The Role of Regulatory T cells in Kidney Transplantation

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"And this was really the way that my whole road experience began, and the things that were to come are too fantastic not to tell."

On the Road, Jack Kerouac

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General Introduction

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GENERAL INTRODUCTION

CONTENTS

1.1 Kidney Transplantation

Worldwide, increasing numbers of patients are affected by end stage renal failure (ESRF). This mainly results from the ageing population and the global epidemic of type 2 diabetes [1]. ESRF is generally caused by a loss of nephrons, which constitute the part of the kidney that filters waste products from the blood (figure 1). For several decades it has been appreciated that once a critical number of nephrons is lost, kidney failure will progress relentlessly towards ESRF, which means that the glomerular filtration rate (GFR) will become less than 60 ml per minute per 1.73 m^2 body surface area [2]. This severely reduced kidney function results in the accumulation of organic waste products, a state that is generally known as uremia [3]. This uremic state is associated with a functionally impaired immune system, resulting in many clinical side effects as an increased incidence of infections and malignancies [4-6].

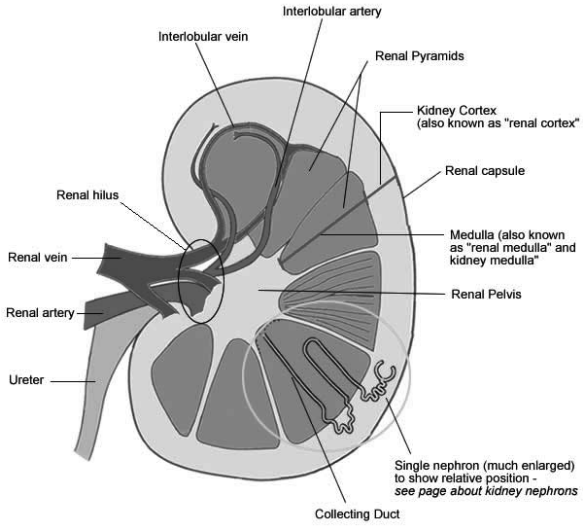

Figure 1: Schematic presentation of the kidney with an enlarged single nephron. The damage to and loss of nephrons generally leads to ESRF.

To prevent patients with ESRF from premature death, they require renal replacement therapy. Subsequently, most patients are treated with dialysis, a procedure replacing essential kidney functions such as removal of waste products and fluids from the blood. Unfortunately, the dialysis procedure itself also affects the immune system and is accompanied with several complications resulting in high morbidity and mortality [7].

Dialysis takes a significant part of the global health-care budgets [8-10]. Compared to dialysis, kidney transplantation has a more favourable outcome on the length and quality of life. Moreover, from an economical perspective, the procedure is also more cost-effective [7,8]. Therefore, from these perspectives, transplantation is more beneficiary for patients suffering from ESRF as well as for society when compared to dialysis. However, the lack of donor kidneys and transplant capacity negatively influence the waiting time before transplantation in many cases [10].

Nowadays, thousands of patients receive a donor kidney each year, which is astonishing since the first human kidney transplantation was performed in 1954 [11,12]. Back then, Ronald Herrick donated a kidney to his identical twin brother Richard who was dying of ESRF (figure 2). Although this was a sensational break through for transplantation medicine, it would take several more years before successful transplantation was achieved between non-identical individuals [12]. The single most important factor that made transplantation a good alternative for dialysis, was the introduction of cyclosporin a potent immunosuppressant in the transplantation clinic [12]. This immunosuppressant provided the opportunity to efficiently suppress the immune response of the recipient to the donor kidney; the basis for successful transplantation at present [13,14]. Moreover, the advances in drug monitoring continuously drive medication to a more individualized protocol. Although this will minimize the side effects of immunosuppressive therapy, long term treatment with these agents is still associated with side effects that significantly diminish quality of life and affect graft and patient survival [15]. Therefore, it is important to develop alternative therapeutic strategies that prevent rejection of the donor kidney.

1.2 Transplantation Tolerance

The ultimate challenge in organ transplantation is to achieve transplant tolerance: the absence of an immune response to the transplanted graft of an otherwise competent immune system without the use of immunosuppressive drugs [16]. This condition is mainly described in transplant patients that by themselves discontinued their immunosuppressive medication. Although there are several clinical studies describing transplant tolerance [17-23], the mechanisms responsible for its development and maintenance in humans remain elusive.

Figure 2: Ronald Herrick (left) donated a kidney to his identical twin brother Richard (right) who was dying of ESRF.

Tolerance can be divided in two categories; central and peripheral tolerance. Central tolerance regulated in the thymus is a process of negative selection of self-reactive T cells. Although this selection process is highly efficient, a number of self-reactive cells overcome the selection barrier [24]. When these self/reactive T cells escape this

thymic selection process, peripheral tolerance will try to silence them in peripheral compartments including the lymph nodes and the spleen [25].
In the setting of kidney transplantation, central tolerance may be induced by treatment of patients with bone marrow transplantation or intrathymic injection with donor antigens before kidney transplantation. Consequently, donor antigens will be present in the thymus, leading to a T cell repertoire that lacks donor reactivity [25-27]. Peripheral tolerance consists of several mechanisms known as deletion, ignorance, induction of T cell anergy and regulation by suppressor cells. The latter mechanism has received accumulating attention in the field of transplantation biology since Sakaguchi and colleagues identified the $CD4^{+}CD25^{bright+}$ regulatory T cell (Treg) in 1995 [28].

1.3 Regulatory T cells

1.3.1 Identification of $CD4^{+}CD25^{bright+}$ Tregs

Regulatory T cells were first discovered in the 1970's by Gershon, who showed that antigen-primed T cells could induce tolerance after transfer to naive mice [29]. These so called suppressor cells were then associated with several diseases like the observed decreased cell-mediated immunity in uremic patients [30,31]. The research techniques in those days, however, did not allow an accurate definition of these cells and for some time the level of investigation by immunologists on this topic was low. Yet, since identification of the $CD4^{+}CD25^{bright+}$ regulatory T cell, the subject has received new attention leading to the discovery of several other human regulatory T cell subsets such as the natural killer T cells and γδ T cells [32,33]. These subsets are interesting but due to difficulties in identification, characterization and isolation of these lymphocyte subsets most attention has been focussed on the $CD4^{+}CD25^{bright+}$ Tregs, which is the most promising lymphocyte subset for cellular immune therapy in patients with autoimmune diseases or after transplantation.
The first experiments with $CD4^{+}CD25^{bright+}$ Tregs were performed with cells from mice and demonstrated their importance in autoimmunity [28,34]. Here, it was shown that elimination or reduction of $CD4^{+}$ T cells with high expression of CD25, the IL-2 receptor α-chain, caused spontaneous development of several autoimmune diseases. Furthermore, upon allogeneic skin transplantation to mice depleted from $CD4^{+}CD25^{bright+}$ T cells, immune responses were much severe than in normal, control mice. These immune responses normalized after reconstitution of $CD4^{+}CD25^{bright+}$ T cells, demonstrating their suppressive, regulatory role in transplantation tolerance [28]. A few years after their identification in animal models, the presence of $CD4^{+}CD25^{bright+}$ T cells with potent regulatory capacities was also established in humans [35-37].
Further analysis of Tregs showed that apart from their high expression of CD25, these cells also express many other markers like CTLA-4, GITR, HLA-DR, CD45RO, CD122 and CD132. However, none of these markers was found to be specific for Tregs only as these markers are also expressed by activated T cells [38]. In 2001, however, immunological similarities between studies with mice having mutations in the Foxp3 gene and in mice depleted from $CD4^{+}CD25^{bright+}$ Tregs were noticed. Soon, two key papers reported that FoxP3 was the key regulatory gene for the development of mouse $CD4^{+}CD25^{bright+}$ Tregs [39,40]. Also, human regulatory $CD25^{+}$ T cells are positive for FoxP3. However, in contrast to rodents it was demonstrated that FoxP3 is

also expressed by human non-regulatory activated T cells [41,42]. Nowadays, the combination of FoxP3 with the IL-7 receptor (CD127) is used to identify human Tregs. Low expression of CD127 inversely correlates with both the expression of FoxP3 and the suppressive function of Tregs [43,44].

1.3.2 Naturally occurring versus adaptive Tregs

When $CD4^{+}CD25^{bright+}$ Tregs were first identified it was thought that all Tregs develop and maturate in the thymus and after their entrance in the periphery circulate through the peripheral compartments, ready for recognition of antigens [45]. It has now been appreciated that the peripheral $CD4^{+}CD25^{bright+}$ Treg population consists of at least two different subsets, which are defined as natural Tregs and adaptive Tregs [46].

Natural Tregs

Indeed, natural Tregs are thymus derived where they may either be selected at the site of the thymic medulla or the thymic cortex [47]. Of interest, it has been shown that these Tregs are positively selected through their T cell receptor (TCR) affinity interactions with self-peptides presented on class II molecules by thymic stromal cells [47]. Due to strong TCR engagement in the thymus, natural Tregs are fully functional when they enter the periphery. Here, they form a population of Tregs that prevent the potential development of self-reactive T cells into effector cells [48].

In vitro analysis of natural Tregs showed that they are anergic, which means that they do not proliferate in culture upon stimulation. This anergic state, however, can be overcome by addition of high doses of IL-2. The latter may reflect their response in vivo to the production of IL-2 by activated effector T cells. In addition, natural Tregs do not produce IL-2 themselves [36,38,45]. In vivo experiments indeed showed that the initiation of their function strongly depends on IL-2 secreting effector T cells. The high expression of CD25 makes Tregs highly sensitive for IL-2, which facilitates rapid induction of their suppressive capacities. Apart from the presence of IL-2, natural Tregs require activation through their TCR and co-stimulation by CD28 to exert their potent suppressive activities as well as other soluble mediators like IL-10 and TGF-β [35,36,48-51].

It is generally accepted, that for potent suppression natural Tregs require cell-cell contact. This is supported by results from experiments with effector cells lacking the co-stimulatory molecules CD80/86, showing that binding of CTLA-4 on Tregs with CD80/86 on effector T cells is essential for suppression [52]. Also, the expression of CTLA-4 was found to be elevated on Tregs upon activation and in vitro blockade of CTLA-4 neutralized their suppressive function [53,54]. Another mechanism that allows Tregs to inhibit immune reactivity is their interaction with antigen-presenting cells (APC). Here, their connection with CD80/86 on APC activates indoleamine 2,3-dioxygenase (IDO) leading to reduced amounts of the essential amino acid trypthophan. This is associated with decreased activation of T cells [55,56]. Finally, several studies indicated that natural Tregs have cytolytic activities [57,58]. This was particularly demonstrated in a recent experimental mouse study, which showed that granzyme-B (GZ-B) is one of the key components of natural Treg cell-mediated suppression. Moreover, Treg activity was correlated with the up-regulation of GZ-B [59].

Given these findings on the suppressive function of Tregs from in vitro and in vivo experiments it is essential that these suppressor cells migrate to the site of action to control immune responses either at the peripheral lymph nodes or at the graft site. The

few studies that investigated whether Tregs migrate to the site of action indicated that the physiology of Tregs parallels that of conventional CD4 T cells. This would indeed allow them to go to those places were their regulation is required, like the donor-kidney or its draining lymph nodes [60-64].

Adaptive Tregs

Adaptive Tregs share many of the features as described for natural Tregs, but there are also important differences. First, these adaptive or induced Tregs are generated in the peripheral compartments. It has been demonstrated that adaptive Tregs can be derived from natural $CD4^+CD25^{bright+}$ Tregs, $CD4^+CD25^-$ precursors and also from $CD4^+$ memory T cells [65-67]. These T cells differentiate into functional adaptive Tregs, after exposure to tissue-specific or foreign antigen in the presence of TGF- β [46,68-70]. Numerous studies demonstrated that induced Tregs by TGF-β also express Foxp3 [71-74]. The suppressive function of these cells was confirmed in several studies. For instance, in a rat cardiac allograft model administration of TGF-β-induced $CD4^+CD25^+$ Tregs resulted in a significant prolongation of cardiac allograft survival [75]. Also, Zheng and colleagues observed that TGF-β induces human $CD4^+$ cells to become suppressor cells. When naïve human $CD4^+$ cells were stimulated with allo-antigen plus TGF-β for one week, the TGF-β-treated but not control cells (without TGF-β) prevented $CD8^+$ T cells from proliferating in response to allo-antigens and from becoming cytotoxic effector cells [76].

In contrast to natural Tregs, the expression of CD25 by the induced, adaptive Tregs is variable, suggesting that they are not as dependent on IL-2 as their natural counterparts [46]. Like natural Tregs, adaptive Tregs also perform their suppressive function most efficiently by cell-cell contact. These regulatory T cells highly express Granzyme-B, suggesting that they exhibit strong cytolytic activities [57,58,77]. Overall, induced, adaptive Tregs provide a unique T cell population that inhibits immune responses in an antigen-specific manner. In organ transplantation, manipulation of the immune system to induce antigen-specific suppressor cells is a next step towards tolerance in patients. It's evident that this requires more research using experimental transplant models and translational studies analyzing patient materials.

1.4 Aims of the Thesis

The objective of this thesis is to phenotypically and functionally characterize $CD4^+CD25^{bright+}$ Tregs in the setting of clinical kidney transplantation. More explicitly we aim to determine whether donor-specific Tregs are generated after clinical kidney transplantation and which factors influence the development of these cells. Furthermore, it has been demonstrated that immunosuppressive drugs like the commonly prescribed calcineurin inhibitors tacrolimus and cyclosporin may affect the development, maintenance and function of $CD4^+CD25^{bright+}$ Tregs. Therefore, we aim to monitor and analyze the characteristics and function of $CD4^+CD25^{bright+}$ T cells from kidney transplant patients on various immunosuppressive regimens.

Chapter 2 describes the effect of end stage renal failure and its treatment by peritoneal and haemodialysis on $CD4^+CD25^{bright+}$ Tregs. In this setting, we analyze their phenotypical and functional properties in a large patient cohort before transplantation. To evaluate the known effects of several immunosuppressive drugs on $CD4^+CD25^{bright+}$ Tregs and their underlying mechanisms we perform an extensive search of the literature in **chapter 3**. A prospective analysis of the phenotypical and

functional development of $CD4^{+}CD25^{bright+}$ Tregs in the first year after clinical kidney transplantation is described in **chapter 4**. Here, patients are treated with different immunosuppressive protocols allowing us to study their effect on the development of $CD4^{+}CD25^{bright+}$ Tregs. In an additional clinical study we investigate the effect of treatment with dacluzimab, a monoclonal antibody directed against the α-chain of the IL-2 receptor (CD25), or steroids on $CD4^{+}CD25^{bright+}$ Tregs in kidney transplant patients on tacrolimus and mycophenolate mofetil. Furthermore, we report whether the function of these cells is associated with rejection episodes after transplantation in **chapter 5**. Finally, in **chapter 6**, an analysis of peripheral blood cells from kidney transplant patients who are converted from tacrolimus/ mycophenolate mofetil to monotherapy with either tacrolimus, rapamycin or mycophenolate mofetil is described. Here, we characterize and analyze their Treg phenotype and function to determine whether monotherapy with these agents may influence the capacity of the peripheral $CD4^{+}CD25^{bright+}$ Tregs to migrate to lymphoid tissues and/or the transplanted kidney.

References

1. El Nahas M. The global challenge of chronic kidney disease. *Kidney Int*. 2005*;* **68**: 2918-29.
2. Stevens LA, Coresh J, Greene T and Levey AS. Assessing kidney function--measured and estimated glomerular filtration rate. *N Engl J Med*. 2006*;* **354**: 2473-83.
3. Meyer TW and Hostetter TH. Uremia. *N Engl J Med*. 2007*;* **357**: 1316-25.
4. Maisonneuve P, Agodoa L, Gellert R *et al.* Cancer in patients on dialysis for end-stage renal disease: an international collaborative study. *Lancet*. 1999*;* **354**: 93-9.
5. Levin A and Foley RN. Cardiovascular disease in chronic renal insufficiency. *Am J Kidney Dis*. 2000*;* **36**: S24-30.
6. Olsen SK and Brown RS, Jr. Hepatitis B treatment: Lessons for the nephrologist. *Kidney Int*. 2006*;* **70**: 1897-904.
7. Wolfe RA, Ashby VB, Milford EL *et al.* Comparison of mortality in all patients on dialysis, patients on dialysis awaiting transplantation, and recipients of a first cadaveric transplant. *N Engl J Med*. 1999*;* **341**: 1725-30.
8. Rabbat CG, Thorpe KE, Russell JD and Churchill DN. Comparison of mortality risk for dialysis patients and cadaveric first renal transplant recipients in Ontario, Canada. *J Am Soc Nephrol*. 2000*;* **11**: 917-22.
9. Meier-Kriesche HU and Kaplan B. Waiting time on dialysis as the strongest modifiable risk factor for renal transplant outcomes: a paired donor kidney analysis. *Transplantation*. 2002*;* **74**: 1377-81.
10. Schieppati A and Remuzzi G. Chronic renal diseases as a public health problem: epidemiology, social, and economic implications. *Kidney Int Suppl*. 2005: S7-S10.
11. Merrill JP, Murray JE, Harrison JH and Guild WR. Successful homotransplantation of the human kidney between identical twins. *J Am Med Assoc*. 1956*;* **160**: 277-82.
12. Morris PJ. Transplantation--a medical miracle of the 20th century. *N Engl J Med*. 2004*;* **351**: 2678-80.
13. Halloran PF. Immunosuppressive drugs for kidney transplantation. *N Engl J Med*. 2004*;* **351**: 2715-29.
14. Taylor AL, Watson CJ and Bradley JA. Immunosuppressive agents in solid organ transplantation: Mechanisms of action and therapeutic efficacy. *Crit Rev Oncol Hematol*. 2005*;* **56**: 23-46.
15. Lopez MM, Valenzuela JE, Alvarez FC *et al.* Long-term problems related to immunosuppression. *Transpl Immunol*. 2006*;* **17**: 31-5.
16. Wood KJ and Sakaguchi S. Regulatory T cells in transplantation tolerance. *Nat Rev Immunol*. 2003*;* **3**: 199-210.
17. Devlin J, Doherty D, Thomson L *et al.* Defining the outcome of immunosuppression withdrawal after liver transplantation. *Hepatology*. 1998*;* **27**: 926-33.
18. VanBuskirk AM, Burlingham WJ, Jankowska-Gan E *et al.* Human allograft acceptance is associated with immune regulation. *J Clin Invest*. 2000*;* **106**: 145-55.
19. Strober S, Benike C, Krishnaswamy S *et al.* Clinical transplantation tolerance twelve years after prospective withdrawal of immunosuppressive drugs:

studies of chimerism and anti-donor reactivity. *Transplantation*. 2000*;* **69**: 1549-54.
20. Takatsuki M, Uemoto S, Inomata Y *et al.* Weaning of immunosuppression in living donor liver transplant recipients. *Transplantation*. 2001*;* **72**: 449-54.
21. Li Y, Koshiba T, Yoshizawa A *et al.* Analyses of peripheral blood mononuclear cells in operational tolerance after pediatric living donor liver transplantation. *Am J Transplant*. 2004*;* **4**: 2118-25.
22. Roussey-Kesler G, Giral M, Moreau A *et al.* Clinical operational tolerance after kidney transplantation. *Am J Transplant*. 2006*;* **6**: 736-46.
23. Martinez-Llordella M, Puig-Pey I, Orlando G *et al.* Multiparameter immune profiling of operational tolerance in liver transplantation. *Am J Transplant*. 2007*;* **7**: 309-19.
24. Sakaguchi S. Regulatory T cells: key controllers of immunologic self-tolerance. *Cell*. 2000*;* **101**: 455-8.
25. Hogquist KA, Baldwin TA and Jameson SC. Central tolerance: learning self-control in the thymus. *Nat Rev Immunol*. 2005*;* **5**: 772-82.
26. Remuzzi G. Cellular basis of long-term organ transplant acceptance: pivotal role of intrathymic clonal deletion and thymic dependence of bone marrow microchimerism-associated tolerance. *Am J Kidney Dis*. 1998*;* **31**: 197-212.
27. Adler SH and Turka LA. Immunotherapy as a means to induce transplantation tolerance. *Curr Opin Immunol*. 2002*;* **14**: 660-5.
28. Sakaguchi S, Sakaguchi N, Asano M *et al.* Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. *J Immunol*. 1995*;* **155**: 1151-64.
29. Gershon RK and Kondo K. Infectious immunological tolerance. *Immunology*. 1971*;* **21**: 903-14.
30. Raskova J and Morrison AB. A decrease in cell-mediated immunity in uremia associated with an increase in activity of suppressor cells. *Am J Pathol*. 1976*;* **84**: 1-10.
31. Guillou PJ, Woodhouse LF, Davison AM and Giles GR. Suppressor cell activity of peripheral mononuclear cells from patients undergoing chronic haemodialysis. *Biomedicine*. 1980*;* **32**: 11-7.
32. Sharif S, Arreaza GA, Zucker P *et al.* Regulation of autoimmune disease by natural killer T cells. *J Mol Med*. 2002*;* **80**: 290-300.
33. Hayday A and Tigelaar R. Immunoregulation in the tissues by gammadelta T cells. *Nat Rev Immunol*. 2003*;* **3**: 233-42.
34. Asano M, Toda M, Sakaguchi N and Sakaguchi S. Autoimmune disease as a consequence of developmental abnormality of a T cell subpopulation. *J Exp Med*. 1996*;* **184**: 387-96.
35. Ng WF, Duggan PJ, Ponchel F *et al.* Human CD4(+)CD25(+) cells: a naturally occurring population of regulatory T cells. *Blood*. 2001*;* **98**: 2736-44.
36. Jonuleit H, Schmitt E, Stassen M *et al.* Identification and functional characterization of human CD4(+)CD25(+) T cells with regulatory properties isolated from peripheral blood. *J Exp Med*. 2001*;* **193**: 1285-94.
37. Dieckmann D, Plottner H, Berchtold S *et al.* Ex vivo isolation and characterization of CD4(+)CD25(+) T cells with regulatory properties from human blood. *J Exp Med*. 2001*;* **193**: 1303-10.
38. Baecher-Allan C, Viglietta V and Hafler DA. Human CD4+CD25+ regulatory T cells. *Semin Immunol*. 2004*;* **16**: 89-98.

39. Fontenot JD, Gavin MA and Rudensky AY. Foxp3 programs the development and function of CD4+CD25+ regulatory T cells. *Nat Immunol*. 2003*;* **4**: 330-6.
40. Hori S, Nomura T and Sakaguchi S. Control of regulatory T cell development by the transcription factor Foxp3. *Science*. 2003*;* **299**: 1057-61.
41. Wang J, Ioan-Facsinay A, van der Voort EI *et al.* Transient expression of FOXP3 in human activated nonregulatory CD4+ T cells. *Eur J Immunol*. 2007*;* **37**: 129-38.
42. Ziegler SF. FOXP3: not just for regulatory T cells anymore. *Eur J Immunol*. 2007*;* **37**: 21-3.
43. Liu W, Putnam AL, Xu-Yu Z *et al.* CD127 expression inversely correlates with FoxP3 and suppressive function of human CD4+ T reg cells. *J Exp Med*. 2006*;* **203**: 1701-11.
44. Seddiki N, Santner-Nanan B, Martinson J *et al.* Expression of interleukin (IL)-2 and IL-7 receptors discriminates between human regulatory and activated T cells. *J Exp Med*. 2006*;* **203**: 1693-700.
45. Itoh M, Takahashi T, Sakaguchi N *et al.* Thymus and autoimmunity: production of CD25+CD4+ naturally anergic and suppressive T cells as a key function of the thymus in maintaining immunologic self-tolerance. *J Immunol*. 1999*;* **162**: 5317-26.
46. Bluestone JA and Abbas AK. Natural versus adaptive regulatory T cells. *Nat Rev Immunol*. 2003*;* **3**: 253-7.
47. Maggi E, Cosmi L, Liotta F *et al.* Thymic regulatory T cells. *Autoimmun Rev*. 2005*;* **4**: 579-86.
48. Salomon B, Lenschow DJ, Rhee L *et al.* B7/CD28 costimulation is essential for the homeostasis of the CD4+CD25+ immunoregulatory T cells that control autoimmune diabetes. *Immunity*. 2000*;* **12**: 431-40.
49. Thornton AM, Piccirillo CA and Shevach EM. Activation requirements for the induction of CD4+CD25+ T cell suppressor function. *Eur J Immunol*. 2004*;* **34**: 366-76.
50. Tai X, Cowan M, Feigenbaum L and Singer A. CD28 costimulation of developing thymocytes induces Foxp3 expression and regulatory T cell differentiation independently of interleukin 2. *Nat Immunol*. 2005*;* **6**: 152-62.
51. Shevach EM. CD4+ CD25+ suppressor T cells: more questions than answers. *Nat Rev Immunol*. 2002*;* **2**: 389-400.
52. Paust S, Lu L, McCarty N and Cantor H. Engagement of B7 on effector T cells by regulatory T cells prevents autoimmune disease. *Proc Natl Acad Sci U S A*. 2004*;* **101**: 10398-403.
53. Read S, Malmstrom V and Powrie F. Cytotoxic T lymphocyte-associated antigen 4 plays an essential role in the function of CD25(+)CD4(+) regulatory cells that control intestinal inflammation. *J Exp Med*. 2000*;* **192**: 295-302.
54. Tang Q, Boden EK, Henriksen KJ *et al.* Distinct roles of CTLA-4 and TGF-beta in CD4+CD25+ regulatory T cell function. *Eur J Immunol*. 2004*;* **34**: 2996-3005.
55. Mellor AL and Munn DH. IDO expression by dendritic cells: tolerance and tryptophan catabolism. *Nat Rev Immunol*. 2004*;* **4**: 762-74.
56. von Boehmer H. Mechanisms of suppression by suppressor T cells. *Nat Immunol*. 2005*;* **6**: 338-44.
57. Grossman WJ, Verbsky JW, Barchet W *et al.* Human T regulatory cells can use the perforin pathway to cause autologous target cell death. *Immunity*. 2004*;* **21**: 589-601.

58. Grossman WJ, Verbsky JW, Tollefsen BL *et al.* Differential expression of granzymes A and B in human cytotoxic lymphocyte subsets and T regulatory cells. *Blood*. 2004*;* **104**: 2840-8.
59. Gondek DC, Lu LF, Quezada SA *et al.* Cutting edge: contact-mediated suppression by CD4+CD25+ regulatory cells involves a granzyme B-dependent, perforin-independent mechanism. *J Immunol*. 2005*;* **174**: 1783-6.
60. Ochando JC, Yopp AC, Yang Y *et al.* Lymph node occupancy is required for the peripheral development of alloantigen-specific Foxp3+ regulatory T cells. *J Immunol*. 2005*;* **174**: 6993-7005.
61. Siegmund K, Feuerer M, Siewert C *et al.* Migration matters: regulatory T-cell compartmentalization determines suppressive activity in vivo. *Blood*. 2005*;* **106**: 3097-104.
62. Lee JH, Kang SG and Kim CH. FoxP3+ T cells undergo conventional first switch to lymphoid tissue homing receptors in thymus but accelerated second switch to nonlymphoid tissue homing receptors in secondary lymphoid tissues. *J Immunol*. 2007*;* **178**: 301-11.
63. Menning A, Hopken UE, Siegmund K *et al.* Distinctive role of CCR7 in migration and functional activity of naive- and effector/memory-like Treg subsets. *Eur J Immunol*. 2007*;* **37**: 1575-83.
64. Tosello V, Odunsi K, Souleimanian NE *et al.* Differential expression of CCR7 defines two distinct subsets of human memory CD4+CD25+ Tregs. *Clin Immunol*. 2008*;* **126**: 291-302.
65. Kingsley CI, Karim M, Bushell AR and Wood KJ. CD25+CD4+ regulatory T cells prevent graft rejection: CTLA-4- and IL-10-dependent immunoregulation of alloresponses. *J Immunol*. 2002*;* **168**: 1080-6.
66. Karim M, Kingsley CI, Bushell AR *et al.* Alloantigen-induced CD25+CD4+ regulatory T cells can develop in vivo from CD25-CD4+ precursors in a thymus-independent process. *J Immunol*. 2004*;* **172**: 923-8.
67. Vukmanovic-Stejic M, Zhang Y, Cook JE *et al.* Human CD4+ CD25hi Foxp3+ regulatory T cells are derived by rapid turnover of memory populations in vivo. *J Clin Invest*. 2006*;* **116**: 2423-33.
68. Apostolou I, Sarukhan A, Klein L and von Boehmer H. Origin of regulatory T cells with known specificity for antigen. *Nat Immunol*. 2002*;* **3**: 756-63.
69. Walker LS, Chodos A, Eggena M *et al.* Antigen-dependent proliferation of CD4+ CD25+ regulatory T cells in vivo. *J Exp Med*. 2003*;* **198**: 249-58.
70. Rouse BT, Sarangi PP and Suvas S. Regulatory T cells in virus infections. *Immunol Rev*. 2006*;* **212**: 272-86.
71. Chen W, Jin W, Hardegen N *et al.* Conversion of peripheral CD4+CD25- naive T cells to CD4+CD25+ regulatory T cells by TGF-beta induction of transcription factor Foxp3. *J Exp Med*. 2003*;* **198**: 1875-86.
72. Zheng SG, Wang JH, Gray JD *et al.* Natural and induced CD4+CD25+ cells educate CD4+CD25- cells to develop suppressive activity: the role of IL-2, TGF-beta, and IL-10. *J Immunol*. 2004*;* **172**: 5213-21.
73. Fantini MC, Becker C, Monteleone G *et al.* Cutting edge: TGF-beta induces a regulatory phenotype in CD4+CD25- T cells through Foxp3 induction and down-regulation of Smad7. *J Immunol*. 2004*;* **172**: 5149-53.
74. Peng Y, Laouar Y, Li MO *et al.* TGF-beta regulates in vivo expansion of Foxp3-expressing CD4+CD25+ regulatory T cells responsible for protection against diabetes. *Proc Natl Acad Sci U S A*. 2004*;* **101**: 4572-7.

75. Watanabe M, Mencel RL, Cramer DV *et al.* Transforming growth factor-beta/interleukin-2-induced regulatory CD4+ T cells prolong cardiac allograft survival in rats. *J Heart Lung Transplant*. 2005*;* **24**: 2153-9.
76. Yamagiwa S, Gray JD, Hashimoto S and Horwitz DA. A role for TGF-beta in the generation and expansion of CD4+CD25+ regulatory T cells from human peripheral blood. *J Immunol*. 2001*;* **166**: 7282-9.
77. Cao X, Cai SF, Fehniger TA *et al.* Granzyme B and perforin are important for regulatory T cell-mediated suppression of tumor clearance. *Immunity*. 2007*;* **27**: 635-46.

CHAPTER 2

End stage renal failure and the regulatory activities of $CD4^{+}CD25^{bright+}FoxP3^{+}$ T-cells

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Abstract

Background: The defensive immune system in patients with end stage renal failure is impaired at multiple levels. This state of immune incompetence is associated with continuous activation of the immune system. An additional explanation for this state of activation may be the disturbed function of $CD4^{+}CD25^{bright+}FoxP3^{+}$ regulatory T-cells.
Methods: The phenotype and function of peripheral regulatory T-cells from patients with end stage renal failure (N=80) and healthy controls (N=17) was studied by flow cytometry, RT-PCR and mixed lymphocyte reaction. Patients were on haemodialysis (N=40), peritoneal dialysis (N=26), or not treated with dialysis yet (N=14). The latter group had a glomerular filtration rate of <20 ml/min/1.73 m^2.
Results: The basal IL-2 mRNA level was high in patient-PBMC ($p=0.0002$ vs healthy controls). The absolute number of $CD4^{+}CD25^{bright+}$ T-cells was low in patients ($p<0.05$ vs healthy controls). Further, proliferation of patient-PBMC upon allogeneic stimulation was impaired ($p<0.0001$ vs healthy controls). The regulatory function of $CD4^{+}CD25^{bright+}$ T-cells was determined in the setting of direct allorecognition. First, the effect of depletion of $CD25^{bright+}$ cells from patient-PBMC on proliferation was low. Second, co-culture of $CD25^{bright+}$ cells with $CD25^{neg/dim}$ cells (1:10 ratio), showed impaired regulatory function ($p<0.001$ vs healthy controls), which was especially pronounced in patients on dialysis. The FOXP3 mRNA level was also low upon stimulation ($p=0.0002$ vs healthy controls).
Conclusions: In line with previous studies, we observed an overactivated but functionally compromised immune system in patients with end stage renal failure. It now appears that in this setting, regulation by $CD4^{+}CD25^{bright+}FoxP3^{+}$ T-cells is also impaired.

Introduction

Patients with end stage renal failure (ESRF) suffer from inadequate responses upon vaccination e.g. hepatitis B [1, 2], susceptibility to infections and an increased incidence of malignancies [1, 3]. Studies have shown that these clinical observations are accompanied by failure of the defensive immune system at multiple levels, despite evidence of activation by markers on immune competent T-cells [4-6]. This state of immune incompetence, associated with continuous activation of the immune system, may result from the high level of uremic toxins that is caused by the renal failure itself and is further intensified by treatment with dialysis [7-10]. However, an additional explanation for this state of activation may be a disturbed function of regulatory T-cells.

The $CD4^+CD25^{bright+}$ T-cell, which specifically expresses the transcription factor $FoxP3^+$, controls immune responses of effector T-cells to auto and foreign antigens [11-13]. $CD4^+CD25^{bright+}FoxP3^+$ T-cells prevent organ-specific auto-immune diseases, control anti-tumor responses, anti-viral responses and immune responses to allo-antigens in the setting of organ transplantation [14-16]. $CD4^+CD25^{bright+}FoxP3^+$ T-cells exert their suppressive function in a cell-cell contact dependent manner by inhibiting the IL-2 and IFN-γ production of effector T-cells [17, 18]. IL-2 is a crucial cytokine for the function, homeostasis and survival of $CD4^+CD25^+FoxP3^+$ T-cells [19, 20]. Previous reports on the IL-2 system in ESRF-patients showed high spontaneous release and expression of IL-2 and its receptor (R) by circulating T-cells [21, 22]. However, after stimulation, the expression of the IL-2R and the production capacity of IL-2 was low compared to those induced in cells from healthy controls [21-24]. Both phenomena may have a significant negative influence on the number and function of $CD4^+CD25^+FoxP3^+$ T-cells in ESRF-patients.

The effect of ESRF on immunoregulatory activities of $CD4^+CD25^+FoxP3^+$ T-cells has not been extensively investigated. In a T-cell dependent murine model of anti-glomerular basement membrane glomerulonephritis, treatment with $CD4^+CD25^+$ T-cells suggested therapeutic value of these suppressor cells [25]. Furthermore, it has been shown that in these animals $CD4^+CD25^+$ T-cells protect against injury of kidney cells [26]. While these animal studies suggest a controlling role for these cells in kidney disease and renal function, it remains unknown whether they influence the function of T effector cells in patients with ESRF. We here postulate that in ESRF-patients the function of $CD4^+CD25^+FoxP3^+$ T-cells is impaired resulting in their characteristic overactivated but functionally compromised immune system.

Therefore, we evaluated the number and function of peripheral $CD4^+CD25^+FoxP3^+$ T-cells from ESRF-patients on haemodialysis (HD), peritoneal dialysis (PD), or not yet on dialysis (ND) and healthy controls (HC). The regulatory function of $CD4^+CD25^{bright+}$ T-cells was determined in the setting of direct allorecognition by mixed lymphocyte reaction (MLR) to overcome the impaired autologous antigen presenting cell (APC) function in ESRF-patients [27, 28].

Material and methods

Subjects

The medical ethical committee (MEC) of Erasmus Medical Centre approved the study protocol and all patients provided informed consent (MEC 2004-264). Inclusion of patients started in February 2004. Peripheral blood samples were obtained from 80 stable patients (table 1) with ESRF and from 17 HC, consisting of 10 males and 7 females with a mean age of 52 ± 8.6 years. Patients were on haemodialysis (HD, N=40) or peritoneal dialysis (PD, N=26) and 14 patients were not yet on dialysis (ND), with an average glomerular filtration rate (GFR) of 11 ± 4.7 ml/min/1.73 m^2. The GFR was calculated with the IDMS-Traceable MDRD Study Equation: GFR = 175 x (Serum creatinine)$^{-1.154}$ x (Age)$^{-0.203}$ x (0.742 if female) x (1.210 if African American). The groups of HD, PD and ND-patients were comparable for their characteristics as presented in table 1. Patients with evidence for active infections or receiving immunosuppressive drugs were not included in the study.

Table 1. Patient characteristics

Demographics	**HD (N = 40)**	**PD (N = 26)**	**ND (N = 14)**
Gender (M/F)	27 / 13	17 / 9	7 / 7
Age (years)	52 ± 16*	52 ± 14*	51 ± 17*
Time on dialysis (months)	20 (3-280)**	12 (3-75)**	
Primary kidney disease			
Hypertensive nephropathy	10	6	3
Immunological disease	6	8	2
Diabetic nephropathy	9	4	1
Polycystic kidney disease	7	1	4
Other	5	3	2
Urological disease	3	4	2

*Mean ± SD, **Median (range), HD=Haemodialysis, 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 (FITC), phycoerythrin (PE), allophycocyanin (APC) or peridinin chlorophyll protein (PerCP). 100µl blood was incubated with 10µl of the dual mAb combination CD45-FITC/CD14-PE; IgG_1-FITC/IgG_{2b}-PE as isotype control. Further we used the mAb CD3-FITC, CD4-PerCP, CD8-APC, CD19-APC, CD16CD56-PE and CD25-PE (epitope B, clone M-A251). 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 FACS lysing solution (BD Biosciences) for 10 min. Cells were washed twice, and analysed on a flow cytometer (FACSCalibur, BD Biosciences) using SimulSet and CELL Quest Pro software (BD Biosciences). The number of leukocytes was determined by the cell counter CASY® model TT. (Schärfe System GmbH, Reutlingen). To establish an

analysis gate that included at least 90% of the lymphocytes, the CD45/CD14 reagent was used. At least 20000 gated lymphocyte events were acquired from each tube.

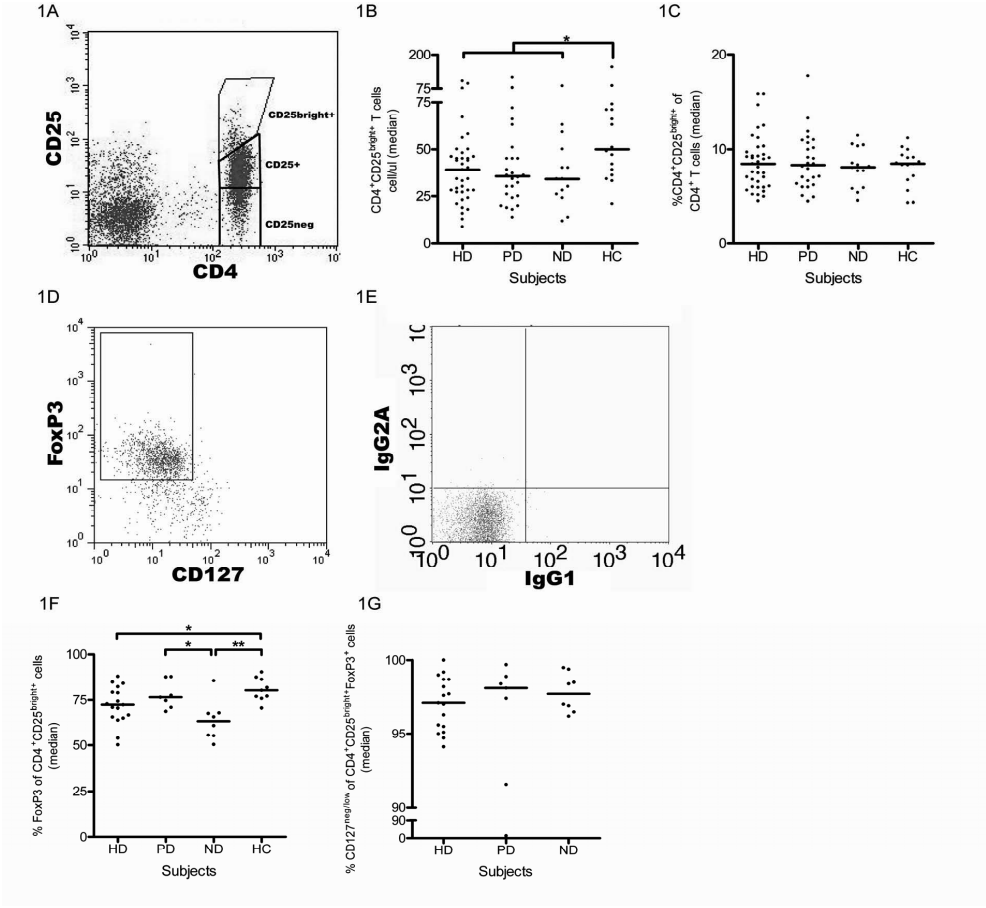

Figure 1. Flow cytometric results from whole blood.
(A) Dotplot of lymphocytes stained for CD4 and CD25 with a representative example of the gated $CD25^{bright+}$, $CD25^{+}$ and $CD25^{neg}$ gated areas. (B) Absolute numbers of peripheral $CD4^{+}CD25^{bright+}$ T cells were significantly lower in all patient groups compared to HC (all groups $p<0.05$). (C) The percentage of $CD4^{+}CD25^{bright+}$ cells of peripheral $CD4^{+}$ T cells was not different in any group of patients when compared to HC. (D) Dotplot of lymphocytes stained for FoxP3 and CD127 with a representative example of the gated $FoxP3^{+}CD127^{+}$ area from the $CD25^{bright+}$ gate in figure 1A. (E) Dotplot of the isotype controls IgG_{2A}-APC and IgG_1-FITC for FoxP3 and CD127 respectively. (F) Whole blood of an additional cohort of patient with ESRF was stained for FoxP3 and CD127. Here, the expression of FoxP3 by $CD4^{+}CD25^{bright+}$ cells was slightly lower when compared to HC in HD and ND-patients ($p<0.05$ and $p<0.01$ respectively). Also this expression of FoxP3 was lower in ND-patients when compared to PD-patients ($p<0.05$). (G) The expression of CD127 by $CD4^{+}CD25^{bright+}FoxP3^{+}$ cells was not different between the groups of patients.
HD=Haemodialysis, PD=Peritoneal Dialysis, ND=No Dialysis, HC=Healthy Controls.

Expression of FoxP3 and CD127

A specific marker for $CD4^{+}CD25^{bright+}$ Treg is FoxP3 and recently it was shown that the expression of CD127 inversely correlates with FoxP3 expression and the suppressive function of Treg [11, 29]. Experiments on our study cohort were performed on fresh materials before the anti-FoxP3 antibody was available for flow cytometric analysis. Therefore, we stained whole blood of an additional cohort of ESRF-patients (N=34) as well as HC (N=9) with CD4-PerCP, CD25-PE (epitope B), CD127-FITC (eBioscience, San Diego, CA, USA) and FoxP3-APC (clone PCH101, eBioscience). The isotype controls for FoxP3 and CD127 were IgG_{2A}-APC and IgG_1-FITC respectively. Patient characteristics were comparable with our study population.

Isolation of peripheral blood lymphocytes

Peripheral blood mononuclear cells (PBMC) were freshly 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 RPMI 1640 (BioWhittaker, Verviers, Belgium) and resuspended in Human Culture Medium (HCM) consisting of RPMI 1640-Dutch Modification (Gibco, BRL, Scotland, UK) supplemented with, 4 mM L-Glutamine (Gibco BRL), 100 IU/ml penicillin (Gibco BRL), 100 µg/ml streptomycin (Gibco BRL) and 10% heat inactivated pooled human serum. The latter consists of serum from 20 subjects (males & females), which was purchased from the blood bank (Sanquin, The Netherlands) and pooled in home. Subjects contained all ABO blood groups and were tested for HLA-antibodies. If the test was positive, the serum was not used. We did not use the same batch for all experiments, but each batch was tested for adequate cell growth prior to our experiments. For the analysis of IL–2 and FOXP3 mRNA expression levels $2x10^6$ PBMC samples were snap-frozen directly after isolation.

Isolation of $CD25^{bright+}$ cells

After isolation, PBMC were washed once and resuspended in 45µl MACS-buffer/$10x10^6$ PBMC prepared according to manufacturers protocol (Miltenyi, Bergisch Gladbach, Germany). PBMC were then incubated with anti-CD25 microbeads (Epitope A, Miltenyi Biotec) followed by a positive selection (POSSELD-program) on the autoMACS® (Miltenyi), resulting in a $CD25^{bright+}$ enriched fraction, which was referred to as the $CD25^{bright+}$ fraction. Cells not selected by the microbeads were referred to as the $CD25^{neg/dim}$ fraction [16]. To control for the autoMACS procedure on cells, $6x10^6$ PBMC were treated by the same protocol in the absence of anti-CD25 microbeads. Purity of the fractions was measured by flow cytometry using CD4-PerCP, CD8-APC, CD25-PE (epitope B, BD Bioscience) and FoxP3-APC (clone PCH101).

Mixed Lymphocyte Reactions

To check for cell viability and proliferative capacity, $5x10^4$ $CD25^{neg/dim}$ cells and PBMC were stimulated with 1 μg/ml Phytohaemagglutinin (PHA; Murex Biotech LTd, Kent, UK) for 3 days. At day 2, ^{3}H-thymidine 0.5 μCi /well was added to the culture and 16 hours later, samples were harvested and radioactivity was measured using a β-counter (PerkinElmer, Oosterhout, The Netherlands).

In the MLR, $5x10^4$ PBMC and $CD25^{neg/dim}$ cells were stimulated with $5x10^4$ HLA-A, B and DR fully mismatched (2-2-2) irradiated (40 Gy) PBMC (allo-Ag). All cultures were performed in HCM, in triplicate in a 96-wells round bottom plate for 7 days. At day 6, ^{3}H-thymidine was added to the cultures and 16 hours later samples were harvested and radioactivity was counted.

Regulation of allo-Ag stimulated responder cells by $CD25^{bright+}$ cells

Regulation of proliferation by $CD25^{bright+}$ cells was quantified both by their depletion from PBMC and by co-culture experiments with $CD25^{neg/dim}$ responder cells. After depletion the increase in proliferation reflects the regulatory capacity of the $CD25^{bright+}$ cells [16]. 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 then calculated as the percentage of inhibition (%IH).

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

FOXP3 and IL-2 mRNA expression

Messenger RNA (mRNA) was extracted from unstimulated PBMC and from allo-Ag stimulated PBMC (7 days) from 16 patients and 17 HC. cDNA transcription and amplification was performed as described before [16]. In brief, total RNA was isolated using the High Pure RNA Isolation kit (Roche Applied Science, Penzberg, Germany), according to the manufacturers instructions. cDNA was synthesized from 500ng RNA with random primers (Promega, Leiden, The Netherlands). Real Time PCR (RT-PCR) was performed on the Taqman (ABI PRISM^{TM} 7700 Sequence detector, Applied Biosystems, Foster City, CA). IL-2 (Hs00174114_m1) and FOXP3 (Hs00203958_m1) mRNA measurements were performed using Assay on Demand (Applied Biosystems) as described before [16, 30]. For the absolute quantification of mRNA expression levels we used the $2^{\wedge(40-Ct)}$ procedure and denoted target expression levels as copy number/500ng RNA [31].

Statistics

To determine if three or more groups were statistically different for a certain parameter, the One-Way ANOVA Dunnett's multiple comparison test was used. Frequency analysis was performed with Chi squared test. Correlation was analyzed with Spearmans Rho. To examine the dependence of one outcome variable on other variables simultaneously, multiple regression was performed. All calculations were done using GraphPad Prism 4.0 or SPSS 11.5. A p-value <0.05 is marked with *, p<0.01 with ** and p<0.001 with ***.

Results

Flow Cytometry

Freshly isolated PBMC samples from 77 out of 80 ESRF-patients and from 17 healthy controls (HC) were evaluated for lymphocyte subsets including the $CD4^+CD25^{bright+}$, $CD4^+CD25^+$ and $CD4^+CD25^{neg}$ T-cells (fig 1A). Our flow cytometric results are summarized in table 2. Absolute numbers of $CD19^+$ B-cells, $CD3^-CD16^+CD56^+$ NK-cells, $CD3^+$ cells and $CD4^+$ T-cells were significantly lower in ESRF-patients than in HC. The absolute number of regulatory T-cells, defined as the $CD4^+CD25^{bright+}$ T-cell population [16, 18], was also lower in ESRF-patients than in HC (fig 1B, p<0.05) as was the case for the $CD4^+CD25^+$ population (p=0.08) and the $CD4^+CD25^{neg}$ population (p<0.01). The percentage of $CD4^+CD25^{bright+}$ cells of $CD4^+$ T-cells was not different from HC (fig 1C) as was the case for the $CD4^+CD25^+$ population and the $CD4^+CD25^{neg}$ population. The ratio of $CD4^+CD25^{bright+}$ T-cells versus $CD4^+CD25^+$ or $CD4^+CD25^{neg}$ T-cells was also not different from HC.

Table 2. Flow cytometric results of whole blood, absolute numbers

Cell Subsets	**HD (N = 37)**	**PD (N = 26)**	**ND (N = 14)**	**HC[#] (N = 17)**
Leuko (*10E9/L)	4,6 (2,5–9)	7,2*** (4,8–12,4)	5,4 (3,6–8,6)	5,0 (3,8–7,3)
B cells (CD19)	44*** (2–318)	77** (18–365)	68* (26–308)	117 (29–469)
NK cells ($CD3^-CD16/56^+$)	131*** (40–703)	153*** (47–389)	181** (38–416)	272 (159–857)
CD3	809 (161–1745)	693* (327–1879)	721 (383–1858)	1011 (464–3020)
CD8	302 (55–768)	220 (59–737)	321 (100–539)	269 (115–1330)
CD4	478* (93–1375)	462* (160–1355)	422** (261–1454)	622 (331–1687)
$CD25^{neg}$	172* (75-687)	99*** (46-494)	122** (71-538)	297 (111-625)
$CD25^+$	274 (62–816)	333 (85–861)	290 (140–916)	368 (200–1147)
$CD25^{bright+}$	39* (9–102)	36* (14–115)	34* (12–84)	50 (21–153)

Absolute numbers in cell/µl, Median (range), *p<0.05 vs HC, **p<0.01 vs HC, *** p<0.001 vs HC, [#] For one HC the staining of CD25 was insufficient to distinguish $CD4^+$ cells for their expression of CD25.
HD=Haemodialysis, PD=Peritoneal Dialysis, ND=No Dialysis, HC = Healthy Controls

To characterize $CD4^{+}CD25^{bright+}$ cells from ESRF-patients in more detail, we stained whole blood of an additional cohort of ESRF-patients with FoxP3 and CD127 (fig 1D-E). In patients, the median proportion of $CD4^{+}CD25^{bright+}$ cells that expressed FoxP3 was 72% (range 50-86%). Overall this was not different from HC (80%, range 71-91%, fig 1F), but further analysis proved that this proportion of $FoxP3^{+}$ was lower in HD and ND-patients when compared to HC ($p<0.05$ and $p<0.01$ respectively). Also the proportion of $CD4^{+}CD25^{bright+}$ cells that expressed FoxP3 was lower in ND-patients when compared to PD-patients (fig 1F, $p<0.05$). Within the $CD4^{+}CD25^{+}$ and $CD4^{+}CD25^{neg}$ fraction, the expression of FoxP3 was much lower (7% (range 2-47%) and 1% (range 0-41%) respectively). This was highly comparable to HC (7% (range 4-11%) and 1% (range 0-2%) respectively). In patients, the percentage of $CD4^{+}CD25^{bright+}FoxP3^{+}$ cells with a $CD127^{neg/low}$ phenotype was 98% (range 13-100%, fig 1G). Within the $CD4^{+}CD25^{+}FoxP3^{+}$ and $CD4^{+}CD25^{neg}FoxP3^{+}$ fraction, the percentage of cells with a $CD127^{neg/low}$ was also high (86% (range 16-100%) and 83% (range 14-100%) respectively). The latter result showed that irrespectively of their expression of CD25, most $CD4^{+}FoxP3^{+}$ T cells have a $CD127^{neg/low}$ phenotype.

Expression levels of IL-2 and FOXP3 mRNA

To determine whether in the studied cohort the IL-2 pathway is activated we measured IL-2 mRNA expression levels in PBMC from 16 patients with ESRF (HD N=7, PD N=5, ND N=4) and 17 HC. RT-PCR analysis indeed showed that the expression level of IL-2 mRNA was significantly higher in patients than in HC (fig 2A, p=0.0002). The most specific marker for $CD4^{+}CD25^{bright+}$ regulatory T-cells is FoxP3 [11]. No difference was found in the expression level of FOXP3 mRNA between patients and HC (fig 2B, p=0.32).

Next, we studied the IL-2 and FOXP3 gene expression level in allo-Ag stimulated PBMC from ESRF-patients and HC. The expression level of IL-2 mRNA was not different between patients (N=14) and HC (N=17, fig 3A, p=0.49), whereas the expression level of FOXP3 mRNA was lower in patients (N=13) than PBMC of HC (N=17, fig 3B, p=0.0002).

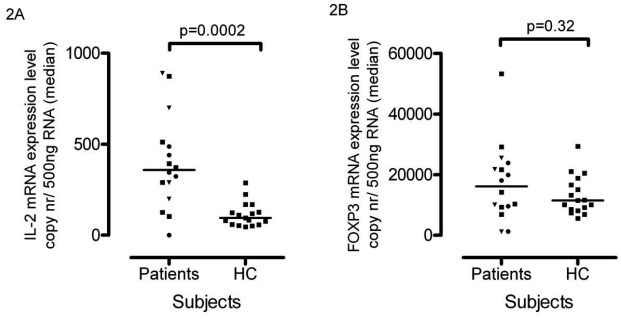

Figure 2. Basal mRNA expression levels of IL-2 and FOXP3 in PBMC
(A) The peripheral mRNA expression level of IL-2 was higher in patients (N=16) as in healthy controls (HC, N=17, p=0.0002), (B) while the mRNA expression level of FOXP3 in PBMC was not significantly different (p=0.32).

■= HD=Haemodialysis, ●= PD=Peritoneal Dialysis ▼= ND=No Dialysis.

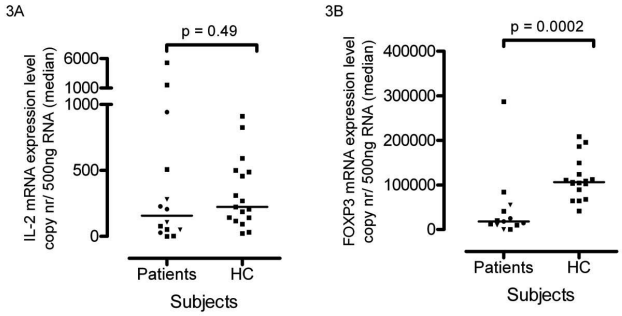

Figure 3. Expression of IL-2 and FOXP3 mRNA in allo-Ag stimulated PBMC
PBMC were cultured with allo-Ag for 7 days. (A) The expression level of IL-2 mRNA after allo-Ag stimulation of PBMC from patients (N=14) and healthy controls (HC, N=17) was comparable (p=0.49). (B) The expression level of FOXP3 mRNA was significantly higher in allo-Ag stimulated PBMC from HC (N=17) than from patients (N=13, p=0.0002).

■= HD=Haemodialysis, ●= PD=Peritoneal Dialysis ▼= ND=No Dialysis.

Proliferative responses

Proliferative responses of freshly isolated PBMC were studied in 60 out of 80 patients. ESRF-patients and HC showed comparable proliferative responses to the mitogen PHA (fig 4A). In contrast, proliferation of PBMC to allo-Ag was lower in ESRF-patients than in HC (fig 4B, p<0.0001). No significant difference was found in the proliferative capacity of PBMC between the different patient groups.

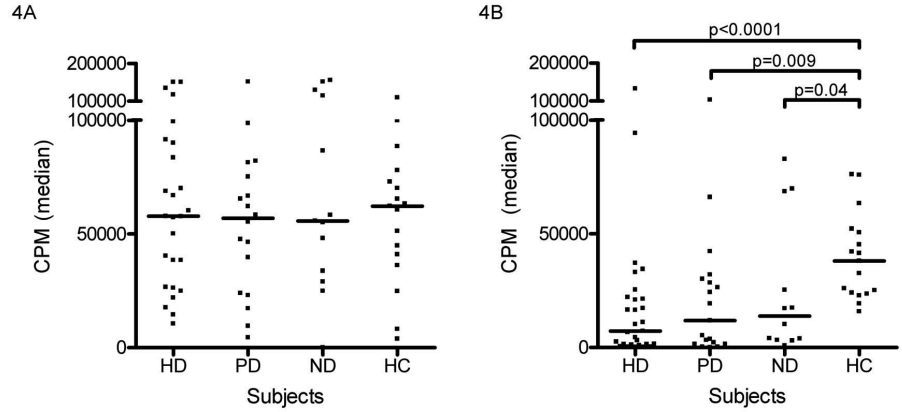

Figure 4. Proliferative responses of PBMC

(A) The proliferative response of freshly isolated PBMC from ESRF-patients and HC to PHA was comparable (HD N=26, PD N=18, ND N=13, HC N=17). (B) Comparing the proliferation of PBMC from patients and HC (N=17) to allo-Ag, demonstrated that proliferation was low by PBMC from HD-patients (N=29 p<0.0001), PD-patients (N=19 p=0.009) and ND-patients (N=12 p=0.04). HD=Haemodialysis, PD=Peritoneal Dialysis, ND=No Dialysis, HC=Healthy Controls.

Phenotypical characterization of isolated fractions by autoMACS

The isolated $CD25^{bright+}$ and $CD25^{neg/dim}$ fractions contained a comparable percentage of $CD4^{+}CD25^{bright+}$, $CD4^{+}CD25^{+}$ and $CD4^{+}CD25^{neg}$ cells for patients and HC (fig 5A-F). We were able to determine the FoxP3 protein expression by isolated fractions from 7 patients (ND N=2, PD N=3, HD N=2). Here, the median expression of FoxP3 by the isolated $CD25^{bright+}$ fraction was 73% (range 40-85%) and of the $CD25^{neg/dim}$ fraction only 3% (1-7%). These percentages are in line with our findings on the expression of FoxP3 in the $CD25^{bright+}$, $CD25^{+}$ and $CD25^{neg}$ fractions of PBMC from our additional patient-cohort (72%, 7% and 1% respectively) as well as from our HC (80%, 7% and 1% respectively).

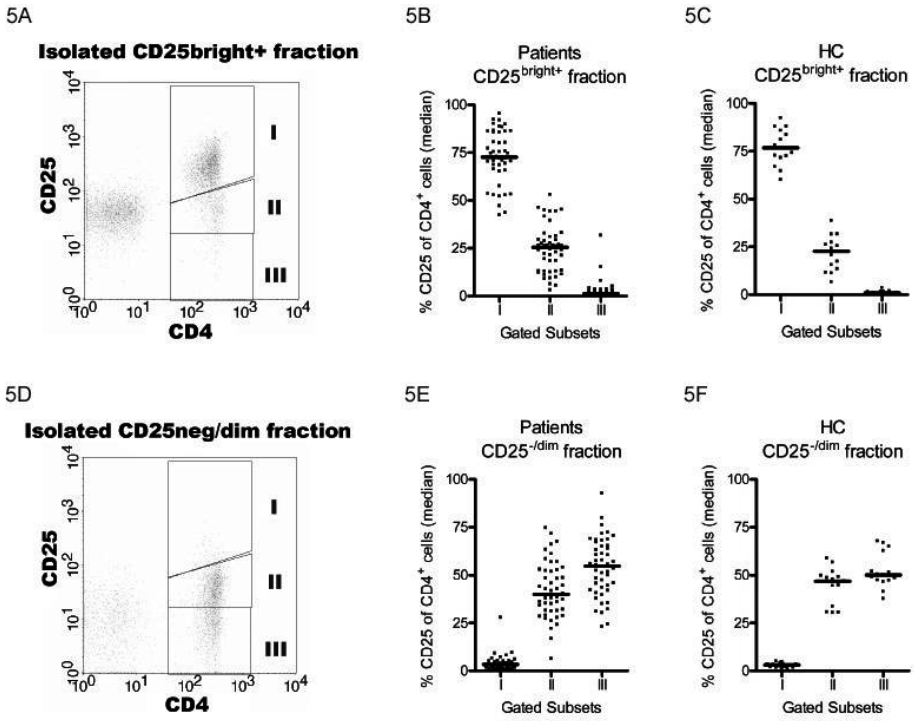

Figure 5. Expression of CD25 by cell fractions isolated by autoMACS
(A) Representative example of the isolated $CD25^{bright+}$ fraction stained for its expression of CD4 and CD25, with gated areas of the $CD25^{bright+}$ (I), $CD25^{+}$ (II) and $CD25^{neg}$ (III) fraction (B-C). Analysis of the isolated $CD25^{bright+}$ fractions from patients and healthy controls (HC) demonstrated that most of these cells resided in gate I (D). Isolated $CD25^{neg/dim}$ fraction (E-F). Analysis of the isolated $CD25^{neg/dim}$ fractions from patients and HC demonstrated that most of these cells resided in gate II and III. Due to the low number of isolated cells this flow cytometric analysis could not be performed for all patients and HC samples.

The regulatory function of $CD4^{+}CD25^{bright+}$ cells

The effect of $CD25^{bright+}$ cell depletion from PBMC on direct alloresponses was determined in the MLR. The increase in proliferation reflects the regulatory capacities

of the depleted fraction. Indeed, in HC we measured a vigorous increase of the proliferative response to allo-Ag after depletion of the $CD25^{bright+}$ fraction (fig 6, p=0.0005). Also in samples obtained from ESRF-patients an increase in the proliferative response was found. However, this effect was only significant in samples from ND-patients (p=0.009) and not for PD-patients (p=0.06) or HD-patients (p=0.13). The suppressive function of the isolated $CD25^{bright+}$ cells was determined in the MLR by adding these cells to $CD25^{neg/dim}$ cells at several ratios. The isolated $CD25^{bright+}$ cells did not proliferate upon stimulation with allo-Ag. In HC, inhibition of proliferation of $CD25^{neg/dim}$ cells by $CD25^{bright+}$ cells was dose dependent (fig 7A). This dose-dependent effect was also found for ND-patients (fig 7B), less outspoken for HD-patients (fig 7C) and not seen for PD-patients (fig 7D). To normalize for the difference in proliferation of PBMC between individuals, the percentage of inhibition (%IH) was calculated. Comparison of the %IH confirmed that the suppressive function of $CD25^{bright+}$ cells was impaired in ESRF-patients (fig 8). This was significant at the ratio of 1:5 and 1:10 (median %IH at a 1:10 ratio: 68% HC vs all patients 42%, p<0.001). The impaired suppressive function of $CD25^{bright+}$ cells in ESRF-patients at a 1:10 ratio was most explicit in patients on dialysis: ND 47%, PD 41% and HD 30%. Importantly, the %IH did not correlate with proliferation of the allo-Ag stimulated $CD25^{neg/dim}$ cells (HC: p=0.16; patients p=0.15), indicating that activated cells do respond to the suppressive signals of $CD25^{bright+}$ cells.

To determine whether the patients underlying disease, age, gender, HD/PD/ND treatment and time on dialysis influenced the function of $CD25^{bright+}$ T-cells, a multiple regression analysis was performed. This analysis showed no association between the number of $CD25^{bright+}$ T-cells, their proportion and function with one of these end-points.

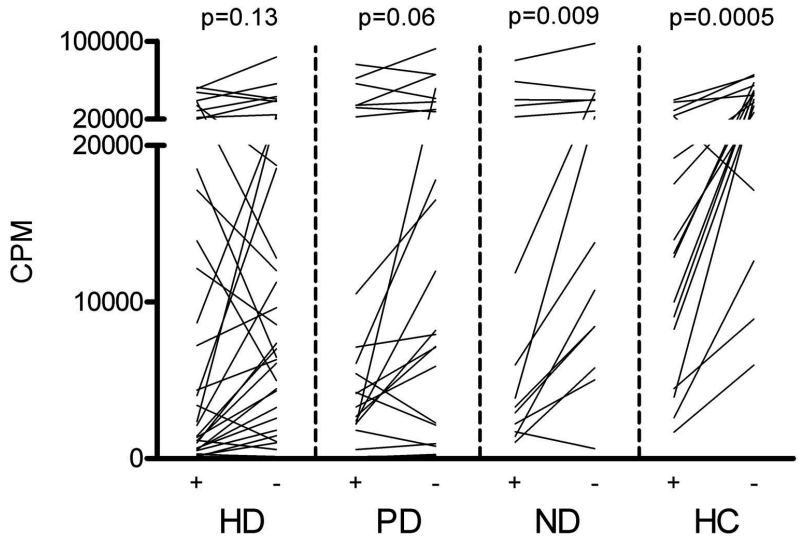

Figure 6. Proliferative responses of PBMC to allo-Ag before and after depletion of $CD25^{bright+}$ cells (+) PBMC that followed the autoMACS procedure as described in materials and methods section p9 and (-) $CD25^{neg/dim}$ cells. Depletion of $CD25^{bright+}$ cells from PBMC resulted in a significant increase in the proliferative response in HC and ND-patients, but not for PD-patients and HD-patients. HD=Haemodialysis (N=33), PD=Peritoneal Dialysis (N=23), ND=No Dialysis (N=14), HC=Healthy Controls (N=17).

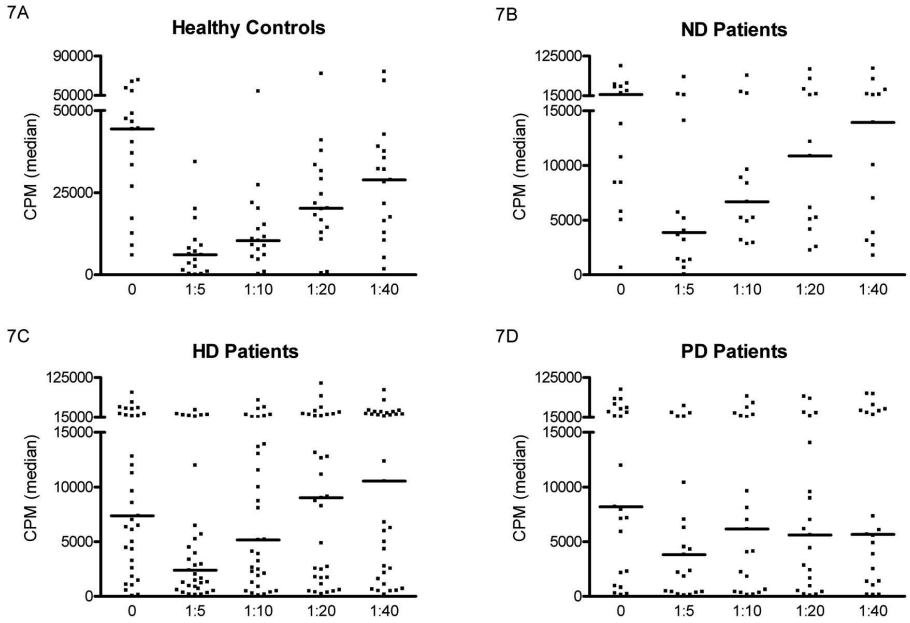

Figure 7. Cocultures of $CD25^{bright+}$ cells with $CD25^{neg/dim}$ responder cells
(A) In HC, adding $CD25^{bright+}$ cells to allo-Ag stimulated $CD25^{neg/dim}$ responder cells at several ratios, showed a dose dependent inhibition. (B) This dose dependent effect was also seen in ND-patients, (C) less outspoken in HD-patients and (D) not seen in PD-patients. The isolated $CD25^{bright+}$ cells did not proliferate upon stimulation.
HD=Haemodialysis (N=33), PD=Peritoneal Dialysis (N=23), ND=No Dialysis (N=14), HC=Healthy Controls (N=17).

Discussion

To assess whether the characteristic overactivated but compromised immune system of ESRF-patients is caused by an impaired function of $CD4^{+}CD25^{bright+}FoxP3^{+}$ T-cells, we analysed their number and regulatory capacities. First, we demonstrated that the number of peripheral $CD4^{+}CD25^{bright+}$ T-cells in ESRF-patients was low, whereas their proportion of $CD4^{+}$ T-cells was not different between ESRF-patients and HC. Second, while the proliferation of PBMC from ESRF-patients upon stimulation with allo-Ag was affected, it appeared that there was also a defect in regulation by $CD4^{+}CD25^{bright+}$ T-cells.

Several explanations for the immunodeficient state observed in ESRF-patients have been described, including a decreased capacity of antigen presentation and a low number of circulating lymphocytes [4-6, 27, 28, 32, 33]. The cause of low numbers of peripheral $CD4^{+}CD25^{bright+}$ T-cells in ESRF-patients is at present unclear. An explanation may be that $CD4^{+}CD25^{bright+}$ T-cells like $CD4^{+}$ T-cells from ESRF-patients show increased susceptibility for apoptosis resulting from their continuous activation by uremic toxins [33-35]. No difference in $CD4^{+}CD25^{bright+}$ cell counts or the ratio of $CD4^{+}CD25^{bright+}$ cells versus $CD4^{+}CD25^{neg}$ and $CD4^{+}CD25^{+}$ cells as well as their percentage of $CD4^{+}$ T-cells was measured between patients on dialysis and

those not yet necessitating RRT. Therefore our results indicate that renal failure itself affects circulating $CD4^{+}CD25^{bright+}$ T cell numbers, but does not disturb the balance between $CD4^{+}$ effector T-cells and Treg. Further analysis of the $CD4^{+}CD25^{bright+}$ cells from our additional cohort demonstrated that their expression of FoxP3 was especially low in ND-patients, while the expression of CD127 by $CD4^{+}CD25^{bright+}FoxP3^{+}$ cells was not different between patient groups. Our findings and other recent results on the expression of CD25 by $CD4^{+}$ T cells [36], are not in line with studies more than 15 years ago, where increased numbers of T-cells expressing CD25 were observed in the periphery of ESRF-patients [21, 37]. This may be explained by improved biocompatibility of dialytic devices leading to less activation.

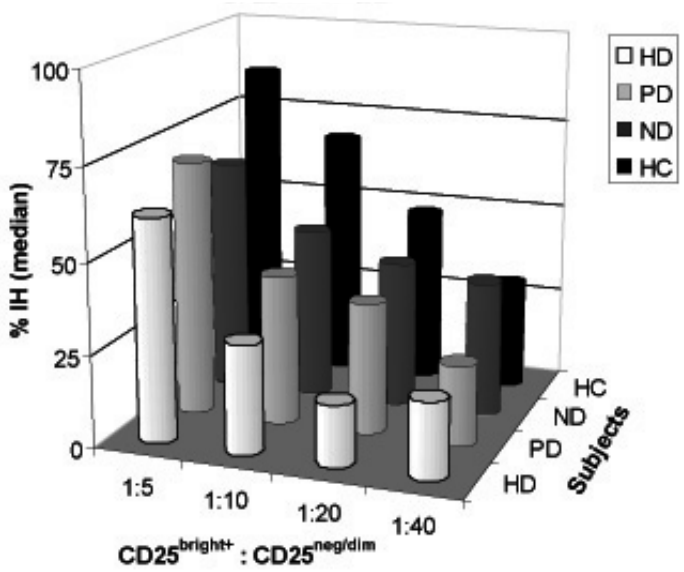

Figure 8. Suppression of $CD25^{neg/dim}$ cells by $CD25^{bright+}$ cells calculated as the percentage of inhibition (%IH)
Inhibition of proliferation of $CD25^{neg/dim}$ cells by $CD25^{bright+}$ cells calculated as the %IH, was dose dependent in HC and all patient groups. Also, the %IH was lower in patients than in HC. This was significant when $CD25^{bright+}$ cells were added to $CD25^{neg/dim}$ cells at the ratio of 1:10 (median %IH: 68% HC vs all patients 42%, $p<0.001$). The impaired suppressive function of $CD25^{bright+}$ cells in ESRD-patients at a 1:10 ratio, was most explicit in patients on dialysis: ND 47%, PD 41% and HD 30%. HD=Haemodialysis (N=33), PD=Peritoneal Dialysis (N=21), ND=No Dialysis (N=13), HC=Healthy Controls (N=17).

Recently, the literature has been overwhelmed by studies analyzing the role of $CD4^{+}$ $CD25^{bright+}$ T-cells in human diseases. Associations were found between impaired $CD4^{+}CD25^{bright+}$ T-cell function and disease activity [38]. However, whether the function of $CD4^{+}CD25^{bright+}$ T-cells is also affected in ESRF-patients remained unclear. The present study showed that $CD4^{+}CD25^{bright+}$ T-cells from ESRF-patients and in particular those on dialysis do not properly exert their suppressive function in the direct pathway of allorecognition. Yet, although this low suppressive capacity of $CD4^{+}CD25^{bright+}$ T-cells is probably not responsible for the functionally compromised immune system, it may still lead to higher T-cell activation.

To generate potent suppressive capacity, $CD4^+CD25^{bright+}$ T-cells require activation through their T-cell receptor (TCR), co-stimulation by CD28 and the presence of IL-2 [39-41]. It has been shown, that the density of the TCR/CD3 antigen receptor complex as well as the expression of CD28 is low on $CD4^+$ T-cells from ESRF-patients [27, 42, 43]. If their expression levels are also affected on $CD4^+CD25^{bright+}FoxP3^+$ T-cells, this may explain their deficient regulatory capacity as observed in this study.

IL-2 is critically involved in $CD4^+CD25^+FoxP3^+$ T-cell function [19, 20, 39], especially by direct targeting of the master gene FoxP3, which controls their function [11, 44]. FoxP3 forms a cooperative complex with nuclear factor of activated T-cells, thereby suppressing the production of IL-2 [45]. Numerous studies have shown that the IL-2 pathway in ESRF-patients is activated but impaired upon activation [22-24]. This was confirmed by high basal expression level of IL-2 mRNA in patient-PBMC from this study, supporting that ESRF-patients have a continuous overactivated immune system. However, despite their significantly higher basal expression level of IL-2 mRNA, alloactivation did not increase their levels beyond the level of the alloactivated control PBMC. This relatively lower inducibility of IL-2 is in line with the already reported lower amount of IL-2 protein in the supernatant of stimulated PBMC from ESRF-patients [21-24]. This low availability of IL-2 could explain the significantly low expression of FOXP3 mRNA by patient-PBMC upon alloactivation and therefore of the impaired regulatory functions. Even low amounts of IL-2 will be bound by the high level of soluble IL-2R, which are abundant in the serum and supernatant of cell cultures from ESRF-patients [21-24]. We are aware that other factors than a deficient IL-2 system may be involved too. In ESRF-patients both IL-6 and TGF-β are upregulated [46, 47]. These factors create a suitable environment for the induction of effector Th17-cells that are able to inhibit the function of $CD4^+CD25^+FoxP3^+$ T-cells [48, 49]. This may also contribute to their decreased functional compartment in ESRF-patients.

The multiple regression analysis showed no significant correlation between one of the clinical parameters and the suppressive function of $CD4^+CD25^{bright+}$ T-cells. Nevertheless, our data indicate that the proliferative capacity of effector T-cells as well as the regulatory capacity of $CD4^+CD25^{bright+}$ T-cells from patients on dialysis is more impaired than from ND-patients. This finding is consistent with the increased disturbance of the defensive immune system in ESRF-patients on dialysis as a result from the continuous non-specific immune cell stimulation by dialytic devices [7, 8]. Therefore, the observed difference in $CD4^+CD25^{bright+}$ T-cell function between patients on dialysis and ND-patients may either result from the duration of ESRF or the dialysis procedure itself. We are aware, however, that the etiological background of ESRF may influence immune regulation [38, 50].

In summary, we observed activation by gene expression markers but a functionally compromised immune system in ESRF-patients. It now appears that in this setting, regulation by $CD4^+CD25^{bright+}FoxP3^+$ T-cells is impaired as well, leading to even higher T-cell activation but still not to improved function.

References

1. Kohler H, Arnold W, Renschin G, Dormeyer HH, Meyer zum Buschenfelde KH. Active hepatitis B vaccination of dialysis patients and medical staff. Kidney Int 1984; 25: 124-128
2. Olsen SK, Brown RS, Jr. Hepatitis B treatment: Lessons for the nephrologist. Kidney Int 2006; 70: 1897-1904
3. Maisonneuve P, Agodoa L, Gellert R, et al. Cancer in patients on dialysis for end-stage renal disease: an international collaborative study. Lancet 1999; 354: 93-99
4. Kurz P, Kohler H, Meuer S, Hutteroth T, Meyer zum Buschenfelde KH. Impaired cellular immune responses in chronic renal failure: evidence for a T cell defect. Kidney Int 1986; 29: 1209-1214
5. Descamps-Latscha B, Chatenoud L. T cells and B cells in chronic renal failure. Semin Nephrol 1996; 16: 183-191
6. Girndt M, Sester U, Sester M, Kaul H, Kohler H. Impaired cellular immune function in patients with end-stage renal failure. Nephrol Dial Transplant 1999; 14: 2807-2810
7. Amore A, Coppo R. Immunological basis of inflammation in dialysis. Nephrol Dial Transplant 2002; 17 Suppl 8: 16-24
8. Opatrny K, Jr. Clinical importance of biocompatibility and its effect on haemodialysis treatment. Nephrol Dial Transplant 2003; 18 Suppl 5: v41-44
9. Yoon JW, Pahl MV, Vaziri ND. Spontaneous leukocyte activation and oxygen-free radical generation in end-stage renal disease. Kidney Int 2007; 71: 167-172
10. Meyer TW, Hostetter TH. Uremia. N Engl J Med 2007; 357: 1316-1325
11. Hori S, Nomura T, Sakaguchi S. Control of regulatory T cell development by the transcription factor Foxp3. Science 2003; 299: 1057-1061
12. Sakaguchi S. Naturally arising Foxp3-expressing CD25+CD4+ regulatory T cells in immunological tolerance to self and non-self. Nat Immunol 2005; 6: 345-352
13. Gavin MA, Rasmussen JP, Fontenot JD, et al. Foxp3-dependent programme of regulatory T-cell differentiation. Nature 2007; 445: 771-775
14. Ghiringhelli F, Larmonier N, Schmitt E, et al. CD4+CD25+ regulatory T cells suppress tumor immunity but are sensitive to cyclophosphamide which allows immunotherapy of established tumors to be curative. Eur J Immunol 2004; 34: 336-344
15. Cabrera R, Tu Z, Xu Y, et al. An immunomodulatory role for CD4(+)CD25(+) regulatory T lymphocytes in hepatitis C virus infection. Hepatology 2004; 40: 1062-1071
16. Velthuis JH, Mol WM, Weimar W, Baan CC. CD4+CD25bright+ regulatory T cells can mediate donor nonreactivity in long-term immunosuppressed kidney allograft patients. Am J Transplant 2006; 6: 2955-2964
17. Thornton AM, Shevach EM. CD4+CD25+ immunoregulatory T cells suppress polyclonal T cell activation in vitro by inhibiting interleukin 2 production. J Exp Med 1998; 188: 287-296
18. Baecher-Allan C, Brown JA, Freeman GJ, Hafler DA. CD4+CD25high regulatory cells in human peripheral blood. J Immunol 2001; 167: 1245-1253

19. Thornton AM, Donovan EE, Piccirillo CA, Shevach EM. Cutting edge: IL-2 is critically required for the in vitro activation of CD4+CD25+ T cell suppressor function. J Immunol 2004; 172: 6519-6523
20. D'Cruz LM, Klein L. Development and function of agonist-induced CD25+Foxp3+ regulatory T cells in the absence of interleukin 2 signaling. Nat Immunol 2005; 6: 1152-1159
21. Beaurain G, Naret C, Marcon L, et al. In vivo T cell preactivation in chronic uremic hemodialyzed and non-hemodialyzed patients. Kidney Int 1989; 36: 636-644
22. Meier P, Dayer E, Ronco P, Blanc E. Dysregulation of IL-2/IL-2R system alters proliferation of early activated CD4+ T cell subset in patients with end-stage renal failure. Clin Nephrol 2005; 63: 8-21
23. Donati D, Degiannis D, Raskova J, Raska K, Jr. Uremic serum effects on peripheral blood mononuclear cell and purified T lymphocyte responses. Kidney Int 1992; 42: 681-689
24. Daichou Y, Kurashige S, Hashimoto S, Suzuki S. Characteristic cytokine products of Th1 and Th2 cells in hemodialysis patients. Nephron 1999; 83: 237-245
25. Wolf D, Hochegger K, Wolf AM, et al. CD4+CD25+ regulatory T cells inhibit experimental anti-glomerular basement membrane glomerulonephritis in mice. J Am Soc Nephrol 2005; 16: 1360-1370
26. Mahajan D, Wang Y, Qin X, et al. CD4+CD25+ regulatory T cells protect against injury in an innate murine model of chronic kidney disease. J Am Soc Nephrol 2006; 17: 2731-2741
27. Girndt M, Sester M, Sester U, Kaul H, Kohler H. Defective expression of B7-2 (CD86) on monocytes of dialysis patients correlates to the uremia-associated immune defect. Kidney Int 2001; 59: 1382-1389
28. Verkade MA, van Druningen CJ, Op de Hoek CT, Weimar W, Betjes MG. Decreased antigen-specific T-cell proliferation by moDC among hepatitis B vaccine non-responders on haemodialysis. Clin Exp Med 2007; 7: 65-71
29. Liu W, Putnam AL, Xu-Yu Z, et al. CD127 expression inversely correlates with FoxP3 and suppressive function of human CD4+ T reg cells. J Exp Med 2006; 203: 1701-1711
30. Baan CC, van der Mast BJ, Klepper M, et al. Differential effect of calcineurin inhibitors, anti-CD25 antibodies and rapamycin on the induction of FOXP3 in human T cells. Transplantation 2005; 80: 110-117
31. Bustin SA. Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays. J Mol Endocrinol 2000; 25: 169-193
32. Litjens NH, van Druningen CJ, Betjes MG. Progressive loss of renal function is associated with activation and depletion of naive T lymphocytes. Clin Immunol 2006; 118: 83-91
33. Yoon JW, Gollapudi S, Pahl MV, Vaziri ND. Naive and central memory T-cell lymphopenia in end-stage renal disease. Kidney Int 2006; 70: 371-376
34. Meier P, Dayer E, Blanc E, Wauters JP. Early T cell activation correlates with expression of apoptosis markers in patients with end-stage renal disease. J Am Soc Nephrol 2002; 13: 204-212
35. Meier P, Spertini F, Blanc E, Burnier M. Oxidized low-density lipoproteins activate CD4+ T cell apoptosis in patients with end-stage renal disease through Fas engagement. J Am Soc Nephrol 2007; 18: 331-342

36. van Riemsdijk IC, Baan CC, Loonen EH, et al. T cells activate the tumor necrosis factor-alpha system during hemodialysis, resulting in tachyphylaxis. Kidney Int 2001; 59: 883-892
37. Stachowski J, Pollok M, Burrichter H, Baldamus CA. Immunodeficiency in ESRD-patients is linked to altered IL-2 receptor density on T cell subsets. J Clin Lab Immunol 1991; 34: 171-177
38. Rouse BT. Regulatory T cells in health and disease. J Intern Med 2007; 262: 78-95
39. Thornton AM, Piccirillo CA, Shevach EM. Activation requirements for the induction of CD4+CD25+ T cell suppressor function. Eur J Immunol 2004; 34: 366-376
40. Salomon B, Lenschow DJ, Rhee L, et al. B7/CD28 costimulation is essential for the homeostasis of the CD4+CD25+ immunoregulatory T cells that control autoimmune diabetes. Immunity 2000; 12: 431-440
41. Tai X, Cowan M, Feigenbaum L, Singer A. CD28 costimulation of developing thymocytes induces Foxp3 expression and regulatory T cell differentiation independently of interleukin 2. Nat Immunol 2005; 6: 152-162
42. Stachowski J, Pollok M, Burrichter H, Spithaler C, Baldamus CA. Does uremic environment down-regulate T cell activation via TCR/CD3 antigen receptor complex? J Clin Lab Immunol 1991; 36: 15-21
43. Cooper AC, Breen CP, Vyas B, Ochola J, Kemeny DM, Macdougall IC. Poor response to recombinant erythropoietin is associated with loss of T-lymphocyte CD28 expression and altered interleukin-10 production. Nephrol Dial Transplant 2003; 18: 133-140
44. Zorn E, Nelson EA, Mohseni M, et al. IL-2 regulates FOXP3 expression in human CD4+CD25+ regulatory T cells through a STAT-dependent mechanism and induces the expansion of these cells in vivo. Blood 2006; 108: 1571-1579
45. Wu Y, Borde M, Heissmeyer V, et al. FOXP3 controls regulatory T cell function through cooperation with NFAT. Cell 2006; 126: 375-387
46. Cheng J, Grande JP. Transforming growth factor-beta signal transduction and progressive renal disease. Exp Biol Med 2002; 227: 943-956
47. Stenvinkel P, Ketteler M, Johnson RJ, et al. IL-10, IL-6, and TNF-alpha: central factors in the altered cytokine network of uremia--the good, the bad, and the ugly. Kidney Int 2005; 67: 1216-1233
48. Bettelli E, Carrier Y, Gao W, et al. Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells. Nature 2006; 441: 235-238
49. Xu L, Kitani A, Fuss I, Strober W. Cutting Edge: Regulatory T Cells Induce CD4+CD25-Foxp3- T Cells or Are Self-Induced to Become Th17 Cells in the Absence of Exogenous TGF-beta. J Immunol 2007; 178: 6725-6729
50. Lindley S, Dayan CM, Bishop A, Roep BO, Peakman M, Tree TI. Defective suppressor function in CD4(+)CD25(+) T-cells from patients with type 1 diabetes. Diabetes 2005; 54: 92-99

CHAPTER 3

Impact of Immunosuppressive Drugs on $CD4^+CD25^+FOXP3^+$ Regulatory T cells: Does In Vitro Evidence Translate to the Clinical Setting?

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Abstract

Success of solid-organ transplantation requires the continuous administration of immunosuppressive drugs to prevent graft rejection. The currently prescribed immunosuppressive medication targets the immune system in a nonspecific fashion, leading to debilitating side effects that diminish patient survival and quality of life. Therefore, it is important to minimize immunosuppression, but this requires the development of alternative therapeutic strategies to induce and maintain transplant tolerance. One such strategy would be to allow and facilitate the induction of alloantigen specific immune regulation by regulatory T cells (Treg). Recent experimental studies indicate that several commonly used immunosuppressive drugs have detrimental effects on the induction and function of Treg, while other drugs seem to spare these cells or may even be beneficial. These differential effects may be explained by differences in signaling pathways between Treg and effector T cells. In this review, we provide a comprehensive overview of the current literature on the effects of immunosuppressive drugs on $CD4^+CD25^+FOXP3^+$ Treg and discuss whether these in vitro data are substantiated by in vivo evidence from the clinic. A greater understanding of the impact of immunosuppression on Treg may help to create future opportunities to manipulate the host allo-immune response and achieve operational tolerance in transplantation.

Introduction

Without immunosuppression, transplanted organs are rapidly rejected by the recipient's immune system. The current immunosuppressive drugs effectively reduce the immune response to alloantigens, resulting in a relatively low incidence of acute rejection. Most immunosuppressive drugs target the intracellular signals involved in T-cell activation following antigen presentation (*1, 2*) as shown in Figure 1. Conventional immunosuppressive drugs include corticosteroids, calcineurin inhibitors (CNI), IL-2 receptor-blocking monoclonal antibodies (mAb), rapamycin, mycophenolate mofetil (MMF), and the T-cell depleting antibodies anti-thymocyte globulines (ATG), the anti-CD3 mAb OKT3 and the anti-CD52 mAb Campath. Characteristics of these compounds and their effect on T cell subsets are summarized in Table 1. Long-term administration of immunosuppressants leads to many debilitating side effects reducing patient and graft survival (*3*). Therefore, it is essential to minimize the use of immunosuppression and accomplish alternative strategies to induce and maintain transplant tolerance. A possible strategy that is now extensively investigated is the induction and maintenance of transplant tolerance by regulatory T cells (Treg), in particular the $CD4^+CD25^+FOXP3^+$ Treg. As immunosuppressive drugs affect effector T cells (Teff), they may also affect Treg. This notion has raised concerns about the influence of immunosuppressive drugs on Treg mediated tolerance and sparked intensive research on this issue.

It has been established that the X chromosome-encoded transcription factor, FOXP3, is highly expressed by Treg and essential for their development (*4, 5*). Though FOXP3 expression can be observed in activated human Teff, this expression is transient and does not seem to induce a Treg phenotype (*6*). Mutations in the FOXP3 gene in humans and mice results in an aggressive multi-organ autoimmune disease (*7, 8*). There is now emerging evidence that upon T cell receptor stimulation in $FOXP3^+$ Treg, different signaling pathways are activated as compared to Teff. These differences in signaling may help explain distinct effects of immunosuppressants on Treg. In the following paragraphs we will discuss experimental as well as clinical studies that investigated the impact of immunosuppression on the generation, proliferation, survival and function of Treg, and how this may affect tolerance after organ transplantation.

Effects of immunosuppressive drugs on Treg: Experimental evidence

The possibility to culture and expand Treg (*9-15*), has opened up the possibility to study the effect of immunosuppressive drugs on these cells in vitro. Although $CD4^+CD25^+FOXP3^+$ Treg can arise from thymic and peripheral origin, both may be important in transplantation tolerance. Most experiments with Treg and immunosuppressive medication focused on the natural occurring Treg, which are generated in the thymus. The adaptive or inducible Treg are thought to be either continuously generated from responding memory Teff in the periphery or to originate directly from the thymus with an naïve phenotype ($CD45RA^{pos}$) (*16, 17*).

Figure 1

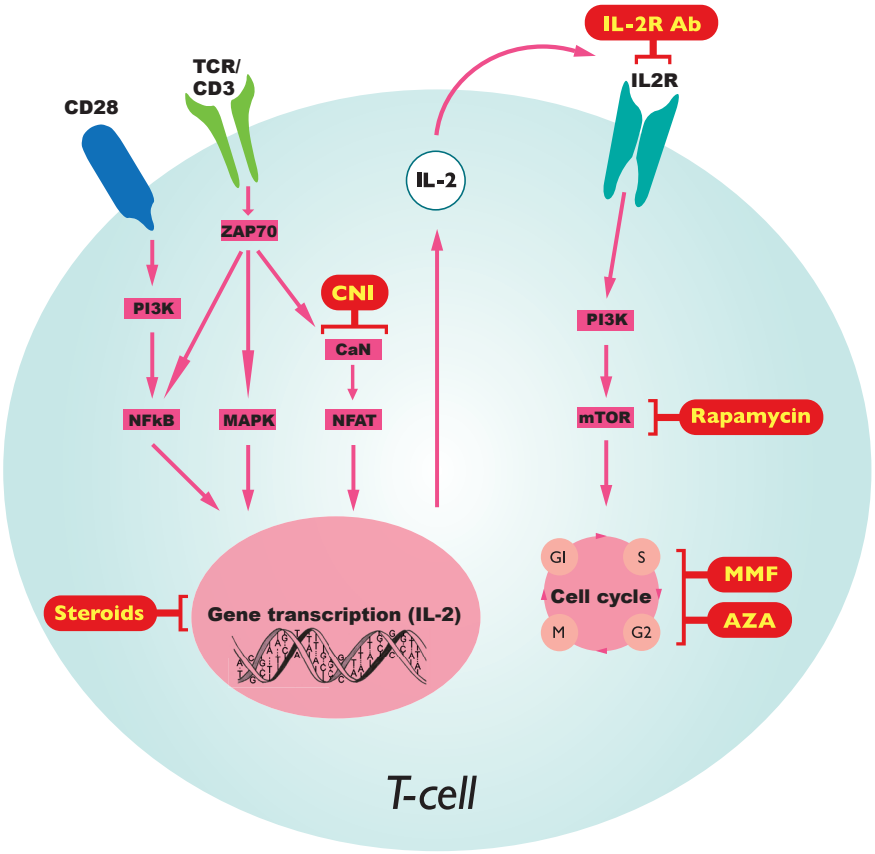

Figure 1. After organ transplantation, the alloactivation of T-cells can be suppressed using different immunosuppressive drugs. These drugs intervene with different signaling pathways involved in T-cell activation and proliferation, e.g. by interaction with the IL-2 pathway. Differential effects of immunosuppressive drugs on regulatory T cells (Treg) and effector T cells (Teff) may be explained by differences in signal transduction pathways. For example IL-2 does not primarily activate PI3K in Treg as in Teff.

Table 1: Immunosuppressive drugs and their effect on T(reg) cells

Drug	*Mechanism of action*	*Specific effect on effector T cells*	*Specific effects on regulatory T cells*
Corticosteroids	Cytosolic receptors, heat shock proteins, interfere with transcription factors	Induce apoptosis, inhibit cytokine production, interfere with migration	Stimulate FOXP3 expression, preserve suppressive activity and cell survival
Tacrolimus (FK506)	Binds FKBP-12 Inhibits calcineurin	Inhibits cell activation, cell death and proliferation	Inhibits FOXP3 expression and possible suppressor function
Cyclosporin A (CsA)	Binds cyclophilins Inhibits calcineurin	Inhibits T-cell activation and proliferation	Inhibits FOXP3 expression and possible suppressor function
CD25 mAbs (daclizumab/ basiliximab)	Binds CD25 as target epitope	Inhibits IL-2 dependent proliferation	Insufficient data
Rapamycin and derivates (sirolimus & everolimus)	Binds FKBP-12 Inhibits mammalian target of rapamycin (mTOR) Blocks p70 S6 kinase	Blocks T-cell proliferation	Does not appear to effect survival and suppressor function
Mycophenolate mofetil (MMF)	Inosine monophosphate dehydrogenase inhibitor (with high affinity for type2)	Selective blockade of lymphocyte proliferation	Does not appear to effect survival and suppressor function
T-cell depleting antibodies (ATG, OKT-3, Campath)	Depletion of T-, B-, NK-cells and monocytes	Complement dependent lyses, activation induced apoptosis, inhibit proliferation	Appear to induce expansion in vitro and spare Treg in vivo

Corticosteroids

Corticosteroids are pleiotropic hormones that are used for their potent anti inflammatory and immunomodulatory action. The most commonly used corticosteroids are prednisone, prednisolone and methylprednisolone. Corticosteroids inhibit the action of transcription factors, like NF-κB and AP-1 that are involved in transcription of cytokine and chemokine genes including IL-2, TNF-α and IFN-γ. It is known that $CD4^+CD25^+FOXP3^+$ T cells highly express the glucocorticoid receptor as well as glucocorticoid-induced TNF receptor (GITR), a potent T-cell co-stimulatory receptor and regulator of Treg function (*18*). The activity of GITR has been implicated in peripheral tolerance since inhibition or a deficiency of GITR increases T-cell proliferation by abrogating the suppressive function of Treg (*19-22*). The first direct evidence that steroids affect Treg came from the observation that the female sex hormone, estrogen, up-regulates FOXP3 expression in mice, both in vitro and in vivo (*23*). Similar results were reported for the synthetic corticosteroid, dexamethasone, which induced FOXP3 expression in short and long-term T-cell cultures, while preserving the suppressive capacity of Treg (*24, 25*). Furthermore, $CD4^+CD25^+$ T cells seemed resistant to

dexamethasone-induced T-cell apoptosis (*18*). In mice, short-term simultaneous administration of dexamethasone and IL-2 expanded FOXP3$^+$ Treg in peripheral lymphoid tissues (*26*). This treatment improved the suppressive capacity of splenic Treg in a way that they were able to prevent the onset of autoimmune disease. Taken together, experimental evidence does not indicate that corticosteroids have a negative impact on Treg but rather improve the survival and function of Treg. One could even speculate whether this effect on Treg may account for some of the anti-inflammatory and immunosuppressive efficacy of steroids.

Calcineurin inhibitors

The CNI, Cyclosporin A (CsA) and Tacrolimus, are potent inhibitors of the phosphatase calcineurin, which is essential for T-cell activation. By inhibiting calcineurin these drugs suppress the production of IL-2 and related cytokines through prevention of downstream activation of the transcription factor, Nuclear Factor of Activated T cells (NFAT) (*27, 28*). Few in vitro studies described the effect of CNI on Treg. Baan et al. showed that in a Mixed Leukocyte Reaction (MLR) the induction of FOXP3 mRNA was inhibited by CNI (*29*). This was confirmed by other studies which observed decreased FOXP3 mRNA and protein (*30, 31*) and a loss of the highly suppressive CD27$^+$ Treg subset in cultures containing CsA (*32*). These later studies report contradicting effects of CsA on the suppressive function of Treg, one observing no effect with human Treg (*32*) and the other finding less suppression with mouse Treg (*30*).

There are now recent in vivo data that show a negative effect of CNI on Treg. Treatment of mice with CsA compromised not only the thymic generation of Treg but also resulted in a sharp reduction of Treg in peripheral immune compartments (*33*). In a mouse bone marrow transplantation model, CsA administration inhibited Treg mediated suppression which was associated with reduced IL-2 production (*30*). Exogenous IL-2 was shown to overturn the reduced suppressive function of Treg by CsA. Together these data suggest that CNI are not beneficial for Treg, but are rather detrimental to their generation, survival and function. This further underlines the importance of IL-2 for Treg function and homeostasis.

IL-2 Receptor-blocking antibodies

The chimeric mAb basiliximab and humanized mAb daclizumab are therapeutic agents directed against the α-chain of the IL-2 receptor (CD25). Both non-depleting mAbs directly interfere with receptor signaling by inhibiting IL-2 binding and subsequent phosphorylation of the IL-2 receptor beta- and gamma-chains. One study showed inhibition of FOXP3 mRNA induction by CD25 mAb daclizumab in allostimulated PBMC (*29*) and another study showed down-regulated FOXP3 protein expression (*34*) and no expansion of CD4$^+$CD25$^+$ T cells after culture with this agent (*35*). However, in direct co-incubations of Treg and Teff, CD25 mAb did not interfere in the suppressive activities of CD4$^+$CD25$^+$ Treg (*36*). A recent study in mice did observe that particular CD25 mAb reduced the percentage of FOXP3$^+$ T cells within the CD4 fraction in vivo (*37*), whereas another study showed no reduction of peripheral Treg numbers but rather inactivated their suppressive function (*38*). Despite the convincing evidence that IL-2 is critical for Treg survival and function, no firm conclusions can be drawn on the effect of anti-CD25 mAb on these immunoregulatory T cells.

Rapamycin

Rapamycin-derivatives, sirolimus and everolimus, are potent inhibitors of the IL-2 signaling pathway. They exert their effect at the level of mammalian target of rapamycin (mTOR), thereby preventing the transgression from G1 to S-phase (*39*). Many studies have investigated the impact of rapamycin on Treg. Initial in vitro studies did not observe an effect of rapamycin on FOXP3 expression during allogenic stimulation, both for human (*29*) and mouse (*30*). The latter study also showed no effect of rapamycin on the suppressive capacity of Treg. Indeed other studies confirmed that rapamycin does not interfere with the suppressive activity of $CD4^+CD25^+$ Treg (*32, 36*). In this setting, $CD4^+CD25^+$ Treg appeared more resistant to the proapoptotic effect of rapamycin than $CD25^-$ T cells (*36*). This was confirmed by culture experiments with rapamycin, were selective expansion of murine and human $CD4^+CD25^+FOXP3^+$ Treg was observed, while at the same time killing Teff or at least preventing their expansion (*32, 40-43*).

Also in vivo, there is evidence that rapamycin treatment has favorable effects on Treg. In a study with mice by Battaglia et al. $CD4^+CD25^+FOXP3^+$ Treg expanded ex vivo in the presence of rapamycin, prevented rejection of β-islet transplants in vivo (*44*). Rapamycin also induces de novo expression of FOXP3 in murine alloantigen specific T cells dose dependently which appeared to be TGF-ß1 dependent (*31*). Since rapamycin can induce the expression of TGF-ß1, this may be an important mechanism contributing to the development of antigen-specific Treg (*45*). Interestingly a recent study suggests that rapamycin can induce regulatory functions in conventional $CD4^+$ T cells in culture (*46*). Furthermore, evidence suggests that rapamycin conditioned dendritic cells are poor stimulators of allogenic T cells, but enrich for antigen specific Treg that can prolong cardiac graft survival in mice (*47*). In conclusion, there is substantial evidence that rapamycin favors Treg survival and function and, by suppressing Teff cells, tips the balance from an aggressive towards a more protective type of allo-immune response.

Mycophenolate mofetil

MMF is a pro-drug of mycophenolic acid (MPA), an inhibitor of inosine monophosphate dehydrogenase. This is the rate-limiting enzyme in de novo synthesis of guanosine nucleotides and T cells in particular are dependent on this pathway for cell division. MMF suppresses proliferation of antigen-stimulated T cells (*48*). In addition, MMF has been shown to decrease the expression of CD25 on these T cells in a dose dependent fashion (*49, 50*). Only one study specifically described the effect of MMF on Treg in vitro (*30*). Here, MPA did not alter the expression of FOXP3 or affect the suppressive capacity of Treg, which is supported by data from our laboratory. Therefore, Treg may function normally in the presence of MMF.

T-cell depleting antibodies

It has been suggested that the clinical benefits of ATG- and Campath (i.e. Alemtuzumab) induction therapy do not completely result form immunodepletion but also from induction of immunoregulatory T-cells. ATG caused a rapid and sustained expansion of Treg in human lymphocytes cultures as well as elevated expression of FOXP3 (*35*). The mode of action of ATG is I. conversion from $FOXP3^-$ to $FOXP3^+$ T cells and II. expansion of already present Treg. Both the converted and expanded cells showed significant suppressive activities. In an

experimental mouse model, treatment with anti-lymphocyte serum spared $CD25^+$ T cells (*39*), and early recovery of Treg following a lymphopenic event in mice seemed to control exuberant antigen-stimulated $CD4^+$ cell cycle progression (*51*). Overall, these data suggest that T-cell depleting antibodies may be useful in Treg expansion protocols and induction of tolerance in vivo.

Signaling pathways in Treg

Differential effects of immunosuppression on Treg may be explained by distinct signaling pathways triggered by the T cell receptor (TCR), cytokines and costimulatory molecule stimulation. Recent transcriptional profiling studies of mouse Treg showed that at least 700 genes are up or down regulated by FOXP3 (*52, 53*). The IL-2 receptor-chain (CD25) is one of the up-regulated genes, which results in the characteristic high expression of CD25 on Treg. Genes that are downregulated by FOXP3 include IL-2 and TCR-signaling molecules Zap70 and ITK. Accordingly, it has been shown that $FOXP3^+$ Treg and $FOXP3^-$ Teff have a distinct response to TCR triggering (*54, 55*). Unlike Teff, human or mice Treg do not proliferate in vitro upon appropriate activation nor produce IL-2, IL-4 and interferon (IFN)-γ cytokines (*54*). Moreover, signaling pathways involved in reorganization of the actin cytoskeleton (e.g. VAV), which is important for sustained signaling by the TCR, are shutdown in Treg (*56*). As a consequence of the suppression of IL-2 production in Treg, their development and survival strongly depends on the presence of exogenous IL-2 (*57, 58*).

Recently it was shown that FOXP3 mediates inhibition of gene expression (e.g. the IL-2 gene) by interfering with the T cell transcriptional factor NFAT. This was demonstrated by the fact that FOXP3 regulated genes (i.e. upregulated in FOXP3 negative cells and downregulated in FOXP3 positive cells) were inhibited by CNI and therefore seem regulated in a calcineurin dependent manner (*52*). Calcineurin is the known activator of NFAT (see Fig. 1). Many of the FOXP3-regulated genes involve NFAT for their transcriptional activation. Based on this notion, it is feasible that CNI treatment may profoundly affect Treg cell programming by direct interference with NFAT:FOXP3 interactions (*27, 59*).

A further relevant observation on signaling in Treg is the finding that IL-2 primarily activates JAK/STAT pathway in Treg rather than the PhosphoInositide 3-Kinase (PI3K) signaling pathway (*60-62*). Activation of the signaling pathway leading from PI3K to mTOR was almost completely absent in both primary Treg and FOXP3-transgenic $CD4^+$ T cells upon IL-2 receptor signaling (*54*). These observations help to explain the differential effects of rapamycin on Treg and Teff, favoring Treg survival and function.

Effects of immunosuppressive drugs on Treg: The clinical evidence

As discussed in previous paragraphs, there is evidence that some of the most widely used immunosuppressive drugs in contemporary transplantation medicine have clear and distinct effects on the survival, expansion and function of $CD4^+CD25^+FOXP3^+$ Treg. However, what remains to be demonstrated is how immunosuppression affects the balance between immunoreactive and immunoregulatory cells in transplant recipients, in vivo. In this part we will review the literature on Treg homeostasis and their allo-suppressive activity in the clinical setting and discuss the limitation of our current knowledge.

There is a general observation that the percentage of circulating $CD4^+CD25^+$ and $CD4^+FOXP3^+$ cells drops in the months and years following transplantation (*63, 64*). Not only peripheral blood levels of Treg are reduced, but also lower FOXP3 mRNA and positive T cells were found in non-inflamed colon tissue from liver transplant recipients on triple therapy compared to healthy controls (*65*). These effects cannot only be attributed to the effect of immunosuppression, since many clinical parameters change after transplantation as well. However, the observation that operationally tolerant liver transplant recipients display a significantly increased proportion of $CD4^+CD25^{high}$ cells compared to recipients on immunosuppression, may support this suggestion (*66, 67*). On the contrary, increased Treg proportions may also reflect the operational tolerant state of these patients and not result from the absence of immunosuppression.

One of the first clinical studies that addressed the effect of specific immunosuppressive drugs on Treg identified $CD4^+CD25^+$ Treg from kidney transplant recipients that can suppress alloantigen-specific responses (*68*). Despite induction therapy with an anti-IL-2 receptor mAb, $CD4^+CD25^+$ Treg could still be generated, suggesting no long-term effect of anti-IL-2 receptor mAb on Treg induction. Also, more recent studies suggest that anti-IL-2 receptor mAb treatment does not affect the number or function of Treg long after renal transplantation (*34*). Only during the first three months after transplantation, when mAb levels were still high, a decreased percentage of $CD4^+CD25^+FOXP3^+$ cells was observed. In contrast, in liver transplantation recipients only the CD25 expression but not the percentage of $CD4^+FOXP3^+$ T cells significantly decreased after a single dose of daclizumab during conversion from CNI to MMF monotherapy (*69*). These differences may be due to the early intensive immunosuppressive treatment following kidney transplantation (quadruple treatment) versus the relative low immunosuppression in liver transplantation (monotherapy). Hence, the effect of frequently applied combination therapies on the development of tolerance should be explored.

CNI versus rapamycin

Also in the clinical setting there is evidence that IL-2 is a critical regulator of Treg homeostasis. In cancer patients treated with exogenous recombinant IL-2, the frequency of $CD4^+FOXP3^+$ Treg increased during therapy, especially in lymphopenic patients (*70, 71*). In transplantation several studies compared patients receiving CNI-based immunosuppression with those on rapamycin. One study showed, that kidney transplant patients receiving CNI maintenance treatment had a significantly lower percentage of peripheral $CD4^+CD25^{high}$ T cells compared to patients receiving rapamycin (*72*). Another study, published by the same authors, about stable liver transplant recipients showed that reduced numbers of peripheral $CD4^+CD25^{high}FOXP3^+$ Treg are associated with high CNI dosing as compared to low CNI dosing and healthy volunteers (*73*). Functionally, $CD4^+CD25^+$ cells from rapamycin treated recipients suppressed the direct immune response, but unfortunately, were not compared to Treg from CNI treated recipients (*72*). Recently, we found that conversion of kidney transplant recipients from tacrolimus/MMF to rapamycin monotherapy, significantly increased the percentage and absolute number of circulating $CD4^+CD25^{high}FOXP3^+$ Treg, while conversion to tacrolimus monotherapy did not have this effect (*74*). These findings suggest a shifted balance between immune reactive and regulatory cells by either CNI or rapamycin, notwithstanding that $CD4^+CD25^+$ cells from CNI treated patients have suppressive capacities (*75*).

Similar findings were observed in rapamycin treated kidney transplant recipients after induction therapy with Campath. A higher proportion of $CD4^+CD25^{high}$ cells was measured

as compared to patients on CsA (*76*). The reported increase in the rapamycin group, however, seems overstated as CD25 expression on all $CD4^+$ cells was increased. Moreover, the determination of CD25 high, intermediate and low positive cells by subjective gating differed between CsA and rapamycin treated recipients. In vitro depletion of $CD4^+CD25^{high}$ cells showed an increase in IFN-γ production by T cells of rapamycin -treated recipients while no changes were seen in the CsA group. This suggests active regulation by Treg in the rapamycin group. Whether the increased proportion of $CD4^+CD25^{high}$ T cells in rapamycin treated recipients contributes to a more tolerogenic immune response towards the graft remains to be determined.

A small study used single-donor islet transplantation to reverse sustained diabetes. They found prolonged increased frequencies of $CD4^+CD25^+$ T cells upon induction therapy with human OKT3 and rapamycin, followed by high dose rapamycin and low tacrolimus after transplantation. Moreover, these cells showed a better suppression of the in vitro proliferative response to donor cells than to third-party cells (*77*), suggesting the induction of donor directed Treg. Also in MMF but CNI free treated kidney transplant patients there is evidence that allo-genic responses can be controlled by donor-specific Treg (*78*).

We have to be aware, however, that almost all transplant patients included in these studies were on double or triple immunosuppression therapy, which complicates the interpretation of their data.

Conclusion: Does in vitro evidence translate to the clinical setting?

Overall, the results from in vitro, animal and clinical studies show that immunosuppressive drugs can have detrimental but also beneficial effects on Treg. Together these data indicate that the use of specific immunosuppression as well as their timing and dosing can be an essential component of strategies to induce and maintain transplant tolerance by Treg. However, despite clear effects observed in vitro, the impact of immunosuppressive drugs on the Treg homeostasis in patients is much less clear and requires more clinical research before firm conclusions can be reached. Having said that, one could speculate that currently the best therapy to promote Treg-mediated transplant tolerance may be treatment with T-cell depleting antibodies prior to transplantation followed by rapamycin treatment possibly combined with corticosteroids. Treatment with CNI should be prevented when possible. Still, the consequence of such a therapy on operational tolerance, especially in the long-term, should be further investigated to extent the limited knowledge we have now.

References

1. Denton MD, Magee CC, Sayegh MH. Immunosuppressive strategies in transplantation. Lancet 1999; 353 (9158): 1083.
2. Taylor AL, Watson CJ, Bradley JA. Immunosuppressive agents in solid organ transplantation: Mechanisms of action and therapeutic efficacy. Crit Rev Oncol Hematol 2005; 56 (1): 23.
3. Lopez MM, Valenzuela JE, Alvarez FC, Lopez-Alvarez MR, Cecilia GS, Paricio PP. Long-term problems related to immunosuppression. Transpl Immunol 2006; 17 (1): 31.
4. Fontenot JD, Gavin MA, Rudensky AY. Foxp3 programs the development and function of CD4+CD25+ regulatory T cells. Nat Immunol 2003; 4 (4): 330.
5. Hori S, Nomura T, Sakaguchi S. Control of regulatory T cell development by the transcription factor Foxp3. Science 2003; 299 (5609): 1057.
6. Gavin MA, Torgerson TR, Houston E, et al. Single-cell analysis of normal and FOXP3-mutant human T cells: FOXP3 expression without regulatory T cell development. Proc Natl Acad Sci U S A 2006; 103 (17): 6659.
7. Bennett CL, Christie J, Ramsdell F, et al. The immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) is caused by mutations of FOXP3. Nat Genet 2001; 27 (1): 20.
8. Brunkow ME, Jeffery EW, Hjerrild KA, et al. Disruption of a new forkhead/winged-helix protein, scurfin, results in the fatal lymphoproliferative disorder of the scurfy mouse. Nat Genet 2001; 27 (1): 68.
9. Zheng SG, Gray JD, Ohtsuka K, Yamagiwa S, Horwitz DA. Generation ex vivo of TGF-beta-producing regulatory T cells from CD4+CD25- precursors. J Immunol 2002; 169 (8): 4183.
10. Chen W, Jin W, Hardegen N, et al. Conversion of peripheral CD4+CD25- naive T cells to CD4+CD25+ regulatory T cells by TGF-beta induction of transcription factor Foxp3. J Exp Med 2003; 198 (12): 1875.
11. Jiang S, Camara N, Lombardi G, Lechler RI. Induction of allopeptide-specific human CD4+CD25+ regulatory T cells ex vivo. Blood 2003; 102 (6): 2180.
12. Zheng SG, Wang JH, Gray JD, Soucier H, Horwitz DA. Natural and induced CD4+CD25+ cells educate CD4+CD25- cells to develop suppressive activity: the role of IL-2, TGF-beta, and IL-10. J Immunol 2004; 172 (9): 5213.
13. Hoffmann P, Eder R, Kunz-Schughart LA, Andreesen R, Edinger M. Large-scale in vitro expansion of polyclonal human CD4(+)CD25high regulatory T cells. Blood 2004; 104 (3): 895.
14. Park HB, Paik DJ, Jang E, Hong S, Youn J. Acquisition of anergic and suppressive activities in transforming growth factor-beta-costimulated CD4+CD25- T cells. Int Immunol 2004; 16 (8): 1203.
15. Earle KE, Tang Q, Zhou X, et al. In vitro expanded human CD4+CD25+ regulatory T cells suppress effector T cell proliferation. Clin Immunol 2005; 115 (1): 3.
16. Valmori D, Merlo A, Souleimanian NE, Hesdorffer CS, Ayyoub M. A peripheral circulating compartment of natural naive CD4 Tregs. J Clin Invest 2005; 115 (7): 1953.
17. Akbar AN, Vukmanovic-Stejic M, Taams LS, Macallan DC. The dynamic co-evolution of memory and regulatory CD4+ T cells in the periphery. Nat Rev Immunol 2007; 7 (3): 231.

18. Chen X, Murakami T, Oppenheim JJ, Howard OM. Differential response of murine CD4+CD25+ and CD4+CD25- T cells to dexamethasone-induced cell death. Eur J Immunol 2004; 34 (3): 859.
19. Shimizu J, Yamazaki S, Takahashi T, Ishida Y, Sakaguchi S. Stimulation of CD25(+)CD4(+) regulatory T cells through GITR breaks immunological self-tolerance. Nat Immunol 2002; 3 (2): 135.
20. McHugh RS, Whitters MJ, Piccirillo CA, et al. CD4(+)CD25(+) immunoregulatory T cells: gene expression analysis reveals a functional role for the glucocorticoid-induced TNF receptor. Immunity 2002; 16 (2): 311.
21. Ronchetti S, Nocentini G, Riccardi C, Pandolfi PP. Role of GITR in activation response of T lymphocytes. Blood 2002; 100 (1): 350.
22. Kanamaru F, Youngnak P, Hashiguchi M, et al. Costimulation via glucocorticoid-induced TNF receptor in both conventional and CD25+ regulatory CD4+ T cells. J Immunol 2004; 172 (12): 7306.
23. Polanczyk MJ, Carson BD, Subramanian S, et al. Cutting edge: estrogen drives expansion of the CD4+CD25+ regulatory T cell compartment. J Immunol 2004; 173 (4): 2227.
24. Karagiannidis C, Akdis M, Holopainen P, et al. Glucocorticoids upregulate FOXP3 expression and regulatory T cells in asthma. J Allergy Clin Immunol 2004; 114 (6): 1425.
25. Dao Nguyen X, Robinson DS. Fluticasone propionate increases CD4CD25 T regulatory cell suppression of allergen-stimulated CD4CD25 T cells by an IL-10-dependent mechanism. J Allergy Clin Immunol 2004; 114 (2): 296.
26. Chen X, Oppenheim JJ, Winkler-Pickett RT, Ortaldo JR, Howard OM. Glucocorticoid amplifies IL-2-dependent expansion of functional FoxP3(+)CD4(+)CD25(+) T regulatory cells in vivo and enhances their capacity to suppress EAE. Eur J Immunol 2006; 36 (8): 2139.
27. Wu Y, Borde M, Heissmeyer V, et al. FOXP3 controls regulatory T cell function through cooperation with NFAT. Cell 2006; 126 (2): 375.
28. Hamawy MM. Molecular actions of calcineurin inhibitors. Drug News Perspect 2003; 16 (5): 277.
29. Baan CC, van der Mast BJ, Klepper M, et al. Differential effect of calcineurin inhibitors, anti-CD25 antibodies and rapamycin on the induction of FOXP3 in human T cells. Transplantation 2005; 80 (1): 110.
30. Zeiser R, Nguyen VH, Beilhack A, et al. Inhibition of CD4+CD25+ regulatory T-cell function by calcineurin-dependent interleukin-2 production. Blood 2006; 108 (1): 390.
31. Gao W, Lu Y, El Essawy B, Oukka M, Kuchroo VK, Strom TB. Contrasting effects of cyclosporine and rapamycin in de novo generation of alloantigen-specific regulatory T cells. Am J Transplant 2007; 7 (7): 1722.
32. Coenen JJ, Koenen HJ, van Rijssen E, Hilbrands LB, Joosten I. Rapamycin, and not cyclosporin A, preserves the highly suppressive CD27+ subset of human CD4+CD25+ regulatory T cells. Blood 2006; 107 (3): 1018.
33. Coenen JJ, Koenen HJ, van Rijssen E, et al. Rapamycin, not cyclosporine, permits thymic generation and peripheral preservation of CD4+ CD25+ FoxP3+ T cells. Bone Marrow Transplant 2007; 39 (9): 537.
34. Kreijveld E, Koenen HJ, Klasen IS, Hilbrands LB, Joosten I. Following anti-CD25 treatment, a functional CD4+CD25+ regulatory T-cell pool is present in renal transplant recipients. Am J Transplant 2007; 7 (1): 249.

35. Lopez M, Clarkson MR, Albin M, Sayegh MH, Najafian N. A novel mechanism of action for anti-thymocyte globulin: induction of CD4+CD25+Foxp3+ regulatory T cells. J Am Soc Nephrol 2006; 17 (10): 2844.
36. Game DS, Hernandez-Fuentes MP, Lechler RI. Everolimus and basiliximab permit suppression by human CD4+CD25+ cells in vitro. Am J Transplant 2005; 5 (3): 454.
37. Couper KN, Blount DG, de Souza JB, Suffia I, Belkaid Y, Riley EM. Incomplete depletion and rapid regeneration of Foxp3+ regulatory T cells following anti-CD25 treatment in malaria-infected mice. J Immunol 2007; 178 (7): 4136.
38. Kohm AP, McMahon JS, Podojil JR, et al. Cutting Edge: Anti-CD25 monoclonal antibody injection results in the functional inactivation, not depletion, of CD4+CD25+ T regulatory cells. J Immunol 2006; 176 (6): 3301.
39. Minamimura K, Gao W, Maki T. CD4+ regulatory T cells are spared from deletion by antilymphocyte serum, a polyclonal anti-T cell antibody. J Immunol 2006; 176 (7): 4125.
40. Battaglia M, Stabilini A, Migliavacca B, Horejs-Hoeck J, Kaupper T, Roncarolo MG. Rapamycin promotes expansion of functional CD4+CD25+FOXP3+ regulatory T cells of both healthy subjects and type 1 diabetic patients. J Immunol 2006; 177 (12): 8338.
41. Strauss L, Whiteside TL, Knights A, Bergmann C, Knuth A, Zippelius A. Selective survival of naturally occurring human CD4+CD25+Foxp3+ regulatory T cells cultured with rapamycin. J Immunol 2007; 178 (1): 320.
42. Keever-Taylor CA, Browning MB, Johnson BD, et al. Rapamycin enriches for CD4(+) CD25(+) CD27(+) Foxp3(+) regulatory T cells in ex vivo-expanded CD25-enriched products from healthy donors and patients with multiple sclerosis. Cytotherapy 2007; 9 (2): 144.
43. May KF, Jr., Chang X, Zhang H, et al. B7-deficient autoreactive T cells are highly susceptible to suppression by CD4(+)CD25(+) regulatory T cells. J Immunol 2007; 178 (3): 1542.
44. Battaglia M, Stabilini A, Roncarolo MG. Rapamycin selectively expands CD4+CD25+FoxP3+ regulatory T cells. Blood 2005; 105 (12): 4743.
45. Dodge IL, Demirci G, Strom TB, Li XC. Rapamycin induces transforming growth factor-beta production by lymphocytes. Transplantation 2000; 70 (7): 1104.
46. Valmori D, Tosello V, Souleimanian NE, et al. Rapamycin-mediated enrichment of T cells with regulatory activity in stimulated CD4+ T cell cultures is not due to the selective expansion of naturally occurring regulatory T cells but to the induction of regulatory functions in conventional CD4+ T cells. J Immunol 2006; 177 (2): 944.
47. Turnquist HR, Raimondi G, Zahorchak AF, Fischer RT, Wang Z, Thomson AW. Rapamycin-conditioned dendritic cells are poor stimulators of allogeneic CD4+ T cells, but enrich for antigen-specific Foxp3+ T regulatory cells and promote organ transplant tolerance. J Immunol 2007; 178 (11): 7018.
48. Allison AC, Eugui EM. Mycophenolate mofetil and its mechanisms of action. Immunopharmacology 2000; 47 (2-3): 85.
49. Thomson AW, Woo J, Yao GZ, Todo S, Starzl TE, Zeevi A. Effects of combined administration of FK 506 and the purine biosynthesis inhibitors mizoribine or mycophenolic acid on lymphocyte DNA synthesis and T cell activation molecule expression in human mixed lymphocyte cultures. Transpl Immunol 1993; 1 (2): 146.
50. Gummert JF, Barten MJ, Sherwood SW, van Gelder T, Morris RE. Pharmacodynamics of immunosuppression by mycophenolic acid: inhibition of both lymphocyte proliferation and activation correlates with pharmacokinetics. J Pharmacol Exp Ther 1999; 291 (3): 1100.

51. Vanasek TL, Nandiwada SL, Jenkins MK, Mueller DL. CD25+Foxp3+ regulatory T cells facilitate CD4+ T cell clonal anergy induction during the recovery from lymphopenia. J Immunol 2006; 176 (10): 5880.
52. Marson A, Kretschmer K, Frampton GM, et al. Foxp3 occupancy and regulation of key target genes during T-cell stimulation. Nature 2007; 445 (7130): 931.
53. Zheng Y, Josefowicz SZ, Kas A, Chu TT, Gavin MA, Rudensky AY. Genome-wide analysis of Foxp3 target genes in developing and mature regulatory T cells. Nature 2007; 445 (7130): 936.
54. Campbell DJ, Ziegler SF. FOXP3 modifies the phenotypic and functional properties of regulatory T cells. Nat Rev Immunol 2007; 7 (4): 305.
55. Rudensky AY, Gavin M, Zheng Y. FOXP3 and NFAT: partners in tolerance. Cell 2006; 126 (2): 253.
56. Tsang JY, Camara NO, Eren E, et al. Altered proximal T cell receptor (TCR) signaling in human CD4+CD25+ regulatory T cells. J Leukoc Biol 2006; 80 (1): 145.
57. Furtado GC, Curotto de Lafaille MA, Kutchukhidze N, Lafaille JJ. Interleukin 2 signaling is required for CD4(+) regulatory T cell function. J Exp Med 2002; 196 (6): 851.
58. Bayer AL, Yu A, Malek TR. Function of the IL-2R for thymic and peripheral CD4+CD25+ Foxp3+ T regulatory cells. J Immunol 2007; 178 (7): 4062.
59. Bettelli E, Dastrange M, Oukka M. Foxp3 interacts with nuclear factor of activated T cells and NF-kappa B to repress cytokine gene expression and effector functions of T helper cells. Proc Natl Acad Sci U S A 2005; 102 (14): 5138.
60. Antov A, Yang L, Vig M, Baltimore D, Van Parijs L. Essential role for STAT5 signaling in CD25+CD4+ regulatory T cell homeostasis and the maintenance of self-tolerance. J Immunol 2003; 171 (7): 3435.
61. Bensinger SJ, Walsh PT, Zhang J, et al. Distinct IL-2 receptor signaling pattern in CD4+CD25+ regulatory T cells. J Immunol 2004; 172 (9): 5287.
62. Zorn E, Nelson EA, Mohseni M, et al. IL-2 regulates FOXP3 expression in human CD4+CD25+ regulatory T cells through a STAT-dependent mechanism and induces the expansion of these cells in vivo. Blood 2006; 108 (5): 1571.
63. Demirkiran A, Kok A, Kwekkeboom J, et al. Low circulating regulatory T-cell levels after acute rejection in liver transplantation. Liver Transpl 2006; 12 (2): 277.
64. Codarri L, Vallotton L, Ciuffreda D, et al. Expansion and tissue infiltration of an allospecific CD4+CD25+CD45RO+IL-7Ralphahigh cell population in solid organ transplant recipients. J Exp Med 2007; 204 (7): 1533.
65. Verdonk RC, Haagsma EB, Jonker MR, et al. Effects of different immunosuppressive regimens on regulatory T-cells in noninflamed colon of liver transplant recipients. Inflamm Bowel Dis 2007; 13 (6): 703.
66. Li Y, Koshiba T, Yoshizawa A, et al. Analyses of peripheral blood mononuclear cells in operational tolerance after pediatric living donor liver transplantation. Am J Transplant 2004; 4 (12): 2118.
67. Martinez-Llordella M, Puig-Pey I, Orlando G, et al. Multiparameter immune profiling of operational tolerance in liver transplantation. Am J Transplant 2007; 7 (2): 309.
68. Salama AD, Najafian N, Clarkson MR, Harmon WE, Sayegh MH. Regulatory CD25+ T cells in human kidney transplant recipients. J Am Soc Nephrol 2003; 14 (6): 1643.
69. Demirkiran A, Metselaar H, Sewgobind V, et al. Conversion from Calcineurin Inhibitor Monotherapy to Mycophenolate Mofetil Changes the Frequency and Phenotype of Circulating CD4+FoxP3+ Regulatory T Cells in Liver Transplant recipients. Am J Transplant 2007; 7 (s2): 227.

70. Zhang H, Chua KS, Guimond M, et al. Lymphopenia and interleukin-2 therapy alter homeostasis of CD4+CD25+ regulatory T cells. Nat Med 2005; 11 (11): 1238.
71. van der Vliet HJ, Koon HB, Yue SC, et al. Effects of the administration of high-dose interleukin-2 on immunoregulatory cell subsets in patients with advanced melanoma and renal cell cancer. Clin Cancer Res 2007; 13 (7): 2100.
72. Segundo DS, Ruiz JC, Izquierdo M, et al. Calcineurin inhibitors, but not rapamycin, reduce percentages of CD4+CD25+FOXP3+ regulatory T cells in renal transplant recipients. Transplantation 2006; 82 (4): 550.
73. San Segundo D, Fabrega E, Lopez-Hoyos M, Pons F. Reduced numbers of blood natural regulatory T cells in stable liver transplant recipients with high levels of calcineurin inhibitors. Transplant Proc 2007; 39 (7): 2290.
74. Hendrikx T, Gurp E, Schoordijk W, et al. Conversion to Steroid free Rapamycin Monotherapy Leads To Expansion of $CD4^{+}CD25^{high}FoxP3^{+}$ T-Cells in Renal Transplant Patients. Am J Transplant 2007; 7 (s2): 279.
75. Baan CC, Velthuis JH, van Gurp EA, et al. Functional CD25(bright+) alloresponsive T cells in fully immunosuppressed renal allograft recipients. Clin Transplant 2007; 21 (1): 63.
76. Noris M, Casiraghi F, Todeschini M, et al. Regulatory T cells and T cell depletion: role of immunosuppressive drugs. J Am Soc Nephrol 2007; 18 (3): 1007.
77. Hering BJ, Kandaswamy R, Harmon JV, et al. Transplantation of cultured islets from two-layer preserved pancreases in type 1 diabetes with anti-CD3 antibody. Am J Transplant 2004; 4 (3): 390.
78. Velthuis JH, Mol WM, Weimar W, Baan CC. CD4+CD25bright+ regulatory T cells can mediate donor nonreactivity in long-term immunosuppressed kidney allograft patients. Am J Transplant 2006; 6 (12): 2955.

CHAPTER 4

Generation of donor-specific regulatory T-cell function in kidney transplant patients

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Abstract

Introduction 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. It remains to be demonstrated, however, whether these cells are generated after clinical transplantation.

Methods We prospectively analysed 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 PBMC and in co-culture with $CD25^{neg/dim}$ responder T-cells in the MLR**.**

Results 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 3^{rd}P-Ag and 4^{th}P-Ag, ($p<0.001$ and $p<0.05$, respectively). Furthermore, functional analysis of $CD25^{brigth+}$ cells showed that the effect of depletion of these cells from PBMC, as well as their suppressive capacities in co-culture with donor-Ag 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-Ag and 3^{rd}P-Ag became apparent at 6 months after transplantation.

Conclusions These findings demonstrate that donor-specific $CD4^{+}CD25^{bright+}$ regulatory T cell function is generated in fully immunosuppressed renal recipients in the first year after transplantation.

Introduction

The ultimate challenge in organ transplantation is to achieve transplant tolerance. While 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 (Tregs) has been frequently suggested (*3-8*).

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

Although these findings suggest an association between transplant tolerance and the presence of Tregs, data providing evidence for the presence of functional donor-specific Tregs after transplantation are only available from stable immunosuppressed kidney transplant recipients (*11-13*). It remains to be demonstrated, however, that Tregs 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. Since these regimens influence T cells (*14*), they may also affect the induction and function of Tregs (*15*). Particularly since 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 Tregs 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 Tregs 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 anti-donor 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 one year. We included 79 patients, which 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 4-6 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 hours 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

males and 7 females 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 (years)	51 ± 17	51 ± 16
Dialysis type HD/ PD/ ND	20/ 12/ 7	19/ 15/ 6
Time on dialysis (months)	11 (0-75)*	17 (0-280)*
Origin of donor kidney Living related/ deceased	25 / 14	27 / 13
1^{st} KTx / $>1^{st}$	34 / 5	35 / 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=Haemodialysis, PD=Peritoneal Dialysis, ND=No Dialysis

Flow cytometric analysis

Blood samples were collected in heparinized tubes and analysed for the presence of T-cell subsets by four-colour flow cytometry using mAbs directly conjugated to fluorescein (FITC), phycoerythrin (PE), allophycocyanin (APC) or peridinin chlorophyll protein (PerCP). 100µl blood was incubated with 10µl of the dual mAb combinations CD45-FITC/CD14-PE; IgG_1-FITC/IgG_{2b}-PE; IgG_1-PerCP/ IgG_1-APC as isotype control. Further we used the mAb CD3-FITC, CD4-PerCP, CD8-APC and CD25-PE. To further determine how Tregs evolve, we added a combination of CD4-PerPC/CD25-PE/CD45RO-APC/CCR7-FITC to 100µl 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 FACS lysing solution (BD Biosciences) during 10 min. Cells were then washed twice, and analysed 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 20000 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.

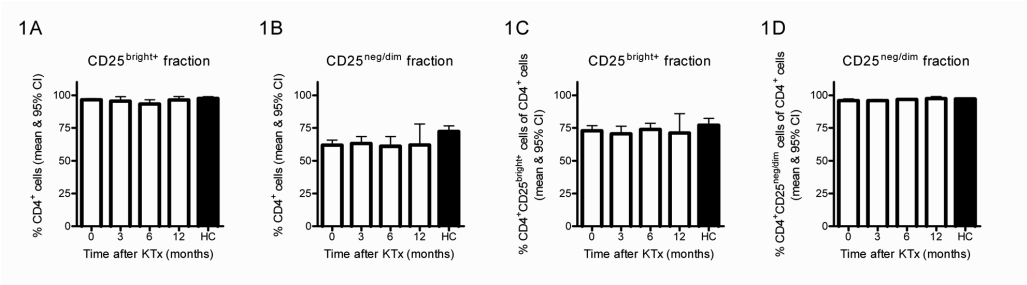

Figure 1. Isolation of $CD25^{bright+}$ cells by autoMACS.
(A-D) All data were normally distributed. (A-B) The average proportion of $CD4^+$ cells in the isolated $CD25^{bright+}$ fraction was 95% and 62% in the $CD25^{neg/dim}$ fraction. (C) In the $CD25^{bright+}$ fraction, the proportion of $CD4^+CD25^{bright+}$ 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 HC (Unpaired t-test). HC = Healthy controls.

Expression of FoxP3 and CD127

FoxP3 is a transcription marker for regulatory cells and in July 2006 it was shown that the expression of CD127 inversely correlates with FoxP3 expression and the suppressive function of Tregs (*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 it's correlation of 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 hours 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.

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. ***i.v. bolus.

Isolation of peripheral blood lymphocytes

Peripheral blood mononuclear cells (PBMC) were isolated from 49ml 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 RPMI 1640 (BioWhittaker, Verviers, Belgium) and resuspended in Human Culture Medium (HCM) consisting of RPMI 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).

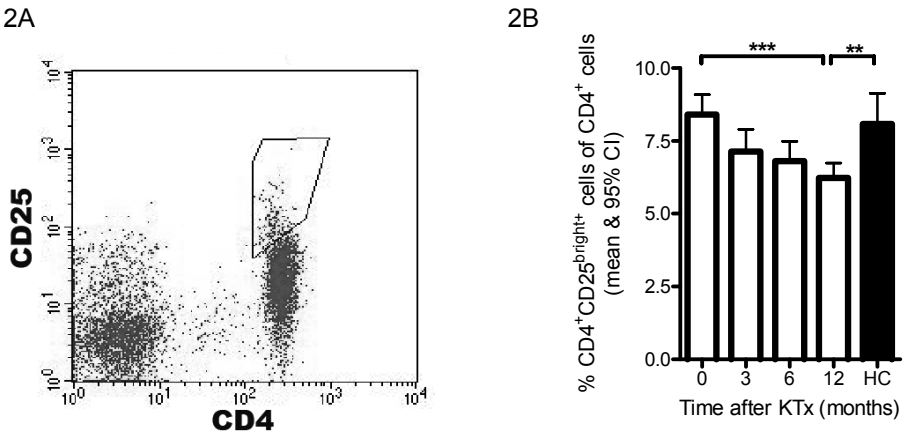

Figure 2. Phenotypic analysis of whole blood
(A) Dotplot of $CD3^+$ T cells stained for CD4 and CD25 gated for the $CD4^+CD25^{bright+}$ cells. (B) The data were normally distributed and statistical analysis showed that the percentage of $CD4^+CD25^{bright+}$ T cells of $CD4^+$ T cells from patients decreased in the first year after transplantation (ANOVA, $p<0.001$) and was lower than healthy controls (HC) at month 12 (Unpaired t-test, $p<0.001$).

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% (Figure 1A-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 to proportions measured in samples from HC (Figure 1A-D).

Mixed Lymphocyte Reactions (MLR)

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-Ag) and $5x10^4$ (40 Gy) HLA A, B and DR fully mismatched third party PBMC (3^{rd}P). Since 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 (4^{th}P). 4^{th}P 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 3^{rd}P and 4^{th}P 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 hours later samples were harvested and radioactivity was measured in counts per minute (CPM) using a β-counter (PerkinElmer, Oosterhout, The Netherlands).

Regulation of allo-Ag 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 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.

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

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 >1000 CPM and the effect of depletion was positive.

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

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 Phytohaemagglutinin (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 hours later the samples were harvested and radioactivity was counted.

Statistical Analysis

All calculations were done using GraphPad Prism 4.0 or SPSS 11.5. Based on 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. To determine if a certain parameter changed significantly over time, One-Way ANOVA was used. To analyse several variables at a fixed time point, Cox or linear regression analysis was performed. A p-value <0.05 is marked with *, $p<0.01$ = ** and $p<0.001$ = ***. 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 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). Patients that suffered from rejection were all treated with anti-rejection therapy and are therefore described separately.

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

Analysis of whole blood samples from patients and healthy controls (HC) was performed for lymphocyte subsets, including Tregs 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 Tregs as well as their proportion, decreased within the first year after KTx (Table 3 & Figure 2B, $p<0.05$ and $p<0.001$, respectively).

We also analysed the expression of CD45RO, CCR7, FoxP3 and CD127 by Tregs. 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), while their proportion increased ($p<0.01$).

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

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 and significance is presented as * $p<0.05$, ** $p<0.01$, *** $p<0.001$. One way ANOVA demonstrated that the number of $CD4^+CD25^{bright+}$ cells and $CD4^+CD25^{bright+}CD45RO^+$ cells significantly decreased over time (both $p<0.05$).

Proliferation of PBMC

The average proliferation of patient-PBMC to the mitogen PHA was >51000 CPM at all tested time points and comparable to proliferation of PBMC from HC (57000 CPM). Before transplantation, proliferation of patient-PBMC to donor-Ag, 3^{rd}P-Ag and 4^{th}P-Ag was significantly lower as compared to proliferation of PBMC from HC to alloantigens (Figure 3, all $p<0.001$). After transplantation, proliferation of PBMC to donor-Ag remained low while increasing proliferation to 3^{rd}P-Ag and 4^{th}P-Ag, was measured (Figure 3, $p<0.001$ and $p<0.05$, respectively). Thus, we observed a proportional hyporesponsiveness towards donor-Ag.

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

The effect of depletion of $CD25^{bright+}$ cells from PBMC on direct alloresponses was determined in MLR. Due to the limited amount of peripheral blood available from our patients, we could not analyse the effect of depletion of $CD25^{bright+}$ cells in cultures stimulated with 4^{th}P-Ag. After depletion of the $CD25^{bright+}$ fraction, we observed an overall increase of the proliferative response in cultures stimulated with donor-Ag and 3^{rd}P-Ag before and after transplantation (Figure 4A). Before transplantation, the average effect of depletion on proliferative responses of alloreactive cells was 51% upon stimulation with donor-Ag and 57% upon stimulation with 3^{rd}P-Ag (Figure 4B). After transplantation, the effect of depletion increased significantly in co-cultures stimulated with donor-Ag and less so with 3^{rd}P-Ag (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-Ag than with 3^{rd}P-Ag (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-Ag. Co-culture experiments proved that the suppressive effect of $CD25^{bright+}$ cells on $CD25^{neg/dim}$ responder cells is a dose dependent phenomenon (Figure 4C-D). Before transplantation, the capacity of $CD25^{bright+}$ cells to suppress donor-Ag or 3^{rd}P-Ag stimulated $CD25^{neg/dim}$ responder cells (ratio 1:10) was significantly lower when compared to HC (Figure 4E, both $p<0.05$). After transplantation,

the average suppressive capacity of $CD25^{bright+}$ T cells improved significantly in cultures stimulated with donor-Ag (Figure 4E, 51% to 75%, $p<0.001$), but not with 3^{rd}P-Ag (50% to 57%, $p=0.49$). Furthermore, at 6 and 12 months after transplantation, the capacity of $CD25^{bright+}$ cells to suppress donor-Ag stimulated $CD25^{neg/dim}$ cells was significantly higher than upon stimulation with 3^{rd}P-Ag (Figure 4E, $p<0.01$ and $p<0.001$ respectively). The results on the %IH 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.

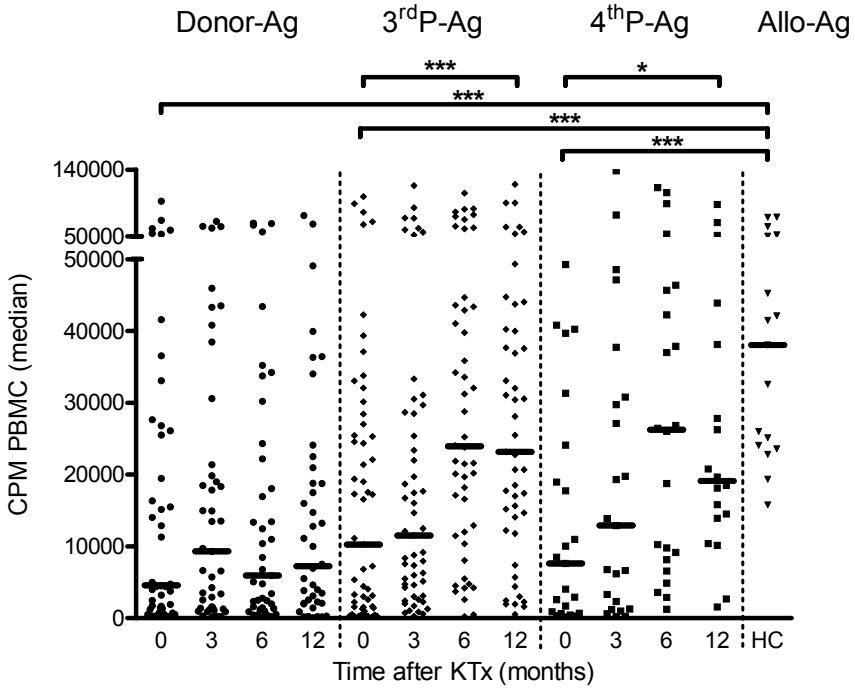

Figure 3. Proliferation of PBMC
The data were not always normally distributed and therefore statistical analysis was based on a nonparametric distribution. Before transplantation, proliferation of patient-PBMC to donor-Ag, 3^{rd}P-Ag and 4^{th}P-Ag was significantly lower as compared to proliferation of PBMC from healthy controls (HC) to alloantigens (Unpaired t-test, all $p<0.001$). After transplantation, proliferation to donor-Ag remained low, while the response to 3^{rd}P-Ag and 4^{th}P-Ag increased (ANOVA, $p<0.001$ and $p<0.05$, respectively).

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). Anti-rejection 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 to be a predictor for rejection prior to transplantation.

We analysed 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-Ag or 3^{rd}P-Ag was not different from non-rejectors at month 12. This is in line with a study from Demirkiran et al. on liver transplant recipients (*23*).

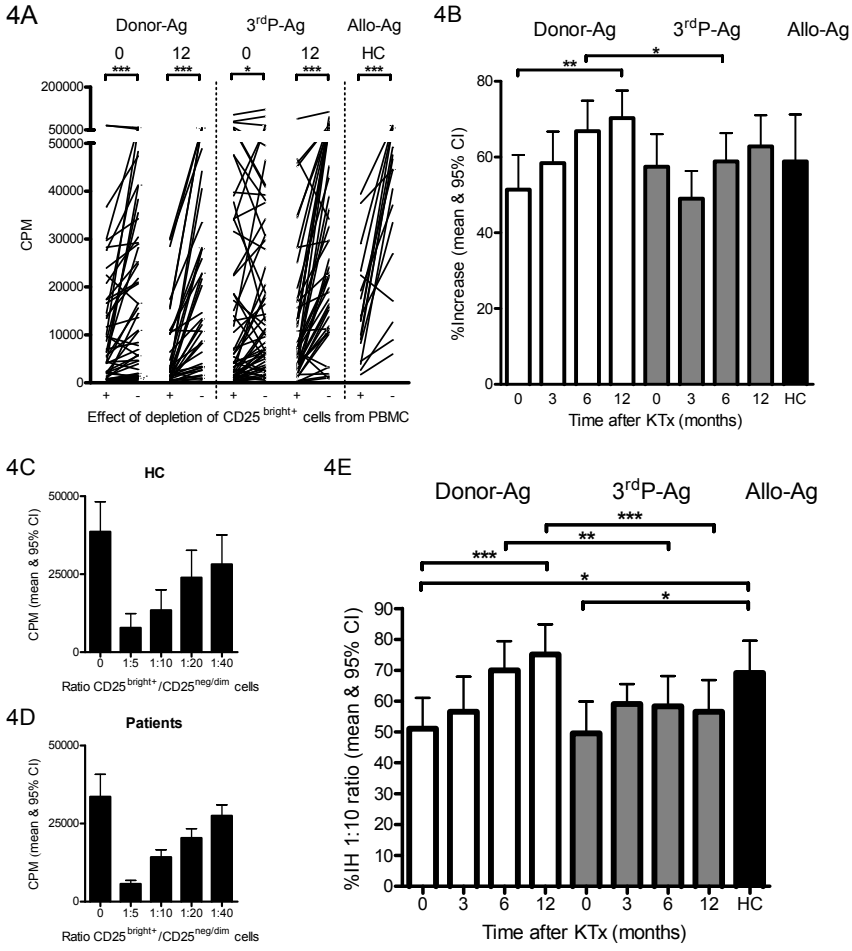

Figure 4. Functional analysis of $CD25^{bright+}$ cells.
Except for the data in figure A, all data were normally distributed. (A) PBMC (+) and $CD25^{neg/dim}$ cells (-). Depletion of $CD25^{bright+}$ cells from PBMC resulted in improved proliferation in donor-Ag and 3^{rd}P-Ag stimulated co-cultures from patients before (0) and 12 months (12) after transplantation (Paired t-test). (B) The effect of depletion was calculated as the %Increase. After transplantation, the effect of depletion increased significantly in co-cultures stimulated with donor-Ag and less vigorously with 3^{rd}P-Ag (ANOVA, $p<0.01$ and $p=0.07$, respectively). At months 6 and 12, the %Increase was higher in co-cultures stimulated with donor-Ag than with 3^{rd}P-Ag (Paired t-test, $p<0.05$ and $p=0.09$, respectively). (C-D) In HC and patients (month 12, 3^{rd}P-Ag stimulated co-cultures), inhibition of allo-Ag stimulated $CD25^{neg/dim}$ cells by $CD25^{bright+}$ cells proved to be dose dependent. (F) The suppressive capacity of $CD25^{bright+}$ cells was calculated as the %IH. Before transplantation, the %IH was significantly lower in co-cultures stimulated with donor-Ag and 3^{rd}P-Ag when compared to HC (Unpaired t-test, both $p<0.05$). After transplantation, the %IH increased in co-cultures stimulated with donor-Ag (ANOVA, $p<0.001$), but not significantly with 3^{rd}P-Ag ($p=0.49$). At months 6 and 12, the %IH was significantly higher in co-cultures stimulated with donor-Ag than with 3^{rd}P-Ag (Paired t-test, $p<0.01$ and $p<0.001$, respectively).

Immunosuppressive drugs: tacrolimus/MMF vs tacrolimus/rapamycin

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

Multivariate analysis

In a multivariate analysis the factors gender, recipient age, dialysis type, time on dialysis, origin of donor kidney, 1st KTx / >1st, 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 analysed their suppressive capacity in the first year after transplantation. In the MLR, depletion of CD25$^{bright+}$ cells from PBMC, as well as 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, while Treg activity was significantly more donor-directed compared to 3rdP-Ag.

Data from in vitro and animal studies indicated that immunosuppressive drugs have detrimental effects on Tregs (*15, 17, 24*). The development of donor-specific Tregs in the present study, however, shows that the immune system can bypass these unfavourable effects in vivo to a certain extent. Apart from the restored kidney function (*25*), an explanation might be that immunosuppressive drugs like cyclosporin and tacrolimus, do not completely 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 (STAT 5), and so induces the expression of the transcription factor for regulatory cells FoxP3 (*8, 28, 29*).

The observed donor-specific hyporesponsiveness as compared to the reactivity against 3rdP-Ag and 4thP-Ag, did not result from better histocompatibility between donor and recipient (*22*). Regulation could be another explanation. Indeed, the suppressive function of Tregs from our patients became increasingly potent to donor-Ag stimulated cultures after transplantation. It has been reported that Tregs 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-antigen could therefore stimulate the peripheral proliferation and accumulation of Tregs. 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 Tregs might be favoured by the lymphopaenic state of transplant patients (Table 3), since stimuli that originate from lymphopaenia favour 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 $CD4^{+}CD25^{bright+}$ Tregs. In contrast, a cross-sectional study from Game and colleagues stated that Tregs do not contribute to the direct pathway of hyporesponsiveness in stable transplant patients (*33*). 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, since 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 favours their expansion in vivo (*34-36*), while MMF and calcineurin inhibitors (CNI) e.g. 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 Tregs. These findings can be explained by the dominant effect of tacrolimus in both arms of treatment. Especially, since CNI based treatment is associated with decreased numbers of Tregs 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 Tregs 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 Tregs 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 Tregs with a memory phenotype, indicating an increased proportion of naive Tregs. Since it has been demonstrated that especially naive Tregs give rise to potent Ag-specific Tregs (*43*), their strong proportional increase may have favoured the development of the observed donor-specific Treg function.

In summary, we prospectively analysed 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.

References

1. VanBuskirk AM, Burlingham WJ, Jankowska-Gan E, et al. Human allograft acceptance is associated with immune regulation. *J Clin Invest* 2000; 106: 145.
2. Strober S, Benike C, Krishnaswamy S, Engleman EG, Grumet FC. Clinical transplantation tolerance twelve years after prospective withdrawal of immunosuppressive drugs: studies of chimerism and anti-donor reactivity. *Transplantation* 2000; 69: 1549.
3. Roussey-Kesler G, Giral M, Moreau A, et al. Clinical operational tolerance after kidney transplantation. *Am J Transplant* 2006; 6: 736.
4. Salama AD, Najafian N, Clarkson MR, Harmon WE, Sayegh MH. Regulatory CD25+ T cells in human kidney transplant recipients. *J Am Soc Nephrol* 2003; 14: 1643.
5. Li Y, Koshiba T, Yoshizawa A, et al. Analyses of peripheral blood mononuclear cells in operational tolerance after pediatric living donor liver transplantation. *Am J Transplant* 2004; 4: 2118.
6. Sakaguchi S. Naturally arising Foxp3-expressing CD25+CD4+ regulatory T cells in immunological tolerance to self and non-self. *Nat Immunol* 2005; 6: 345.
7. Martinez-Llordella M, Puig-Pey I, Orlando G, et al. Multiparameter immune profiling of operational tolerance in liver transplantation. *Am J Transplant* 2007; 7: 309.
8. Long E, Wood KJ. Understanding FOXP3: Progress Towards Achieving Transplantation Tolerance. *Transplantation* 2007; 84: 459.
9. Louis S, Braudeau C, Giral M, et al. Contrasting CD25hiCD4+T cells/FOXP3 patterns in chronic rejection and operational drug-free tolerance. *Transplantation* 2006; 81: 398.
10. Brouard S, Mansfield E, Braud C, et al. Identification of a peripheral blood transcriptional biomarker panel associated with operational renal allograft tolerance. *Proc Natl Acad Sci U S A* 2007; 104: 15448.
11. Velthuis JH, Mol WM, Weimar W, Baan CC. CD4+CD25bright+ regulatory T cells can mediate donor nonreactivity in long-term immunosuppressed kidney allograft patients. *Am J Transplant* 2006; 6: 2955.
12. Bestard O, Cruzado JM, Mestre M, et al. Achieving donor-specific hyporesponsiveness is associated with FOXP3+ regulatory T cell recruitment in human renal allograft infiltrates. *J Immunol* 2007; 179: 4901.
13. Akl A, Jones ND, Rogers N, et al. An investigation to assess the potential of CD25(high)CD4(+) T cells to regulate responses to donor alloantigens in clinically stable renal transplant recipients. *Transpl Int* 2008; 21: 65.
14. Halloran PF. Immunosuppressive drugs for kidney transplantation. *N Engl J Med* 2004; 351: 2715.
15. Demirkiran A, Hendrikx TK, Baan CC, van der Laan LJ. Impact of Immunosuppressive Drugs on CD4+CD25+FOXP3+ Regulatory T Cells: Does In Vitro Evidence Translate to the Clinical Setting? *Transplantation* 2008; 85: 783.
16. Thornton AM, Donovan EE, Piccirillo CA, Shevach EM. Cutting edge: IL-2 is critically required for the in vitro activation of CD4+CD25+ T cell suppressor function. *J Immunol* 2004; 172: 6519.
17. Baan CC, van der Mast BJ, Klepper M, et al. Differential effect of calcineurin inhibitors, anti-CD25 antibodies and rapamycin on the induction of FOXP3 in human T cells. *Transplantation* 2005; 80: 110.
18. Maloy KJ, Powrie F. Fueling regulation: IL-2 keeps CD4+ Treg cells fit. *Nat Immunol* 2005; 6: 1071.

19. D'Cruz LM, Klein L. Development and function of agonist-induced CD25+Foxp3+ regulatory T cells in the absence of interleukin 2 signaling. *Nat Immunol* 2005; 6: 1152.
20. E. van Gurp. Renal function following kidney transplantation is equally good regardless of whether tacrolimus is combined with sirolimus or mycophenolate mofetil [abstract]. *Transpl Int* 2007; 20: 25.
21. Liu W, Putnam AL, Xu-Yu Z, et al. CD127 expression inversely correlates with FoxP3 and suppressive function of human CD4+ T reg cells. *J Exp Med* 2006; 203: 1701.
22. Rodriguez DS, Jankowska-Gan E, Haynes LD, et al. Immune regulation and graft survival in kidney transplant recipients are both enhanced by human leukocyte antigen matching. *Am J Transplant* 2004; 4: 537.
23. Demirkiran A, Kok A, Kwekkeboom J, et al. Low circulating regulatory T-cell levels after acute rejection in liver transplantation. *Liver Transpl* 2006; 12: 277.
24. Coenen JJ, Koenen HJ, van Rijssen E, et al. Rapamycin, not cyclosporine, permits thymic generation and peripheral preservation of CD4+ CD25+ FoxP3+ T cells. *Bone Marrow Transplant* 2007; 39: 537.
25. Meyer TW, Hostetter TH. Uremia. *N Engl J Med* 2007; 357: 1316.
26. Lohr J, Knoechel B, Abbas AK. Regulatory T cells in the periphery. *Immunol Rev* 2006; 212: 149.
27. Yates J, Rovis F, Mitchell P, et al. The maintenance of human CD4+ CD25+ regulatory T cell function: IL-2, IL-4, IL-7 and IL-15 preserve optimal suppressive potency in vitro. *Int Immunol* 2007; 19: 785.
28. Antov A, Yang L, Vig M, Baltimore D, Van Parijs L. Essential role for STAT5 signaling in CD25+CD4+ regulatory T cell homeostasis and the maintenance of self-tolerance. *J Immunol* 2003; 171: 3435.
29. Zorn E, Nelson EA, Mohseni M, et al. IL-2 regulates FOXP3 expression in human CD4+CD25+ regulatory T cells through a STAT-dependent mechanism and induces the expansion of these cells in vivo. *Blood* 2006; 108: 1571.
30. Walker LS, Chodos A, Eggena M, Dooms H, Abbas AK. Antigen-dependent proliferation of CD4+ CD25+ regulatory T cells in vivo. *J Exp Med* 2003; 198: 249.
31. Brouard S, Dupont A, Giral M, et al. Operationally tolerant and minimally immunosuppressed kidney recipients display strongly altered blood T-cell clonal regulation. *Am J Transplant* 2005; 5: 330.
32. Gavin MA, Clarke SR, Negrou E, Gallegos A, Rudensky A. Homeostasis and anergy of CD4(+)CD25(+) suppressor T cells in vivo. *Nat Immunol* 2002; 3: 33.
33. Game DS, Hernandez-Fuentes MP, Chaudhry AN, Lechler RI. CD4+CD25+ regulatory T cells do not significantly contribute to direct pathway hyporesponsiveness in stable renal transplant patients. *J Am Soc Nephrol* 2003; 14: 1652.
34. Zeiser R, Nguyen VH, Beilhack A, et al. Inhibition of CD4+CD25+ regulatory T-cell function by calcineurin-dependent interleukin-2 production. *Blood* 2006; 108: 390.
35. Coenen JJ, Koenen HJ, van Rijssen E, Hilbrands LB, Joosten I. Rapamycin, and not cyclosporin A, preserves the highly suppressive CD27+ subset of human CD4+CD25+ regulatory T cells. *Blood* 2006; 107: 1018.
36. Game DS, Hernandez-Fuentes MP, Lechler RI. Everolimus and basiliximab permit suppression by human CD4+CD25+ cells in vitro. *Am J Transplant* 2005; 5: 454.
37. Gao W, Lu Y, El Essawy B, Oukka M, Kuchroo VK, Strom TB. Contrasting Effects of Cyclosporine and Rapamycin in De Novo Generation of Alloantigen-Specific Regulatory T Cells. *Am J Transplant* 2007.

38. Muthukumar T, Dadhania D, Ding R, et al. Messenger RNA for FOXP3 in the urine of renal-allograft recipients. *N Engl J Med* 2005; 353: 2342.
39. Dijke IE, Velthuis JH, Caliskan K, et al. Intragraft FOXP3 mRNA expression reflects antidonor immune reactivity in cardiac allograft patients. *Transplantation* 2007; 83: 1477.
40. Veronese F, Rotman S, Smith RN, et al. Pathological and clinical correlates of FOXP3+ cells in renal allografts during acute rejection. *Am J Transplant* 2007; 7: 914.
41. Valmori D, Merlo A, Souleimanian NE, Hesdorffer CS, Ayyoub M. A peripheral circulating compartment of natural naive CD4 Tregs. *J Clin Invest* 2005; 115: 1953.
42. Menning A, Hopken UE, Siegmund K, Lipp M, Hamann A, Huehn J. Distinctive role of CCR7 in migration and functional activity of naive- and effector/memory-like Treg subsets. *Eur J Immunol* 2007; 37: 1575.
43. Hoffmann P, Eder R, Boeld TJ, et al. Only the CD45RA+ subpopulation of CD4+CD25high T cells gives rise to homogeneous regulatory T-cell lines upon in vitro expansion. *Blood* 2006; 108: 4260.

CHAPTER 5

Clinical rejection and persistent immune regulation in kidney transplant patients

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ABSTRACT
We evaluated whether the regulatory function of CD4+CD25^{high+}FoxP3+ T-cells from patients on tacrolimus and mycophenolate mofetil (MMF) is affected by preceding steroid and anti-CD25 mAb induction therapy and whether this function is associated with rejection after kidney transplantation. Kidney recipients (N=15) were randomized to receive either anti-CD25 mAb induction (i.e., daclizumab) or steroids for 4 months. We analyzed the presence and suppressive activity of CD4+CD25^{high+}FoxP3+ peripheral T-cells in samples obtained pre and 4-6 months after transplantation. Anti-CD25 mAb therapy and treatment with steroids did not significantly affect protein expression of FoxP3. However, at the functional level, significant differences were found in the regulatory activities of CD4+CD25^{high+} T-cells from the anti-CD25 group vs those from the steroid group. At 4-6 months after transplantation, the regulatory activities of CD4+CD25^{high+} T-cells were comparable to those before anti-CD25 mAb therapy; 49 ± 13% (mean ± SEM) vs 40 ± 14% at a 1:20 ratio (CD25^{high+}: CD25$^{-/dim}$), respectively. In contrast, the regulatory capacities of CD4+CD25$^{bright+}$ T-cells from the steroid patient group became significantly impaired. The percentage inhibition of the anti-donor response decreased from 57 ± 12% before transplantation to 12 ± 7% after transplantation ($p<0.01$). Five out of 15 patients experienced a rejection episode. At 4-6 months after transplantation, the CD25^{high+} cells from these rejectors (who all received daclizumab induction therapy) had clear regulatory function, while suppression by CD25^{high+} cells from non-rejectors (N=10) was significantly lower. The percentage inhibition of the anti-donor response was 48 ± 14% (mean ± SEM) vs 10 ± 7%, respectively, $p=0.02$.
Anti-CD25 mAb induction therapy does not negatively influence the regulatory function of CD4+CD25^{high+}FoxP3+ T-cells from kidney transplant recipients on tacrolimus and MMF. The majority of these patients experienced an acute rejection episode, which suggests that immune activation is required for persistent immunoregulatory function.

Key words: CD4+CD25^{high+} regulatory T-cell, kidney transplantation, patients, anti-CD25 Ab induction therapy, steroids, rejectors

INTRODUCTION

The CD4+ T-cell subpopulation that constitutively coexpresses the IL-2Rα chain ($CD4^+CD25^+$) and the forkhead transcription factor FoxP3, has been shown to play a critical role in controlling immune responses to both self antigens and non-self antigen. These naturally occurring $CD4^+CD25^+FoxP3^+$ regulatory T-cells suppress the proliferation of antigen activated T-cells by inhibition of Thelper (Th)-1 cytokines of the effector T-cell population through a cell-cell contact-dependent mechanism, their inability to produce IL-2, and their anergic phenotype *in vitro* [1-3]. At the same time, IL-2 produced by the activated T-cells is necessary for the function, homeostasis and survival of these $CD4+CD25^{high+}$ FoxP3+ regulatory T-cells [4-7]. In addition, regulatory T-cells can be induced in the periphery upon conversion from CD4+CD25- T-cells into $CD4+CD25^{high+}$FoxP3+ T-cells in response to antigen and TGF-ß. In organ transplantation, both thymus derived and induced $CD4+CD25^{high+}$FoxP3+T-cells may limit donor-specific immune responses [3,8]. Like immunological genes, the FoxP3 promoter carries NF-AT and AP-1 binding sites, which are induced after triggering of the T-cell receptor [9]. These transcription factors regulate FOXP3 promoter activity, mRNA and protein expression.

IL-2 is a prominent player in anti-donor responses and exerts its function after binding to its specific cytokine receptor, i.e. CD25. Subsequently, treatment with selective immunosuppressive agents i.e. daclizumab and baxiliximab have been given to organ transplant patients to block the interaction of IL-2 with its high affinity IL-2 receptor (IL-2R) [10,11]. Apart from selective immunosuppression transplant patients are treated with non-specific drugs like steroids, calcineurin inhibitors (CNI's; e.g. tacrolimus and cyclosporin) and T-cell proliferation inhibitors (i.e., mycophenolate mofetil (MMF)). Glucocorticosteriods (GC) bind to intracytoplasmatic receptors that translocate GC to the nucleus where steroids affect GC response elements and subsequently block the production of pro-inflammatory cytokines. Tacrolimus, blocks the transcription of cytokines like IL-2, while the prodrug MMF that releases mycophenolic acid inhibits inosine monophosphate dehydrogenase, a key enzyme in purine synthesis required for lymphocyte proliferation.

Contrasting effects of immunosuppressants like steroids, anti-CD25 monoclonal antibodies (mAb) and CNI's to regulate the expression of the FOXP3 gene have been described [9, 13-15]. These experimental studies showed that anti-CD25 mAb's and CNI's, have detrimental effects on the induction of FOXP3 expression, while the studies in steroid treated asthmatic patients suggest that this agent induced FoxP3 gene/protein expression in T-cells. The pivotal function of FoxP3 in the control of immune responses was demonstrated in mice lacking this gene. These mice develop fatal autoimmune pathology, while in humans the absence of FoxP3 activity due to genetic disorders, is associated with multiorgan autoimmune diseases with fatal consequences [16,17]. Thus, by affecting FoxP3 expression and/or function, immunosuppressive drugs may alter the regulatory function of $CD4+CD25^{high+}$ T-cells in patients after transplantation. In kidney transplant patients the function of these FOXP3 T cells is controversial. Functional regulatory FoxP3 T cells have been found in stable kidney transplant patients, while these cells are also abundantly present in the kidney and urine during rejection [18-20]. These findings suggest that FOXP3+ T cells control anti-donor responses at various stages of the immune response. They prevent the activation and expansion of donor reactive T cells and are involved in damage control during rejection.
Here, we studied FoxP3 protein expression and function by $CD4+CD25^{high+}$ T-cells in kidney transplant patients were treated with anti-CD25 mAb or steroids in combination with tacrolimus and MMF to unravel the effects of immunosuppressive medication on the

mechanisms controlling immune reactivity *in vivo*. In addition, the function of CD4+CD25^{high+} T-cells in patients who rejected their transplanted kidney was studied.

MATERIAL AND METHODS

Patients

Analysis of the regulatory function of peripheral CD4+CD25^{high+} T-cells was performed in materials obtained from consecutive renal allograft recipients (N=15) in our center, as part of a multi-center study [11]. Patients were treated with tacrolimus, mycophenolate mofeltil (MMF), steroids for only 3 days and daclizumab induction therapy (N=7, anti-CD25 group). Daclizumab (1mg/kg body weight) was administrated i.v. 1 h before transplantation and once again on days 10-14 after transplantation. The steroid group was treated with tacrolimus, MMF and steroids for 4 months (N=8). These patients received prednisolone 0.3 mg/kg/day orally for the first 2 weeks, and thereafter the prednisolone dose was gradually tapered to zero in 4 months. Concominant therapy was identical in both groups. All patients received 100 mg of prednisolone i.v. during the first 3 days after transplantation. Tacrolimus was started on day 1 or 2 after transplantation at 0.15 mg/kg twice daily and the dose was subsequently adjusted to achieve target whole blood trough levels of 15-20 ng/mL from days 0-14, 10-15 ng/mL from weeks 3-6, and 5-10 ng/mL after week 7. MMF was administrated at 1000 mg twice daily, and decreased to 750 mg twice daily at 2 weeks after transplantation. Rejection was treated with 1-gram methylprednisolone i.v. on three consecutive days. No recurrent rejection episodes were observed. Details of the treatment and of the clinical outcome patients were reported by ter Meulen et al. [11].

Flow cytometry

Peripheral blood mononuclear cells (PBMC) were isolated from heparin blood samples from 15 kidney transplant patients before and at 1 – 4 weeks and 4 – 6 months after transplantation using Ficoll-Isopaque gradients. To determine the frequency of CD4+CD25^{high+} T-cells, PBMC were analyzed for the presence of CD4+CD25^{high+} T-cells by 4-colour flow cytometry using mAbs directly conjugated to fluorescein (FITC) or phycoerythrin (PE). Twenty µl of the following mAb combination was added to 10^6 PBMC: CD4-PERCP/CD25-PE (clone M-A251, epitope B/FoxP3-APC (clone PCH101). The appropriate isotype controls were included in each straining procedure. The FoxP3 antibody was purchased from eBioscience, (San Diego, CA), all other antibodies were from BD Biosciences (San Jose, CA). PBMC were incubated with the antibodies for 30 min at room temperature. After washing, cells were analysed on the FACSCalibur with Cell Quest Pro software (BD Biosciences). To establish an analysis gate that included at least 90% of the lymphocytes, the CD45/CD14 (BD Biosciences) reagent was used. At least 5000 gated lymphocyte events were acquired from each tube.

Regulatory T-cell function

PBMC were isolated from heparin blood samples from 15 kidney transplant patients before and at 4 – 6 months after transplantation using Ficoll-Isopaque gradients. To determine the regulatory, suppressive function of CD4+CD25^{high+} T-cells the effects of CD25^{high+} depletion and reconstitution on direct alloresponses were determined in mixed lymphocyte reactions (MLR). Peripheral cells from the 15 patients receiving either daclizumab induction therapy

(N=7) or steroids for 4 months (N=8) were tested for their suppressive activities against donor and third party allostimulators. $CD25^{high+}$ T-cells were isolated by autoMACS® (Miltenyi Biotec, Bergisch Gladbach, Germany) from the PBMC. The $CD25^{high+}$ T-cells were defined as the population, which highly expressed CD25 in combination with slightly less CD4 expression (figure 1). Cells not selected by the microbeads were designed as the $CD25^{-/dim}$ fraction. Purity of the fractions was measured by flow cytometry using CD25-PE (epitope B, BD PharMingen, San Diego, CA) and CD4-PerCP (BD Biosciences) mAbs. For the co-culture experiments, 100 µl of 5 x 10^4 responder $CD25^{-/dim}$ and $CD25^{high}$ cells were added to 100 µl 5 x 10^4 irradiated (45 Gy) donor cells and third party cells (2-2-2- HLA mismatch with both recipient and donor) in a round bottom 96-well plate and cultured for 7 days. Each proliferation assay was carried out in triplicate. Culture medium consisted of RPMI-1640 Dutch Modification (Gibco, Paisley, Scotland) supplemented with 10% pooled heat inactivated human serum, 4 mM L-glutamine, 100 IU penicillin and 100 µg/ml streptomycin. Proliferation was measured after ^{3}H-thymidine (0.5 µCi/well) incubation for the last 8 h before harvesting. Radioactivity was determined using a Betaplate counter (LKB, Bromma, Sweden).

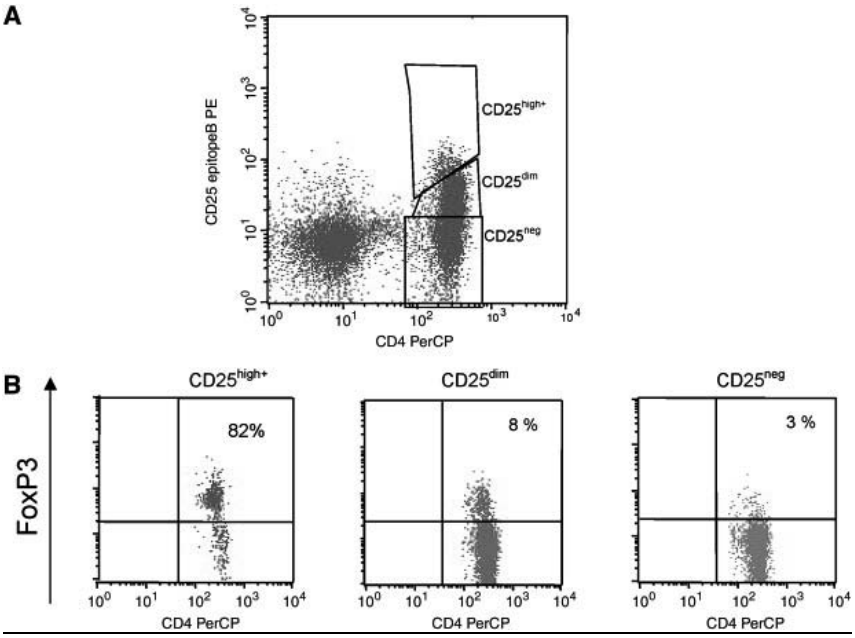

Fig. 1. (A) Dot plot of $CD3^+$ T-cells stained for CD4 and CD25 gated for the $CD4^+CD25^{high+}$ cells, $CD4^+D25^{dim}$ cells and $CD4^+CD25^{neg}$ cells. (B) In this representative example 82% of the $CD4^+CD25^{high+}$ expressed FoxP3, 8% of the $CD4^+D25^{dim}$ cells, and 3% of the $CD4^+D25^{neg}$ cells.

Statistics

For the determination of levels of statistical significance, two-sided probability values according to the signed Wilcoxon test or if appropriate, to the Student's t-test was calculated.

RESULTS

Clinical data

In the multi center study, 364 patients were included for clinical analysis [11]. Patients were randomly randomized to one of the treatment groups in a 1 : 1 ratio to receive either two doses of daclizumab and, for the first 3 days, prednisolone (anti-CD25 mAb group), or steroids (steroid group). All patients received tacrolimus and MMF. The incidence of biopsy-confirmed acute rejection at 12 months was not different between the anti-CD25 mAb group (15%) and the steroid group (14%). Also, graft survival at 12 months was comparable in the two groups (anti-CD25 mAb group: 91%; steroid group: 90%). At time of analyses our patients did have no clinical evidence of rejection as judged by the absence of increasing serum creatinine levels or proteinuria. Five out of 15 patients experienced a biopsy proved acute rejection episode prior to the analyses for functional peripheral $CD25^{high+}$ regulatory T-cells. Rejectors all received anti-CD25 mAb induction therapy. The demographics of the 15 patients from whom the regulatory capacities of $CD4+CD25^{high+}$ T-cells are studied are summarized in table 1.

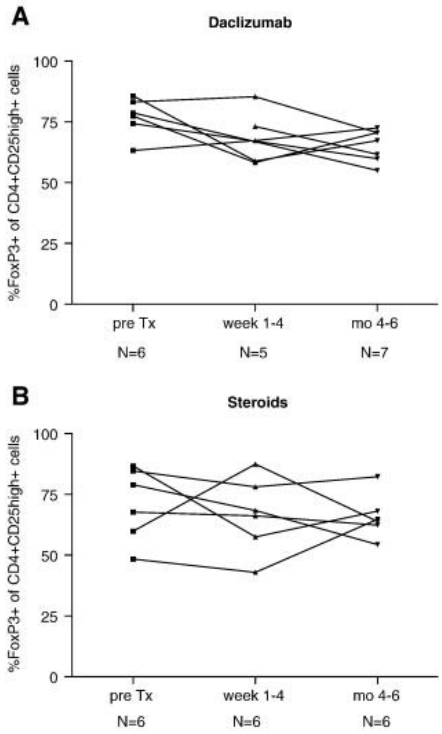

Fig. 2. Flow cytometric analysis of $CD4^{+}D25^{high+}$ cells for FoxP3 expression. The proportion of FoxP3 positive $CD4^{+}D25^{high+}$ T-cells remained stable over time in the daclizumab (anti-CD25 mAb treated) group (A) and steroid group (B).

Characterization of peripheral $CD4+CD25^{high+}$ T-cells

The humanized anti-CD25 antibody daclizumab is a non-depleting antibody; it covers the CD25 molecule and the cells remain present in the circulation. By flow cytometry it is not possible to stain T-cells with anti-CD25 mAb that bind to the same epitope as daclizumab for

at least the first 10 weeks after transplantation [11]. Therefore, CD25 cells were stained with the Ab binding epitope B. A typical example of CD25 epitope B expression during daclizumab therapy is depicted in figure 1. The percentage $CD25^{high+\ (epi\ B)}$ of CD4+ T-cells for the total group of 15 patients pre-transplantation varied from 1-5%, which is in line with the literature [20-22]. After transplantation these percentages did not significantly change. Moreover, between the 2 study groups, the anti-CD25 mAb group (N=7) and the steroid group (N=8), no statistically significant difference in the frequency of $CD25^{high+\ (epi\ B)}$ CD4+ T-cells was found at 1 – 4 weeks and 4 – 6 months after transplantation. These percentages varied from 1 - 6%. To determine whether treatment with anti-CD25 mAb and steroids influenced the expression of FoxP3, the regulatory T-cell marker, the $CD25^{high+}$ positive T-cells were analyzed in the first period after transplantation. As shown in the example, of the $CD25^{high+}$ T-cells 82% expressed FoxP3, 8% of the $CD25^{dim}$ positive cells and 3% of the CD25 negative cells (figure 1B).

In the first weeks after transplantation, when the CD25 epitope A molecule is blocked by daclizumab, no significant effect on FoxP3 levels of the $CD25^{high+}$ T-cell population was found (figure 2A). Also in the steroid group the proportion of FoxP3 positive cells in these $CD25^{high+}$ T-cells remained constant over time (figure 2B). In addition, when we analyzed the top 2% of gated $CD25^{high+}$ T-cells again no difference was measured in the frequency of FoxP3 positive cells between patients treated with anti-CD25 mAb or steroids at week 1-4 (figure 3). Thus, $CD4+CD25^{high+}$FoxP3+ T-cells remain present in the circulation of anti-CD25 mAb and steroid treated patients on tacrolimus and MMF early after kidney transplantation. Furthermore, the % peripheral FoxP3+CD4+$CD25^{high+}$ T-cells was comparable between rejectors (N=5) and non-rejectors (N=10) at the analyzed time points, i.e., before transplantation, at week 1-4 and at months 4 –6 after transplantation (data not shown).

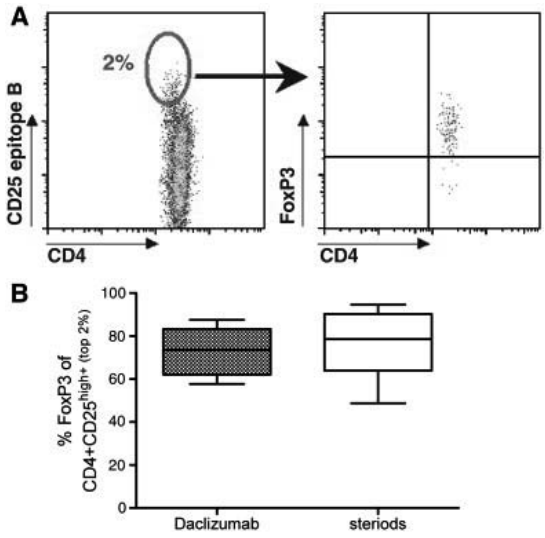

Fig. 3. Flow cytometric analysis of $CD4^{+}D25^{high+\ (top2\%)}$ cells for FoxP3 expression. (A) Analysis of $CD4^{+}D25^{high+\ (top2\%)}$ cells for FoxP3 expression. (B) No difference was measured in the frequency of FoxP3 positive $CD4^{+}D25^{high+(top2\%)}$ cells between patients treated with daclizumab or steroids at weeks 1–4 after kidney transplantation.

Table 1: Demographics of renal allograft recipients.

Patient	Donor origin	Number of HLA MM (A–B–DR)	Immunosuppressive medication group[a]	Acute rejection (Y/N, day pTx[b])
1	CAD	0–0–0	Daclizumab	Y (day 7)
2	CAD	1–1–0	Daclizumab	N
3	CAD	1–0–1	Daclizumab	Y (day 135)
4	L(U)RD	1–1–1	Daclizumab	Y (day 7)
5	L(U)RD	0–2–1	Daclizumab	N
6	CAD	1–1–2	Daclizumab	Y (day 22)
7	L(U)RD	1–0–1	Daclizumab	Y (day 6)
8	CAD	2–1–2	Steroids	N
9	L(U)RD	1–1–1	Steroids	N
10	L(U)RD	2–2–1	Steroids	N
11	CAD	0–0–1	Steroids	N
12	CAD	1–1–0	Steroids	N
13	L(U)RD	2–2–2	Steroids	N
14	L(U)RD	1–1–1	Steroids	N
15	CAD	1–0–0	Steroids	N

All patients received steroids during the first 3 days, tacrolimus, and mycophenolate mofetil.CAD, cadaveric donor; L(U)RD, living (un) related donor; HLA MM, HLA mismatches; Y, yes; N, no.[a] Concomitant therapy was identical in both groups. [b] pTx, posttransplantation.

Regulation by peripheral CD4+CD25^{high+} T-cells

To test the immunoregulatory function of CD4+CD25^{high+} cells from the patients treated with daclizumab and the steroid group in allogeneic responses, the CD25^{high+} cells were depleted from PBMC and their function was tested in co-culture experiments with anti-donor and anti-third party stimulated CD25 $^{-/dim}$ responder cells. An example of these cell populations is depicted in figure 4.

Depletion of the CD25^{high+} suppressive cell fraction from PBMC before and after transplantation resulted in a significant increase in the response to donor antigens and third party antigens (all $p<0.01$, figure 5). The magnitude of the increased proliferation upon depletion was the same before and after transplantation and not different for the donor and third party stimulated PBMC. Regulation by CD25^{high+} T-cells was confirmed in co-culture experiments with isolated CD25$^{-/dim}$ responder T-cells (figure 6). These isolated CD25^{high+} cells did not proliferate upon allogeneic stimulation, characteristic for CD25^{high+} regulatory T-cells. Purity of the CD25^{high+} cell fraction was >80%. In both the ati-CD25 mAb and

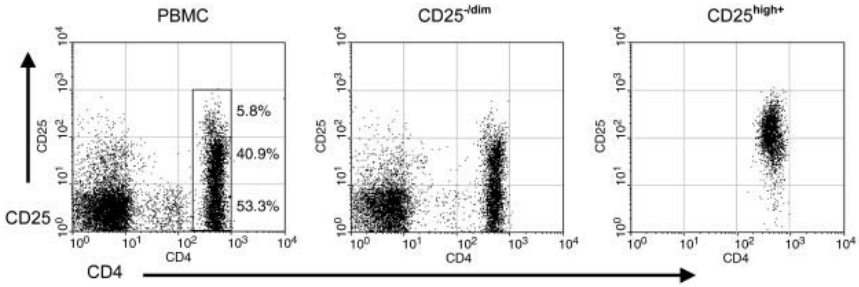

Fig. 4. Flow cytometric analysis of PBMC, $CD25^{-/dim}$ and $CD25^{high+}$ populations. The PBMC, $CD25^{-/dim}$ and $CD25^{high+}$ obtained after MACS-separation were stained for the expression of CD4 and CD25 epitope B to check the purity of the obtained fractions. A representative example is shown.

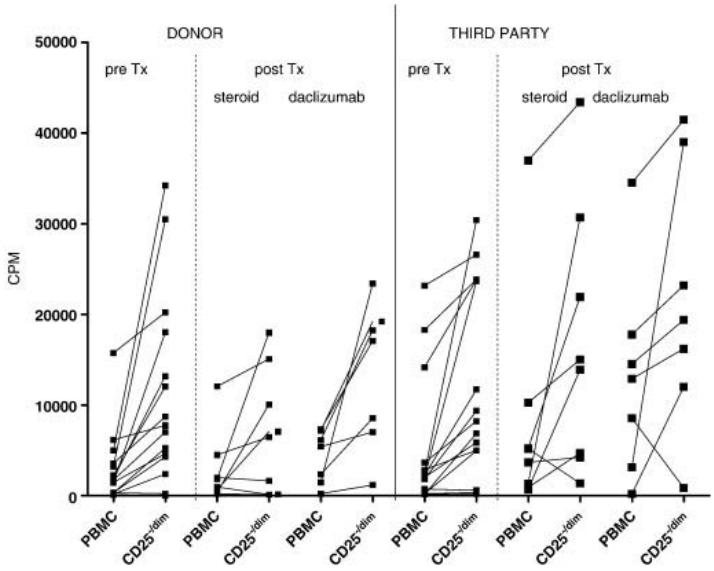

Fig. 5. Depletion of $CD4^{+}D25^{high+}$ T-cells. Proliferative response to alloantigens of PBMC before and after the depletion of $CD25^{high+}$ T-cells. Depletion of $CD25^{high+}$ T-cells resulted in a significant increase in the proliferate response of patient samples taken before and at 4–6 months after transplantation when stimulated with donor antigens and third party antigens ($p < 0.01$ for pre Tx and post Tx donor stimulated cells and $p < 0.01$ for third party stimulated cells). Proliferation of PBMC and $CD25^{-/dim}$ cells was measured by incorporation of tritiated thymidine at day 7.

steroids treated patients the proportion of $CD25^{high+}$ cells expressing FoxP3 was > 85% and comparable between the treatment groups. Reconstitution of $CD25^{high+}$ T-cells resulted in dose-dependent inhibition for the $CD25^{-/dim}$ cells stimulated with donor antigens or with third party alloantigens at both time points. However, after transplantation, at a 1:20 $CD25^{high+}$: $CD25^{-/dim}$ ratio, the percentage inhibition was lower than before transplantation irrespective whether the patient cells are stimulated with donor or third party alloantigen (both p=0.02). Before transplantation the anti-donor response the proliferative response was inhibited by 52 ± 10% (mean ± SEM) and after transplantation by 30 ± 9% (p=0.02, figure 6). To define whether this difference resulted from differences in immunosuppressive therapy, we

analyzed the daclizumab vs the steroid group. Treatment with daclizumab shortly after transplantation, did not significantly affect the regulatory activities of the CD25 cells at 4-6 months after transplantation. The regulatory capacities of CD25 cells were comparable to those before induction therapy with daclizumab, 49 ± 13% (mean ± SEM) vs 40 ± 14%, respectively. In contrast, the cells obtained from patients who received steroids for 4 months, the regulatory capacities were significantly impaired (figure 7). At a 1 : 20 ratio the percentage inhibition of the anti-donor response decreased from 57 ± 12% (mean ± SEM) before transplantation to 12 ± 7% at 4–6 months after transplantation ($p<0.01$).

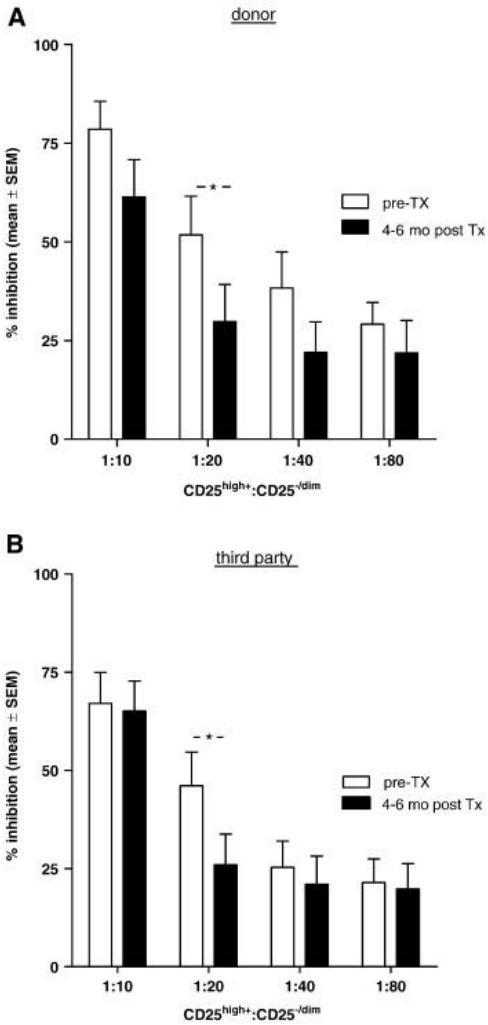

Fig. 6. $CD4^{+}D25^{high+}$ T-cells dose dependently inhibits proliferation. Isolated $CD4^{+}D25^{high+}$ T-cells were co-cultured with the responder $CD25^{-/dim}$ cells of the patient's ($N = 15$) different ratios ranging from 80:1 to 10:1. A dose-dependent inhibition of proliferation against donor antigens (A) and third party antigens (B) was seen, before and at 4–6 months after transplantation. However, after transplantation, at a 1:20 $CD25^{high+}$:$CD25^{-/dim}$ T-cell ratio, the inhibition percentage was lower than before transplantation irrespective whether the patient cells are stimulated with donor or third party alloantigen (both $p = 0.02$). Data are depicted as mean ± SEM.

To study whether rejection is associated with regulatory T cell function, we evaluated the function of rejectors (N=5) vs non-rejectors (N=10). At 4-6 months after transplantation, the $CD25^{high+}$ cells from rejectors had clear regulatory function, while the suppression by $CD25^{high+}$ cells from non-rejectors was significantly lower. The percentage inhibition of the anti-donor response was 48 ± 14% (mean ± SEM) vs 10 ± 7%, respectively, p=0.02, figure 8.

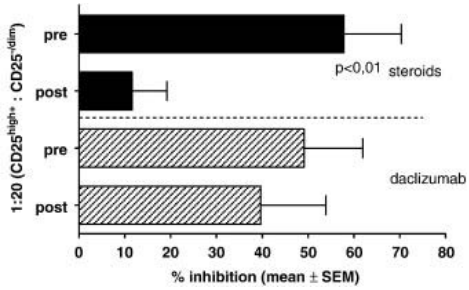

Fig. 7. At 4 to 6 months after transplantation, the regulatory capacities of $CD25^{high+}$ cells was comparable to those obtained before induction therapy with daclizumab, 49% (mean) vs 40%, respectively. In contrast, in the steroids group, the regulatory capacities were significantly impaired. At a 1 to 20 ratio the percentage inhibition of the anti-donor response dropped from 57% (mean) before transplantation to 12% after transplantation ($p < 0.01$).

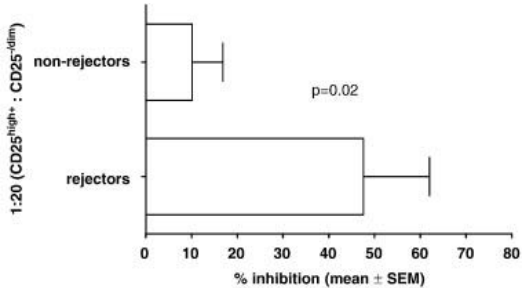

Fig. 8. The function of $CD25^{high+}$ cells from rejectors ($N = 5$) vs non-rejectors ($N = 10$) was analyzed. At 4–6 months after transplantation, the $CD25^{high+}$ cells from rejectors had clear regulatory function, while the suppression by $CD25^{high+}$ cells from non-rejectors was significantly lower ($p = 0.02$). The percentage inhibition of the anti-donor response was 48 ± 14% (mean ± SEM) vs 10 ± 7%, respectively.

DISCUSSION

In the present study, we analyzed whether anti-CD25 mAb therapy with the humanized anti-IL-2Rα mAb daclizumab and steroids influence the protein expression of the transcription factor FoxP3 and function of the natural arising $CD4+CD25^{high+}$ regulatory T-cells in kidney transplant patients on tacrolimus and MMF. In addition, the function of $CD4+CD25^{high+}$ T-cells was studied in relation to rejection.

While no significant changes on the percentage of $CD4+CD25^{high+}$ and the proportion of FoxP3 expressing cells during treatment with anti-CD25 mAb or steroids were measured, the regulatory capacity of $CD25^{high+}$ lymphocytes from patients treated with steroids for 4 months, tacrolimus and MMF was weak. The observation that anti-CD25 mAb induction therapy or steroids do not influence the percentage of peripheral $FoxP3+CD4+CD25^{high+ (epi B)}$ T cells in kidney transplant patients on tacrolimus and MMF shows that the combination of

these drugs do not have a measurable effect on their development and maintenance. This suggests that during blockade of the IL-2 pathway by CNI's, anti-CD25 mAb and steroids, other common γ-chain dependent cytokines compensate for the lack of IL-2. The paper by Vang et al. reports that in a mouse model in the absence of IL-2, indeed other common γ-chain dependent cytokines like IL-7 and IL-15 redundantly govern CD4+Foxp3+ regulatory T cell development [23]. Moreover, Vlad et al. reported that the peripheral levels of FOXP3 mRNA in heart transplant patients were not adversely affected by the anti-CD25 mAb daclizumab [21]. Alternatively, the IL-2 route is only partly inhibited by these immunosuppressants and as a result, IL-2 can still bind and signal via the IL-2Rβ, the receptor essential for the development and maintenance of FoxP3+ cells *in vivo* [24,25]. After transplantation the function of $CD4+CD25^{high+}$FoxP3+ T cells from patients on tacrolimus/MMF treated with steroids declined in contrast to those from patients receiving induction therapy with anti-CD25 mAb (figure 7). Thus, not even in combination with tacrolimus/MMF, anti-CD25 mAb impair the *ex vivo* function of $CD4+CD25^{bright+}FoxP3^{+}$ T-cells. These results are in line with findings reported by others [19,26,27]. Several explanations for these findings can be given, I. the mAb competes with IL-2 for CD25 and residual IL-2 binding may be sufficient for regulatory cell function, II. IL-2 triggers regulatory function after binding to epitope B of the CD25 molecule not covered by daclizumab, the IL-2R β-chain and the common γ-chain and III. other common γ-chain dependent cytokines like IL-15 take over the function of IL-2 [19,26,27]. The adverse effect of steroids on the function of FoxP3+ regulatory T cells from patients on CNI's and MMF could be the consequence of it's interaction with NF-AT, NF-κB and AP-1 [9]. Steroids bind to intracytoplasmatic receptors that translocate steroids to the nucleus where it negatively regulates the activities of these transcription factors. Moreover, high doses of steroids inhibit GC receptor independent effects that affect pivotal but currently unknown factors crucial for the function of FoxP3 T-cells [9,28]. In addition, steroids may interact with tacrolimus and MMF that has a synergistic and harmful effect on FoxP3 T-cells [29]. Thus, the combination of immunosuppressive drugs that target the activities of major transcription factors, key for the regulation of FoxP3 promoter activity affect, the function of $CD4+CD25^{high+}$ FoxP3+ T-cells significantly, while their function remains intact when primarily cytokine pathways are targeted by CNI's and non-depleting anti-CD25 mAb's.

We speculate that in case of steroids, CNI's and MMF treatment, the immune system cannot bypass the tremendous effects of these drugs on FoxP3+ regulatory T-cells. This also explains why immune regulation is mainly measured in cells obtained from patients treated with the less potent immunosuppressive combination of CNI's with non-depleting anti-CD25 mAb's. The latter combination does not completely prevent the T cell mediated acute rejection response, which at the same time induces immune regulation to control the anti-donor reactivity. Thus, again suggesting that immune regulatory T cells are involved in damage control during rejection. The regulatoryactivities of $CD25^{high+}$ T cells or the generation of these cells is an intrinsic part of activation. This, it is conceived that patients with high immune activity resulting in acute rejection episodes are the same patients with high regulatory activities.

In conclusion, anti-CD25 mAb induction therapy does not negatively influence the regulatory function of $CD4+CD25^{high+}$FoxP3+ T-cells from kidney transplant recipients on tacrolimus and MMF. The vast majority of these patients experienced an acute rejection episode, which suggests that immune activation is required for persistent immunoregulatory function.

REFERENCES

1. Sakaguchi S, Sakaguchi N, Asano M, et al. Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. J Immunol 1995; 155;1151-64.
2. Fontenot JD, Gavin MA and Rudensky AY. Foxp3 programs the development and function of CD4+CD25+ regulatory T cells. Nat Immunol 2003; 4; 330-36.
3. Kingsley CI, Nadig SN, Wood KL. Transplantation tolerance: lessions from experimental rodent models. Transplant Int 2007; 20; 828-41.
4. Furtado GC, De Lafaille MAC, Kutchukhidze N, Lafaille JJ. Interleukin 2 signaling is required for CD4(+) regulatory T cell function. J Exp Med 2002; 196; 85157.
5. Thornton AM, Donovan EE, Piccirillo CA, Shevach EM. IL-2 is critically required for the in vitro activation of CD4+ CD25+ T cell suppressor function. J Immunol 2004; 172; 6519-23.
6. Fontenot JD, Rasmussen JP, Gavin MA, Rudensky AY. A function of interleukin 2 in Foxp3-expressing regulatory T cells. Nat Immunol 2006; 6;1142-51.
7. D'Cruz LM, Klein L. Development and function of agonist-induced CD25+Foxp3+ regulatory T cells in the absence of interleukin 2 signaling. Nat Immunol. 2005; 6; 1152-59.
8. Kang SM, Tang O, Bluestone JA. CD4+CD25+ regulatory T cells in transplantation: progress, challenges and prospects. Am J Transplant. 2007; 7;1457-63.
9. Mantel PY, Ouaked N, Ruckert B, et al. Molecular mechanisms underlying FOXP3 induction in human T-cells. J Immunol 2006; 176;3593-602.
10. Vincenti F, Kirkman R, Light S, et al. Interleukin-2-receptor blockade with daclizumab to prevent acute rejection in renal transplantation. Daclizumab Triple Therapy Study Group. N Engl J Med 1998; 338;161-65.
11. Meulen ter CG, Riemsdijk van I, Hené RJ, et al. Steroid-withdrawal at 3 days after renal transplantation with anti-IL-2 receptor α therapy: A prospective, randomized, multicenter study. Am J Transplant 2004; 4;803-10.
12. Karagiannidis C, Akdis M, Holopainen P, et al. Glucocorticiods upregulate FOXP3 expression and regulatory T-cells in asthma. J Allergy Clin Immunol 2004; 114:1425-33.
13. Baan CC, van der Mast BJ, Klepper M, et al. Differential effect of calcineurin inhibitors, anti-CD25 antibodies and rapamycin on the induction of FOXP3 in human T cells. Transplantation 2005; 80;110-17.
14. Chen X, Oppenheim J, Winkler-Pickett RT, Ortaldo JR, Zack Howard OM. Glucocorticoid amplifies IL-2 dependent expansion of functional FoxP3+CD4+CD25+ T regulatory cells in vivo and enhances their capacity to suppress EAE. Eur J Immunol 2006; 36;2139-49.
15. Louis S, Braudeau C, Giral M, et al. Contrasting CD25hiCD4+T cells/FOXP3 patterns in chronic rejection and operational drug-free tolerance. Transplantation 2006; 81;398-07.
16. Kim JM, Rasmussen JP, Rudenski AY. Regulatory T cells prevent catastrophic autoimmunity throughout the lifespan of mice. Nat Immunol 2007; 8;191-97.
17. Gambineri E, Torgerson TR, Ochs HD. Immune dysregulation, polyendocrinopathy, enteropathy, and X-linked inheritance (IPEX), a syndrome

systemic autoimmunity caused by mutations in FOXP3, a critical regulator of T-cell homeostasis. Curr Opin Rheumatol 2003; 15;430-35.
18. Velthuis JHL, Mol WM, Weimar W, Baan CC. $CD4^{+}$ $CD25^{bright+}$ regulatory T cells can mediate donor non-reactivity in long-term immunosuppressed kidney allograft patients. Am J Transplant 2006; 6;2955-64.
19. Muthukumar T, Dadhania D, Ding R, et al. Messenger RNA for FOXP3 in the urine of renal-allograft recipients. N Engl J Med 2005; 353;2342-51.
20. Veronese F, Rotman S, Smith RN, Kawai T, et al. Pathological and Clinical Correlates of FOXP3(+) Cells in Renal Allografts during Acute Rejection. Am J Transplant 2007; 7;914-22.
21. Vlad G, Ho EK, Vasilescu ER, et al. Anti-CD25 treatment and FOXP3-positive regulatory T cells in heart transplantation. Transpl Immunol. 2007; 18;13-21.
22. Akl A, Jones ND, Rogers N, et al. An investigation to assess the potential of CD25(high)CD4(+) T cells to regulate responses to donor alloantigens in clinically stable renal transplant recipients. Transpl Int. 2008; 21;65-73.
23. Vang KB, Yang J, Mahmud SA, Burchill MA, Vegoe AL, Farrar MA. IL-2, -7, and -15, but not thymic stromal lymphopoeitin, redundantly governs CD4+Foxp3+ regulatory T cell development. J Immunol. 2008; 181;3285-90.
24. Baan CC, Boelaars-van Haperen MJAM. Van Riemsdijk IC, van der Plas AJ, Weimar W. IL-7 and IL-15 bypass the immnuosuppressive action of anti-CD25 monoclonal antibodies. Transplant Proc 2001; 33; 2244-46.
25. Soper DM, Kasprowicz DJ, Ziegler SF. IL-2Rß links IL-2R signaling with Foxp3 expression. Eur J Immunol 2007; 37 ;1817-26.
26. Game DS, Hernandez-Fuentes MP, Lechler RI. Everolimus and Basiliximab permit suppression by human CD4+CD25+ cells in vitro. Am J Transplant 2005; 5;454-64.
27. Kreijveld E, Koenen HJPM, Klasen IS, Hilbrands LB, Joosten I. Following anti-CD25 treatment, a functional CD4+CD25+ Treg pool is present in renal transplant recipients. Am J Transplant 2007; 1;249-55.
28. Karin M. New twists in gene regulation by glucocorticoid receptor: is DNA binding dispensable? Cell 1998; 93;487-90.
29. Lam S, Partovi N, Ting LSL, Ensom MHH. Corticosteroid interactions with cyclosporine, tacrolimus, mycophenolate, and sirolimus: fact or fiction? Annals of Pharmacotherapy 2008; 42;1037-47.

CHAPTER 6

Monotherapy rapamycin allows an increase of $CD4^{+}CD25^{bright+}FoxP3^{+}$ T cells in renal recipients

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Abstract

$CD4^+CD25^{bright+}FoxP3^+$ regulatory T cells (Tregs) may control donor-specific allogeneic responses in kidney transplant recipients. Recent evidence demonstrated that three phenotypical Treg-subsets, naive ($CCR7^+CD45RO^-$), central-memory ($CCR7^+CD45RO^+$) and effector-memory ($CCR7^-CD45RO^+$), are essential for the development and function of antigen-specific suppression in the lymphoid and peripheral tissues. Also, it has been appreciated that Tregs are affected by immunosuppressive agents. In clinical practice, however, the effect of a single-drug remains to be determined.

Therefore, we analyzed the effect of several immunosuppressive agents on the number, phenotype and function of peripheral Tregs from 46 stable kidney transplant recipients. These patients were converted to monotherapy with tacrolimus (N=15), rapamycin (N=17) or mycophenolate mofetil (N=14). Blood was obtained at inclusion and 6 months thereafter.

The number of Tregs increased significantly in patients on monotherapy with rapamycin ($p<0.001$), which was caused by increased numbers of Tregs with a central-memory and an effector-memory phenotype (both $p<0.05$). At six months after conversion, however, the suppressive function of Tregs did not significantly change in co-cultures stimulated with donor-Ag.

Therefore monotherapy with rapamycin allows the signals that are needed to increase the number of functional Tregs with a memory phenotype, thereby enhancing the potential capacity to regulate donor-specific responses in the lymphoid and the peripheral tissues.

Introduction

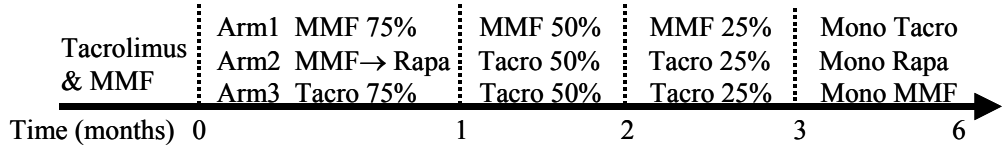
Recently, much research has focused on the role of $CD4^+CD25^{bright+}FoxP3^+$ regulatory T cells (Tregs) in organ transplantation [1-9]. These studies show that donor-specific Tregs may develop after transplantation and actively contribute to donor-specific hyporesponsiveness in vivo [1-3,7,9]. Therefore, Tregs may be essential to achieve graft acceptance.

For their development and function, Tregs require dynamic interactions with antigen presenting cells and T effector cells [10,11]. Increased levels of FoxP3 mRNA were found in the urine of kidney transplant recipients during acute rejection, while Veronese and colleagues demonstrated that $FoxP3^+$ cells infiltrated the kidney during acute cellular rejection [12,13]. This implies that Tregs from organ transplant recipients migrate to the lymphoid tissues as well as the graft [12-15]. Insights into the mechanisms and molecules involved in the trafficking of Tregs have recently been provided in patients suffering from ovarian cancer [16]. In line with other studies, the latter study shows that like conventional T cells, Tregs can be distinguished according to their in vivo differentiation stage into naive cells ($CD45RO^-$) and memory cells ($CD45RO^+$) [16-24]. Upon activation, naive-Tregs give rise to $CD45RO^+$ Tregs [17,25]. Moreover, CD45RO was identified as an important marker for Tregs with potent suppressive capacities [25,26]. Similar to conventional T cells, $CD45RO^+$ Tregs can be divided in central-memory Tregs (CM; $CCR7^+CD45RO^+$), which migrate to the draining lymph nodes and effector-memory Tregs (EM; $CCR7^-CD45RO^+$), which travel to inflamed tissues [16-23]. This suggests that together CM and EM-Tregs control immune responses both in the lymphoid and in the peripheral tissues [27].

At present, transplant patients receive life-long immunosuppressive medication to prevent and treat rejection. At the same time, these drugs may cause a variety of complications such as nephrotoxicity and hypertension and allow opportunistic infections and malignancies to develop [28-30]. Experimental models suggest that immunosuppressive drugs may also affect the development and function of Tregs [31,32]. Here, especially the effect of these drugs on the IL-2 pathway seems important, since this pathway appears to be crucial for the development, homeostasis and function of Tregs [33,34]. Therefore, the effect of calcineurin inhibitors (CNI) that inhibit the production of IL-2 [28], and in contrast, inhibitors of the mammalian target of rapamycin (mTOR) that block the early expansion of alloreactive T cells but spare IL-2 production, has been studied [32,35]. Based upon the results from additional experiments [36-38], it has been suggested that immunosuppressive drugs can affect the composition of Tregs in kidney transplant patients and thereby their migration to draining lymph nodes and the allograft. In clinical practice, however, patients are treated with a combination of drugs and therefore, the effect of a single-drug remains to be determined.

Here, we prospectively analyzed the number, phenotype (e.g. CD45RO and CCR7) and function of $CD4^+CD25^{bright+}FoxP3^+$ regulatory T cells from 46 stable kidney transplant recipients on monotherapy. Patients were converted from triple therapy (tacrolimus, mycophenolate mofetil (MMF) and prednisone) to monotherapy tacrolimus, rapamycin or MMF. This provided us with the unique opportunity to determine the individual in vivo effect of a single drug on Tregs in kidney transplant patients.

Figure 1



Flow chart for conversion of patients treated with tacrolimus/MMF to monotherapy tacrolimus (arm 1), rapamycin (arm 2) or MMF (arm 3). Full conversion to monotherapy with either drug was achieved at month 3.

Materials and Methods

Subjects

The study was performed at Erasmus MC and all patients provided informed consent according to the rules of our local medical ethics committee. We included 46 stable patients (table 1), defined as the absence of proteinuria (>0.25 g/l), no increase in serum creatinine levels and no biopsy proven acute rejection (BPAR) in the last 3 months prior to inclusion. Patients were 2.8 ± 1.4 years after kidney transplantation (KTx) and without malignancies or active infections such as CMV. 15 patients were on treatment with statins for hyperlipidemia [39]. As a result, none of our patients presented hyperlipidemia at baseline. From transplantation until study inclusion, immunosuppressive therapy consisted of standard triple therapy with calcineurin inhibitors (CNI), MMF and prednisone. Patients were then randomly assigned to one of the treatment groups with stratification for cadaveric/ living transplant and in cohorts of 10. Randomization was carried out by opening a sealed envelope. In arm 1 patients were converted to monotherapy with tacrolimus (N=15), in arm 2 to rapamycin (N=17) and in arm 3 to MMF (N=14). The study medication schedule is shown in figure 1. In all arms of treatment patients also received prednisone for the first month whereafter prednisone was fully withdrawn. The target trough levels for month 6 were 5-10 ng/ml for tacrolimus, 8-12 ng/ml for rapamycin and >2 ug/ml for MMF. Peripheral blood samples were obtained at inclusion and 6 months thereafter. There were no significant differences in patient characteristics between the arms of treatment at baseline (table 1). Also, we included 15 healthy controls (HC), consisting of 9 males and 6 females with a mean age of 51 ± 8 years. These HC were age and gender matched for our patient population.

Flow cytometric analysis

Peripheral blood was collected in heperanized tubes and analyzed for the presence of T-cell subsets by four-color flow cytometry using mAbs directly conjugated to fluorescein (FITC), phycoerythrin (PE), allophycocyanin (APC) or peridinin chlorophyll protein (PerCP). 100µl blood was incubated with 10µl of the dual mAb combinations CD45-FITC/CD14-PE; IgG_1-FITC/IgG_{2b}-PE; IgG_1-PerCP/ IgG_1-APC as isotype control. Further we used the mAb CD3-FITC, CD4-PERCP, CD8-APC and CD25-PE. Also, PBMC were stained for CD3-PerCP, CD4-FITC, CD25-PE (epitope B) and intracellular FoxP3-APC (clone PCH101, eBioscience, San Diego, CA, USA). To determine how Tregs evolve, we added a combination of CD4-PerPC/CD25-PE/CD45RO-APC/CCR7-FITC to 100µl whole blood. Except for FoxP3,

Table 1. Patient characteristics at inclusion

Demographics	Arm 1 Tacrolimus (N=15)	Arm 2 Rapamycin (N=17)	Arm 3 MMF (N=14)
Gender (M / F)	8 / 7	11 / 6	10 / 4
Age (years)	50 ± 12	54 ± 10	54 ± 16
Time after KTx (months)	30 ± 13	32 ± 18	39 ± 20
Donor origin Living related / deceased	11 / 4	10 / 7	9 / 5
HLA-A/B mm	2.3 ± 1.1	2.1 ± 1.2	2.5 ± 0.7
HLA-DR mm	1.1 ± 0.7	1.0 ± 0.8	0.9 ± 0.6
Creatinine (μmol/L)	114 ± 22	118 ± 35	114 ± 41
Proteinuria (g/L)	0.08 (0.04-0.14)*	0.07 (0.02-0.21)*	0.08 (0.02-0.23)*
GFR (ml/min/1.73 m^2)	58 ± 14	58 ± 18	62 ± 17
CMV positive pre versus post KTX	8 / 9	13 / 14	8 / 8
BPAR	3	0	3
Statins	5	4	6
Tacrolimus (ng/mL)	6.6 ± 1.8	5.7 ± 2.0	5.5 ± 2.2
MPA (μg/mL)	2.1 ± 1.8	1.9 ± 1.0	2.0 ± 1.0

Mean ± SD, * Median (range), GFR = glomerular filtration rate, CMV = Cytomegalovirus, BPAR = Biopsy Proven Acute Rejection, MPA = mycophenolic acid.

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 FACS lysing solution (BD Biosciences) for 10 min. Cells were washed twice, and analysed 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 20000 gated lymphocyte events were acquired from each tube. Cells with a $CCR7^{+}CD45RO^{-}$ phenotype were considered to be naive cells, $CCR7^{+}CD45RO^{+}$ cells central-memory (CM) and $CCR7^{-}CD45RO^{+}$ cells effector-memory (EM).

Isolation of $CD4^{+}CD25^{bright+}FoxP3^{+}$ cells

Defrosted patient-PBMC were washed twice and resuspended in 42μl 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). We previously demonstrated that this is an effective method to isolate $CD4^{+}CD25^{bright+}FoxP3^{+}$ Tregs [9].

Regulation of donor-Ag stimulated responder cells by $CD25^{bright+}$ cells

$5x10^4$ defrosted patient-PBMC were incubated with $5x10^4$ irradiated (40 Gy) donor PBMC (donor-Ag). Co-cultures were performed in Human Culture Medium (HCM) consisting of RPMI 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), in triplicate, in a 96-wells round bottom plate for 7 days.

Isolated $CD25^{bright+}$ T cells were added to donor stimulated patient-PBMC at a 1:10 ratio to determine their regulatory, suppressive capacities [9]. At day 6, ^{3}H-thymidine 0.5 μCi/well was added to the culture and 16 hours later samples were harvested and radioactivity was measured in counts per minute (CPM) using a β-counter (PerkinElmer, Oosterhout, The Netherlands).
Inhibition by the $CD25^{bright+}$ cells of the proliferative response was calculated as the percentage of inhibition (%IH).

$$\%IH = \frac{\text{CPM PBMC} - (\text{CPM PBMC} + CD25^{bright+}\text{ cells})}{\text{CPM PBMC}} * 100$$

Proliferation of mitogen stimulated cells

We determined the capacity of PBMC ($5x10^4$) to proliferate upon stimulation with 1 μg/ml Phytohaemagglutinin (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 hours later the samples were harvested and radioactivity was counted.

Statistical analysis

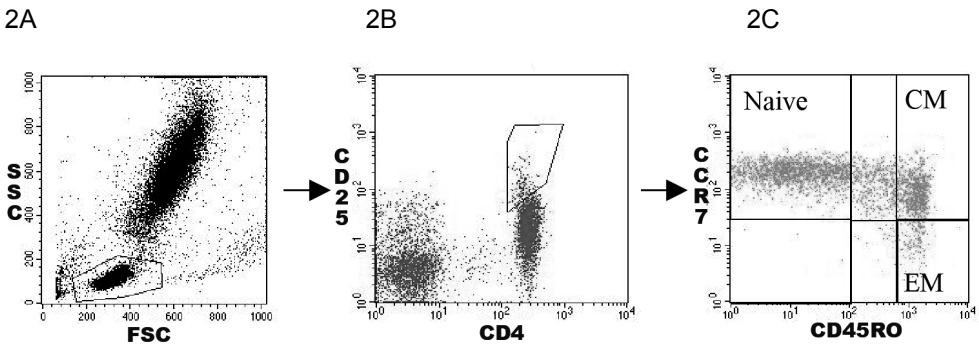
Statistics were performed using GraphPad Prism 4.0 or SPSS 11.5 software. To analyze several variables at the same time, linear regression was performed. To analyze differences between before and after treatment with monotherapy, we performed paired analysis (Wilcoxon signed rank test). A p-value <0.05 is marked with one asterisk * and $p<0.01$ = **.

Table 2. Patient characteristics 6 months after conversion

Demographics	Arm 1 Tacrolimus	Arm 2 Rapamycin	Arm 3 MMF
Patient survival	100%	100%	100%
Graft survival	100%	100%	100%
Rejection incidence	0 (0%)	0 (0%)	3 (21%)
Creatinine** (μmol/L)	122 ± 29	121 ± 41	124 ± 29
Proteinuria** (g/L)	0.09 (0.03-2.49)*	0.12 (0.02-1.56)*	0.08 (0.02-0.16)*
GFR** (mL/min/1.73 m^2)	54 ± 14	58 ± 19	56 ± 16
Medication** level	8.6 ± 1.6 (ng/mL)	7.6 ± 1.7 (ng/mL)	5.0 ± 2.1 (μg/mL)

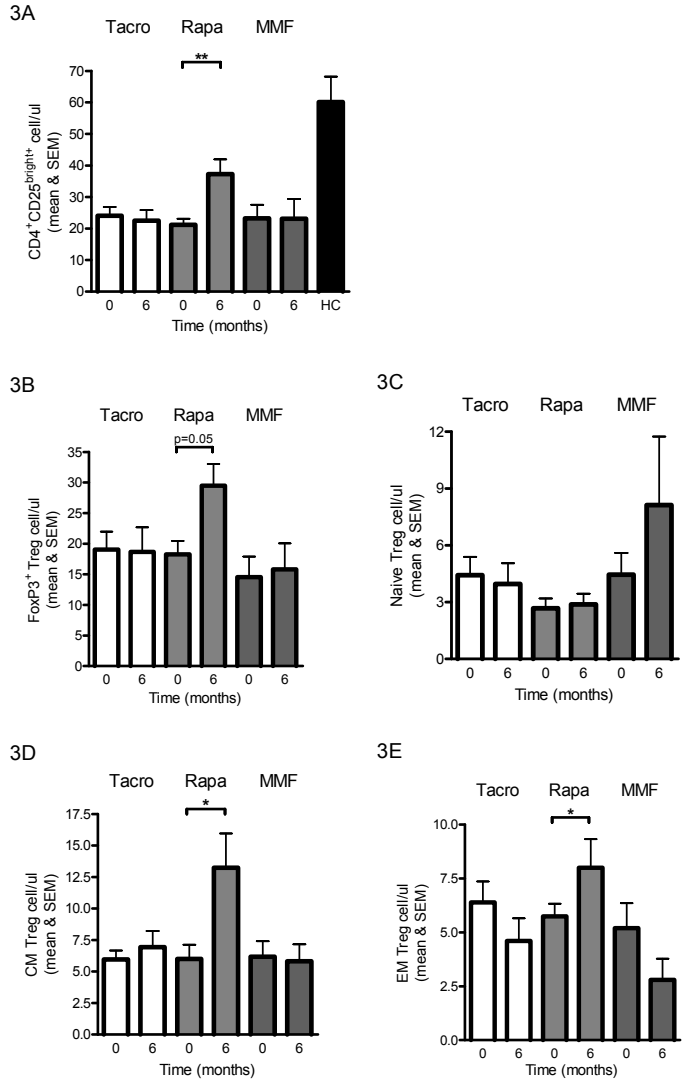
Mean ± SD, * Median (range), GFR = glomerular filtration rate, ** Data of patients that completed the study.

Figure 2



(A) Mononuclear cells were gated with forward scatter (FSC) and sideward scatter (SSC). (B) From the gate in 2A cells with a $CD4^+CD25^{bright+}$ phenotype (Treg) were gated. (C) Based on their expression of CCR7 and CD45RO, cells from the gate in 2B were then distinguished into naive-Treg ($CCR7^+CD45RO^-$), central-memory Treg (CM; $CCR7^+CD45RO^+$) and effector-memory Treg (EM; $CCR7^-CD45RO^+$).

Figure 3



(A-E) At baseline (time 0), the absolute number of Tregs and their subsets were not significantly different between the arms of treatment. (A) The absolute number of Tregs was significantly lower at baseline when compared to healthy controls (HC; all $p<0.01$). After conversion, the number of Tregs increased significantly in patients treated with rapamycin ($p<0.01$). (B) The number of Tregs with a $FoxP3^+$ phenotype only increased in patients treated with rapamycin ($p=0.05$). (C) The number of naive-Tregs did not significantly change in either arm of treatment, however, in patients treated with MMF the number seemed to increase. (D-E) The number of CM and EM-Tregs only increased in patients converted to monotherapy rapamycin (both $p<0.05$).

Results

Patients

Thirty-nine of the 46 randomized patients (85%) completed the study, while 7 (15%) were withdrawn due to adverse clinical events (rapamycin 2/17; MMF 5/14). Clinical characteristics at 6 months after conversion are summarized in table 2. Rejection incidence was high in patients on monotherapy MMF (21%), but not on rapamycin or tacrolimus (both 0%). At month 6, the average study drugs trough levels were within or below target levels. The creatinine and protein levels were not different between the arms of treatment at month 6. Compared to baseline, however, the protein level slightly increased in patients treated with rapamycin monotherapy from 0,07 g/L (mean, range: 0,02-0,21 g/L) to 0,12 g/L (mean, range: 0,02-1,56 g/L, $p<0.01$, table 1 and 2). This was clinically relevant since they received treatment with ACE inhibitors. Also, two patients developed hyperlipidemia during the study and were therefore treated with statins [39].

Flow cytometric results

Flow cytometric analysis demonstrated that, irrespective of their treatment, patients were lymphopenic before and after conversion when compared to HC (table 3). Tregs were defined as the $CD4^{+}CD25^{bright+}$ T-cell population in combination with slightly less CD4 expression (figure 2) [3,9]. At inclusion, the absolute number of Tregs was not different between the arms of treatment (figure 3A). At month 6, their number was strongly increased in patients treated with monotherapy rapamycin (figure 3A; $p<0.01$). Also, the intracellular expression of FoxP3 by Tregs was measured. Analysis showed that the number of $FoxP3^{+}$ Tregs only increased in those patients converted to monotherapy rapamycin (figure 3B; $p=0.05$).

Treatment with monotherapy may also affect the phenotypical characteristics of Tregs. Therefore we analyzed the expression of CCR7 and CD45 by Tregs to distinguish their naive, EM and CM subsets (figure 2). At month 6, the number of naive-Tregs seemed to be increased in patients treated with MMF, but due to low statistical power this was not significant (figure 3C). In contrast, the number of CM-Tregs and EM-Tregs increased significantly in patients treated with monotherapy rapamycin (figure 3D & E; both $p<0.05$), while in patients treated with monotherapy tacrolimus or MMF the number of EM-Tregs decreased (figure 3E).

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

Proliferation of PBMC to the mitogen PHA was >30000 CPM at all tested time points (data not shown).

The suppressive capacity of the isolated $CD4^{+}CD25^{bright+}FoxP3^{+}$ cells was determined in co-culture experiments with donor-stimulated patient-PBMC. Before conversion, the average capacity of $CD4^{+}CD25^{bright+}FoxP3^{+}$ cells to suppress the anti-donor response was 37% (range: 1-85%, figure 4). At 6 months after conversion, the capacity of the $CD4^{+}CD25^{bright+}FoxP3^{+}$ cells to suppress the anti-donor response of PBMC from patients on

monotherapy with rapamycin, tacrolimus or MMF, was comparable to the percentage measured before conversion and not different between the various therapies (figure 4).

Table 3: Flow cytometric results of whole blood

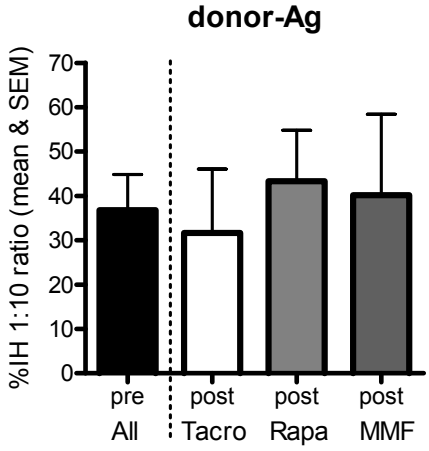
	Tacrolimus		Rapamycin		MMF		HC
Cell subsets	**Pre**	**Month 6**	**Pre**	**Month 6**	**Pre**	**Month 6**	
Lymphocytes	1130 ± 114*	1191 ± 119*	1012 ± 89**	1064 ± 67**	1076 ± 140*	1007 ± 188*	1749 ± 194
$CD3^+$	851 ± 98	861 ± 90	815 ± 77	816 ± 61	794 ± 95	633 ± 159*	1150 ± 159
$CD8^+$	308 ± 56	314 ± 42	375 ± 49	384 ± 52	340 ± 51	211 ± 38	375 ± 75
$CD4^+$	525 ± 62	531 ± 74*	416 ± 50**	412 ± 34**	445 ± 55*	404 ± 134*	772 ± 94

Absolute numbers per μl blood (mean ± SEM), t-test was performed for patients vs HC.

Multivariate analysis

In a multivariate analysis, the factors gender, recipient age, time after KTx, origin of donor kidney, HLA mismatch of donor vs recipient, treatment with statins before and during the study period, history of BPAR, and CMV infection after KTx were not associated with the number of Tregs, their phenotypical subsets or suppressive function over time.

Figure 4



Data are representative for experiments from 19 patients (Tacro N=6, Rapa N=7, MMF N=6). At 6 months after conversion, the capacity of the $CD4^+CD25^{bright+}FoxP3^+$ cells to suppress the anti-donor response of PBMC from patients on monotherapy with tacrolimus, rapamycin or MMF was comparable to the percentage measured before conversion, and not different between the various arms of treatment (ANOVA, p=0.82).

Discussion

We prospectively analyzed peripheral Tregs from 46 renal recipients to investigate whether in a clinical transplant setting monotherapy with tacrolimus, rapamycin or MMF affects the number, phenotype and function of these cells. This is the first study that allows conclusions on the in vivo effect of a single immunosuppressive drug on $CD4^{+}CD25^{bright+}FoxP3^{+}$ regulatory T cells.

We demonstrated that in kidney transplant recipients Tregs can be distinguished into naive and memory populations and that based on their expression of CCR7 the memory Treg-compartment contains a CM population and an EM population [16].

Before conversion, the low number of peripheral Tregs may result from treatment with tacrolimus and MMF. Tacrolimus has been shown to decrease the thymical output of Tregs in mice, while MMF inhibited Treg-expansion [40,41]. Further analysis revealed that within the Treg compartment the number of naive, CM and EM-Tregs was low. Naive-Tregs give rise to potent memory-Tregs in vitro and their low number before conversion suggests little capacity to generate memory-Tregs [25]. We are aware, however, that the number of naive-Tregs decreases with age and therefore the latter effect may be less substantial in our patients [20]. Another explanation for the low number of circulating Tregs at baseline might be their increased migration to the lymph nodes and transplanted kidney [12,13,15].

Analysis of patients that completed the study showed that the number of $CD4^{+}CD25^{bright+}FoxP3^{+}$ cells only increased in patients treated with monotherapy rapamycin. The differential effects of immunosuppressive drugs are probably best explained by their different mechanisms of action. First, tacrolimus almost completely inhibits the production of IL-2 by effector T cells [28,34,42,43]. MMF does not directly inhibit the production of IL-2 but by preventing cell-division and down regulating the receptor for IL-2 (CD25), it may severely affect the number of Tregs [28,32]. Rapamycin does not prevent the production of IL-2 nor does it affect the expression of CD25 [28,32]. Furthermore, it's lack of effect on Tregs may be explained by the almost complete absence of the signaling pathway leading to mTOR [44].

The observed increase in peripheral Tregs from patients treated with rapamycin was due to an enlargement of the CM and EM compartment. Whether the number of naive-Tregs remains low by little output from the thymus, or by rapid differentiation into memory-Tregs remains to be determined.

Recently it was shown, that CM and EM-Tregs do not seem to differ in their regulatory capacity [16]. Their differential expression of CCR7, however, directs them to the lymphoid or the peripheral tissues to perform their suppressive function [16,17]. Therefore, the strong increase in the number of CM as well as EM-Tregs in patients treated with monotherapy rapamycin, suggests that compared to baseline, their capacity to control allogeneic responses is enhanced both in the lymphoid and the peripheral tissues.

To determine the effect of conversion to either monotherapy with tacrolimus, rapamycin or MMF on the function of $CD4^{+}CD25^{bright+}FoxP3^{+}$ cells, we analyzed their suppressive capacity in co-culture experiments. At six months after conversion to monotherapy with tacrolimus, rapamycin or MMF, the regulatory activities of $CD4^{+}CD25^{bright+}FoxP3^{+}$ cells from these 3 patient groups were comparable. Therefore, this study suggests that within the time frame of 6 months, neither monotherapy with tacrolimus, rapamycin or MMF, has a beneficial effect on the suppressive function per cell. Yet, the higher number of $CD4^{+}CD25^{bright+}FoxP3^{+}$ cells in combination with unaltered function suggests a higher potential of Tregs in patients on rapamycin in comparison to patients on tacrolimus or MMF.

In conclusion, this study demonstrated that monotherapy rapamycin facilitates the signals that are needed to increase the number of functional peripheral Tregs with a memory phenotype that have the potential to home to the lymphoid and the peripheral tissues.

References

1. Game DS, Hernandez-Fuentes MP, Chaudhry AN and Lechler RI. CD4+CD25+ regulatory T cells do not significantly contribute to direct pathway hyporesponsiveness in stable renal transplant patients. *J Am Soc Nephrol*. 2003*;* **14**: 1652-61.
2. Li Y, Koshiba T, Yoshizawa A *et al.* Analyses of peripheral blood mononuclear cells in operational tolerance after pediatric living donor liver transplantation. *Am J Transplant*. 2004*;* **4**: 2118-25.
3. Velthuis JH, Mol WM, Weimar W and Baan CC. CD4+CD25bright+ regulatory T cells can mediate donor nonreactivity in long-term immunosuppressed kidney allograft patients. *Am J Transplant*. 2006*;* **6**: 2955-64.
4. Braudeau C, Racape M, Giral M *et al.* Variation in numbers of CD4+CD25highFOXP3+ T cells with normal immuno-regulatory properties in long-term graft outcome. *Transpl Int*. 2007*;* **20**: 845-55.
5. Kingsley CI, Nadig SN and Wood KJ. Transplantation tolerance: lessons from experimental rodent models. *Transpl Int*. 2007*;* **20**: 828-41.
6. Sanchez-Fueyo A, Domenig CM, Mariat C *et al.* Influence of direct and indirect allorecognition pathways on CD4+CD25+ regulatory T-cell function in transplantation. *Transpl Int*. 2007*;* **20**: 534-41.
7. Bestard O, Cruzado JM, Mestre M *et al.* Achieving donor-specific hyporesponsiveness is associated with FOXP3+ regulatory T cell recruitment in human renal allograft infiltrates. *J Immunol*. 2007*;* **179**: 4901-9.
8. Akl A, Jones ND, Rogers N *et al.* An investigation to assess the potential of CD25highCD4+ T cells to regulate responses to donor alloantigens in clinically stable renal transplant recipients. *Transpl Int*. 2008*;* **21**: 65-73.
9. Hendrikx TK, van Gurp EA, Mol WM *et al.* End-stage renal failure and regulatory activities of CD4+CD25bright+FoxP3+ T-cells. *Nephrol Dial Transplant*. 2009
10. Lohr J, Knoechel B and Abbas AK. Regulatory T cells in the periphery. *Immunol Rev*. 2006*;* **212**: 149-62.
11. Ochando JC, Homma C, Yang Y *et al.* Alloantigen-presenting plasmacytoid dendritic cells mediate tolerance to vascularized grafts. *Nat Immunol*. 2006*;* **7**: 652-62.
12. Muthukumar T, Dadhania D, Ding R *et al.* Messenger RNA for FOXP3 in the urine of renal-allograft recipients. *N Engl J Med*. 2005*;* **353**: 2342-51.
13. Veronese F, Rotman S, Smith RN *et al.* Pathological and clinical correlates of FOXP3+ cells in renal allografts during acute rejection. *Am J Transplant*. 2007*;* **7**: 914-22.
14. Walker LS, Chodos A, Eggena M *et al.* Antigen-dependent proliferation of CD4+ CD25+ regulatory T cells in vivo. *J Exp Med*. 2003*;* **198**: 249-58.
15. Dijke IE, Velthuis JH, Caliskan K *et al.* Intragraft FOXP3 mRNA expression reflects antidonor immune reactivity in cardiac allograft patients. *Transplantation*. 2007*;* **83**: 1477-84.
16. Tosello V, Odunsi K, Souleimanian NE *et al.* Differential expression of CCR7 defines two distinct subsets of human memory CD4+CD25+ Tregs. *Clin Immunol*. 2008*;* **126**: 291-302.
17. Sallusto F, Geginat J and Lanzavecchia A. Central memory and effector memory T cell subsets: function, generation, and maintenance. *Annu Rev Immunol*. 2004*;* **22**: 745-63.

18. Ochando JC, Yopp AC, Yang Y *et al.* Lymph node occupancy is required for the peripheral development of alloantigen-specific Foxp3+ regulatory T cells. *J Immunol*. 2005*;* **174**: 6993-7005.
19. Siegmund K, Feuerer M, Siewert C *et al.* Migration matters: regulatory T-cell compartmentalization determines suppressive activity in vivo. *Blood*. 2005*;* **106**: 3097-104.
20. Seddiki N, Santner-Nanan B, Tangye SG *et al.* Persistence of naive CD45RA+ regulatory T cells in adult life. *Blood*. 2006*;* **107**: 2830-8.
21. Lee JH, Kang SG and Kim CH. FoxP3+ T cells undergo conventional first switch to lymphoid tissue homing receptors in thymus but accelerated second switch to nonlymphoid tissue homing receptors in secondary lymphoid tissues. *J Immunol*. 2007*;* **178**: 301-11.
22. Siewert C, Menning A, Dudda J *et al.* Induction of organ-selective CD4+ regulatory T cell homing. *Eur J Immunol*. 2007*;* **37**: 978-89.
23. Menning A, Hopken UE, Siegmund K *et al.* Distinctive role of CCR7 in migration and functional activity of naive- and effector/memory-like Treg subsets. *Eur J Immunol*. 2007*;* **37**: 1575-83.
24. Valmori D, Merlo A, Souleimanian NE *et al.* A peripheral circulating compartment of natural naive CD4 Tregs. *J Clin Invest*. 2005*;* **115**: 1953-62.
25. Hoffmann P, Eder R, Boeld TJ *et al.* Only the CD45RA+ subpopulation of CD4+CD25high T cells gives rise to homogeneous regulatory T-cell lines upon in vitro expansion. *Blood*. 2006*;* **108**: 4260-7.
26. Jonuleit H, Schmitt E, Stassen M *et al.* Identification and functional characterization of human CD4(+)CD25(+) T cells with regulatory properties isolated from peripheral blood. *J Exp Med*. 2001*;* **193**: 1285-94.
27. Kang SM, Tang Q and Bluestone JA. CD4+CD25+ regulatory T cells in transplantation: progress, challenges and prospects. *Am J Transplant*. 2007*;* **7**: 1457-63.
28. Halloran PF. Immunosuppressive drugs for kidney transplantation. *N Engl J Med*. 2004*;* **351**: 2715-29.
29. Lopez MM, Valenzuela JE, Alvarez FC *et al.* Long-term problems related to immunosuppression. *Transpl Immunol*. 2006*;* **17**: 31-5.
30. Gutierrez-Dalmau A and Campistol JM. Immunosuppressive therapy and malignancy in organ transplant recipients: a systematic review. *Drugs*. 2007*;* **67**: 1167-98.
31. Golshayan D, Buhler L, Lechler RI and Pascual M. From current immunosuppressive strategies to clinical tolerance of allografts. *Transpl Int*. 2007*;* **20**: 12-24.
32. Demirkiran A, Hendrikx TK, Baan CC and van der Laan LJ. Impact of immunosuppressive drugs on CD4+CD25+FOXP3+ regulatory T cells: does in vitro evidence translate to the clinical setting? *Transplantation*. 2008*;* **85**: 783-9.
33. Thornton AM, Donovan EE, Piccirillo CA and Shevach EM. Cutting edge: IL-2 is critically required for the in vitro activation of CD4+CD25+ T cell suppressor function. *J Immunol*. 2004*;* **172**: 6519-23.
34. Maloy KJ and Powrie F. Fueling regulation: IL-2 keeps CD4+ Treg cells fit. *Nat Immunol*. 2005*;* **6**: 1071-2.
35. Battaglia M, Stabilini A and Roncarolo MG. Rapamycin selectively expands CD4+CD25+FoxP3+ regulatory T cells. *Blood*. 2005*;* **105**: 4743-8.
36. Koprak S, Matheravidathu S, Springer M *et al.* Down-regulation of cell surface CXCR6 expression during T cell activation is predominantly mediated by calcineurin. *Cell Immunol*. 2003*;* **223**: 1-12.

37. Sordi V, Bianchi G, Buracchi C *et al.* Differential effects of immunosuppressive drugs on chemokine receptor CCR7 in human monocyte-derived dendritic cells: selective upregulation by rapamycin. *Transplantation*. 2006*;* **82**: 826-34.
38. Jones DL, Sacks SH and Wong W. Controlling the generation and function of human CD8+ memory T cells in vitro with immunosuppressants. *Transplantation*. 2006*;* **82**: 1352-61.
39. Mira E, Leon B, Barber DF *et al.* Statins induce regulatory T cell recruitment via a CCL1 dependent pathway. *J Immunol*. 2008*;* **181**: 3524-34.
40. Zeiser R, Nguyen VH, Beilhack A *et al.* Inhibition of CD4+CD25+ regulatory T-cell function by calcineurin-dependent interleukin-2 production. *Blood*. 2006*;* **108**: 390-9.
41. Coenen JJ, Koenen HJ, van Rijssen E *et al.* Rapamycin, not cyclosporine, permits thymic generation and peripheral preservation of CD4+ CD25+ FoxP3+ T cells. *Bone Marrow Transplant*. 2007*;* **39**: 537-45.
42. Baan CC, van der Mast BJ, Klepper M *et al.* Differential effect of calcineurin inhibitors, anti-CD25 antibodies and rapamycin on the induction of FOXP3 in human T cells. *Transplantation*. 2005*;* **80**: 110-7.
43. D'Cruz LM and Klein L. Development and function of agonist-induced CD25+Foxp3+ regulatory T cells in the absence of interleukin 2 signaling. *Nat Immunol*. 2005*;* **6**: 1152-9.
44. Campbell DJ and Ziegler SF. FOXP3 modifies the phenotypic and functional properties of regulatory T cells. *Nat Rev Immunol*. 2007*;* **7**: 305-10.

CHAPTER 7

Summary and Discussion

Summary

Immunological tolerance, like immune reactivity, is an active process. The checks and balances of immune reactive and immune suppressive cells determine whether there is immune reactivity or non-responsiveness (tolerance). Tolerance to self and foreign antigens is regulated by $CD4^{+}CD25^{bright+}FoxP3^{+}$ T cells (Tregs). Accordingly, cells with immune suppressive properties may also control immune reactivity in patients before and after kidney transplantation. Moreover, development of donor-specific Tregs in transplant patients reflects active suppression of alloreactivity and will be crucial to adjust the immunosuppressive medication given for life. In theory, Tregs are a target for immunosuppressive drugs. Most of these agents potentially hamper the suppressive, regulatory T cells and as a result could have an inhibitory effect to tolerance induction after transplantation.

In **chapter 1**, we described a general introduction on kidney transplantation, tolerance and $CD4^{+}CD25^{bright+}$ regulatory T cells. In addition, the features of Tregs were summarized and we emphasized why it is important to study this cell population in the setting of clinical transplantation.

The objective of this thesis was to elucidate whether donor-specific Tregs are generated in immunosuppressed kidney transplant patients. Therefore, $CD4^{+}CD25^{bright+}$ T cells were phenotypically and functionally characterized in patients before and after kidney transplantation.

Chapter 2 shows the analysis of immune regulatory mechanisms in patients with end stage renal failure (ESRF). Patients with ESRF suffer from impaired cell-mediated immunity resulting in inadequate responses upon vaccination, susceptibility to infections and an increased incidence of malignancies. Imbalanced regulation could be responsible for this immunosuppressed state. Therefore we studied the phenotype and function of peripheral regulatory T-cells from patients with ESRF and healthy controls by flow cytometry, RT-PCR and mixed lymphocyte reaction. Patients were on haemodialysis, peritoneal dialysis, or not treated with dialysis yet. The immune competent cells from patients with ESRF show multiple signs of activation. We confirmed this observation by high expression of the basal IL-2 mRNA level in patient-PBMC. In contrast, upon allogeneic stimulation their proliferative responses were impaired. When we analyzed the characteristics and function of $CD4^{+}CD25^{bright+}$ T cells in our ESRF patient population, their absolute number was low. The regulatory function of $CD4^{+}CD25^{bright+}$ T cells was determined in the setting of direct allorecognition. Here, the effect of depletion of $CD25^{bright+}$ cells from patient-PBMC on proliferation was minimal compared to the effect measured in cultures of healthy controls. Furthermore, co-culture of $CD25^{bright+}$ cells with $CD25^{neg/dim}$ cells, showed impaired regulatory function, which was especially pronounced in patients on dialysis. Also, the FOXP3 mRNA expression level of these co-cultures with patient-PBMC was low, which indicated decreased presence or activity by $CD4^{+}CD25^{bright+}$ T cells. Therefore, we concluded that the immune system in ESRF-patients is indeed overactivated but functionally compromised. In addition, our data showed that these patients also suffer from impaired regulation by $CD4^{+}CD25^{bright+}FoxP3^{+}$ T cells.

Chapter 3 provides a comprehensive overview on the effects of immunosuppressive medication on $CD4^{+}CD25^{bright+}FoxP3^{+}$ immune regulatory T cells.

Overall, the results from in vitro, animal and clinical studies showed that immunosuppressive drugs could have detrimental but also beneficial effects on

immunoregulatory T cells. Furthermore, they suggested that the use of specific immunosuppression as well as their timing and dosing is an essential component of strategies to induce and maintain transplant tolerance by Tregs. However, despite clear effects observed in vitro and in experimental transplantation models, the impact of immunosuppressive drugs on Treg homeostasis in patients is not completely understood. In general, they indicate that several drugs may contribute to the development of donor-specific Tregs in vivo. However, the effect of frequently applied combination therapies on the development of Tregs should be further explored. In addition, many studies describe the results from few patients and do not provide information of phenotypical *and* functional properties of Tregs. Especially studies analyzing the development of Tregs in a prospective setting are lacking. These studies would gain detailed insight in those transplantation related conditions that induce and maintain the function of donor-specific Tregs.

Several studies showed that Treg markers, indicative for active immune regulation, are present in immunosuppression free, tolerant transplant patients. We performed a prospective study to investigate whether and when donor-specific Tregs develop in immunosuppressed patients after kidney transplantation. In **chapter 4** we described the analysis of the percentage and function of peripheral Tregs from patients in the first year after kidney transplantation. Our results demonstrated that the percentage of peripheral $CD4^{+}CD25^{bright+}FoxP3^{+}$ T cells decreased after transplantation. Functional experiments showed that the reactivity of patient-PBMC to 3rd party-antigens significantly improved, while reactivity against donor-antigens was not affected. Functional analysis further demonstrated potent donor-specific regulatory activities by $CD4^{+}CD25^{bright+}FoxP3^{+}$ T cells after transplantation. This was shown by depletion of $CD4^{+}CD25^{bright+}FoxP3^{+}$ T cells from PBMC, as well as by co-culture experiments where the capacity of $CD4^{+}CD25^{bright+}FoxP3^{+}$ T cells to control the proliferation of anti-donor reactive $CD25^{neg/dim}$ T cells significantly increased over the first year. Donor-specific T cell function was further proved by higher anti-donor regulatory activities by the $CD4^{+}CD25^{bright+}FoxP3^{+}$ T cells than those controlling 3rd party-antigen stimulated responder T cells. These findings demonstrate that donor-specific $CD4^{+}CD25^{bright+}$ regulatory T cell function is generated in fully immunosuppressed renal recipients in the first year after transplantation.

IL-2 is an important cytokine for the survival and function of Tregs, which also explains their high expression of the IL-2 receptor, CD25. In transplant medicine, however, several patients are treated with CD25 mAb induction therapy (i.e., daclizumab). This prevents activation of the IL-2 pathway following CD25 treatment. In **Chapter 5** we investigated whether IL-2 blockade affects the regulatory function of $CD4^{+}CD25^{high+}FoxP3^{+}$ T cells. Patients received either induction therapy with daclizumab or steroids for four months and both groups received standard therapy with tacrolimus and mycophenolate mofetil. The presence and suppressive activity of $CD4^{+}CD25^{high+}FoxP3^{+}$ peripheral T cells was analyzed pre- and 4-6 months after transplantation. mRNA analysis showed that anti-CD25 mAb therapy and treatment with steroids did not significantly affect protein expression of FoxP3. Functional analysis from co-culture experiments stimulated with donor-antigen demonstrated that the regulatory activities of $CD4^{+}CD25^{high+}$ T cells from the anti-CD25 group were not different before versus after transplantation. In contrast, the regulatory capacities of $CD4^{+}CD25^{bright+}$ T cells from the steroid patient group became significantly impaired. We also analyzed whether the suppressive function of Tregs was different between rejectors and non-rejectors. At 4-6 months after transplantation, the $CD25^{high+}$ cells from rejectors (who all received daclizumab induction therapy) had clear regulatory function, while suppression by $CD25^{high+}$ cells from non-rejectors was significantly lower. Therefore we concluded that anti-CD25 mAb

induction therapy does not negatively influence the regulatory function of $CD4^{+}CD25^{high+}FoxP3^{+}$ T cells from kidney transplant recipients on tacrolimus and MMF. The majority of these patients experienced an acute rejection episode, which suggests that immune activation is required for persistent immunoregulatory function.

Chapter 6 describes a study where stable kidney transplant recipients on tacrolimus/mycophenolate mofetil (MMF) are converted to monotherapy with rapamycin, tacrolimus or MMF. In this setting we prospectively analyzed the number, phenotype and function of regulatory T cells.

Recent evidence demonstrated that phenotypical Treg-subsets, naive ($CCR7^{+}CD45RO^{-}$), central-memory ($CCR7^{+}CD45RO^{+}$) and effector-memory ($CCR7^{-}CD45RO^{+}$), are essential for the development and function of antigen-specific suppression in the lymphoid and peripheral tissues. It is unclear, however, how monotherapy with immunosuppressive drugs influences these characteristics of peripheral Tregs in clinical transplantation. At 6 months after conversion to rapamycin, analysis of PBMC from patients who completed the study, showed a significant increase in the number of Tregs. This increase in Treg numbers was not found when patients were converted to MMF or tacrolimus. The increase of Tregs in patients receiving rapamycin was due to an increase in Tregs with a central-memory or effector-memory phenotype. The functional capacity of these cells remained unaltered. Therefore we conclude that the higher number of $CD4^{+}CD25^{bright+}FoxP3^{+}$ T cells in combination with unaltered function suggests a higher immuneregulatory potential by Tregs in patients on rapamycin in comparison to patients on tacrolimus or MMF.

Discussion

In this thesis we investigated the phenotypical and functional characteristics of Tregs in patients with end stage renal failure (ESRF) before and after kidney transplantation.

End Stage Renal Failure and Regulatory T cells

In chapter 2 we concluded that the immunosuppressed state of patients with ESRF could also be the result of imbalanced regulation by Tregs. This implicates that like effector T cells, Tregs are also affected by uremia and dialysis. This is important knowledge since these Tregs prevent organ-specific auto-immune diseases, control anti-tumor responses and anti-viral responses [1-3]. At present, animal studies demonstrated that cell therapy with Tregs may protect against kidney injury by mechanisms independent of other lymphocytes [4-6]. This could become an important strategy to prevent the development of ESRF and requires additional studies.

Kidney Transplantation and Regulatory T Cells

The aim of this thesis was to determine whether donor-specific Tregs develop after clinical kidney transplantation. In a large prospective study (chapter 4) we demonstrated development of donor-specific Tregs in kidney transplant recipients. In two smaller studies (chapter 5 & 6) we also investigated the generation of donor-specific Tregs under different immunosuppressive regimens and at other time points after transplantation. In both studies no evidence of induction of donor-specific Tregs was found. This may be due to the lower number of patients in both studies but also to factors as study duration as well as the moment of study inclusion (before or 2 years after transplantation). To establish if certain constant factors in each study affected the phenotypical or functional development of Tregs, statistical

analysis was performed. Significant factors proved to be immunosuppressive therapy and clinical rejection.

Based on the literature overview in chapter 3, we already suggested that various immunosuppressive strategies would affect the development of donor-specific Tregs differently. The results from our clinical studies in this thesis confirm these experimental findings and a recent overview further supports this conclusion [7]. However, in chapter 3 we also suggested that CNI are probably not beneficial for Tregs, but rather detrimental to their generation, survival and function. Surprisingly, our large prospective clinical study in chapter 4 demonstrated that donor-specific Treg-function does develop in kidney transplant patients that are treated with CNI based therapy. In line with our finding, a recent study concluded that a low dose of tacrolimus (target trough level of 3 to 7 ng/ml) favored the induction of Tregs in solid organ transplantation [8]. Their target trough levels were comparable with our target trough levels of 4-8 ng/ml. From our studies, it remains unclear which immunosuppressive protocol most efficiently allows the development of donor-specific Tregs after transplantation. Therefore more prospective studies should be performed to determine the optimal immunosuppressive strategy for donor-specific Treg induction.

Clinical rejection has been mentioned as a factor that may lead to increased suppressive capacities of Tregs. Immune activation may therefore be required to trigger immune regulation. This theory is supported by studies that showed that Tregs are abundantly present in the periphery and graft during rejection [1,9]. Also, high levels of FOXP3 mRNA were present in urine of kidney transplant patients suffering from rejection [10]. We described a significant difference in the function of Tregs between rejectors and non-rejectors in chapter 5. More potent suppressor $CD4^{+}CD25^{high+}$ T cells were found in the peripheral blood of rejectors than in patients who did not reject their allograft. However, all rejectors were treated with daclizumab, which suggests that the drug itself may be a confounding factor here. In our study from chapter 4 no correlation with rejection was found, this could be due to the low number of rejectors, which complicates such an analysis. Taken together, the results of our clinical studies in this thesis do suggest that immune activation may trigger immune regulation.

Speaking of clinical transplantation studies, most of the studies that investigated the phenotype and or function of Tregs were performed using in vitro or animal models. Studies that analyzed Tregs in human transplant recipients were mostly retrospective and/or performed at a single moment in time only. This makes it difficult to determine how and when human donor-specific Tregs develop after transplantation. Therefore future studies should analyze Tregs in transplant recipients in a prospective setting to study the kinetics by which donor-specific Tregs develop.

Conclusion

In conclusion, fully immunosuppressed kidney transplant patients generate functional donor-specific $CD4^{+}CD25^{bright+}FoxP3^{+}$ regulatory T cells. The optimal immunosuppressive protocol by which transplant patients develop the most potent donor-specific suppressor T cells remains to be elucidated. Based on the literature and our findings, we hypothesize that T cell depletion induction therapy combined with rapamycin and low dose CNI may favor the number and development of donor-specific Tregs. A large prospective clinical study should evaluate such an immunosuppressive protocol, which is essential to specify strategies to induce transplant tolerance. This “Holy Grail” in transplant medicine would significantly increase the quality of life and (kidney transplant) survival of our patients, which is of course from the beginning to the end, the ultimate goal of our research.

References

1. Velthuis JH, Mol WM, Weimar W and Baan CC. CD4+CD25bright+ regulatory T cells can mediate donor nonreactivity in long-term immunosuppressed kidney allograft patients. *Am J Transplant*. 2006*;* **6**: 2955-64.
2. Ghiringhelli F, Larmonier N, Schmitt E *et al.* CD4+CD25+ regulatory T cells suppress tumor immunity but are sensitive to cyclophosphamide which allows immunotherapy of established tumors to be curative. *Eur J Immunol*. 2004*;* **34**: 336-44.
3. Cabrera R, Tu Z, Xu Y *et al.* An immunomodulatory role for CD4(+)CD25(+) regulatory T lymphocytes in hepatitis C virus infection. *Hepatology*. 2004*;* **40**: 1062-71.
4. Mahajan D, Wang Y, Qin X *et al.* CD4+CD25+ regulatory T cells protect against injury in an innate murine model of chronic kidney disease. *J Am Soc Nephrol*. 2006*;* **17**: 2731-41.
5. Wang YM, Zhang GY, Wang Y *et al.* Foxp3-transduced polyclonal regulatory T cells protect against chronic renal injury from adriamycin. *J Am Soc Nephrol*. 2006*;* **17**: 697-706.
6. Wang YM, Hu M, Wang Y *et al.* Regulatory T cells in renal disease. *Int J Clin Exp Med*. 2008*;* **1**: 294-304.
7. Feng G, Chan T, Wood KJ and Bushell A. Donor reactive regulatory T cells. *Curr Opin Organ Transplant*. 2009*;* **14**: 432-8.
8. Wang Z, Shi B, Jin H *et al.* Low-dose of tacrolimus favors the induction of functional CD4(+)CD25(+)FoxP3(+) regulatory T cells in solid-organ transplantation. *Int Immunopharmacol*. 2009*;* **9**: 564-9.
9. Veronese F, Rotman S, Smith RN *et al.* Pathological and clinical correlates of FOXP3+ cells in renal allografts during acute rejection. *Am J Transplant*. 2007*;* **7**: 914-22.
10. Muthukumar T, Dadhania D, Ding R *et al.* Messenger RNA for FOXP3 in the urine of renal-allograft recipients. *N Engl J Med*. 2005*;* **353**: 2342-51.

Samenvatting
en
Discussie

Samenvatting

Het immuunsysteem is een complexe verzameling van cellen en moleculen die het lichaam laat functioneren in een omgeving vol ziekteverwekkers, zoals bacteriën en virussen. De cellen van het immuunsysteem zijn onder te verdelen in immuun-reactieve cellen en immuun-suppressieve cellen. De immuun-reactieve cellen zorgen ervoor dat ziekteverwekkers worden aangevallen en opgeruimd en immuun-suppressieve cellen onderdrukken de immuun-reactieve cellen. De zoektocht naar een evenwicht tussen beide soorten cellen in ons lichaam is een continu en actief proces. Dit betekent dat er in de praktijk het ene moment sprake kan zijn van immuun-reactiviteit en op een ander moment, als de immuun-suppressieve cellen het overwicht hebben, geen respons optreedt tegen dezelfde ziekteverwekker. Dit laatste kan bijvoorbeeld gebeuren wanneer een ziekteverwekker al lange tijd in ons lichaam zit en het lichaam "gewend" raakt aan de aanwezigheid van deze ziekteverwekker. In een dergelijke situatie veroorzaakt de ziekteverwekker vaak geen of weinig schade. Wanneer het immuunsysteem niet (meer) actief reageert op een ziekteverwekker dan noemen we dat non-responsiviteit ofwel tolerantie. Dit zou het geval kunnen zijn bij orgaantransplantatie.

Alle cellen in ons lichaam en ook ziekteverwekkers als bacteriën en virussen hebben op het oppervlak grote moleculen zitten die we antigenen noemen. Wanneer een cel of ziekteverwekker in ons lichaam terecht komt met een antigeen op het oppervlak dat ons lichaam niet kent, dan ziet ons immuunsysteem dat als lichaamsvreemd en zal het aanvallen. Tolerantie voor lichaamseigen en lichaamsvreemde antigenen wordt gereguleerd door de eerder genoemde immuun-suppressieve cellen. Een belangrijke immuun-suppressieve cel is de $CD4^{+}CD25^{bright+}FoxP3^{+}$ regulatoire T cel (Treg). Over deze immuun-suppressieve cel gaat dit proefschrift. Deze cellen kunnen ook immuun-reactiviteit reguleren in patiënten voor en na een orgaantransplantatie. Wanneer Tregs ook nog specifiek de immuunrespons tegen cellen van het donororgaan onderdrukken (donor-specifiek), dan is er sprake van een extra krachtige onderdrukking van immuun-reactiviteit tegen het donororgaan. In deze situatie zou een patiënt wel eens minder of geen immunosuppressieve medicatie (onderdrukt het immuunsysteem) meer hoeven te gebruiken om afstoting van het donororgaan tegen te gaan. Dat is gunstig, want deze medicijnen zorgen op de lange termijn voor allerlei bijwerkingen. Het probleem is echter, dat de ontwikkeling van donor-specifieke Tregs tijd nodig heeft. Je moet dus na een transplantatie wel gebruik maken van medicatie om afstoting te voorkomen. Mogelijk heeft deze medicatie ook invloed op Tregs en staat daardoor het ontstaan en de overleving van deze suppressieve cellen in de weg.

De doelstelling van dit proefschrift was om uit te zoeken of donor-specifieke Tregs daadwerkelijk ontstaan na niertransplantatie in de aanwezigheid van immunosuppressieve medicatie. Om dat vast te stellen hebben wij de uiterlijke (fenotypische) en functionele kenmerken van Tregs van patiënten voor en na niertransplantatie onderzocht.

Hoofdstuk 1 betreft een algemene introductie over patiënten met chronisch nierfalen (CN) en orgaantransplantatie. Daarnaast beschrijven wij hier de eigenschappen van Tregs en waarom het belangrijk is deze te bestuderen binnen het kader van klinische niertransplantatie.

Hoofdstuk 2 beschrijft de resultaten van een onderzoek naar Tregs van patiënten die lijden aan CN en wachten op een niertransplantatie.

Het is bekend dat het immuunsysteem van patiënten met CN minder goed werkt. Dit blijkt onder andere uit een verminderde respons op vaccinaties en een verhoogde kans op de ontwikkeling van infecties of kanker. Een verstoorde balans tussen immuun-reactieve en immuun-suppressieve cellen zou dit kunnen verklaren. Om vast te stellen of deze hypothese

correct is, onderzochten wij de karakteristieken van Tregs uit het perifere bloed van patiënten met CN en van gezonde controles. Hierbij werd gebruik gemaakt van technieken als flow cytometry, RT-PCR en celkweken. De studiepopulatie bestond uit patiënten met CN die reeds dialyseerden (hemodialyse of peritoneale dialyse) en patiënten die nog geen gebruik maakten van dialyse.

Uit de literatuur is bekend dat immuun-reactieve cellen van patiënten met CN, verschillende verschijnselen van activatie vertonen zoals een hoge expressie van IL-2 mRNA. Ook de immuun-reactieve cellen van onze patiënten vertoonden een hoge expressie van IL-2 mRNA, wat de bevindingen uit de literatuur bevestigt. Een dergelijke activatie doet vermoeden dat deze cellen ook in staat zijn om hun functie effectief uit te voeren. Onze resultaten lieten echter zien dat na stimulatie in celkweken deze cellen niet zo goed delen (prolifereren) als die van gezonde controles. Analyse van Tregs toonde aan dat het aantal van deze cellen in het perifere bloed van patiënten met CN laag is. Celkweken met Tregs van patiënten toonden aan dat de functie van deze cellen significant slechter was dan van gezonde controles. Dit was extra duidelijk bij Tregs van patiënten die dialyseerden. FoxP3 is een specifieke marker voor Tregs. In celkweken van patiënten was de expressie van FOXP3 mRNA laag, wat duidt op de een laag aantal en weinig activiteit van Tregs.
Samenvattend concludeerden wij dan ook dat het immuun-systeem van patiënten met CN weliswaar geactiveerd is, maar niet goed functioneert. Daarnaast toonden onze data aan dat ook de functie van Tregs is aangetast.

Hoofdstuk 3 geeft een overzicht van de literatuur over de effecten van immunosuppressieve medicatie op Tregs.

In het algemeen kan men stellen dat de resultaten van studies in vitro, met proefdieren en in de kliniek, laten zien dat immunosuppressieve medicatie negatieve maar ook positieve effecten kan hebben op Tregs. Van belang is dat naast de specifieke soort van medicatie, ook de dosering en het tijdstip van starten en stoppen met medicatie invloed heeft op Tregs. Met al deze componenten moet dus rekening worden gehouden bij het bedenken van een strategie om donor-specifiek Tregs te induceren en in stand te houden na transplantatie.

Het probleem is echter, dat er op dit moment vooral resultaten bekend zijn van in vitro- en proefdierstudies. Het effect van medicatie op Tregs bij mensen is veel minder goed onderzocht. Duidelijk is echter wel dat sommige immunosuppressieve medicijnen mogelijk een positief of in ieder geval geen negatief effect hebben op de ontwikkeling van donor-specifieke Tregs bij transplantatiepatiënten. Het is daarom noodzakelijk dat met name het effect van in de kliniek veel toegepaste medicatie op Tregs wordt onderzocht. Ook is er behoefte aan studies die zowel de uiterlijke kenmerken van Tregs als de functie van deze cellen tegelijk bestuderen. Tevens is er gebrek aan prospectief onderzoek dat op basis van duidelijk vastgestelde criteria deze cellen bij niertransplantatiepatiënten onderzoekt. De resultaten van dergelijke studies zijn hard nodig om de condities in kaart te brengen die een rol spelen bij de inductie (aanmaak) en het in stand houden van donor-specifieke Tregs bij transplantatiepatiënten.

Verschillende studies hebben aangetoond dat Tregs mogelijk actief immuun-reactiviteit reguleren in tolerante transplantatiepatiënten die geen immunosuppressieve medicatie gebruiken. Wij voerden een prospectieve studie uit om te onderzoeken of donor-specifieke Tregs ontstaan na niertransplantatie in de aanwezigheid van immunosuppressieve medicatie.

Hoofdstuk 4 beschrijft de resultaten van dit onderzoek gedurende het eerste jaar na transplantatie. Deze toonden aan dat het percentage van Tregs in het perifere bloed van patiënten in dit eerste jaar significant daalt. Functionele experimenten (celkweken) lieten zien

dat immuun-reactieve cellen gedurende dat jaar steeds beter gaan reageren op lichaamsvreemde cellen, maar niet tegen cellen van de donor. Analyse van de regulatoire capaciteit van Tregs gaf aan dat deze significant toenam in celkweken met cellen van de donor, maar niet in celkweken met andere lichaamsvreemde cellen. Op basis hiervan kon worden vastgesteld dat er sprake was van donor-specifieke regulatie door Tregs. Wij concludeerden dan ook dat in het eerste jaar na niertransplantatie, sprake is van de ontwikkeling van potente donor-specifieke Tregs in het perifere bloed van patiënten, ondanks de aanwezigheid van immunosuppressieve medicatie.

In **Hoofdstuk 5** hebben we onderzocht of het blokkeren van de koppeling tussen het cytokine IL-2 en de IL-2 receptor daadwerkelijk de functie van Tregs beïnvloedt. Wat bedoelen we hiermee?

Een cytokine is een eiwit dat door een cel gemaakt wordt en dat een boodschap overbrengt aan een andere cel. IL-2 is zo'n cytokine en belangrijk voor de overleving en functie van Tregs. De IL-2 receptor zit op het oppervlakte van een Treg en bindt met IL-2 om zo de boodschap van IL-2 aan de cel door te geven. De IL-2 receptor wordt ook CD25 genoemd. Op het oppervlakte van Tregs is veel CD25 aanwezig, omdat het voor Tregs belangrijk is om de boodschap van IL-2 te ontvangen.

Er bestaan medicijnen voor transplantatiepatiënten, monoclonale antilichamen genaamd, die zich hechten aan CD25. Eén van deze monoclonale antilichamen is daclizumab. Doordat dacluzimab zich hecht aan CD25, kan IL-2 zich niet meer hechten en ontvangt de Treg dus niet de boodschap van IL-2. De kort durende behandeling met daclizumab in de eerste periode na transplantatie wordt ook wel inductie therapie genoemd. Om te onderzoeken of het blokkeren van de IL-2 receptor daadwerkelijk de functie van Tregs beïnvloedt kreeg de helft van de patiënten inductie therapie met daclizumab gevolgd door therapie met tacrolimus en mycophenolate mofetil. De andere helft van de patiënten werd behandeld met steroïden, tacrolimus en mycophenolate mofetil. De karakteristieken van Tregs werden gemeten voor en 4 tot 6 maanden na transplantatie. Analyse van FOXP3 mRNA, een specifieke marker voor Tregs, liet zien dat in beide groepen de expressie van FOXP3 niet significant werd beïnvloed. Resultaten van celkweken van patiënten uit de daclizumab groep toonden aan, dat de capaciteit van Tregs om immuun-activiteit tegen donor-cellen te onderdrukken niet veranderde na transplantatie. Echter, de regulatoire capaciteit van Tregs van patiënten die behandeld werden met steroïden was significant lager na transplantatie. Op basis hiervan concludeerden wij dat inductie therapie met daclizumab geen negatief effect heeft op de functie van Tregs bij niertransplantatiepatiënten in de eerste 4 tot 6 maanden in combinatie met tacrolimus en mycophenolate mofetil.

In de daclizumab groep waren veel patiënten die last hadden van afstotingsverschijnselen (rejectors). Analyse van de functie van Tregs van rejectors versus non-rejectors stelde vast dat de suppressieve capaciteit van Tregs van non-rejectors significant lager was dan van rejectors. Op basis hiervan concludeerden wij dat rejectie geen negatieve invloed lijkt te hebben op het handhaven van immuunregulatie door Tregs na transplantatie bij patiënten die inductie therapie met daclizumab ondergaan.

Hoofdstuk 6 beschrijft een prospectief onderzoek met stabiele niertransplantatiepatiënten. Deze patiënten kregen standaard medicatie bestaande uit tacrolimus met mycophenolate mofetil (MMF). Bij inclusie werden patiënten omgezet naar medicatie met maar één immunosuppressief medicijn namelijk; rapamycine, tacrolimus of MMF. Dit gaf ons de mogelijkheid om voor het eerst het effect van monotherapie met deze medicijnen op Tregs van niertransplantatiepatiënten te onderzoeken.

In enkele recente studies werd aangetoond dat Tregs uit verschillende subsets van cellen bestaan. Op basis van oppervlaktemarkers van deze cellen (CCR7 en CD45RO) werden hierbij de volgende populaties onderscheiden; naïeve Tregs ($CCR7^{+}CD45RO^{-}$), central-memory (CM) Tregs ($CCR7^{+}CD45RO^{+}$) en effector-memory (EM) Tregs ($CCR7^{-}CD45RO^{+}$). Deze verschillende Treg-populaties bleken gezamenlijk essentieel te zijn voor de ontwikkeling en functie van antigeen-specifieke regulatie in de lymphoïde en periphere weefsels. Er was echter geen enkele studie die had onderzocht wat het effect was van verschillende immunosuppressieve medicijnen op deze populaties bij niertransplantatiepatiënten. De resultaten van onze studie lieten zien dat 6 maanden na conversie naar monotherapie met rapamycine, het aantal Tregs significant toenam. Dit was niet zo bij patiënten die geconverteerd waren naar monotherapie met tacrolimus of MMF. De toename van het aantal Tregs van patiënten die rapamycine kregen, werd veroorzaakt door een absolute stijging binnen de CM en EM subsets, maar niet van naïeve Tregs. De regulatoire capaciteit van de Tregs van patiënten met monotherapie rapamycine veranderde niet na transplantatie. Op basis van deze resultaten concludeerden wij dat conversie van therapie met tacrolimus/MMF naar monotherapie rapamycine leidt tot een verhoogde potentie van immuunregulatie door Tregs in patiënten met een niertransplantaat.

Discussie

In dit proefschrift werden de fenotypische en functionele karakteristieken van Tregs onderzocht bij patiënten met chronisch nierfalen voor en na niertransplantatie.

Chronisch nierfalen en Regulatoire T cellen

In hoofdstuk twee werd geconcludeerd dat het dysfunctioneren van het immuunsysteem bij patiënten met CN veroorzaakt zou kunnen worden door een verstoorde balans tussen immuun-reactieve en immuun-suppressieve cellen. Het is bekend dat de functie van immuun-reactieve cellen bij deze patiënten waarschijnlijk aangetast is door de slechte nierfunctie. Dit leidt tot een ophoping van afvalstoffen in het bloed (uremie) en de dialyse procedure. Mogelijk hebben beide factoren ook effect op Tregs. Kennis van een dergelijke invloed is van belang aangezien Tregs geassocieerd zijn met bescherming tegen orgaan-specifieke auto-immuunziekten en controle van anti-tumor en virale reacties [1-3]. Proefdierstudies hebben daarbij aangetoond dat celtherapie met Tregs mogelijk beschermt tegen nierschade door middel van mechanismen die onafhankelijk zijn van andere lymfocyten [4-6]. Een dergelijke vorm van therapie met Tregs zou daarom een belangrijke strategie kunnen worden om de ontwikkeling van CN tegen te gaan. Additionele studies om deze mogelijkheid te onderzoeken zijn dan ook noodzakelijk.

Niertransplantatie en Regulatoire T cellen

De doelstelling van dit proefschrift was om na te gaan of donor-specifieke Tregs daadwerkelijk ontstaan na niertransplantatie in de aanwezigheid van immunosuppressieve medicatie. In een groot prospectief onderzoek (hoofdstuk 4) werd de ontwikkeling van donor-specifieke Tregs inderdaad vastgesteld bij niertransplantatiepatiënten in de aanwezigheid van immunosuppressieve medicatie. Tevens werden twee kleinere prospectieve studies uitgevoerd (hoofdstuk 5 & 6), waarbij andere immunosuppressieve protocollen werden gebruikt en patiënten ook op andere tijdspunten na transplantatie werden geïncludeerd. In deze twee studies vonden wij geen bewijs voor de ontwikkeling van donor-specifieke Tregs. Een factor die het verschil tussen deze bevindingen kan verklaren is allereerst de statistische kracht (power) die in de kleinere studies minder groot is door de inclusie van minder patiënten. Andere factoren zijn bijvoorbeeld de studieduur en het moment van inclusie van patiënten voor danwel na transplantatie. Om vast te stellen of bepaalde factoren in de drie prospectieve studies constant van invloed bleken op de fenotypische danwel functionele karakteristieken van Tregs, werd een statistische analyse uitgevoerd. Hieruit bleken zowel de vorm van immunosuppressieve medicatie als klinische rejectie significante factoren van invloed te zijn.

Dat immunosuppressieve medicatie negatieve maar ook positieve effecten kan hebben op Tregs werd reeds vastgesteld in het literatuuroverzicht in hoofdstuk 3. De resultaten van onze prospectieve studies met niertransplantatiepatiënten bevestigen deze conclusie. Een recente review uit de literatuur komt eveneens tot deze conclusie [7]. In hoofdstuk 3 werd tevens gesteld dat een bepaalde vorm van medicatie, namelijk de calcineurine inhibitors (CNI), waarschijnlijk een negatieve invloed heeft op de ontwikkeling van donor-specifieke Tregs. Het is daarom opmerkelijk dat uit de resultaten van de grote prospectieve studie uit hoofdstuk 4 bleek, dat patiënten die CNI's gebruiken als basistherapie wel degelijk donor-specifieke Treg-functie ontwikkelen. Volledig in lijn met deze bevinding zijn de resultaten van een recente publicatie. Hierin wordt vastgesteld dat een lage dosering van tacrolimus bij orgaantransplantatie (streefspiegels 3 tot 7 ng/ml) de inductie van Tregs positief beïnvloedt [8]. De streefspiegels in dit onderzoek zijn vergelijkbaar met die van onze studie van 4 tot 8 ng/ml. Onze klinische studies geven inzicht in de factoren die de ontwikkeling van donor-specifieke Tregs na transplantatie beïnvloeden. Het blijft echter onduidelijk wat nu het beste

protocol is voor therapie met immunosuppressieve medicatie om de ontwikkeling van donor-specifieke Tregs zo efficiënt mogelijk te laten verlopen. Aanvullend prospectief onderzoek is dan ook gewenst om de meeste optimale strategie vast te stellen.

Ook klinische rejectie werd genoemd als factor die kan leiden tot een verhoogde suppressieve capaciteit van Tregs. Dit suggereert dat immuun-activatie leidt tot verbeterde immuun-regulatie. Een dergelijke theorie wordt ondersteund door studies die hebben aangetoond dat Tregs sterk vertegenwoordigd zijn in het perifere bloed en het transplantaat gedurende rejectie [1,9]. Daarnaast werden verhoogde levels van FOXP3 mRNA gevonden in de urine van patiënten met een niertransplantaat tijdens rejectie [10]. In hoofdstuk 5 beschreven wij een significant verschil in Treg functie tussen rejectors en non-rejectors. Opvallend was echter dat alle rejecties plaats vonden bij patiënten die behandeld werden met daclizumab. De invloed van de soort medicatie kan hier dus een belangrijke verstorende variable (confounder) zijn. Bovendien werd de regulatoire capaciteit van de Tregs van afstoters niet beter dan voor transplantatie. Een correlatie tussen rejectie en Treg functie werd niet gevonden in hoofdstuk 4. Hierbij moet wel worden vermeld dat het aantal patiënten met een afstotingsperiode in hoofdstuk 4 laag was. Op basis van de resulaten van klinische studies in de proefschrift concluderen wij dat immuun-activatie mogelijk leidt tot verbeterde immuun-regulatie of deze in stand houdt, maar niet direct leidt tot de ontwikkeling van donor-specifieke Tregs.

Tenslotte is het van belang om te benadrukken dat het meeste onderzoek naar Tregs gedaan is met in vitro studies en proefdiermodellen. De studies, die Tregs bestudeerden van patiënten zijn veelal retrospectief en/of gebaseerd op één moment in de tijd (cross-sectioneel). Door de opzet van deze studies is het moeilijk vast te stellen welke veranderingen Tregs van transplantatiepatiënten ondergaan en of donor-specifieke Tregs zich daadwerkelijk ontwikkelen. Toekomstige studies moeten daarom, net zoals de studies in dit proefschrift, prospectief plaatsvinden op basis van wel overwogen criteria.

Conclusie

Patiënten die een niertransplantaat hebben ontvangen en standaard immunosuppressieve medicatie krijgen, ontwikkelen functionele donor-specifieke Tregs. Welk immunosuppressief medicatieprotocol moet worden gebruikt door transplantatiepatiënten om tot de meest optimale ontwikkeling van potente donor-specifieke Tregs te komen moet verder worden onderzocht. Echter, op basis van de literatuur en de bevindingen van de studies in dit proefschrift stellen wij de volgende strategie voor. Start met inductie therapie die leidt tot T-cel depletie en hanteer daarna een combinatie van rapamycine met een lage dosering CNI's. Een groot prospectief onderzoek is essentieel om vast te stellen of een dergelijk protocol inderdaad leidt tot de ontwikkeling van donor-specifieke Tregs. Met de resultaten van een dergelijk onderzoek kunnen strategiën om tolerantie voor het donororgaan te bewerkstelligen verder worden gespecificeerd. Tolerantie, de zogenaamde "Holy Grail" binnen de transplantatie geneeskunde zou de kwaliteit van leven en de (transplantaat) overleving van onze patiënten significant verhogen en dat is uiteindelijk altijd het ultieme doel van medisch wetenschappelijk onderzoek.

Referenties

1. Velthuis JH, Mol WM, Weimar W and Baan CC. CD4+CD25bright+ regulatory T cells can mediate donor nonreactivity in long-term immunosuppressed kidney allograft patients. *Am J Transplant*. 2006*;* **6**: 2955-64.
2. Ghiringhelli F, Larmonier N, Schmitt E *et al.* CD4+CD25+ regulatory T cells suppress tumor immunity but are sensitive to cyclophosphamide which allows immunotherapy of established tumors to be curative. *Eur J Immunol*. 2004*;* **34**: 336-44.
3. Cabrera R, Tu Z, Xu Y *et al.* An immunomodulatory role for CD4(+)CD25(+) regulatory T lymphocytes in hepatitis C virus infection. *Hepatology*. 2004*;* **40**: 1062-71.
4. Mahajan D, Wang Y, Qin X *et al.* CD4+CD25+ regulatory T cells protect against injury in an innate murine model of chronic kidney disease. *J Am Soc Nephrol*. 2006*;* **17**: 2731-41.
5. Wang YM, Zhang GY, Wang Y *et al.* Foxp3-transduced polyclonal regulatory T cells protect against chronic renal injury from adriamycin. *J Am Soc Nephrol*. 2006*;* **17**: 697-706.
6. Wang YM, Hu M, Wang Y *et al.* Regulatory T cells in renal disease. *Int J Clin Exp Med*. 2008*;* **1**: 294-304.
7. Feng G, Chan T, Wood KJ and Bushell A. Donor reactive regulatory T cells. *Curr Opin Organ Transplant*. 2009*;* **14**: 432-8.
8. Wang Z, Shi B, Jin H *et al.* Low-dose of tacrolimus favors the induction of functional CD4(+)CD25(+)FoxP3(+) regulatory T cells in solid-organ transplantation. *Int Immunopharmacol*. 2009*;* **9**: 564-9.
9. Veronese F, Rotman S, Smith RN *et al.* Pathological and clinical correlates of FOXP3+ cells in renal allografts during acute rejection. *Am J Transplant*. 2007*;* **7**: 914-22.
10. Muthukumar T, Dadhania D, Ding R *et al.* Messenger RNA for FOXP3 in the urine of renal-allograft recipients. *N Engl J Med*. 2005*;* **353**: 2342-51.

Dankwoord

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Geachte professor Metselaar, doctor van Gelder en van der Laan, beste Herald, Teun en Luc. Ik ben vereerd dat jullie alledrie deel zullen uitmaken van de grote commissie tijdens mijn verdediging. Dank voor jullie aanwezigheid en ik kijk er naar uit om jullie vragen te beantwoorden.

Eveline van Gurp, Ahmet Demirkan en Luc van der Laan, Co-auteurs
Beste Eveline, een duo promotie was het doel. Ik in het lab, jij in de kliniek. Helaas heb je voortijdig het Erasmus MC verlaten, een andere bestemming riep. Desalniettemin hebben we veel samengewerkt en wil ik je bedanken voor al je inspanningen, er zijn zoals je in dit boekje ziet een paar mooie publicaties uit voort gekomen. Ahmet, al enige tijd gepromoveerd en in opleiding tot chirurg. Samen schreven we een mooi review (hoofdstuk 3), nu dus eindelijk ook verschenen in mijn boekje! Luc, niet alleen lid van de grote commissie maar

uiteraard mede auteur van hoofdstuk 3 en daarnaast ook veelvuldig aanwezig geweest bij literatuurbesprekingen en dergelijke. Dank voor de stimulerende discussies ter verdieping van onze wetenschap over Tregs en in het algemeen.

Dolf Segers, Paranimf
Segers, we leerden elkaar kennen in 1997 in Maastricht. Zelfde studie, studentenvereniging en jaarclub. Al na een jaar verliet je Maastricht om geneeskunde te gaan studeren in Rotterdam. We hielden contact en in 2004 kwamen we elkaar op het Erasmus MC weer tegen. We starten hier allebei met een promotietraject en hebben gedurende die periode veel gesproken over "promoveren". Het was fijn om een vriend dichtbij te hebben die mij begreep en altijd dicht in de buurt was voor een bakkie. Ik mis die momenten. Als mijn paranimf sta je tijdens de verdediging van dit boekje naast mij en die plek komt je meer dan toe. Ook in ons sociale leven sta je altijd voor me klaar, ik mocht zelfs Mireille "lenen" om te leren salsa dansen! Wat een vertrouwen! Terwijl je wordt opgeleid tot cardioloog, komt ook de verdediging van jouw proefschrift steeds dichterbij. Ik wens je veel succes met de afronding en heb er het volste vertrouwen in dat wij ook daarna nog vele mooie momenten zullen vieren!

Jurjen Velthuis, Paranimf
Jur, je werkte zelf pas net als wetenschappelijk medewerker op het transplantatielab toen je mij als student kreeg toegewezen. Daar zat je niet echt op te wachten en mijn kennis en vaardigheden van de medische biologie waren op dat moment niet geweldig. Daar wees jij mij ook fijntjes op. „Jurjen, wat is dnase?" „ Ja hallo, het eindigt op ase, dus wat dacht je van een enzym?!?!?!"
Wat begon als een enigszins geforceerde werkrelatie groeide uit tot een blijvende vriendschap. Uiteindelijk waren wij vaak samen als laatsten op het lab aan het werk of dronken een biertje om bv. het weekend in te luiden. Je stond altijd klaar en open voor discussie en suggesties. Toen je besloot in Leiden te gaan werken voelde dat voor mij op meerdere manieren als een groot verlies. We hebben echter altijd contact gehouden en dit proefschrift is zeker ook jouw verdienste. Thanks voor je inzet en vriendschap!

Esmé Dijke en Jeroen Gerrits, AIO's
Zoals zoveel junior onderzoekers in academische centra zat ook ik in een klein kamertje opgesloten met meerdere AIO's. We hadden kleine bureautjes en zaten dicht op elkaar. Esmé en Jeroen, jullie zaten er toen ik aankwam en gingen ongeveer gelijk met mij weg. Drie heel verschillende mensen die alle aspecten van het AIO bestaan, maar ook prive ruim 4 jaar met elkaar hebben gedeeld. Je kunt het eigenlijk alleen begrijpen als je het hebt meegemaakt, ik zou er een boek over kunnen schrijven. Esmé, je bent creatief, scherp, kunt zaken fantastisch regelen en veeeeeeeeeeeeeeel beter volleyballen dan ik. Het ga je goed als post-doc in Edmonton! Jeroen, we dronken samen veel lekkere sterke koffie, zongen meerdere malen *jij bent de zon* tijdens karaoke en waren vaak roomies tijdens de congressen. Na je korte avontuur bij farma wens ik je heel veel succes tijdens je opleiding tot klinisch chemicus! Allebei ontzettend bedankt voor alles, ik zal jullie zeker niet uit het oog verliezen.

Petros, Dennis en Marthijn, "oude" AIO's
Toen ik op het lab kwam werken waren daar ook drie jonge mannen die geneeskunde hadden gestudeerd en volop bezig waren met het promotietraject. Op de een of andere manier moet ik een beetje denken aan de drie musketiers. Jullie hielpen mij op weg in Rotterdam en lieten mij zien waar ik naartoe werkte. Petros, griekse borrelaar, we beleefden samen de olympische spelen in Athene en hebben heel veel plezier gehad. Dennis, ik heb genoten van

je wetenschappelijke inzicht maar ook van de manier waarop je de geboorte van je dochters beleefde en vaak een snotterende papa was. Marthijn, filosoof en sportman, altijd relativerend. Ik zal nooit vergeten hoe jij op zondagochtenden als ik binnekwam als een mol in het donker bedachtzaam naar je scherm zat te staren.

Varsha en Meindert, "nieuwe" AIO's
Terwijl de drie musketiers langzaam verdwenen namen jullie de lege plaatsen in. Varsha, als vrouw was je een welkome verschijning in het AIO-hok dat tot dan toe voornamelijk door mannen werd gedomineerd. Ambitieus en vrolijk stapte jij de wereld van de Tregs in en mede door jou kreeg ik halverwege mijn onderzoekstraject weer nieuwe energie! Je kwam voor dezelfde problemen te staan als ik en het was prettig om daar met jou over te discussiëren. Je bent al een heel eind met je onderzoek, veel succes met de laatste loodjes, het wordt vast een prachtig boekje! Meindert, buiten het feit dat je gewoon een leuke vent bent die van wetenschap houdt, vond ik in jou dan eindelijk degene die zich binnen de academie ook graag met andere zaken bemoeide. Zo organiseerden we samen de PhD-informatiemarkt en speelden zaadcel op de Parade. Overigens is een stamcel wat mij betreft nog steeds een wannabe Treg, maar jou proefschrift over stamcellen bij transplantatie zal ongetwijfeld vernieuwend zijn!

Mariska, Wenda en Wendy, de allerbeste analisten!
Zonder jullie had ik het nooit gered. Ten eerste hebben jullie mij van alles geleerd en daarnaast heel erg veel testen voor mij gedaan en geanalyseerd. Mariska, je bent gewoon geweldig. Je weet waar je het over hebt, bent kundig, zelfstandig en een hele leuke vrouw. Ze mogen ontzettend blij zijn met jou op het lab. Wendaat! Als je iemand van de industrie nodig hebt dan weet jij wie je moet bellen. Je hebt ongelofelijk veel voor mij aan het flowcytometrisch werk gedaan zelfs toen je met een hernia thuis zat en ik vond het met jou altijd gezellig. En dan, last but not least, Wendy! MLRs, flowcytometry, PCRs etc, van alles heb jij voor mij gedaan en je deed niet moeilijk als het eens wat later werd. Soms irriteerde ik je als ik een ADHD momentje had, maar we hebben zeker ook gelachen en succes gehad. Lieve fantastische dames, dit proefschrift is zeker ook het resultaat van jullie inzet!

Annemarie en Tineke, research verpleegkundigen
Toen ik op het Erasmus MC kwam had ik nog nooit van een researchverpleegkundige gehoord, maar ik had al snel door dat die onmisbaar zijn voor goed translationeel onderzoek. Jullie waren mijn contact met de kliniek in de dagelijkse praktijk. Als ik bloed wilde hebben van een patiënt, dan zorgden jullie daarvoor, of niet. Waar dat dan weer door kwam, dat was altijd weer een verassing en leidde wel eens tot irritaties. Aan de andere kant leer je elkaar daardoor ook weer beter kennen. Tineke, je bent op een gegeven moment op een andere afdeling gaan werken maar je hebt me enorm geholpen, heel erg bedankt daarvoor. Annemarie, met jou heb ik langer samen mogen werken en ik moet zeggen met heel erg veel plezier. Je bent een heerlijk mens met wie je veel plezier kunt hebben en die heel duidelijk kan zijn als het om HAAR patiënten gaat. Ik hoop voor de patiënten, artsen en onderzoekers op het Erasmus MC dat je daar nog heel lang blijft werken. Dank voor je tomeloze inzet, ook zonder jou was dit proefschrift er niet geweest.

Annelies, Annemiek, Cees, Corne, Joke, Len, Martin Ho, Martin Hu, Monique, Nicole, Nicolle, Perikles, Rens, Ronella, Sander en Thea, wetenschappelijk medewerkers en analisten
Iedereen van jullie heeft wel op de één of andere manier een steentje bijgedragen. Annelies, je kwam pas laat in mijn promotietraject binnen, maar bracht plezier op het lab en ik waardeer je betrokkenheid met patiënten. Annemiek, je gaf mij bv. tips over de PCR data en o.a. door jou voelde ik mij meteen welkom op het lab in Rotterdam. Cees, je vertrok al vroeg, maar leerde mij nog wel even alles over veegproeven en statistische modellen die helaas nooit in gebruik zijn genomen. Corné, ook jij vertrok helaas al vroeg nadat ik begon, maar was een vrolijke noot op het lab en altijd in voor discussie over de voetbaluitslagen. Joke, jij maakte mij wegwijs op het lab, was nooit te beroerd om even te helpen en altijd geïnteresseerd. Len, je leerde mij nog een beetje facsen en was vooral verschrikkelijk gezellig tijdens de borrel, waarna je met pensioen ging. Samen met Karin heb ik nog een nachtje gelogeerd bij jou en Ria in het Loire district. Dank voor alle gezelligheid! Martin Ho, aka Jaap Stam. Je kwam binnen als postdoc om met stamcellen te werken en bracht meteen kennis en sfeer op het lab. Martin Hu, je werkte je een slag in de rondte voor Nicolle Litjens en gedurende die periode heb ik regelmatig veel plezier met je gehad. Monique, je kwam zo'n beetje samen met Martin Ho binnen en bleek een ontzettend aardig en slim mens te zijn met tomeloze inzet voor wetenschap. Ik ben blij je dat laatste jaar nog te hebben mogen leren kennen. Nicole, zowel op het lab als thuis een goede moeder. Houdt alles in de gaten en heeft altijd wel een mening. Het ga je goed! Nicolle, vaak als eerste op het lab en als laatste weer weg. Slimme meid, mooie publicaties en een hart van goud. Keep up the good work! Perikles, na Petros de tweede Griek en je keerde al snel weer terug naar Greece. Op de sportschool hief je heel wat meer kilotjes dan ik! Rens, je bent een vrolijke jonge vent. Ik wens je veel succes verder met je carrière op het lab. Ronella, je bent lekker kritisch en duidelijk, dank daarvoor. Ook zorgde jij samen met de borrel-cie voor de juiste borrels en was een van de weinigen die regelmatig op vrijdag een biertje meedronk! PS: je hebt een fantastische dochter! Sander, als stille kracht op het lab altijd behulpzaam en je levert goed werk. Daarnaast stiekem heel goed in staat om een feestje te vieren, ik zal ons Big Lebowsky feestje niet snel vergeten. Thea, ik kon lekker met je discussiëren over andere zaken dan wetenschap, dank daarvoor. Ook niet te onderschatten, als je goeie zin had kon het heel gezellig worden op een feestje!

Erasmus MC
Er zijn onwaarschijnlijk veel andere mensen die mij hebben geholpen. Met name de werknemers van de afdeling niertransplantatie, de poli, de verpleging, de prikzusters. Allemaal ontzettend bedankt!

Patiënten
Waarschijnlijk krijgen zij dit boekje nooit te zien, maar het is geen pretje om naast alle lopende onderzoeken extra bloed af te staan voor wetenschappelijk onderzoek. Allemaal heel erg bedankt voor jullie bijdrage.

Epar/ Promeras, PhD association of Erasmus MC/Rotterdam
Naast wetenschappelijk onderzoek kon ik het niet nalaten nog wat andere zaken binnen het Erasmus MC en de universiteit te organiseren. Alle leden van EPAR en Promeras hartelijk dank voor de brainstorm sessies, activiteiten die we hebben opgezet en vele gezellige momenten.

Ad, Loes, Sjef en Wouter, ouders en broers
Toen ik begon aan mijn promotietraject waren er een aantal belangrijke zaken aan het veranderen in mijn leven. Zowel voor mij als voor jullie was dat niet altijd even makkelijk. Desalniettemin hebben jullie mij altijd gesteund door dik en door dun. Pa, het is niet iedere promovendus gegeven om een ouder te hebben die echt begrijpt wat een PhD traject inhoudt. Met je eigen PhD op zak en de begeleiding van je eigen AIO's begreep jij dat maar al te goed. Je was geïnteresseerd, kritisch en vol adviezen, maar bleef gelukkig ook gewoon mijn vader. Die combinatie, dat was en is ontzettend fijn. Mama, de wetenschappelijke kant was dan misschien niet jouw "erea of expertise", maar dat maakte je rol niet minder belangrijk. Altijd luisterde je aandachtig, zocht samen met mij naar oplossingen, gaf mij motivatie als ik het niet meer zag zitten of leidde mijn aandacht eens even van dat onderwerp af. Dank voor je betrokkenheid en beleving, ik ben ontzettend blij dat jij mijn moeder bent. Broers, met jullie ging het vaak niet over mijn promotie en als dat wel zo was, dan was het al snel genoeg. Dat was prima. Er zijn namelijk nog talloze andere zaken in het leven die vaak veel interessanter zijn om te bespreken dan wetenschappelijk onderzoek en werk zeker met je broers. Let's keep it that way!

Karin, mijn allerliefste, leukste en mooiste vriendin ooit en toekomstige vrouw!
Ik was net een paar maanden aan het werk op het Erasmus MC toen ik jou leerde kennen. Als er dus iemand is die weet hoe ik die jaren als PhD student en de afronding van dit proefschrift heb beleeft, dan ben jij dat. Regelmatig kwam ik verslagen thuis als een experiment weer eens was mislukt of een artikel afgewezen. Ik weet heel goed dat het ook voor jou niet altijd makkelijk is geweest. Toch was jij dan vaak degene die mij de energie en motivatie gaf om door te zetten. Zeker op het einde, toen ik echt geen zin meer had, motiveerde jij mij om achter die PC te gaan zitten en dit boekje af te maken. Mede dankzij jou is dat dus ook gelukt! Dat is maar goed ook, want we hebben onze tijd nu hard nodig voor andere projecten waaronder onze feestdag volgend jaar als we gaan trouwen! Ik ben onzettend gelukkig dat jij er net als ik voor gekozen hebt om samen te beleven wat de toekomst ons te bieden heeft.
Dankjewel voor alles, ik hou van je, dikke kus,

Thijs

Curriculum Vitae

Curriculum Vitae

Thijs Kodzo Hendrikx, de auteur van dit proefschrift werd geboren op 7 mei 1979 in Dzodze, gelegen in het oosten van Ghana. In 1997 behaalde hij het VWO diploma op het Strabrecht College te Geldrop, waarna hij begon aan de studie Gezondheidswetenschappen aan de Universiteit van Maastricht. Na in het eerste jaar zijn propedeuse te hebben gehaald, koos hij voor de afstudeerrichting Biologische Gezondheidskunde waarvoor hij zijn afstudeerstage liep op het transplantatielaboratorium van het Erasmus MC te Rotterdam. Hier onderzocht hij onder begeleiding van dr. Jurjen Velthuis en dr. Carla Baan, de regulatoire capaciteit van transplantaat infiltrerende lymphocyten van harttransplantatie patiënten. In september 2004 behaalde hij zijn docteraal examen en direct aansluitend trad hij in dienst als promovendus op het transplantatielaboratorium van het Erasmus MC te Rotterdam. Onder begeleiding van dr. Carla Baan en Prof. Willem Weimar werkte hij aan het in dit proefschrift beschreven onderzoek. Sinds 14 juli 2008 is hij werkzaam als Medical Advisor bij Wyeth Pharmaceuticals in Hoofddorp, sinds 16 oktober 2009 onderdeel van Pfizer Inc..

PhD Portfolio

PhD Portfolio

Name PhD student	Thijs Kodzo Hendrikx
Erasmus MC Department	Internal Medicine – Transplantation
PhD Period	September 2004 – Juli 2008
Promotor	Prof. dr. W. Weimar
Copromotor	dr. C.C. Baan

PhD Training

General academic skills
- Irradiation hygiene expertise level 5B, Erasmus MC, Rotterdam, 2004
- Infection prevention for Paramedics, Erasmus MC, Rotterdam, 2005

Research skills
- Classical methods in Biostatistics, NIHES, University of Rotterdam, 2007
- Good clinical practice (GCP), Erasmus MC, Rotterdam, 2008

In-depth courses
- Advanced course in Immunology (MolMed), University of Rotterdam, 2005

(Inter)national conferences and workshops	*presentations*
- American transplant congress 2008, Toronto.	Oral 1, Poster 2
- Nefrologiedagen Dutch society of Nephrology 2008, Veldhoven.	Oral 1
- Bootcongress Dutch society of transplantation 2008, Zeewolde.	Oral 2
- Euroscicon meeting 2008: Analyzing the phenotype and function of regulatory T cells, Glenwyn Garden City.	Poster 1
- European society of transplantation 2007, Praque.	Oral 1, Poster 2
- American transplant congress 2007, San Francisco.	Oral 2
- MolMed day 2007, University of Rotterdam, Rotterdam.	Poster 1
- Bootcongress Dutch society of transplantation 2007, Zeewolde.	Oral 1
- NAT 2006 "Innovative Therapeutics in Transplantation", Nantes.	Oral 1
- Plan research dag 2006, Erasmus MC, Rotterdam.	Oral 1
- Bootcongress Dutch society of transplantation 2006, Zeewolde.	Oral 2
- Dutch society of immunology 2005, Noordwijkerhout.	
- Euroscicon meeting 2005: Analyzing the phenotype and function of regulatory T cells, London.	
- European society of transplantation 2005, Geneve.	
- Bootcongress Dutch society of transplantation 2005, Kerkrade.	
- Bootcongress Dutch society of transplantation 2004, Texel.	

Additional activities
- Active member of Promeras, PhD association of Erasmus MC
  - -Board member (treasurer/chairman)
  - -Organizer of the PhD information market 2006 and 2007
  - -Member of the PhD committee of Erasmus MC
  - -Editor of “Cubic”, PhD magazine of the University of Rotterdam

Publicatielijst

Publicatielijst

1 Velthuis JH, Hesselink DA, Hendrikx TK, van der Mast BJ, Klepper M, de Greef GE, Baan CC, Weimar W. 2007. Kinetic analysis reveals potency of CD4+ CD25bright+ regulatory T-cells in kidney transplant patients. *Transpl Immunol;*18:159-65

2 Demirkiran A, Hendrikx TK, Baan CC, van der Laan LJ. 2008. Impact of Immunosuppressive Drugs on $CD4^{+}CD25^{+}FOXP3^{+}$ Regulatory T cells: Does In Vitro Evidence Translate to the Clinical Setting? *Transplantation;* 85:783-9

3 Sewgobind VD, Kho MM, van der Laan LJ, Hendrikx TK, van Dam T, Tilanus HW, IJzermans JN, Weimar W, Baan CC. 2009. The effect of rabbit anti-thymocyte globulin induction therapy on regulatory T cells in kidney transplant patients. *Nephrol Dial Transplant;* 24:1635-44

4 Hendrikx TK, van Gurp EA, Mol WM, Schoordijk W, Sewgobind VD, IJzermans JN, Weimar W, Baan CC. 2009. End stage renal failure and the regulatory activities of $CD4^{+}CD25^{bright+}FoxP3^{+}$ T-cells. *Nephrology Dialysis Transplantation;* 24:1969-78

5 Hendrikx TK, van Gurp EA, Sewgobind VD, Mol WM, Schoordijk W, Klepper M, Velthuis JH, Geel A, IJzermans JN, Weimar W, Baan CC. 2009. Generation of donor-specific regulatory T-cell function in kidney transplant patients. *Transplantation;* 87:376-83

6 Hendrikx TK, Klepper M, Ijzermans J, Weimar W, Baan CC. 2009. Clinical rejection and persistent immune regulation in kidney transplant patients. *Transplant Immunology;* 21:129-35

7 Hendrikx TK, Velthuis JH, Klepper M, van Gurp E, Geel A, Schoordijk W, Baan CC, Weimar W. 2009. Monotherapy rapamycin allows an increase of $CD4^{+}CD25^{bright+}FoxP3^{+}$ T cells in renal Recipients. *Transplant International;* 22:884-91