er een donorspecifieke hyporesponsiviteit van patiënten PBMC die gestimuleerd werden met donorcellen en een significante toename in de groei bij stimulatie met 3$^{e}$ partij- en 4$^{e}$ partijcellen. Zoals eerder gesteld, onderdrukken T-reg de immuunreacties van T-eff cellen. T cellen in PBMC kunnen we indelen in een niet-delende T-reg populatie en een delende T-eff populatie. Functionele analyse van CD25$^{bright}$ T cellen toonde aan dat de groei van T cellen toeneemt als zij verwijderd zijn uit de PBMC populatie. Deze toename in de groei werd significant groter in de tijd. In suppressie assays verbeterde de suppressieve capaciteit van T-reg zichtbaar significant in de tijd. Bovendien werd het verschil in T-eff groei op donor en 3$^{e}$ partij cellen evenals de remming hiervan door T-reg zichtbaar op 6 maanden na transplantatie. Hoewel tacrolimus en rapamycin dezelfde aangrijpingspunten hebben op T cellen, zagen we geen verschil in de beide armen van therapie. Uit **hoofdstuk 3** concluderen we dat in niertransplantatiepatiënten onder invloed van immunosuppressieve therapie donorspecifieke CD4$^{+}$CD25$^{bright}$ T cellen worden geïnduceerd in het eerste jaar na klinische niertransplantatie.

In de **hoofdstukken 4, 5 en 6** staan konijn anti-thymocyten globulines centraal. In de literatuur is beschreven dat ATG CD4$^{+}$CD25$^{bright}$FoxP3$^{+}$ T cellen kan vormen uit humane CD25$^{neg}$ T-eff cellen. Dit *in vitro* fenomeen zou zeer gunstig zijn voor de patiënt, omdat deze door ATG-gestuurde inductie van T-reg in de patiënt kan zorgen voor een toename van (donorspecifieke) T-reg ten koste van T-eff cellen die zorgen voor transplantaatafstoting.

In **hoofdstuk 4** beschrijven wij in een prospectieve studie het effect van ATG-inductie therapie op het fenotype, de frequentie en de functie van perifere immunoregulatoire CD4$^{+}$CD25$^{bright}$FoxP3$^{+}$CD127$^{-/low}$ T cellen in niertransplantatiepatiënten. De studiegroep werd behandeld met ATG-inductie therapie, tacrolimus, MMF en steroïden. De resultaten van de studiegroep zijn vergeleken met die van een controlegroep zonder ATG-inductie therapie. Pretransplantatie vertoonde 6% van de CD4$^{+}$ T cellen in het bloed het FoxP3$^{+}$CD127$^{-/}$

$^{low}$ fenotype. Op 1 week na transplantatie en ATG-inductie therapie waren geen meetbare $CD4^+FoxP3^+CD127^{-/low}$ T cellen meer aanwezig. Na 4 weken vond langzaam herstel plaats van dit aantal, maar in de eerste 26 weken na ATG-behandeling bleef het aantal significant lager dan vóór transplantatie. Op 14 weken observeerden we binnen de $CD4^+FoxP3^+$ T cellen een significante verschuiving naar het $CD45RO^+CCR7^+$ (central memory) fenotype. Bij het analyseren van de functie van PBMC en T-eff cellen zagen we op 26 weken na transplantatie een significante afname in de groei van PBMC en T-eff cellen tegen donor- en 3$^e$ partij cellen bij patiënten in de ATG-groep vergeleken bij pretransplantatie. De $CD25^{bright}$ T cellen waarvan 90% uit $FoxP3^+CD127^{-/low}$ T cellen bestaat, konden deze immuunreacties goed onderdrukken. Op basis van deze resultaten concluderen wij dat ATG-inductie therapie T-eff cellen en T-reg elimineert uit het bloed en lichaam, maar dat de T-reg die na regeneratie aanwezig zijn in het bloed hun suppressieve activiteit behouden.

We vroegen ons aan de hand van deze resultaten af waarom de T-reg aantallen niet juist verhoogd waren na ATG-behandeling omdat *in vitro* is aangetoond dat ATG T-reg kan genereren. Onze hypothese was dan ook dat de T-eff cellen van pretransplantatie bij patiënten met eindstadium nierfalen dusdanig verstoord zijn in hun functie, dat zij het vermogen om in een T-reg te veranderen wellicht verloren hebben. In een *in vitro* studie beschreven in **hoofdstuk 5** hebben we daarom T-reg inductie bestudeerd in patiënten met eindstadium nierfalen die kandidaat zijn voor niertransplantatie en ATG-inductie therapie. De ATG-geïnduceerde T-reg hebben we gekarakteriseerd en vergeleken met natuurlijke $CD4^+CD25^{bright}FoxP3^+CD127^{-/low}$ T cellen die in het bloed aanwezig zijn voor transplantatie en door de thymus zijn gevormd. $CD25^{neg}$ cellen afkomstig uit PBMC zijn voor 24 uur geïncubeerd met ATG of met controle immunoglobulines uit een konijn die niet gericht zijn tegen humane thymocyten. De ATG-geïnduceerde $CD4^+CD25^+$ en $CD8^+CD25^+$ waren -in dezelfde mate als natuurlijke $CD4^+CD25^{bright}$ T cellen- in staat om de groei van T-eff cellen tegen 3$^e$ partij cellen te onderdrukken. Echter, het percentage FoxP3 binnen de top 2% van deze geïnduceerde $CD25^+$ T cellen was significant lager dan in de natuurlijke $CD4^+CD25^{bright}$ cellen. Tenslotte hebben we de expressie van een aantal moleculen gekwantificeerd die aanwezig zijn in natuurlijke $CD4^+CD25^{bright}$ T cellen of die wellicht iets onthullen over het mechanisme dat ATG-geïnduceerde T-reg uitoefenen. Voordat een eiwit ontstaat, wordt eerst messenger RNA (mRNA) gevormd, dat van het gen in het DNA afkomt. mRNA is dus een soort tussenstadium dat noodzakelijk is om van het gen in het DNA een eiwit te genereren. De mRNA-niveaus van IL-10, IL-27 IFN-γ, perforine en granzyme B (de laatste 4 eiwitten veroorzaken celschade, 'cytotoxiciteit' genaamd) waren hoger in ATG-geïnduceerde T-reg dan in natuurlijke $CD25^{bright}$ T cellen, terwijl de mRNA-expressie van CTLA4, TGF-β en RORγt lager was. Er kan dus gesteld worden dat ATG *in vitro* functionele T-reg uit $CD25^{neg}$ cellen van patiënten met eindstadium nierfalen induceert. Ten opzichte van natuurlijke T-reg hebben deze geïnduceerde T-reg een verschillend fenotype, en overeenkomsten en verschillen op mRNA profielen. De regulatoire activiteit is echter vergelijkbaar. ATG draagt dus bij aan

de mechanismen die de immuunreactiviteit temperen en daarom kan de hierboven gestelde hypothese kan dus worden verworpen.

Als T-reg inductie mogelijk is in patiënten met eindstadium nierfalen, was onze volgende hypothese dat wellicht de immunosuppressieve therapie die gecombineerd wordt met ATG-inductie therapie verantwoordelijk is voor het uitblijven van T-reg inductie in onze niertransplantatiepatiënten. In de literatuur wordt gesproken over het belang van IL-2 voor de generatie, homeostase en functie van T-reg. Daarom zouden calcineurine remmers, die de IL-2 productie onderdrukken, mogelijk ook de inductie van T-reg kunnen verstoren. In **hoofdstuk 6** vroegen wij ons af of ATG T cellen naar T-reg kan converteren in aanwezigheid van geneesmiddelen die hun inductie en functie kunnen beïnvloeden zoals calcineurine remmers. Om dit te onderzoeken hebben wij $CD25^{neg}$ cellen uit PBMC van gezonde individuen geïncubeerd met ATG of controle immunoglobulines voor een periode van 24-uur, maar nu in aan- en afwezigheid van tacrolimus. Opmerkelijk genoeg werden er ook $FoxP3^+$ T cellen geïnduceerd in aanwezigheid van tacrolimus. Blokkade van het effect van IL-2 op T cellen (d.m.v. anti-CD25 en anti-IL-2 antilichamen) beïnvloedde de frequentie van ATG-geïnduceerde $FoxP3^+$ T cellen ook niet. De ATG-tacrolimus geïnduceerde $CD25^+$ T cellen beschikten over voldoende suppressieve capaciteit om de groei van T-eff cellen te onderdrukken die gestimuleerd zijn met bestraalde cellen van een ander individu. Echter, in tegenstelling tot de natuurlijke $CD25^{bright}$ T cellen, vertoonden deze geïnduceerde T-reg hoge expressie van IL-10, IL-27, IFN-$\gamma$, perforine en granzyme B, terwijl FoxP3 significant lager tot expressie werd gebracht. Deze mRNA resultaten zijn bevestigd in niertransplantatiepatiënten die ATG-inductie therapie hebben gekregen na transplantatie. De bevindingen zoals beschreven in deze studie hebben geleid tot de volgende conclusie. Tacrolimus heeft geen negatief effect op de inductie en functie van ATG-geïnduceerde $CD4^+CD25^+$ T cellen en mogelijk induceert ATG ook T-reg in patiënten die behandeld worden met een combinatie van ATG-inductie therapie en tacrolimus.

In **hoofdstuk 7** hebben wij ons gefocust op de signalen die van buitenaf aan de T cel worden gegeven en noodzakelijk zijn voor T cel groei en activatie. Deze signalen zijn afkomstig van groeifactoren en cytokines. Voornamelijk de signalen van IL-2 zijn belangrijk voor de groei, differentiatie en functie van T-eff cellen en T-reg. Cytokines van de IL-2 familie hebben allen hun eigen bindingsreceptor op het celoppervlak, maar in de cel maken zij allen gebruik van dezelfde $\gamma$-receptor, waardoor het signaal van buitenaf door alle groeifactoren van de IL-2 familie op dezelfde manier de cel door geleid wordt. Eenmaal in de cel, wordt het signaal door de cel geleid via Janus Kinasen (Jak) eiwitten die aan het $\gamma$-gedeelte binden. Deze Jaks geven het signaal door aan STAT (Signal Transducers and Activators of Transcription) eiwitten die het signaal daarna naar de celkern leiden. Jak-STAT signalering leidt tot de vorming van nieuwe eiwitten die de T cel nodig heeft voor zijn groei en functie. In deze studie hebben we onderzocht of de Jak-remmer CP-690,550 de regulatoire activiteit van $CD4^+CD25^{bright}FoxP3^+CD127^{-/low}$ T cellen beïnvloed. De Jak-activiteit in aan- en afwezigheid van de Jak-remmer hebben we bepaald aan de hand van de activatie (fosforylatie) van STAT5. Kwantificatie van gefosforyleerd

STAT5 geeft een indicatie van de activatie status van Jak3 en kan dus worden gebruikt om het inhiberende effect van CP-690,550 te bepalen. Met behulp van 'fosfo-specifieke flow cytometrie' hebben we de gefosforyleerde eiwitten in kaart gebracht. Om T-reg functie te bepalen zijn suppressie assays in af- en aanwezigheid van CP-690,550 uitgevoerd met het materiaal van gezonde individuen en niertransplantatiepatiënten die CP-690,550 therapie hebben gekregen gedurende 29 dagen na transplantatie en daarnaast anti-CD25 inductie therapie, MMF en prednison. Een controlegroep kreeg standaardtherapie bestaande uit anti-CD25 inductie therapie, cyclosporine, MMF en prednisone.

De IL-2 geïnduceerde fosforylatie van STAT5 was significant hoger in de $CD4^+CD25^{bright}$ T cellen dan in $CD25^{-/dim}$ T-eff cellen. In aanwezigheid van een klinische relevante dosis CP-690,550 van 100 ng/mL, werd deze IL-2 geïnduceerde STAT5 fosforylatie gedeeltelijk geremd in de $CD4^+CD25^{bright}$ T cellen, terwijl deze bijna geheel geremd werd in $CD25^{-/dim}$ T-eff cellen. De dosis waarbij 50% remming van de STAT fosforylatie optreedt, de $IC_{50}$ was 2-3x hoger voor T-reg dan voor $CD25^{-/dim}$ T-eff cellen. De groei van T-eff cellen in respons op bestraalde PBMC van een ander individu werd dosisafhankelijk geremd door CP-690,550. Onder invloed van klinische relevante doses van CP-690,550 waren T-reg in staat om deze groei van T-eff cellen nog meer te onderdrukken. De suppressieve capaciteit van T-reg van niertransplantatiepatiënten die behandeld zijn met CP-690,550 is vergelijkbaar met die van voor transplantatie, en met de suppressieve capaciteit van T-reg van patiënten uit de controlegroep. Deze bevindingen laten zien dat de Jakremmer CP-690,550 op effectieve wijze T-eff groei onderdrukt en tegelijkertijd de suppressieve activiteit van T-reg behoudt.

**Hoofdstuk 8** beschrijft het effect van conversie van immunosuppressieve geneesmiddelen na levertransplantatie. In deze studie betreft het de conversie van een calcineurine remmer naar de T-cel proliferatieremmer MMF na transplantatie. Patiënten met slechte nierfunctie werden geconverteerd naar MMF en ontvingen ook een dosis van de IL-2 receptor blocking antilichaam Daclizumab. Controle patiënten continueerden de behandeling met de calcineurine remmer. Daclizumab behandeling resulteerde in afdekking van het IL-2 bindende deel van de CD25 moleculen op 1 maand na transplantatie. Wanneer CD25-expressie gekwantificeerd werd aan de hand van het IL-2 onafhankelijke gedeelte, waarbij IL-2 binding geen beperkende factor is voor de kwantificatie van CD25, bleek dat er een significante daling was in het percentage $CD4^+CD25^+$ T cellen. Echter, er was geen daling in het aantal $CD4^+FoxP3^+$ cellen. Zes maanden na MMF-conversie nam het percentage $CD4^+CD25^{bright}FoxP3^+$ T cellen zelfs toe met 125%. mRNA-analyse van FOXP3 bevestigde deze verrijking van FoxP3 in het perifere bloed. Opmerkelijk genoeg steeg na MMF-conversie het aantal CD25-moleculen per cel op $CD4^+FoxP3^+$ cellen, maar niet op $CD4^+FoxP3^{neg}$ cellen vergeleken bij pre-conversie. Op deze manier zorgt MMF therapie voor herstel van circulerende T-reg aantallen in het bloed na het onderdrukkende effect van calcineurine remmers. Door dit effect kan MMF therapie dus mogelijk de T-reg-gemedieerde suppressie van immuunreactiviteit tegen het transplantaat kunnen bevorderen.

## CONCLUSIE

Immunosuppressieve therapie wordt vaak gekozen op basis van de mate van effectiviteit in de preventie van transplantaatafstoting en de minste bijwerkingen. Deze medicatie is niet specifiek gericht tegen de immuunreacties tegen het transplantaat, maar ook tegen immuunreacties tegen bacteriën en virussen. Regulatoire T cellen zijn juist in staat om die immuunresponsen die gericht zijn tegen het transplantaat te onderdrukken. Er valt dus winst te behalen voor de patiënt als de regulatie van immuunreacties tegen het transplantaat niet negatief en mogelijk zelfs positief wordt beïnvloed door de immunosuppressieve therapie. Met behulp van immunosuppressieve geneesmiddelen die T-reg-aantallen of T-regfunctie stimuleren, is het mogelijk om het immuunsysteem te sturen richting donorspecifieke immuunregulatie. De (combinatie van) immuunosuppressieve geneesmiddelen die wij hebben onderzocht, vertonen geen nadelig effect op de functie van regulatoire T cellen. Donorspecifieke regulatoire T cellen ontstaan in het eerste jaar na transplantatie onder standaardtherapie. ATG-inductie therapie in combinatie met rapamycine, CP-690,550 of MMF kan de aanmaak van deze donorspecifieke regulatoire T cellen wellicht versnellen. In de toekomst zouden we deze immunosuppressiva kunnen gebruiken om het immuunsysteem actief te sturen in de richting van donorspecifieke regulatoire T cellen.

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# Chapter 10

## Dankwoord/Acknowledgements

## Curriculum Vitae Auctoris

## PhD Portfolio

## Abbreviations

## DANKWOORD/ACKNOWLEDGEMENTS

'A goal without a plan is just a wish'
(Antoine de Saint-Exúpiry, schrijver)

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Transplantatielab

Nicole, bedankt voor je nuttige adviezen en suggesties tijdens de werk- en projectbesprekingen. Ook bedankt voor je hulp bij de laatste loodjes; met name het direct inschakelen van analytische hulp toen de druk zijn hoogtepunt bereikte en er veel werk in korte tijd af moest komen; dit betekent echt veel voor mij! Martin Hoogduijn, aka 'Ho', jij was altijd in voor een goed gesprek, en dat waardeer ik zeer. Dank voor je goede ideëen tijdens werk- en projectbesprekingen en thanks voor de gezelligheid tijdens lunchpauzes en congressen! Wenda, bedankt voor het inwerken op de FACS en het meedenken bij de analyses; ik heb er veel van geleerd! Dankzij jouw rappe bestellingen waren de monoklonalen altijd op voorraad en konden we er flink tegenaan met onze experimenten, dank daarvoor! Joke, jij bent altijd erg behulpzaam geweest; ik kon jou altijd alles vragen op het lab. Als je het mij vraagt ben jij echt onmisbaar voor het lab! Ik wil je ook bedanken voor de gezelligheid op congressen en labweekenden! Ronella, dank voor je uitleg en hulp met de cytotoxiciteits assays, maar ook dank voor de gezelligheid op de bootcongressen en labborrels! Annemiek, bedankt voor je hulp bij de laatste loodjes. Het deed mij goed, dat je even wilde bijspringen! Ook dank voor al je suggesties bij de mRNA isolaties en PCRs! Marjolein, dank voor je vriendelijkheid en interesse, dat vond ik erg fijn, suc6 verder! Elly, jij bent een leuk mens; altijd vrolijk! Dank voor de (brabantse) gezelligheid op het lab! André, ik waardeer jouw vriendelijkheid en behulpzaamheid, het ga je goed! Nicolle, jou kan ik eigenlijk niet genoeg bedanken! Je hebt mij geholpen met analyses, metingen, referenties en protocollen. Ook mocht ik weleens stofjes van jou lenen; dank daarvoor. Suc6 met je onderzoek! Dat er nog meer mooie papers mogen komen!

Jurjen Velthuis. Beste Jurjen, toen ik net op het lab was begonnen vond ik jou maar een strenge leermeester. FACSen, MACSen, overal werd ik bij op de vingers gekeken; 'zorg ervoor dat je het apparaat HELEMAAL onder de knie hebt' kreeg ik te horen. Later, toen ik 's avonds laat weleens alleen achter de FACS of MACS zat, realiseerde ik mij de waarde van die uitspraken; dank voor deze wijze lessen; ze zijn goed tot mij doorgedrongen! Ik vond je oprechte interesse in mijn Treg project erg fijn en ik wil je daarom ook bedanken voor je betrokkenheid en je ideeën, maar ook voor de goede gesprekken tijdens de treinreizen. Het ga je goed Jurjen!

Martin Huisman. Beste Martin, wij zijn tegelijkertijd begonnen op het transplantatielab en dat creëerde een band tussen ons. We maakten altijd veel plezier op het lab. Helaas ging jij na twee jaar weg; naar een ander lab in het EMC. We bleven contact houden en hebben nog steeds regelmatig een update! Dank voor deze vriendschap en natuurlijk heel erg bedankt voor het ontwerpen van de prachtige cover van dit proefschrift!

Nefrologen
Dr. Michiel Betjes, dr. Ajda Rowshani en drs. Jacqueline van de Wetering, ik wil jullie heel erg bedanken voor de nuttige suggesties omtrent mijn project bij de werk- en projectbesprekingen. De discussies die wij tijdens deze besprekingen gevoerd hebben, waren erg leerzaam en stimulerend.

Researchverpleegkundigen
Annemarie Geel en Hanneke Tanck-Vernhout, bedankt voor jullie inzet bij de ATG-studie. In het bijzonder het coördineren van de afspraken van de patiënten en natuurlijk het bloedprikken. Annemarie ook bedankt voor de gezelligheid tijdens het labweekend in Berlijn, je peptalks en bemoedigende woorden!

Nierpolikliniek
Marja, Heidi en Sylvana bedankt voor het bewaren van de bloedbuizen. Ik kon altijd op jullie rekenen! Mijn dank gaat ook uit naar de prikzusters voor het prikken van de patiënten!

De dames van de D-vleugel die altijd in zijn voor een praatje, wil ik bedanken voor hun betrokkenheid en interesse!

Alle orgaantransplantatie patiënten en bloedbankdonoren wil ik uiteraard ook hartelijk bedanken voor hun medewerking aan de verschillende studies die beschreven staan in dit proefschrift. Zonder hen was het niet mogelijk geweest om dit boekje te schrijven.

Tregmeeting
Mark, André, Janneke, Fleur en Colin bedankt voor de interesse, de goede ideeën, waardevolle tips en de vele discussies over Tregs, waar ik veel van geleerd heb.

Maag-, Darm, Leverlab (MDL)
Ieder lab heeft zijn eigen cultuur, zo ook het MDL-lab. Ik heb er veel geleerd in de tijd dat ik er zat en later kon ik er altijd terecht als ik iets wilde lenen. Ik wil alle MDL'ers bedanken voor hun input tijdens presentaties op stafvergaderingen, researchbesprekingen etc. en vooral voor alle gezelligheid tijdens borrels en congressen! Lieve Alice, jij hebt mij in de eerste maanden wegwijs gemaakt op het MDL-lab en je hebt mij de FoxP3-kleuringen en het FACSen geleerd! Helaas vertrok jij kort daarna. Mij staat nog bij dat jij altijd goedgehumeurd en goedlachs was. Bedankt voor de fijne samenwerking! Anthonie, al sinds mijn inwerkperiode op het MDL-lab was jij altijd bereid om een handje te helpen. Ik kon altijd met alle vragen bij jou terecht. Ook dank voor je hulp bij het sorten van de Tregs! Patrick, jij was altijd erg behulpzaam en je dacht vaak met mij mee! Bedankt daarvoor! Suc6 met jouw promotie! Het wordt vast een prachtig boekje! Brenda, bedankt voor de gezelligheid op borrels congressen! Suc6 met je carrière! Angela, Suomi, Özlem en Viviana; bedankt voor alle plezierige momenten tijdens congressen en suc6 met het afronden van jullie promotietraject! In het bijzonder wil ik Thanya bedanken voor het meedenken over de ATG *in vitro* studie; dit was zeer nuttig! Ook dank voor alle gezelligheid op congressen; waarbij ik onze excursie naar de Niagara Watervallen nooit zal vergeten!

Prof.dr. Herald Metselaar, bedankt voor de brainstormsessie over mijn project omtrent levertransplantaties. Helaas is het bij een brainstormsessie gebleven. Lara; dank voor je coördinatie van de PROTECT-studie en het verzamelen van de klinische data.

De labdag 'crew' 2007
Piet Kramer, Bas, Lisette, Martin Kroos, Arnold en Katy. Ik vond het erg gezellig om in 2007 met jullie samen de labdag te organiseren. Het is ons gelukt om er een geslaagde dag van te maken! Een speciaal dankwoord richt ik aan Lisette; dank voor alle waardevolle tips omtrent mijn promotie en voor mijn carrière daarna! Suc6 verder als postdoc!

Prof.dr. Andries Hoitsma, ik heb veel bewondering voor u als goeroe in de transplantatiewereld. Bedankt voor de mooie en bemoedigende woorden op congressen. Toen ik nog maar 2 jaar oud was, promoveerde u op de behandeling van afstoting na niertransplantatie met rATG therapie! Hartelijk dank voor uw proefschrift met èchte handtekening!

Vrienden/familie

Lieve Junita, aka 'girly girl'; wij kennen elkaar sinds mijn afstudeeronderzoek in Leiden; jij studeerde biomedische wetenschappen in Leiden. Het klikte vanaf het begin erg goed tussen ons; we zijn allebei vrij 'down-to-earth' (doe maar gewoon, dan doe je al gek genoeg) en we kunnen heerlijk babbelen over girls-stuff (!). Ik kan altijd erg lachen met jou! Veel suc6 in de farmaceutische wereld en dat onze vriendschap nog lang mag voortbestaan!

Beste Vishal, als goede vriend en AIO in het Sophia kinderziekenhuis heb je mij (als Amsterdamse) ervan weten te overtuigen om op een project in Rotterdam te solliciteren. Dank voor deze stimulans! Ik vond het altijd fijn om met jou te kletsen over alle AIO perikelen en natuurlijk ook over de vele andere onderwerpen die de revue passeerden als we weer eens uren aan de telefoon hingen! Suc6 met jouw promotie!

Mijn vriendinnen Sabriena, Hajar, Iris en Chiu Ting. Lieve meiden, we zijn allemaal (soms letterlijk) een andere kant op gegaan na onze studie, maar we wisselen nog regelmatig onze ervaringen uit en het is fijn dat ik met jullie echt over vanalles en nog wat kan kletsen! Ik wil jullie graag bedanken voor deze vriendschap, waarvan we dit jaar het 10-jarig jubileum vieren! Op naar de volgende 10 jaar!

Lieve pa, jij was altijd geïnteresseerd in mijn promotietraject en je begreep door je werk in het ziekenhuis ook goed wat promoveren inhield. Ik kon daardoor altijd de verschillende facetten van mijn promotietraject met jou bespreken, dat was erg fijn. Bedankt voor je hulp bij de stellingen en voor je bemoedigende woorden. Ik heb dan ook echt goed doorgezet en het resultaat ligt hier!

Dit proefschrift draag ik op aan mijn moeder, die mij altijd heeft gesteund. Zonder haar steun had ik dit niet kunnen bereiken. Lieve mam, jij hebt mij geleerd in mijzelf te geloven en zelfverzekerd door het leven te gaan; daar ben ik je erg dankbaar voor. Jouw zorg voor mij begon 's ochtends vroeg bij het opstaan (ontbijtje) en ging weer verder als ik 's avonds thuiskwam (avondeten), elke dag weer opnieuw. Ook kan ik altijd alles met jou bespreken en je om advies vragen en dat doet mij goed! Dank voor je onuitputtelijke steun en liefde! Mam, ik weet dat je trots bent op mij; maar ik ben ook trots op jou, omdat ik de sterke vrouw in jou bewonder!

Varsha

## CURRICULUM VITAE AUCTORIS

Varsha Devi Kareshma Devi was born in Amsterdam on the 1st of November 1982. In 2000 she completed secondary school (Atheneum) at the Fons Vitae Lyceum in Amsterdam and started with the study Medical Biology at the University of Amsterdam. In 2004, she wrote the literature essay 'Immunopathology and Treatment of Acute Humoral Graft Rejection after Renal Transplantation' which was supervised by Prof.dr. R.J.M. ten Berge of the Department of Nephrology of the Academic Medical Centre in Amsterdam and Prof.dr. R. Aalberse of the Sanquin Blood Bank in Amsterdam. In 2005, she performed her graduation research project at the Department of Cardiology of the University Medical Center in Leiden that was supervised by Prof.dr. A. van der Laarse, where she studied the genetic predisposition of restenosis in the coronory arteries of the heart in patients with a sudden heart attack. By the end of 2005 she graduated and obtained her Master of Science degree. She started in 2006 with her PhD research project at the transplantation laboratory at the Department of Internal Medicine (Supervisors: Prof.dr. Willem Weimar and Dr. Carla C. Baan) in collaboration with the Department of Surgery (Supervisor: Dr. Luc J.W. van der Laan) of the Erasmus Medical Centre in Rotterdam. In her PhD project she investigated the influence of clinical kidney- and liver transplantation and immunosuppressive drugs on the frequency and function of $CD4^{+}CD25^{bright}$ regulatory T cells in the blood. The results of this work are described in this thesis. During her PhD period she presented her work on several national and international congresses, even in the land 'Down Under'. In 2009, she got the opportunity to present part of her work during the 'late-breaking' session on the American Transplant Congress, where only high potential findings with a great clinical relevance are presented. Since May 2010, she works as an 'In-house Clinical Research Associate' at Genzyme Europe B.V. in Almere.

## PHD PORTFOLIO

*Summary of PhD training and teaching activities*

| | |
|---|---|
| **Name PhD student:** | Varsha D.K.D. Sewgobind |
| **Erasmus MC Department** : | Internal Medicine/Transplantation laboratory/Surgery |
| **Research School:** | Molecular Medicine/NIHES |
| **PhD period:** | Jan 2006 – Mrt 2010 |
| **Promotor:** | Prof.dr. W. Weimar |
| **Co-promotors:** | Dr. C.C. Baan/Dr. L.J.W. van der Laan |

| **General academic skills** | **Year** |
|---|---|
| - Research Integrity | 2006 |
| - Stralingshygiëne 5B | 2006 |
| **Research skills** | |
| - Statistics (Classical Methods of Data Analysis, CC02, NIHES) | 2006 |
| **In-depth courses** | |
| - Advanced Course in Molecular Immunology (Postgraduate School of Molecular Medicine) | 2007 |
| - Basiscursus Regelgeving en Organisatie voor Klinisch Onderzoekers (Good Clinical Practice) | 2009 |
| **Presentations** | |
| - **Oral**, Annual meeting Nederlandse Vereniging voor Immunologie, Noordwijkerhout | 2006 |
| - **Oral**, 11th Molecular Medicine Day, Rotterdam | 2007 |
| - **Oral**, 7th American Transplant Congress, San Francisco, USA | 2007 |
| - **Poster**, 'Top 10 Best Poster', 13th European Society of Transplantation Congress, Prague, Czech Republic | 2007 |
| - **Poster**, Wetenschapsdagen Internal Medicine Erasmus MC, Antwerpen, Belgium | 2008 |
| - **Poster**, European Scientific Conferences Meeting, London, UK | 2008 |
| - **Oral**, 20e Bootcongres, Nederlandse Transplantatie Vereniging, Zeewolde | 2008 |
| - **Oral**, 8th American Transplant Congress, Toronto, Canada | 2008 |
| - **Oral**, XXII International Congress of the Transplantation Society, Sydney, Australia | 2008 |
| - **Poster**, Wetenschapsdagen Internal Medicine Erasmus MC, Antwerpen, Belgium | 2009 |
| - **Oral**, 21e Bootcongres, Nederlandse Transplantatie Vereniging Zeewolde | 2009 |
| - **Oral**, III Basic Science Meeting ESOT, Brussels, Belgium | 2009 |
| - **Oral**, 'late-breaking' session' 9thAmerican Transplant Congress, Boston, USA | 2009 |
| - **Poster**, 9thAmerican Transplant Congress, Boston, USA | 2009 |

- **Oral**, 14th European Society of Transplantation Congress, Paris, France 2009
- **Oral**, Wetenschapsdagen Internal Medicine Erasmus MC, Antwerpen, Belgium 2010
- **Oral**, 22e Bootcongres, Nederlandse Transplantatie Vereniging, Rotterdam 2010

**(Inter)national conferences**

- 18e Bootcongres, Nederlandse Transplantatie Vereniging 2006
- Annual meeting, Nederlandse Vereniging voor Immunologie 2006
- 19e Bootcongres, Nederlandse Transplantatie Vereniging 2007
- 7th American Transplant Congress, San Francisco, USA 2007
- 20e Bootcongres, Nederlandse Transplantatie Vereniging 2008
- 8th American Transplant Congress, Toronto, Canada 2008
- European Scientific Conferences Meeting, London 2008
- XXII International Congress of the Transplantation Society, Sydney, Australia 2008
- III Basic Science Meeting European Society of Transplantation, Brussels, Belgium 2009
- 21e Bootcongres, Nederlandse Transplantatie Vereniging 2009
- 9th American Transplant Congress, Boston, USA 2009
- 14th European Society of Transplantation Congress, Paris, France 2009

**Seminars and workshops**

- MolMed Masterclass mucosal immunology, 'Imaging tolerance and Tregs *in vivo*', Erasmus MC 2008
- Klinisch Review Symposium Nederlands Transplantatie Vereniging, Utrecht 2006
- Klinisch Review Symposium Nederlands Transplantatie Vereniging, Utrecht 2007
- Klinisch Review Symposium Nederlands Transplantatie Vereniging, Utrecht 2008
- 'Tolerance induction in Transplantation', LIFI minisymposium, LUMC, Leiden 2008

**Other**

- Internal presentations at the Departments of:
  Nefrologie, 2006
  Maag- darm- lever (MDL) ziekten, -
  'Stafdag' Heelkunde 2009

**Grants**

| | |
|---|---|
| - Travelgrant Novartis B.V. (t.b.v ATC) | 2007 |
| - Travelgrant Nederlandse Transplantatie Vereniging (t.b.v Bootcongres NTV) | 2008 |
| - Travelgrant Genzyme B.V. (t.b.v ATC) | 2008 |
| - Travelgrant Novartis B.V. (t.b.v ICTS) | 2008 |
| - Travelgrant Nederlands Vereniging voor Immunologie (t.b.v Basic Science ESOT/TTS) | 2009 |
| - Travelgrant Novartis B.V. (t.b.v ATC) | 2009 |

**Excursions**

| | |
|---|---|
| - Member of the Transplantation laboratory 'Labweekend' Organising Committee | 2006 |
| - Member of the Internal Medicine 'Labday' Organising Committee | 2007 |

**Other**

| | |
|---|---|
| - Invited article for Daniel den Hoed, 'Cancer Magazine' (Molecular Medicine School) | 2007 |

## ABBREVIATIONS

| | |
|---|---|
| **APC** | Allophycocyanin |
| **BSA** | Bovine Serum Albumin |
| **CML** | Cytotoxic T lymphocyte Mediated Lysis |
| **CNI** | Calcineurin Inhibitor |
| **CPM** | Counts per Minute |
| **CsA** | Cyclosporine A |
| **FITC** | Fluorescein Isothiocyanate |
| **FMO** | Fluorescence Minus One |
| $\gamma_c$ | gamma chain |
| **gMFI** | geometric Mean Fluorescence Intensity |
| **HC** | Healthy Controls |
| **HCM** | Human Culture Medium |
| **HLA** | Human Leukocyte Antigens |
| **IH** | Inhibition |
| **Jak** | Janus family of tyrosine kinases |
| **KTx** | Kidney Transplantation |
| **MACS** | Magnetic Cell Sorting |
| **MFI** | Median Fluorescence Intensity |
| **MLR** | Mixed Lymphocyte Reaction |
| **MMF** | Mycophenolate Mofetil |
| **PBMC** | Peripheral Blood Mononuclear Cells |
| **PE** | Phycoerythrin |
| **PERCP** | Peridinin chlorophyll protein |
| **PHA** | Phytohemagglutinin |
| **post-Tx** | post-transplantation |
| **pre-Tx** | pre-transplantation |
| **P-STAT5** | phosphorylated STAT5 |
| **rATG** | Rabbit Anti-Thymocyte Globulins |
| **rIgG** | Rabbit Immunoglobulins |
| **SD** | Standard Deviation |
| **SEM** | Standard Error of the Mean |
| **STAT** | Signal Transducer and Activator of Transcription |
| **Teff** | Effector T cells |
| **Treg** | Regulatory T cells |
| **Tx** | Transplantation |