Immune Modulation after T-Cell Depletion Therapy in Kidney Transplant Patients

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It is not the strongest of the species that survive,
nor the most intelligent, but the one most responsive to change

Charles Darwin

Contents

Chapter 1

General introduction and outline of the thesis

BACKGROUND

Chronic kidney disease is caused by a heterogeneous group of disorders; i.e. primary kidney diseases, diabetes, hypertension, obesity and cardiovascular disease, which often leads to kidney failure necessitating renal replacement therapy (end-stage renal disease). Kidney function can be partially replaced by dialysis and more effectively by kidney transplantation (1, 2).

When a kidney is transplanted into a genetically non-identical recipient it will be recognized as foreign and attacked by the recipient's immune system. Eventually this will lead to allograft rejection. To prevent rejection, transplant patients depend on life-long immunosuppressive medication. Over the last 30 years, the incidence of acute rejection in the early post-transplant period significantly decreased by improved immunosuppressive drugs. This success consequently improved graft survival early after transplantation, in contrast, long term graft survival rates remain largely unchanged (2-4). Immunosuppressive therapy is complicated by severe side effects, including development of infections, malignancies, cardiovascular diseases and diabetes mellitus, mainly caused by the non-specific immunosuppressive effects and side effects of the given drugs (5). In addition, some of the immunosuppressants are nephrotoxic. Therefore, it is important to find the right balance between sufficient immunosuppression, to prevent graft rejection, and a minimal immunosuppressive dosing regimen to prevent drugs related complications.

IMMUNE RESPONSES AFTER ORGAN TRANSPLANTATION

The chirurgical procedures of kidney donation and transplantation are accompanied by inflammatory and stress responses of donor tissue, processes that have a profound effect on the recipients' immune system. After transplantation, cells of the innate immune system; i.e., macrophages, natural-killer (NK) cells, dendritic cells (DC) and granulocytes respond to these inflammatory changes by producing inflammatory mediators, such as cytokines, by activating the complement system and by stimulating antigen-presenting cells (APCs) to migrate from the transplant into the recipients' lymphoid tissues. In the lymphoid tissue, APC activate cells of the immune system that subsequently migrate to the transplanted organ where they mediate the rejection response towards the allograft (6, 7).

Next to the innate immune system, the adaptive immune system, existing of T cells and B cells, is responsible for antigen specific immune responses. This response exists of four distinct phases; a recognition, activation, proliferation and an effector phase, after the latter memory cells are formed. In the recognition phase, T cells interact with APCs that present allopeptides bound to the major histocompatibility complex (MHC) molecule or the human MHC called Human Leucocyte Antigens (HLA). After organ trans-

plantation, there are three distinct antigen presentation pathways; the direct, indirect and semi-direct pathway. In the direct pathway recipient T cells interact with allogeneic HLA molecules presented by donor-derived APCs. Indirect allorecognition occurs when allogeneic peptides are processed and presented by recipient APCs. In semi-direct allorecognition, alloantigen is presented by recipient APCs that captured intact donor HLA molecules presenting the alloantigen (6, 7). As a consequence of allorecognition, the T-cell receptor (TCR) complex, in combination with the CD3 molecule, transduces a signal into the cell, representing the first signal of T-cell activation (Fig. 1) (5).

The second signal of T-cell activation, required for optimal activation, occurs after binding of co-stimulatory molecules to their ligands. One of the best characterized co-stimulatory molecules is CD28 expressed on T cells and binding CD80 and CD86 expressed by APC (8). This interaction triggers a cascade of intracellular signaling molecules; i.e., the calcineurin-nuclear factor of activated T cells (NFAT) signaling pathway, the RAS-mitogen-activated protein (MAP) kinase pathway and the nuclear factor-κB pathway (NF-κB). The joined action of these pathways results in the transcription of various genes leading to the production of cytokines, chemokines and upregulation of cell surface proteins like cytokine receptors and co-stimulation and co-inhibitory molecules (5, 8).

The produced cytokines provide signal 3 needed for T-cell activation, differentiation and proliferation (5). Cytokines are soluble factors that have important cellular functions and exert their biological effects by signaling via the JAK (Janus tyrosine kinases) - STAT (signal transduction and activators of transcription) pathway. When cytokines engage their cytokine receptor; JAK molecules become activated and phosphorylate the cytokine receptor after which STAT molecules can bind. Subsequently, STAT molecules become phosphorylated (pSTAT), form dimers and interact with genes, important in T-cell activation, T-cell proliferation and T-cell differentiation into effector cells (9, 10). Furthermore, cytokines, in particular interleukin (IL)-2 and IL-15 activate the mammalian 'target of rapamycin' (mTOR) pathway and deliver growth signals through the phophoinositide-3-kinase (PI-3K) that is a major trigger for T-cell proliferation and differentiation.

The differentiation of T cells into effector cells is mediated by the local environment, resulting in different T helper and cytotoxic effector cells (12, 13). These cells fulfill the effector phase of the immune response, tailored to the threat encountered. Within the CD4+ T helper (Th) cells there are distinct lineages based upon their cytokine profile, chemokine receptor expression, master regulator and STAT signaling molecules; Th1, Th2, Th17, Th22, Th9 and follicular (Tfh) cells (12, 13). These Th cell populations provide help to cells of the innate immune system, to CD8 + cytotoxic T cells and to B cells, all contributing to the rejection process. CD8+ cytotoxic T-cells release granules containing cytotoxic molecules, such as perforin and granzyme A and B, that after secretion mediate lysis in target cells; B cells can function as an APC, produce cytokines and differentiate

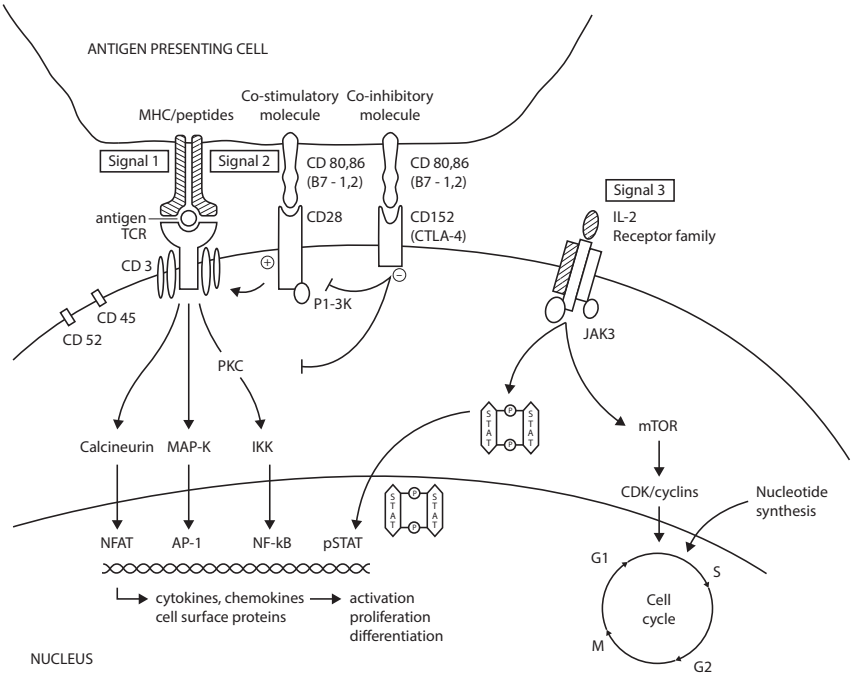

Figure 1. Signaling cascades in T-cell activation. The first signal in T-cell activation is provided when antigen, bound to a MHC molecule, binds the TCR of the T cell. The second signal is initiated by binding of co-stimulatory molecules to their ligands on the APC. Together signal 1 and 2 activate the calcineurin-nuclear factor of activated T cells (NFAT) signaling pathway, the RAS-mitogen-activated protein (MAP) kinase pathway and the nuclear factor-κB pathway (NF-κB). Cytokines provide signal 3 that activates the JAK-STAT pathway. Furthermore it activates the mammalian 'target of rapamycin' (mTOR) pathway (figure adapted from Halloran 2004 and Hunt 2008 (5, 11)).

into antibody producing plasma cells that after activation of the complement system damage the allograft (6, 7).

CD4+ cells are also important for the regulation and control of immune responses (13). These regulatory T cells (Treg) can be thymic-derived, naturally occurring Treg (nTreg) or can acquire a suppressive phenotype in the periphery, thereby becoming an induced regulatory T cell (iTreg). Both Treg populations highly express CD25 and the transcription factor FoxP3, and both depend on IL-2 for homeostasis, expansion, maintenance and function (14). Although the importance of Treg is well established, the biological significance of iTreg is still unsolved. Treg perform their suppressive functions via various mechanisms including the production of the immunosuppressive cytokines IL-10, TGF-β, IL-34, IL-35, affecting the APC function of dendritic cells and via cell-cell contact by i.a. CTLA-4 (cytotoxic T lymphocyte-associated antigen 4), Lag3 (Lymphocyte-activation gene 3), GITR (glucocorticoid induced TNRF-related protein) and ICAM (intracellular adhesion molecule) (15, 16).

After the effector phase a small number of T and B cells differentiate into memory T cells that persist and confer lifelong immune protection. Memory T cells rapidly and vigorously respond to antigen as they do not depend on co-stimulation for activation. Based upon the expression of the homing receptor chemokine (C-C motif) receptor 7 (CCR7) and CD45RO, an isoform of the leucocyte common antigen expressed on memory cells, three distinct memory T-cell subsets can be characterized: central memory (CM), effector memory (EM) and effector memory expressing CD45RA (EMRA) T cells (17). Central memory cells provide reactive memory as these cells home to secondary lymphoid tissue were they can rapidly proliferate and differentiate into effector cells in response to antigenic stimulation. Central memory cells itself have little or no effector functions. Effector memory cells migrate to tissue sites where they exert their effector functions. EMRA T cells are differentiated effector memory cells expressing CD45RA. These cells are mostly seen in the CD8+ T-cell population and exert cytotoxic activity but are also susceptible for apoptosis (17, 18).

IMMUNOSUPPRESSIVE DRUGS

After kidney transplantation, patients are on immunosuppressive medication to prevent allograft rejection. According to the KDIGO guidelines, immunosuppressive therapy consists of a calcineurin inhibitor (CNI), the lymphocyte proliferation inhibitor mycophenolate mofetil (MMF) and steroids, which can be combined with an induction agent (19). With this strategy the incidence of allograft rejection decreased to 10-20% the first six months after transplantation (3).

The most frequently given calcineurin inhibitor is tacrolimus that engages FK506-binding-protein 12 to create a complex that inhibits calcineurin leading to the blockade of NFAT which is required for the transcription of genes encoding multiple cytokines including IL-2. Although effective, tacrolimus is nephrotoxic and associated with other undesired clinical symptoms like hypertension and diabetes mellitus. Accurate dosing is therefore important. Mycophenolic acid is the active component of MMF and blocks the purine synthesis thereby affecting the proliferation of lymphocytes (20). The third agent given to prevent graft rejection are the commonly used corticosteroids that inhibit the action of various factors involved in the transcription of multiple cytokine and chemokine genes, including IL-2, tumor necrosis factor-α (TNF- α) and interferon-γ (IFN-γ). Long-term use of corticosteroids is however associated with multiple complications like hypertension, dyslipidemia and diabetes (21).

In addition to maintenance immunosuppressive therapy by CNI, MMF and steroids, induction therapy can be given to kidney transplant patients at the time of transplantation. This strategy is based on the observation that shortly after transplantation, more powerful immunosuppression is needed to prevent acute rejection (22). Addition-

ally, induction therapy allows reduced dosages of nephrotoxic CNIs the first days after transplantation, decreasing the risk for CNI mediated delayed graft function (5, 22). The most commonly used induction agents are basiliximab, rabbit antithymocyte globulin (rATG) and alemtuzumab. Basiliximab is a non-depleting humanized monoclonal antibody directed against CD25, the α-chain of the IL-receptor (IL-2Rα). Binding blocks the engagement of IL-2 to activated cells, thereby inhibiting activation and proliferation of these cells (23, 24). The other commonly used induction agent is rATG, a polyclonal antibody preparation derived from the serum of rabbits who are immunized with human thymocytes. RATG recognizes CD3+ T cells, B cells, and NK cells as immunogens and depletes them from the circulation via several mechanisms; I. activation of the classical complement pathway resulting in complement dependent cytotoxicity, II. antibody binding which results in antibody-dependent cell-mediated cytotoxicity by NK cells and macrophages, and III. rATG triggers the induction of surface Fas (CD95) and Fas-ligand (L) expression, resulting in Fas/Fas-L interaction and apoptosis (25-27). In addition, rATG also has immunosuppressive effects by influencing T-cell migration by binding adhesion and homing molecules, by blocking co-stimulation molecules, by affecting dendritic cell function and by inducing regulatory T cells (25, 26, 28-30). Another T-cell depleting agent used in the transplantation clinic is alemtuzumab (Campath-1H), a humanized monoclonal antibody preparation, directed against the cell surface antigen CD52 a glycosyl-phosphatidylinositol (GPI) linked protein, expressed on multiple lymphoid cells; T cells, B cells, NK cells and on monocytes, macrophages and dendritic cells. Ligation of alemtuzumab to CD52, induces lymphocyte destruction by complement dependent lysis, antibody-dependent cell-mediated cytotoxicity and apoptosis (31). In contrast to rATG treatment, treatment with alemtuzumab is accompanied by less severe infusion related side-effects, especially when given subcutaneously (32).

Despite the improved immunosuppressive strategies, allograft rejection still occurs. Treatment of allograft rejection exists of high dose corticosteroids. However, when allograft rejection persists, patients are treated with T-cell depleting agents. The most commonly used T-cell depleting agent to treat allograft rejection is rATG. However due to the minimal side effects and comparable effectiveness, alemtuzumab appeared to be an attractive alternative (32).

T-CELL DEPLETION AND T-CELL HOMEOSTASIS

Following T-cell depletion, the T-cell compartment is reconstituted by two mechanisms; I. thymopoiesis; the production of new, naive T cells in the thymus and II, homeostatic proliferation, the expansion of residual T cells (Fig. 2A, B). Thymopoiesis is the maturation and education process of hematopoietic stem cells, which originate in the bone marrow, into T cells. After progenitor cells leave the bone marrow, they enter the thymus

via the circulation, here positive and negative selection takes place. During positive selection, only cells with a TCR, capable of binding MHC class I or II expressed by stromal cells, even with weak affinity, will receive survival signals. During negative selection all thymocytes with high affinity for self-peptides undergo apoptosis. At this stage some cells are also selected to become Treg. These processes lead to functional T cells that do not react to naive self-antigens, thereby preventing auto-immunity (33, 40). Because the thymus involutes with increasing age, homeostatic proliferation is thought to be the main mechanism of T-cell reconstitution in adults. After lymphodepletion, residual T cells proliferate in response to antigen and/or in response the homeostatic cytokines IL-7 and IL-15 (34, 35). These homeostatic cytokines are normally produced at low levels

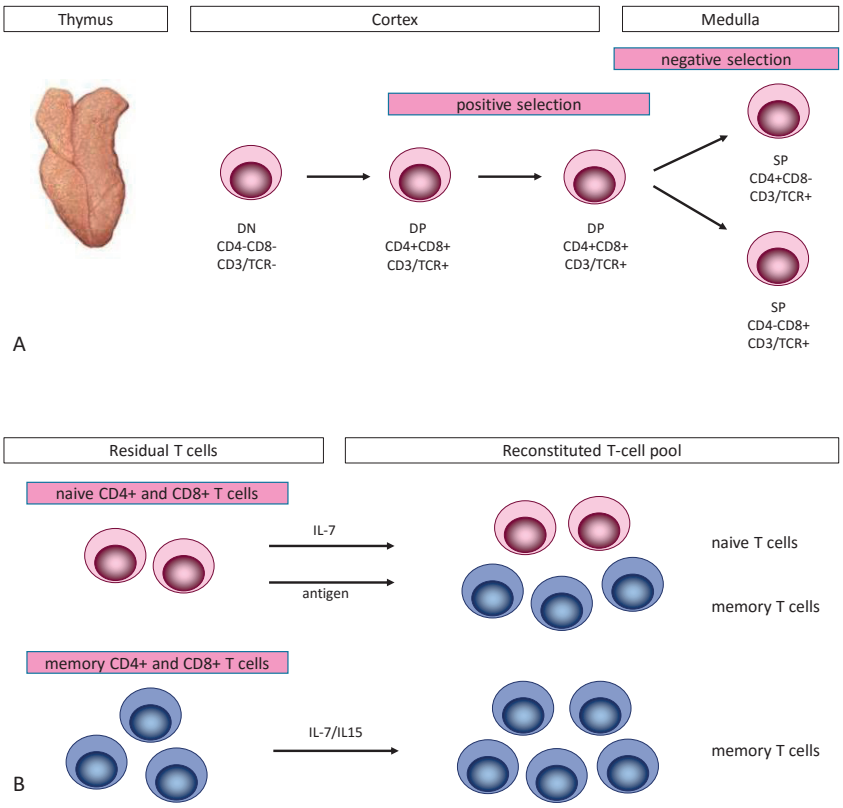

Figure 2. Immune reconstitution. **(A)** Thymopoiesis, the maturation of thymocytes. During differentiation immature, double negative (DN) thymocytes, not expressing CD4 and CD8, generate a CD3/TCR complex. After upregulation of CD4 and CD8, double positive (DP) thymocytes undergo positive selection. Hereafter the DP thymocytes undergo lineage commitment, maturing into CD8+ or CD4+ , single positive (SP) T cells. During migration to and in the medulla negative selection takes place (40). **(B)** Homeostatic proliferation of residual naïve and memory CD4+ and CD8+ T-cells after T-cell depletion therapy is the result of antigen and cytokine stimulation; i.e. IL-7 and IL-15 (34)

for the survival, maintenance and proliferation of T cells. However, after T-cell depletion the level of IL-7 increases as the consumption of IL-7 by T cells is diminished resulting in high cytokine levels, an incentive for T-cell expansion. Although both naive and memory T cells can undergo homeostatic proliferation, memory T cells are most frequently found in the repopulated T-cell population (34, 36-39).

In general, memory T cells can initiate a rapid and sustained alloresponse and are relatively resistant to immunosuppressive drugs. Therefore, these cells are thought to be a barrier to transplantation (39, 41). The predominance of these cells after immune reconstitution however, does not result in increased necessity of immunosuppressive therapy. In contrast, these patients can be treated with reduced dosages of immunosuppressive drugs (42-46). In vitro, decreased T-cell reactivity to donor, third party and recall antigens, was measured by mixed lymphocyte reactions and interferon-γ enzyme-linked ImmunoSpot assays in kidney transplant patients (30, 47-49). Immune modulatory mechanisms like induction of regulatory T cells and the induction of functionally impaired memory cells are hypothesized to explain this phenomenon. Existing literature indeed showed increased percentages of suppressive Treg which may explain, at least in part, this hypothesis (30, 36). Furthermore, terminally differentiated and functionally impaired CD28-CD8+ T cells were found, cells that compete for "immune space" with functional CD4+ and CD8+ T cells (38). Also T-cell anergy, where T-cells become functionally impaired by activation without co-stimulation, might play a role (8, 50). The role of T-cell exhaustion, the hierarchical loss of T-cell effector functions induced by persistent antigen stimulation (Fig. 3), might also explain poor responses to donor-antigen after T-cell depletion therapy (50, 51). The role of T-cell exhaustion in organ transplantation has not elucidated yet and is part of this thesis.

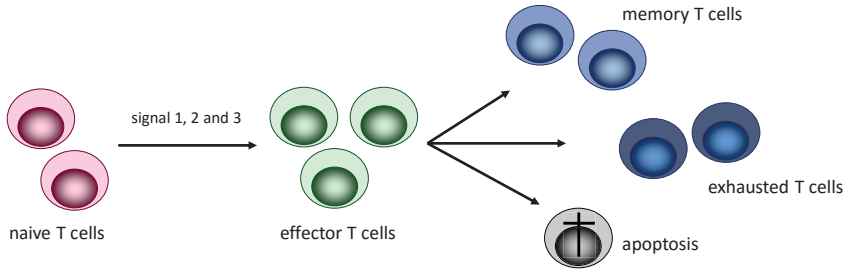

Figure 3. T-cell exhaustion. In response to antigen naive T cells differentiate into effector T cells. When the antigen disappears from the circulation memory cells are formed. Recently it was discovered that when antigen persists functionally impaired, exhausted T cells are induced.

AIM AND OUTLINE OF THE THESIS

The mechanisms responsible for T-cell reconstitution after T-cell depletion therapy, are not fully elucidated. Also the exact underlying mechanisms of the decreased need for immunosuppression after T-cell depletion therapy in kidney transplant recipients are unknown. The overarching aim of this thesis is to better understand the process of T-cell reconstitution and to characterize the mechanisms by which T-cell depletion therapy is modulating the immune system after kidney transplantation. This knowledge will help us understand the influence of immunosuppressive medication on immune processes occurring after transplantation and will help us design less toxic and more efficient immunosuppressive treatment strategies.

As the optimal dose of rATG induction therapy is not yet known, **chapter 2** questions the effects ultra-low, low and standard dosages of rATG induction therapy on T-cell, B-cells and NK-cell numbers. **Chapter 3** describes the kinetics and mechanisms of T-cell reconstitution after rATG induction therapy, which were compared with patients treated with the non-depleting anti-CD25 mAb basiliximab, or no induction therapy. Moreover, the incentives for homeostatic proliferation; antigen or the homeostatic cytokines IL-7 and IL-15, were analysed. **Chapter 4** focuses on the repopulation mechanisms of Tregs after rATG and basiliximab induction therapy. We studied the origin of the repopulated Treg (induced or thymic derived) as well as their suppressive, regulatory functions in response to donor and third party antigens. Knowing the repopulation mechanisms of T cells and their phenotypical characteristics after rATG and basiliximab induction therapy, we questioned their responses to the homeostatic cytokine IL-7 and the immune modulator IL-2 in **Chapter 5**. In addition, in-depth analysis of T-cell exhaustion features will be performed. As T-cell depletion therapy is not only given as induction therapy but also as anti-rejection therapy, we describe in **Chapter 6** the kinetics of T-cell reconstitution and T-cell function when the T-cell depleting alemtuzumab is given as anti-rejection therapy during immune activation. In the field of transplantation there is a clear-cut need for methods that provide information at the molecular activation level of T cells. A method that recently became available for clinical research and diagnostics and offers these applications is phospho-specific flow cytometry. In **Chapter 7** we discuss this novel technique to monitor the activity of intracellular immune signaling pathways at the single-cell level in T cells of transplant patients. In **Chapter 8** the results obtained in this thesis are summarized and discussed.

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Chapter 2

The effect of low and ultra-low dosages Thymoglobulin on peripheral T, B and NK cells in kidney transplant recipients

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ABSTRACT

Introduction

Rabbit Anti-Thymocyte Globulin (rATG) is a polyclonal antibody preparation, used to prevent and treat acute rejection episodes after organ transplantation. However, despite more than 40 years of clinical use, the optimal dose of rATG is still not defined. To find a better balance between efficacy and infectious complications, we embarked on a controlled study and monitored the effect of low and ultra-low dosages Thymoglobulin (Genzyme) on peripheral T, B, and NK cells.

Patients and methods

Kidney transplant recipients received either 0.5 mg/kg, 1.0 mg/kg or 2.0 mg/kg on the first 3 consecutive days post-transplantation. Thus, total doses were 1.5 mg/kg, 3.0 mg/kg and 6.0 mg/kg. A total of 40 patients were enrolled, including 11 controls. All patients were treated with Prednisolon, Advagraf (Astellas) and Mycophenolate Mofetil (Roche). T (CD3+), B (CD19+) and NK (CD3-CD16+56+) cells were analyzed by flow cytometry. Baseline cell counts were compared to forty age and sex matched healthy persons. Post-transplantation cell counts of the 3 Thymoglobulin groups were compared to the 11 control patients, who received no induction therapy.

Results

Absolute numbers of T, B, and NK cells were comparable in all patients pre-transplantation, but T and B cells were lower than in healthy persons ($p=0.007$ and $p=0.0003$, Mann Whitney test). In the first week, T cells and NK cells were significantly lower in all Thymoglobulin groups compared to controls. B cells were not affected. One month after Thymoglobulin NK cells had returned to control numbers in all groups, while T cells had already recovered to control counts in the 1.5 mg/kg group. During follow-up, T cells in the 3.0 mg/kg group also returned to control values, but at one year the patients in the 6.0 mg/kg group still had significantly lower T cells ($p=0.03$). Patient and graft survival, rejection and infection incidence and renal function did not differ between groups.

Conclusion

Patients with end stage renal disease have significantly lower peripheral T and B cell counts than healthy persons. (Ultra-) low Thymoglobulin schedules deplete peripheral lymphocytes in a dose dependent way. Knowledge of the duration of this depletion contributes to finding the optimal immunosuppressive strategy for kidney transplant recipients.

INTRODUCTION

Rabbit Antithymocyte Globulin (rATG) is a lymphocyte depleting polyclonal antibody preparation, used to treat and to prevent acute rejection after organ transplantation. However, the optimal dose of rATG is still not defined despite the clinical use of anti-lymphocyte agents for more than 40 years.

The concept to prepare a serum specifically active against one cell type was developed by Metchnikoff in 1899. After more than 50 years of animal studies, the first use of a rabbit anti-human-lymphocyte globulin to prevent skin alloreactivity was described by Monaco in 1967 (1). One year later Kashiwagi et al. reported the administration of an anti-lymphocyte globulin in human kidney transplant recipients (2). In the following decade various rabbit or horse anti-lymphocyte preparations were developed in the USA and Europe. At the moment, the most widely used depleting agent to prevent acute rejection of organ transplants is Thymoglobulin (Genzyme, Cambridge, USA) (3,4).

The main goal of induction therapy is to reduce the risk of acute rejection in the first weeks after organ transplantation. Indeed, induction therapy with Thymoglobulin does result in a lower acute rejection rate, but also in a higher risk of infections and malignancies (5–8). The most recent meta-analysis of the Cochrane database reported a benefit of ATG therapy over IL-2 receptor antibodies for BPAR at 1 year, but at the cost of a 75% increase in malignancy and a 32% increase in cytomegalo virus disease (9). These complications reflect over-immunosuppression and are associated with depletion of peripheral T cells (10) and low pre-transplant thymic function (11). To reduce these Thymoglobulin related side effects, it seems appropriate to explore the potential of lower dosage of Thymoglobulin. However, only few studies are reported using induction doses lower than 6 mg/kg (12–15) and none described total dosages lower than 3 mg/kg. Only two, Wong and Pankewycz, monitored lymphocyte counts after induction therapy, but neither had a proper control group.

OBJECTIVE

As peripheral lymphocyte counts reflect the overall immunosuppressive state, we studied the effect of 6 mg/kg, 3 mg/kg and 1.5 mg/kg Thymoglobulin on peripheral T, B and NK cell counts. The control group consisted of kidney transplant recipients who did not receive induction therapy.

PATIENTS AND METHODS

Patients

Between September 2008 and July 2009, we performed a prospective study in which patients were enrolled successively in dose cohorts. We enrolled 40 consecutive kidney

transplant recipients who fulfilled at least one of the following criteria: a deceased donor kidney, a non-first living donor kidney transplant, detectable anti-HLA antibodies by Complement Dependent Cytotoxicity test in the current serum or an HLA mismatch on the DR-locus. No patient selection was made. As induction therapy rabbit Anti-Thymocyte Globulin (Thymoglobulin, Genzyme, Cambridge, USA) was given on days 1, 2 and 3 after kidney transplantation. Three different low doses of Thymoglobulin were given. The first group (10 patients) received a total dose of 1.5 mg/kg (0.5 mg/kg/day), the second group (9 patients) 3 mg/kg (1.0 mg/kg/day) and the last group (10 patients) 6 mg/kg (2.0 mg/kg/day). The control group (11 patients) received no induction therapy. Thymoglobulin was dissolved in 500 ml NaCl 0.9% and administered during a 6 hour period, through a peripheral venous cannula. Thirty minutes before the start of all three Thymoglobulin doses, 50 mg Prednisolon and 4 mg Clemastine were administered intravenously.

All patients were treated with steroids, Advagraf (Astellas Pharma Inc, Tokyo, Japan) and Mycophenolate Mofetil (Roche, Basel, Switzerland), according to the schedule shown in Figure 1.

Oral Co-trimoxazol 480 mg daily was given as Pneumocystis pneumoniae prophylaxis for four months. CMV IgG negative recipients with a CMV IgG positive donor were given prophylax is with oral Valganciclovir for four months. Valganciclovir dose was adapted to renal function: Valganciclovir 450 mg was given twice weekly when MDRD-GFR was 10–25 ml/min., on alternate days when MDRD-GFR was 25–35 ml/min and once daily when MDRD-GFR was above 35 ml/min.

Blood samples were drawn pre-transplantation, thrice weekly in week one and two, at every outpatient visit until baseline values were reached and at one year post transplantation. Baseline values were compared to forty age and sex matched healthy persons. No extra blood samples, visits or other tests were taken from patients other than routine monitoring. Side effects were recorded in the patients' charts and the investigators' database.

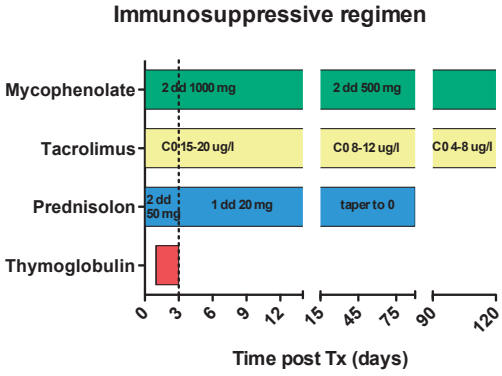

Figure 1. The immunosuppressive regimen in all groups. C0: target trough level.

Flow cytometry

EDTA blood was analyzed for CD3+ T cells, CD3-CD19+ B cells and CD3-CD16+ CD56+ NK cells using BD TruCOUNT™ technology in combination with BD MultiTEST (Catalog No. 342416, BD Biosciences, San Jose, CA, USA). BD MultiTEST determines absolute counts of human T, natural killer, and B lymphocytes in erythrocyte-lysed whole blood. This reagent contains CD3 fluorescein isothiocyanate (FITC), CD16+CD56 phycoerythrin (PE), CD45 peridinin chlorophyll protein (PerCP) and CD19 allophycocyanin (APC) monoclonal antibodies allowing direct fluorescence gating of the lymphocyte population to reduce contamination of unlysed or nucleated red blood cells in the gate. According to the manufacturer's specifications (BD Biosciences) 20 µl MultiTEST reagent and 50 µl well-mixed whole EDTA blood were added to a TruCOUNT Tube (BD Biosciences), releasing a known number of fluorescent beads and mixed gently. Incubation was conducted for 15min at room temperature followed by lysis of red blood cells. Lysation of red blood cells was determined by adding 450 µl 1X FACS lysing solution (BD Biosciences, containing diethylene glycol and formaldehyde), gently mixing and incubation for 15 min in the dark at room temperature. Samples were immediately measured on a FACSCantoII (BD Biosciences). By FACSCanto clinical software and BD Multiset™ software the absolute number (cells/ml) of positive cells in each sample was determined by comparing cellular events to bead events. Detection limitb20 CD3+ T cells/mm3.

Statistics

Mann–Whitney tests were used to compare baseline values to healthy persons and to compare each Thymoglobulin group to the control group at one week, one month and one year after transplantation. No cell counts after treatment of acute rejection were included in the analysis. Statistical analyses were performed in Graphpad Prism 5.01.

Patient and graft survival, rejection rate, infection rate and occurrence of malignancy were evaluated at one year post-transplant. Because clinical outcome was not the aim of our study, our patient numbers were low and statistical analyses were not performed on clinical data.

RESULTS

The four patient groups did not significantly differ in gender, recipient and donor age, number of DR mismatches, panel reactive antigen (PRA) percentage, number of previous transplants or living/deceased donor ratio (Table 1). All patients received the intended Thymoglobulin dose. Mycophenolate mofetil was given in two fixed doses (Fig. 1), Prednisolon was tapered to 0 in the course of 3 months and tacrolimus dose was adjusted to the trough levels also shown in Figure 1. No surveillance biopsies were taken.

Table 1. Patient characteristics.

Thymoglobulin dose	Control	1.5	3.0	6.0
No. of patients	11	10	9	10
Males	7	8	6	6
Recipient age[#] (yr)	59 20-70	51 29-74	51 27-57	49 30-67
Donor age[#] (yr)	52 33-76	56 40-65	56 32-71	54 40-68
DR-MM 0 / 1 / 2[$]	2 / 8 / 1	2 / 7 / 1	0 / 7 / 2	0 / 7 / 3
PRA*	0 (0-4%)	0 (0-83%)	2 (0-36%)	0 (all 0%)
No. of non-first transplants	3	2	4	2
Living / deceased donors	7 / 4	7 / 3	9 / 0	10 / 0

No: number. Yr: years. [#]: median + range. [$]: number of patients with 0, 1 or 2 mismatches on DR-locus. PRA: current panel reactive antigen, *: median + range.

Table 2 shows patient and graft survival, acute and chronic rejection rates and the number of viral and bacterial infections leading to hospitalization at one year post transplant. Acute rejection rate was highest in the 1.5 mg/kg group and lowest in the 3 mg/kg group. All acute and chronic rejections were biopsy proven and defined according to the Banff 07 classification (16). In the control group 2 patients had an acute rejection episode, at 5 and 7 months, one leading to graft loss. One patient had a chronic humoral rejection at 1 year, stabilized after treatment. In the 1.5 mg/kg group, 4 patients had 5 acute rejection episodes, which occurred at 2 weeks, 2, 4 and 5 months, one had led to graft loss. In the 3.0 mg/kg group no rejection occurred. In the 6 mg/kg group 1 patient had an acute humoral rejection one week post-transplantation, stabilized after treatment.

One patient, who was in the 1.5 mg/kg group, suffered from graft loss due to rejection, multiple infections and heart failure and died after stopping hemodialysis.

Table 2: Clinical parameters

Thymoglobulin dose	Control	1.5	3.0	6.0
No. of patients	11	10	9	10
Patient survival	11	9	9	10
Graft survival	9	9	8	10
Acute rejection	2	4	0	1
Chronic rejection	1	0	0	0
Infections[#]	10	10	8	9
bact. / viral[$]	8 / 2	10 / 0	4 / 4	5 / 4
Serum creatinine*	147	162	127	128
(µmol/l)	66-200	73-572	94-203	57-282

All data are absolute numbers at 1 year post-transplant. No.: number. [#]: Infections leading to hospitalization. bact.: bacterial. [$]: viral infections, including CMV reactivations. *: Median serum creatinine + range.

Four grafts were lost. Two in the control group, 1 due to rejection and 1 never functioned. One in the 1.5 mg/kg group due to rejection. One in the 3.0 mg/kg group due to hemolytic uremic syndrome, 4 months post-transplantation.

In the first post-transplant year, one patient in the 6 mg/kg group developed a basal cell carcinoma.

Pre-transplantation, absolute numbers of T, B and NKcells were comparable in all patient groups, however, T and Bcells, but not NK cells, were significantly lower in patients than in age and sex matched healthy persons (Fig. 2). In the first week, T cells and NK cells were significantly lower in all Thymoglobulin groups compared to the control patient group, who did not receive induction therapy. B cells were not statistically significantly affected by Thymoglobulin. At one month, T cells had recovered to control values in the 1.5 mg/kg group only, while in the 3 and 6 mg/kg groups T cell absolute numbers remained significantly lower than in the controls. NK cells in all Thymoglobulin groups had returned to control values within one month. B cells were significantly lower in the 6 mg/kg group only. T cells in the 3 and 6 mg/kg groups, as well as B cells in the 6 mg/kg group, gradually increased over the following months. At one year, only patients who received 6 mg/kg had lower T cell counts, while the 3 mg/kg group had recovered to control values. B and NK cell counts were comparable to the controls in all Thymoglobulin groups studied (Fig. 3).

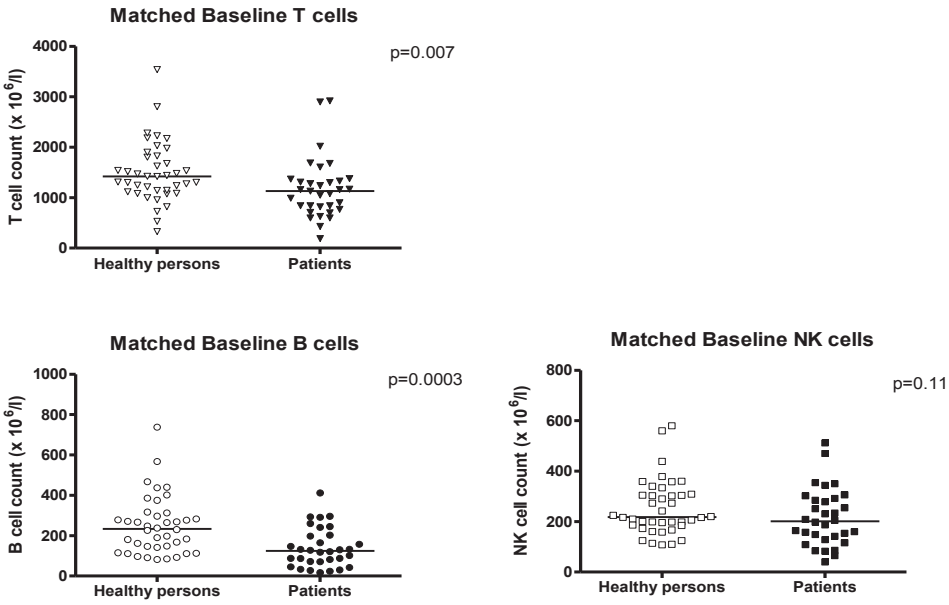

Figure 2. T, B and NK cell counts before transplantation compared to healthy persons. Line at median.

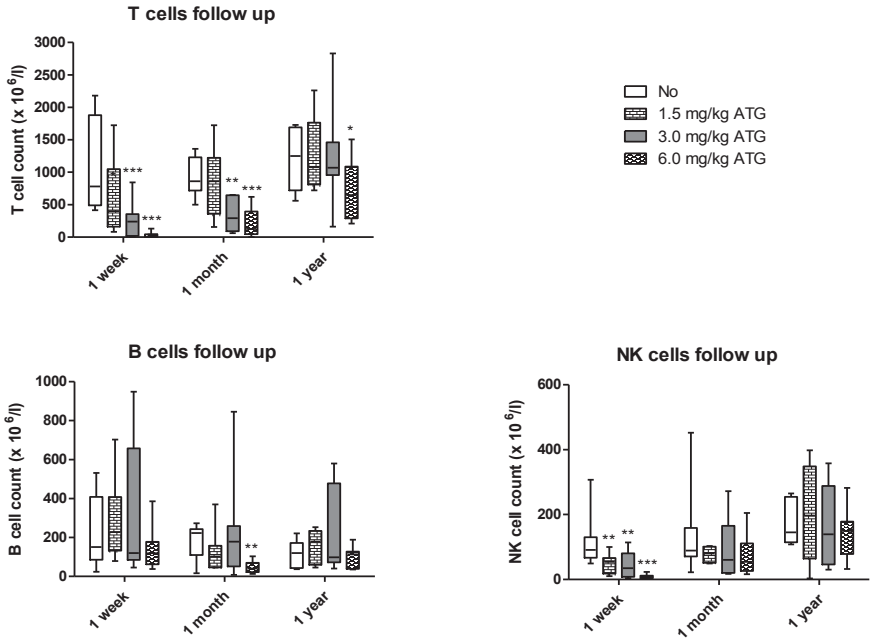

Figure 3. T, B and NK cell counts post-transplantation. Whiskers indicating total range. *: p<0.05, **: p≤0.01, ***: p≤0.001 when compared to the group without Thymoglobulin.

DISCUSSION

In the present study we found, in accordance with other investigators, a depletion of T cells after Thymoglobulin induction treatment for months to over a year (17–20). However, in these studies high total dosages of 6 to 13.5 mg/kg were given, while others used a different rATG preparation namely ATG-Fresenius (21,22). Only few reports also mentioned a short-lived effect on NK cells (23–25), while only modest changes in B cells were observed (20,23,25). Most investigators compared cell counts after rATG preparations to cell counts before transplantation only, neglecting the effect of maintenance immunosuppression on reconstitution of lymphocytes. Others compared patients to healthy persons.

Previously, we have also demonstrated that a total dose of 6 mg/kg Thymoglobulin depletes T and NK cells, but not B cells. In that study, NK cells had reached baseline values at 14 weeks after transplantation. T cells recovered more gradually to only 50% of pre-Thymoglobulin numbers at 26 weeks post-transplantation (26).

In the present study, we used low and ultra-low dosages of Thymoglobulin and a control group consisting of kidney transplant recipients treated with the same immunosuppressive regimen without induction therapy. This way, the duration of lymphocyte depletion should be dependent on the induction therapy, only.

T and B cell counts in renal insufficient patients, before start of immunosuppression, turned out to be significantly lower than in healthy persons. It is well known that patients suffering from end stage renal disease have a higher incidence of infections. Impaired cellular and humoral responses are reported by several investigators (27,28), as well as lower absolute lymphocyte counts compared to healthy persons (29,30). Thus, healthy persons are not suitable as control group.

We found that all dosages had an effect on peripheral lymphocyte counts. Even the ultra-low total dose of 1.5 mg/kg rATG resulted in depletion of peripheral T and NK cells for at least one week. We showed again that the effect of Thymoglobulin on NK cells is only short lived. The variable effect of Thymoglobulin on B cells could be the result of batch to batch variability in presence of B cell specific antibodies, considering the very low numbers of B cells among human thymocytes used to immunize the rabbits (20,31).

The T cell depleting effect of a total dose of 6 mg/kg Thymoglobulin lasts at least for one year after transplantation. A definite explanation for this long-lasting effect has not been found yet. A study on pharmacokinetics and pharmacodynamics reported that recipients of a total dose of 6 mg/kg Thymoglobulin showed a clearance of "active ATG" (the fraction of total rabbit immunoglobulin with the capacity to bind to human lymphocytes) to levels below 1 µg/ml, considered sub-therapeutic, by a median of 17 days (range 8–36 days) and a half-life of 6 days (32). Persistence of "active ATG" in the circulation is therefore not the explanation. One hypothesis is that slow thymic regeneration and output, especially in elderly people, attributes to the delayed recovery of T cells.

Another hypothesis could be that ATG persistently binds to lymphoid tissue. However, there are no reports that Thymoglobulin binds to human thymus and even in the only cynomolgus monkey study no depletion of thymocytes was seen (33). Thus, the question why T lymphocyte reconstitution after Thymoglobulin is delayed much longer than circulating rabbit immunoglobulin is detected remains.

CONCLUSION

Patients with end stage renal disease have significantly lower peripheral T and B cell counts than healthy persons. We found that a total dose of 3 mg/kg significantly lowered T cells for one month, but at one year T cell count had recovered to baseline values. The goal of induction therapy is to suppress the immune system especially in the first few months post-transplantation, but preferably not far beyond that period. Knowledge of the dose dependent duration of lymphocyte subset depletion after Thymoglobulin infusion contributes to finding the optimal immunosuppressive strategy for kidney transplant recipients.

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Chapter 3

Kinetics of homeostatic proliferation and thymopoiesis after rATG induction therapy in kidney transplant patients

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ABSTRACT

Background

Lymphocyte-depleting therapy is associated with long-lasting effects on repopulated T cells and subsequent increased rates of infections and malignancies. The mechanisms of T cell repopulation and their posttransplantation kinetics are not fully understood.

Methods

We studied thymopoiesis by CD31+ naive T cells (recent thymic emigrants) and homeostatic proliferation by Ki-67+ T cells in rabbit antithymocyte globulin (rATG) treated patients the first 6 months after transplantation. Patients receiving basiliximab or no induction therapy served as controls.

Results

At 6 months after transplantation, T cell numbers were lower than before transplantation in rATG-treated patients, whereas T cell numbers remained stable in both control groups. In this time period, thymopoiesis was similar between the three treatment groups; CD8+ T cells showed the highest percentage of recent thymic emigrants. At month 1, percentages of Ki-67+ naive and memory CD4+ and CD8+ T cells were the highest in rATG-treated patients, but these percentages declined in the months thereafter. When CD31 was used to distinguish between cytokine- and antigen-driven proliferation in naive T cells, we found evidence for cytokine-dependent proliferation. Cytokine-dependent proliferation was also shown by in vivo increased percentages of phosphorylated STAT5 and high expression levels of the interleukin-7 receptor-α and interleukin-15 receptor-α by T cells.

Conclusion

These findings demonstrate that, in the first month after rATG therapy, cytokine-induced homeostatic proliferation is involved in T-cell repopulation of both naive and memory T cells. At later time points, the contribution of homeostatic proliferation diminished, which explains the observed incomplete T-cell recovery.

INTRODUCTION

Induction therapy with lymphocyte-depleting agents is given to transplant patients to prevent acute rejection. The currently most used agent is the polyclonal rabbit antithymocyte globulin (rATG; i.e., thymoglobulin), which consists of multiple antibodies directed toward cell surface markers on T cells and non-T cells (1-3). Reconstitution of the T-cell pool after depletion therapy is a slow and incomplete process, resulting in changes in the phenotype and function of T cells (4-9), reflecting an immunocompromised state of the patient (10-12).

The primary processes involved in the immune reconstitution are (a) thymopoiesis, the output of new naive T cells called recent thymic emigrants (RTE), and (b) homeostatic proliferation, the expansion of residual T cells. Homeostatic proliferation can be the result of T-cell receptor (TCR) triggering and of the signal transducer and activator of transcription 5 (STAT5) activating cytokines interleukin (IL)-7 and IL-15 (13-15). There are, however, unresolved issues about the contribution of these two processes as well as on their posttransplantation kinetics after rATG induction therapy (6, 16, 17). In addition, the triggers for homeostatic proliferation are studied extensively in animal models, although it remains unknown whether the lymphopenia-induced homeostatic proliferation in transplant recipients results from antigen or cytokine stimulation (13, 14). Understanding the molecular mechanisms by which rATG therapy results in a functionally compromised immune system may explain some of the clinical responses of these immunocompromised patients (18-20). Furthermore, it may also influence future therapeutic strategies in kidney transplant patients ameliorating the host defense to infections and malignancies without compromising the graft.

We here studied the posttransplantation kinetics of the two primary processes involved in T-cell reconstitution, thymopoiesis and homeostatic proliferation, in patients treated with rATG induction therapy. Flow cytometric analyses were performed to phenotype the first repopulated naive and memory CD4+ and CD8+ T cells at 1, 3, and 6 months after induction therapy. We used Ki-67 to measure homeostatic proliferation in naive and memory T-cell subsets; central memory (CM), effector memory (EM), and EMRA T cells. CD31 was used to characterize RTEs and to discriminate between naive cells proliferating upon cytokines (CD31+) and TCR engagement (CD31-) (21). Phosphorylated STAT5 (pSTAT5) in memory T cells and the expression of the IL-7 receptor-α chain (IL-7Rα) and the IL-15Rα were measured to confirm cytokine activation. Patients treated with the nondepleting agent basiliximab that blocks the IL-2Rα and patients not receiving induction therapy served as control groups.

PATIENTS AND METHODS

Patients

Patients (n=8) were given intravenous induction therapy consisting of 2 mg/kg rATG (thymoglobulin; Genzyme/Sanofi, Paris, France) on days 1, 2, and 3 after transplantation, or patients (n=8) received 20 mg basiliximab (Simulect; Novartis Pharma, Basel, Switzerland), monoclonal antibodies targeting the IL-2 receptor, on days 0 and 4 after transplantation. In addition, a third group of kidney transplant patients on immunosuppression but without induction therapy was studied (n=4). The study protocol was approved by the Medical Ethics Committee of the Erasmus Medical Centre (MEC-2010-022). All patients received triple maintenance immunosuppressive therapy consisting of tacrolimus (Prograf; Astellas Pharma, Tokyo, Japan), mycophenolate mofetil (CellCept; Roche, Basel, Switzerland), and steroids for 3 months. Tacrolimus, mycophenolate mofetil, and steroids were given from day 1 after transplantation. There were no differences in patient age, gender, and cytomegalovirus status between the different treatment groups (Table 1).

Table 1. Patient characteristics

Induction Therapy	**rATG**	**Basiliximab**	**None**
No. of patients	8	8	4
Male/Female	7/1	7/1	2/2
Recipient age (yrs)[#] (range)	52 (29-69)	47 (19-68)	61 (24-72)
DR-MM 0/1/2[$]	3/0/5	1/4/3	4/0/0
No. of first transplants	7	5	4
Living/Deceased donors	7/1	5/3	4/0
Pre-emptive/ Dialysis	6/2	1/7	1/3
CMV			
donor-recipient:			
pos-pos	3	3	2
pos-neg	3	1	0
neg-pos	1	2	1
neg-neg	1	2	1
Primary disease:			
DM	1	2	1
Hypertension	3	1	0
PKD	2	1	1
Glomerulopathy	2	2	1
Urologic problem	0	3	0
ATN	0	0	1

Patient characteristics. No: number. [#]: median + range. [$]: number of patients with 0, 1 or 2 mismatches on DR-locus. CMV: cytomegalo virus donor-recipient combination, pos: IgG positive, neg: IgG negative. DM: diabetes mellitus. PKD: polycystic kidney disease. ATN: acute tubulus necrosis.

In the rATG group, one patient died after the first month. In the basiliximab group, one patient died after the third month, and another patient who received rATG antirejection therapy was excluded from analysis beyond that point. There were no deaths or rejections in the patient group without induction therapy.

Blood (heparinized and EDTA) was drawn before and 1, 3, and 6 months after transplantation. Heparinized lithium blood was used to perform whole-blood phosphospecific flow cytometry and to isolate the peripheral blood mononuclear cells (PBMCs) by standard density gradient centrifugation. PBMCs were stored at -140-C until analysis.

Flow Cytometry

Absolute Numbers of CD3+, CD4+, and CD8+ T cells

Absolute counts of CD3+ T cells and CD4+ and CD8+ T cells were measured in EDTA blood using the BD MultiTest 6 color TBNK reagent and BD TruCount tubes in combination with BD FACSCantoII flow cytometer (BD Biosciences, San Jose, CA). In brief, 20 µL of MultiTest reagent containing CD3 fluorescein isothiocyanate (FITC), CD16+CD56-phycoerythrin (PE), CD45 peridinin chlorophyll protein-Cy5.5 (PerCPCy5.5), CD4 PE-Cy7, CD19 allophycocyanin (APC), and CD8 APC-Cy7 monoclonal antibodies, were pipetted into a BD TruCount tube containing a known number of fluorescent beads. EDTA blood (50 µL) was added and incubated for 15 min in the dark at room temperature. Hereafter, red blood cells were lysed by 500 µL FACS lysing solution (BD Biosciences) for 15 min. Samples were measured on a FACSCantoII (BD Biosciences). By FACSDiva software, the absolute number (cells/µL) in each sample was determined.

T-Cell Subsets and STAT5 Phosphorylation

To measure the different T-cell subsets and the phosphorylation status of STAT5, we used phosphospecific flow cytometry. Staining was conducted using 200 µL heparin blood for 30 min in the dark with CD3-AmCyan, CD8-FITC, and CCR7-PE-Cy7 antibodies (BD Biosciences). After lysation, fixation, and permeabilization by 70% methanol, PBMCs were washed and stained with CD4-PerCP, STAT5-PE (BD Biosciences), and CD45RO-Pacific Blue (PB) (Biolegend, San Diego, CA). IgG1-PE served as an isotype control.

Measurement of Markers for Homeostatic Proliferation and Thymopoiesis

PBMCs were thawed and stained with the following fluorochrome-conjugated antibodies for 30 min in the dark at room temperature: CD3-AmCyan, CD4-PB, CD8-APC-Cy7, and CCR7-PE-Cy7 (BD Biosciences) and CD45RO-APC and CD31-PE (Biolegend). IgG2a-APC, IgG2a-PECy7, IgG1-FITC, and IgG1-PE served as isotype controls. Next, cells were washed, fixated, and permeabilized followed by an intracellular staining for Ki-67-FITC (BD Biosciences). A minimum of 100 events per T-cell subset was analyzed by the

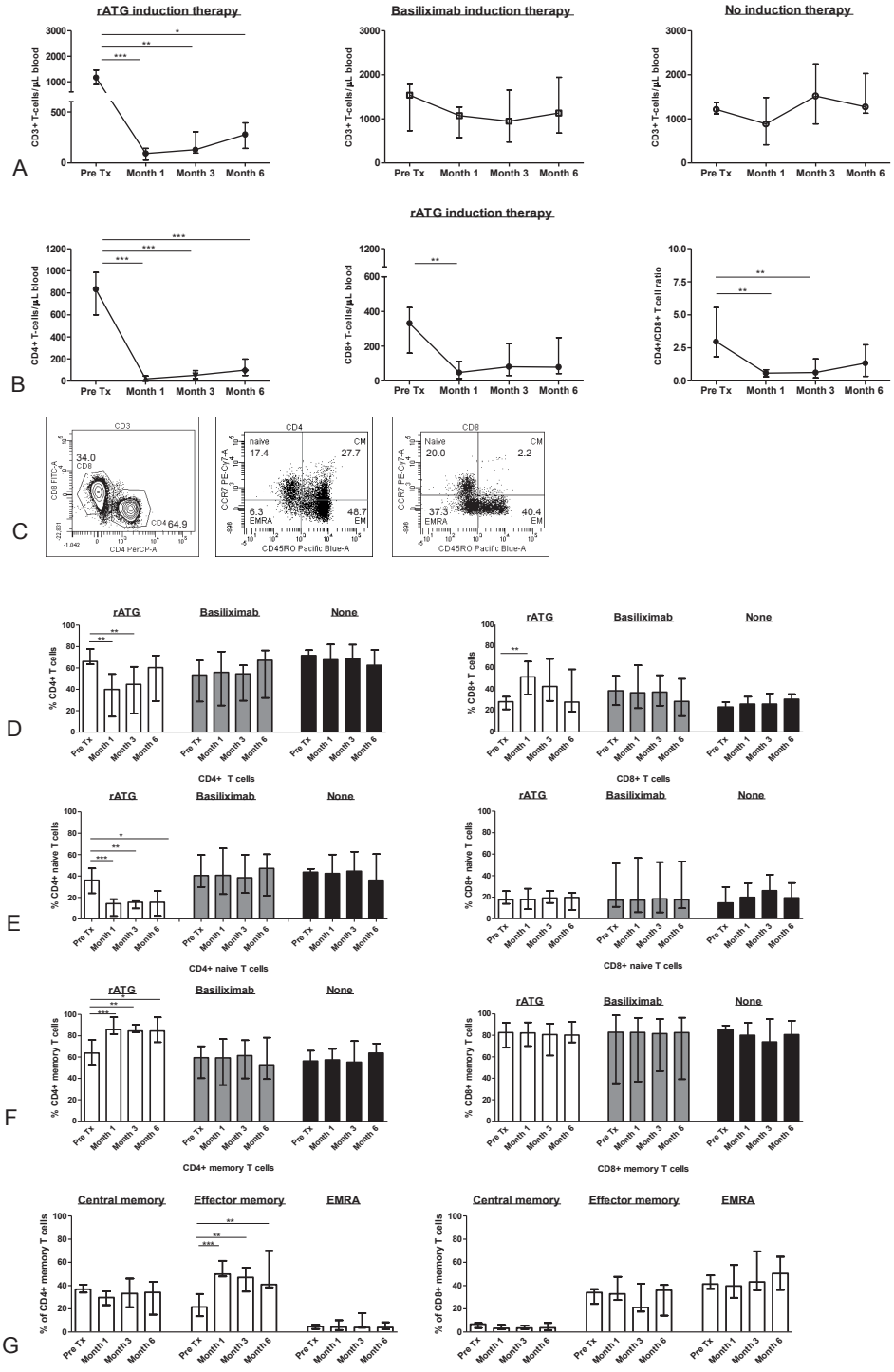

A
rATG induction therapy
Basiliximab induction therapy
No induction therapy
CD3+ T-cells/µL blood
Pre Tx
Month 1
Month 3
Month 6
B
rATG induction therapy
CD4+ T-cells/µL blood
CD8+ T-cells/µL blood
CD4+/CD8+ T cell ratio
C
CD3
CD8
CD4
34.0
64.9
CD4 PerCP-A
CD8 FITC-A
naive
CM
17.4
27.7
6.3
EMRA
48.7
EM
CCR7 PE-Cy7-A
CD45RO Pacific Blue-A
Naive
20.0
2.2
37.3
40.4
D
rATG
Basiliximab
None
% CD4+ T cells
% CD8+ T cells
CD4+ T cells
CD8+ T cells
E
% CD4+ naive T cells
% CD8+ naive T cells
CD4+ naive T cells
CD8+ naive T cells
F
% CD4+ memory T cells
% CD8+ memory T cells
CD4+ memory T cells
CD8+ memory T cells
G
Central memory
Effector memory
EMRA
% of CD4+ memory T cells
% of CD8+ memory T cells

Figure 1. Phenotype of the repopulated T cells. Whole-blood measurement of the absolute number of T cells and the percentage of the different T-cell subpopulations. **(A)** Absolute number of total CD3+ T cells in the different treatment groups. **(B)** Absolute number of the CD4+ and CD8+ T cells and the CD4+/CD8+ T-cell ratio after rATG induction therapy. **(C)** Gating strategy used for determination of the CD4+ and CD8+ T-cell subsets; naive (CD45RO-CCR7+), CM (CD45RO+CCR7+), EM (CD45RO+CCR7-), and EMRA (CD45RO-CCR7-) T cells. **(D)** Percentage of CD4+ and CD8+ T cells within the total CD3+ T-cell population in patients treated with rATG (white), patients treated with basiliximab (gray), or patients treated without (black) induction therapy. **(E)** Percentage of naive T cells within the CD4+ and CD8+ T-cell population in patients treated with rATG (white), basiliximab (grey), or no (black) induction therapy. **(F)** Percentage of memory T cells within CD4+ and CD8+ T cells in patients treated with rATG (white), basiliximab (gray), or no (black) induction therapy. **(G)** Subdivision of the CD4+ and CD8+ memory T cells; CM, EM, and EMRA T-cell subsets for rATG-treated patients. Error bars represent median±interquartile range. *P<0.05; **P<0.01; *** P<0.001.

FACSCantoII followed by analysis using BD FACSDiva Software. Consequently fewer data points are depicted in the figures. Data are presented by proportions of cells.

Measurement of the IL-7R and IL-15R

PBMCs from were thawed and stained for 30 min in the dark at room temperature with the following fluorochrome-conjugated antibodies: CD3-AmCyan, CD4-PerCP, CD8-APC-Cy7, CD45RO-PB, and CCR7-PECy7 (BD Bioscience) and CD127-FITC and IL-15Rα-PE (eBioscience, San Diego, CA). IgG1-FITC and IgG2a-PE (BD Biosciences) served as isotype controls.

Statistics

Statistical analyses were performed using GraphPad Prism version 5.01. For determination of the levels of statistical significance, the two-sided probability values according to the Mann-Whitney U test and Fisher's exact test were used. A linear regression model was used for the correlation between RTEs and age. $P<0.05$ was considered statistically significant.

RESULTS

Phenotype of the Repopulating T Cells

Before transplantation, the absolute number of CD3+ T cells was comparable between patients treated with rATG, basiliximab, or no induction therapy. At 1 month, we observed a significant decrease in the number of CD3+ T cells in the rATG treatment group ($PG<0.001$), which was followed by a slow and incomplete recovery ($P<0.05$). The number of CD3+ T cells in basiliximab-treated patients and patients without preconditioning remained constant over time (Fig. 1A). For rATG-treated patients, the numbers of CD4+ and CD8+ T cells are depicted in Figure 1B. Both subsets showed a significant decrease in the number of cells at 1 month ($P<0.01$) and they did not return to baseline level for the

whole study period. However, at month 3, CD8+ T cells were not significantly different from their pre-transplantation level, whereas CD4+ T cells remained decreased for the whole study period (P<0.001). This difference in repopulation rate of CD4+ and CD8+ T cells is in line with the literature (4, 16), resulting in a decreased CD4/CD8 T-cell ratio (baseline vs. months 1 and 3; P<0.01) (Fig. 1B).

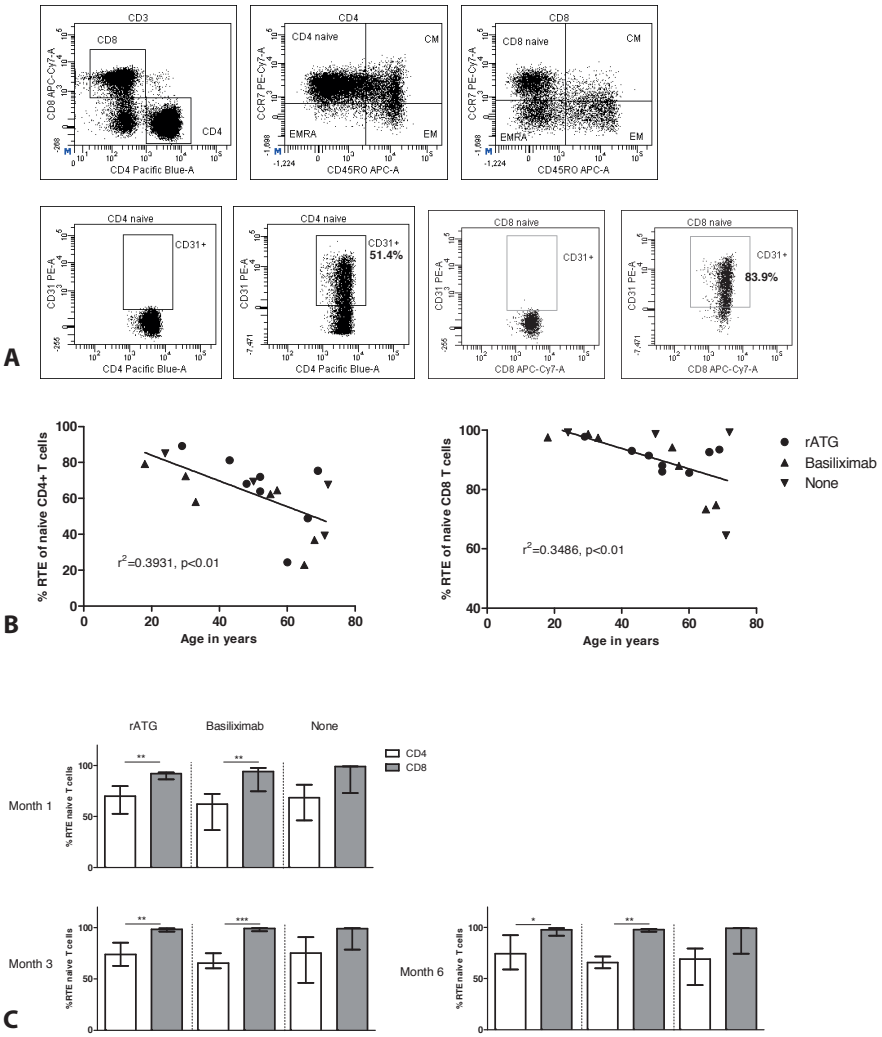


Figure 2. Thymopoiesis. **(A)** Percentage CD31+ RTE CD4+ and CD8+ T cells were determined by FACS analysis. **(B)** CD4+ and CD8+ RTEs were inversely correlated with age at 1 month after transplantation as tested by the linear regression model: CD4+ T cells (r2=0.3931, PG<0.01) and CD8+ T cells (r2=0.3486, PG<0.01). **(C)** Percentage of RTEs within the CD4+ (white) and CD8+ (gray) naive T cells for patients treated with rATG (left), basiliximab (middle), and no (right) induction therapy at 1, 3, and 6 months after transplantation. Error bars represent median±interquartile range. *P<0.05; **P<0.01; ***P<0.001.

The phenotype of the repopulated CD4+ and CD8+ T cells was further analyzed as shown in Figure 1C, which shows the gating strategy to define CD4+ and CD8+ naive (CD45RO-CCR7+) and memory T-cell subsets; CM (CD45RO+CCR7+), EM (CD45RO+CCR7-), and EMRA (CD45RO-CCR7-). The proportion of CD4+ and CD8+ T cells resembled the findings of the absolute numbers shown in Figure 1B; the percentage of CD4+ T cells decreased, where the percentage of CD8+ T cells increased after rATG induction therapy ($P<0.01$) (Fig. 1D). When analyzing the distribution of naive and memory CD4+ and CD8+ over time, we observed a decrease in the proportion of naive and an increase in the proportion of memory T cells in the CD4+ but not in the CD8+ T-cell compartment ($P<0.05$) (Fig. 1E,F). As depicted in Figure 1G, the increase in the proportion of CD4+ memory T cells of the rATG group was the result of EMT cells ($P<0.01$) and not of the CM or the EMRA subpopulation.

Thymopoiesis

To study thymopoiesis, we measured the percentage RTEs, defined as CD31+ naive T cells (Fig. 2A) (21, 22). Both CD4+ and CD8+ RTEs are known to be inversely correlated with age in healthy individuals (21-23). As shown in Figure 2B, CD31+CD4+ and CD31+CD8+ naive T cells are correlated with age after transplantation (CD4: r^2=0.3931,PG0.01; CD8: r^2=0.3486, PG0.01). The percentage CD4+and CD8+ RTEs in the different treatment arms are depicted in Figure 2C. No significant differences within the percentage RTEs between rATG and the other treatment groups were found. However, the proportion of CD31+CD8+ naive T cells was significantly higher than the proportion of CD31+CD4+ naive T cells in rATG- and basiliximab treated patients for all time points studied ($P<0.05$).

Homeostatic Proliferation

Homeostatic proliferation after rATG induction therapy was studied in CD4+ and CD8+ T-cell subsets by analyzing the expression of Ki-67, an antigen only present in the G1-M phase of cell division (Fig. 3A). One month after induction therapy, higher percentages of Ki-67-expressing CD4+ naive, CM, and EM T cells were measured in rATG than in basiliximab-treated patients ($P<0.05$) and in patients without preconditioning (Fig. 3B). A similar pattern was found for the CD4+ EMRA T cells. At 3 months after rATG induction therapy only in the CD4+ naive and CM subset, higher percentages of Ki-67+ T cells were found, which declined thereafter.

A comparable profile of Ki-67 expression was observed for the CD8+ T-cell subsets (Fig. 3C). At 1 month, we measured higher percentages of Ki-67+ naive, CM, EM, and EMRA T cells in rATG-treated patients than in basiliximab treated patients (PG<0.05). These percentages of Ki-67+ naive, CM, and EMRA T cells were also higher than the no induction therapy group ($P<0.05$). At 3 months, the percentages of Ki-67+ T cells in the treatment groups were comparable. At 6 months, a small increase of Ki-67+ memory

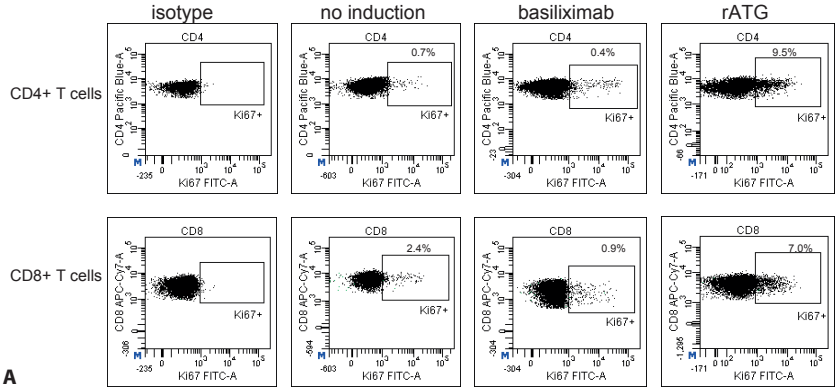
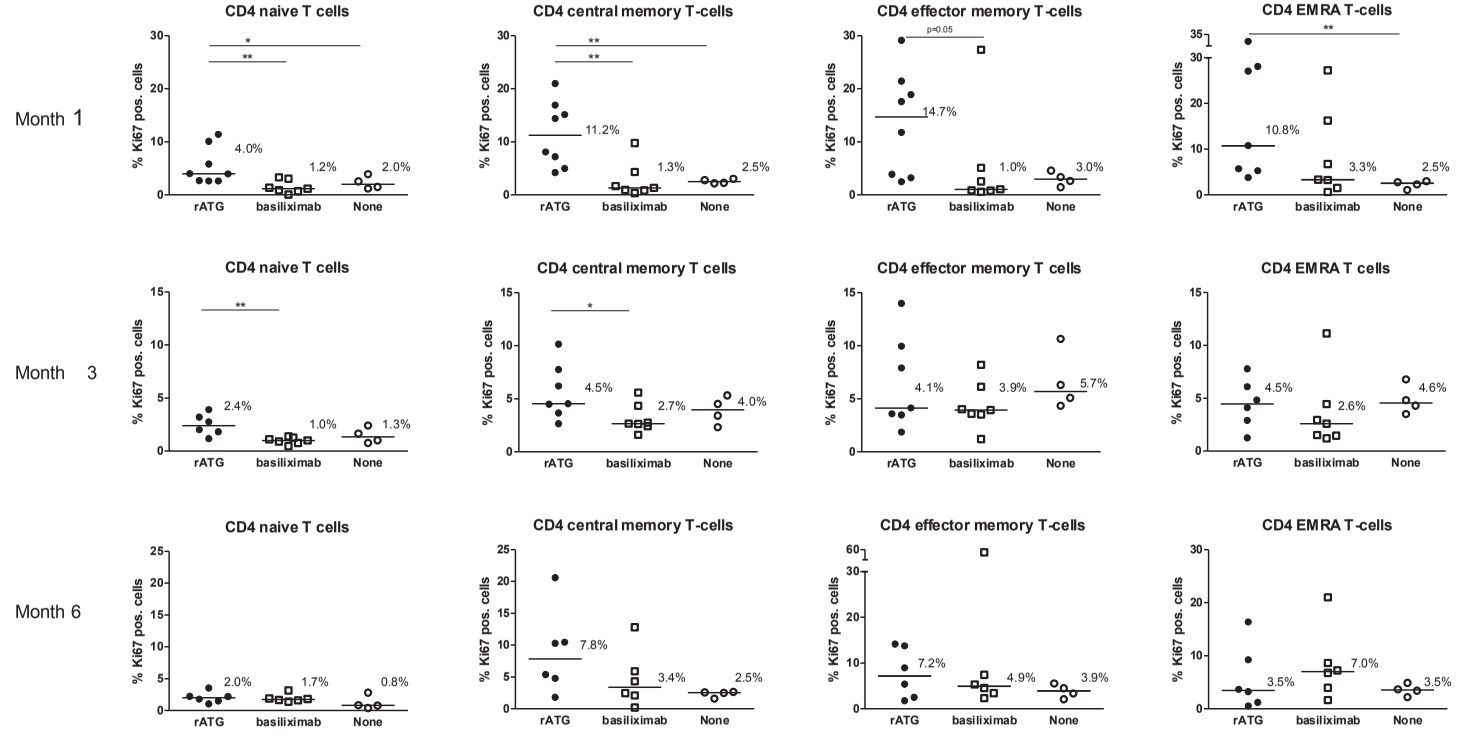


Figure 3. Homeostatic proliferation. **(A)** We measured homeostatic proliferation by the expression of the proliferation marker Ki-67. Typical examples of the different treatment arms, 1 month after transplantation, are shown. **(B)** Homeostatic proliferation in patients treated with rATG (●), basiliximab (□) and without (○) induction therapy in the different CD4+ T-cell subsets; naive, CM, EM, and EMRA T cells at 1, 3, and 6 months after transplantation. **(C)** Homeostatic proliferation in patients treated with rATG (●), basiliximab (□) and without (○) in the different CD8+ T-cell subsets; naive, CM, EM, and EMRA T cells at 1, 3, and 6 months after transplantation. A minimum of 100 events per T-cell subset was analyzed; consequently sometimes fewer data points are depicted in the figure. *P<0.05; **P<0.01; *** P<0.001.

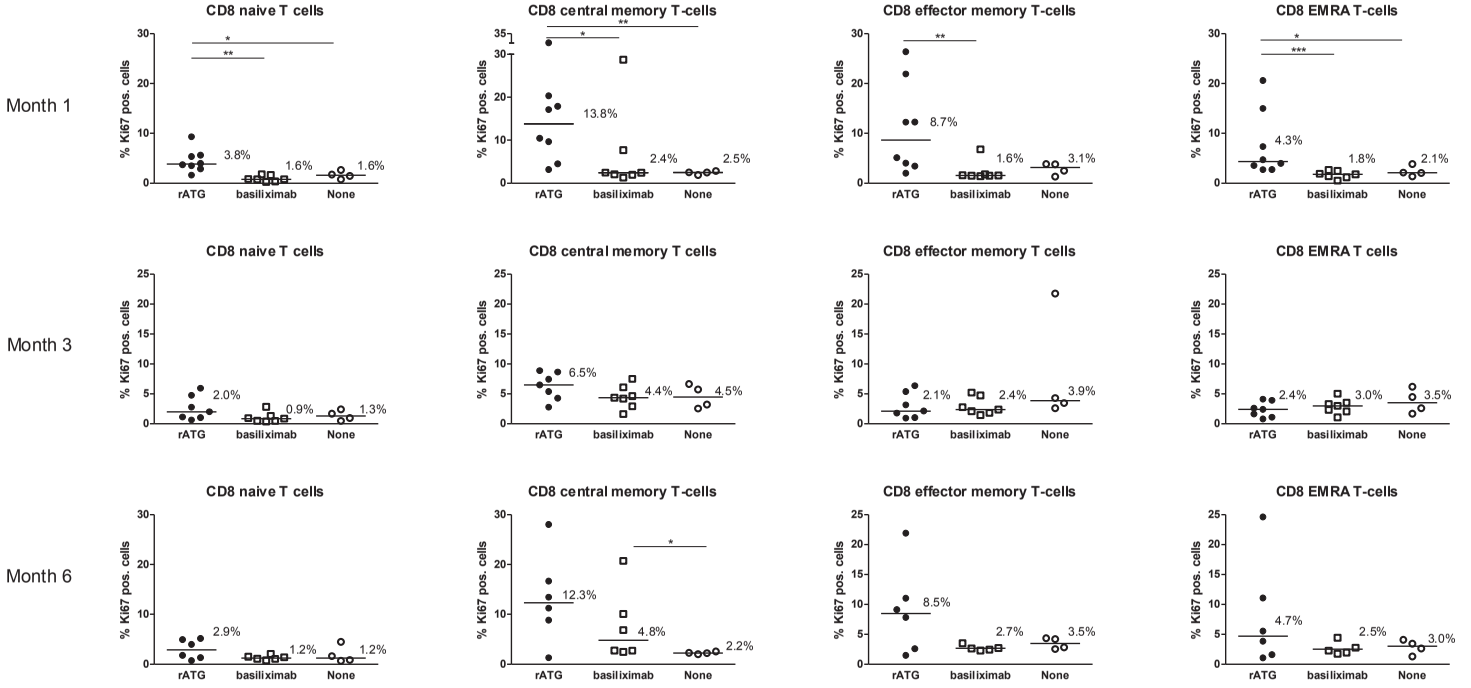
CD4+ and CD8+ T cells was seen in the rATG group (Fig. 3B,C), which may be the result of steroid withdrawal. Overall, the percentages of Ki-67+ memory T cells exceeded those of the naive T cells in both CD4+ and CD8+ populations.

Cytokine- and Antigen-Induced Proliferation

There are two triggers for homeostatic proliferation: the STAT5-dependent homeostatic cytokines IL-7 and IL-15 and contact of the T-cell receptor (TCR) with major histocompatibility complex-peptide-ligands (antigen) (14). To determine which of these two stimuli mediates proliferation of naive T cells in rATG-treated patients, we used the expression of the cell surface molecule CD31. CD31 is lost upon T-cell stimulation by antigen but not when cells are stimulated with IL-7/IL-15 resulting in two subpopulations of proliferating T cells: Ki-67+CD31+ naive T cells dividing upon cytokines and Ki-67+CD31- naive T cells dividing upon TCR-contact. In Figure 4A, the proportion of Ki-67+ CD31+ naive T cells is depicted for the different treatment groups. One month after transplantation, the proportion of Ki-67+CD31+ naive T cells of both CD4+ and CD8+ T cells is higher in the rATG group than in the other treatment groups. This effect declined at 3 months for the CD8+ T cells and at 6 months for the CD4+ T cells. The percentages of Ki-67+CD31- naive T cells are depicted in Figure 4B. For the study period, the percentages of Ki-67+CD31- naive T cells were comparable for the three treatment groups.


Month 1
Month 3
Month 6
CD4 naive T cells
CD4 central memory T-cells
CD4 effector memory T-cells
CD4 EMRA T-cells
CD4 central memory T cells
CD4 effector memory T cells
% Ki67 pos. cells
rATG
basiliximab
None
4.0%
1.2%
2.0%
11.2%
1.3%
2.5%
p=0.05
14.7%
1.0%
3.0%
10.8%
3.3%
2.5%
2.4%
1.0%
1.3%
4.5%
2.7%
4.0%
4.1%
3.9%
5.7%
4.5%
2.6%
4.6%
2.0%
1.7%
0.8%
7.8%
3.4%
2.5%
7.2%
4.9%
3.9%
3.5%
7.0%
3.5%

B


Month 1
Month 3
Month 6
CD8 naive T cells
CD8 central memory T-cells
CD8 effector memory T-cells
CD8 EMRA T-cells
CD8 central memory T cells
CD8 effector memory T cells
CD8 EMRA T cells
% Ki67 pos. cells
rATG
basiliximab
None
3.8%
1.6%
1.6%
13.8%
2.4%
2.5%
8.7%
1.6%
3.1%
4.3%
1.8%
2.1%
2.0%
0.9%
1.3%
6.5%
4.4%
4.5%
2.1%
2.4%
3.9%
2.4%
3.0%
3.5%
2.9%
1.2%
1.2%
12.3%
4.8%
2.2%
8.5%
2.7%
3.5%
4.7%
2.5%
3.0%

C

The marker CD31 cannot be used to study cytokine triggered homeostatic proliferation in memory T cells; hence, we measured the phosphorylation status of STAT5, the downstream target after IL-7R and IL-15R activation. As shown in Figure 4C and D, rATG-treated patients showed a clearly different profile than the other treatment arms, with at month 1 increased percentages of pSTAT5+CD4+ and CD8+ memory T cells. This normalized at the later time points.

To further define the involvement of cytokines in this process, we measured the expression of the IL-15Rα and IL-7Rα (CD127) on CD4+ and CD8+ T cells (Fig. 5A,B). After rATG therapy, CD4+ T cells expressed higher levels of the IL-15Rα than the two control groups. The IL-15Rα expression profile among the different T-cell subsets was comparable for all treatment groups with the highest expression by CD4+ EM and CD8+ CM T cells (Fig. 5C,D). Figures 5E and F show the results for the IL-7Rα, which was expressed at a similar level for all treatment groups. This cytokine receptor was also highly expressed by CD4+ and CD8+ central and EM T cells for all treatment groups.

DISCUSSION

The beneficial effects of induction therapy with T-cell depleting rATG in the prevention and treatment of rejection are well defined (24); however, this therapy is associated with an incomplete T-cell repopulation resulting in a long-lasting impaired immune system (4). This study focuses on the mechanisms and stimuli of T-cell reconstitution and their posttransplantation kinetics in kidney transplant patients who received T-cell depletion therapy.

Our data demonstrated that rATG induction therapy had no effect on thymopoiesis; however, a higher output of CD8+ RTEs than of CD4+ RTEs was observed. Thymopoiesis is the route for de novo production of T cells, although, with increasing age, the thymus involutes and thymopoiesis declines (25). Multiple studies in patients suffering from T-cell depletion, that is, patients with HIV infection on highly active antiviral therapy, after chemotherapy or after bone marrow or kidney transplantation, showed a substantial and maintained output of naive T cells during life, and even increased thymopoiesis was reported (16, 26-32). In our study, we did not observe an effect of T-cell depletion therapy on thymopoiesis, defined as the percentage of RTEs. This difference may be explained by a difference in control groups. We compared our rATG-treated patients to patients treated with basiliximab or no induction therapy, overcoming the potential effect on thymopoiesis of maintenance immunosuppression and stress-related events, such as surgery, in kidney transplant patients, whereas others studied the process just over time (16, 26-28, 30, 31).

Because, in adults, the role of thymopoiesis is restricted, homeostatic proliferation is thought to be the major contributor to immune reconstitution after rATG induction

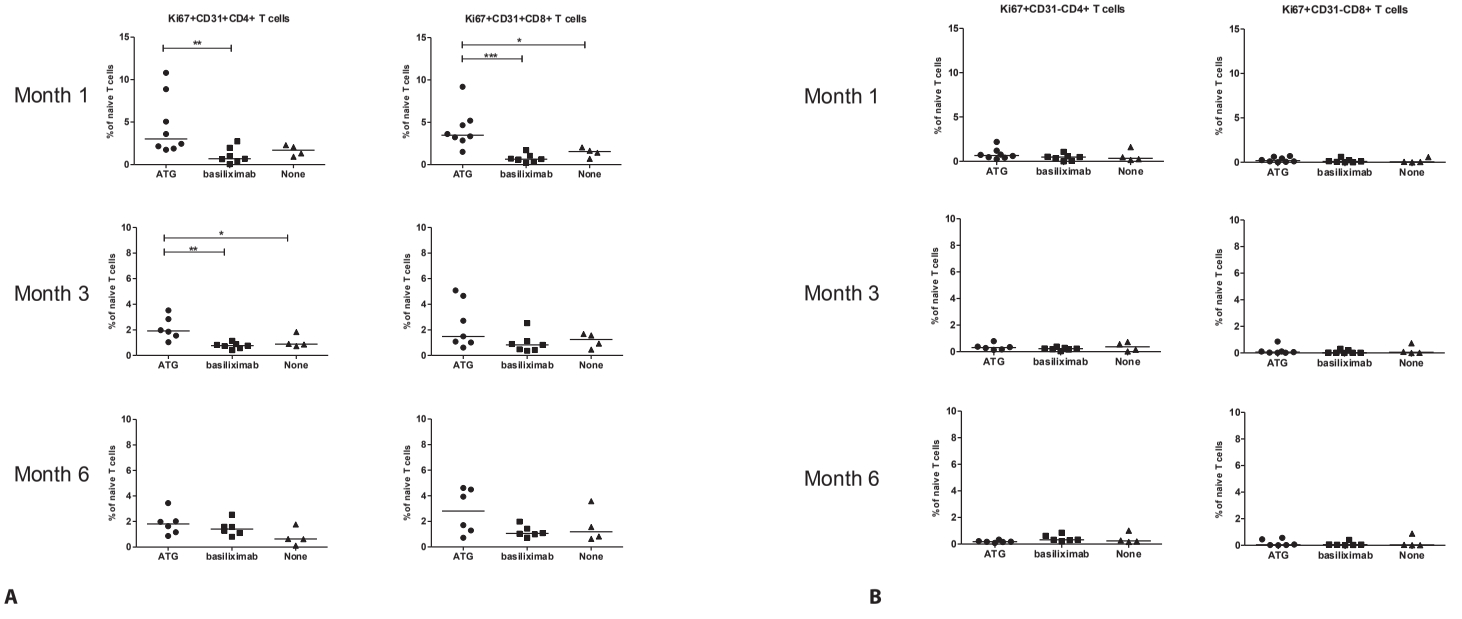
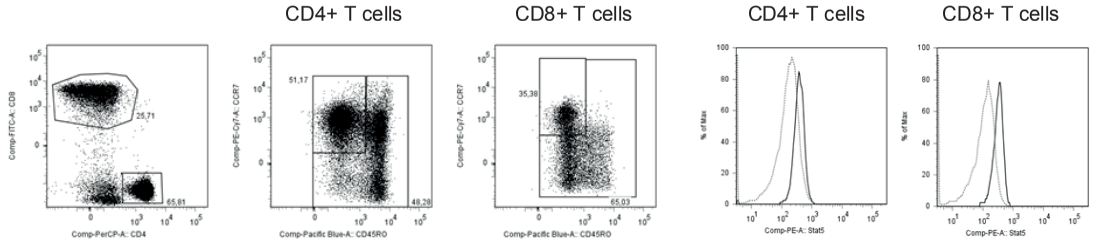
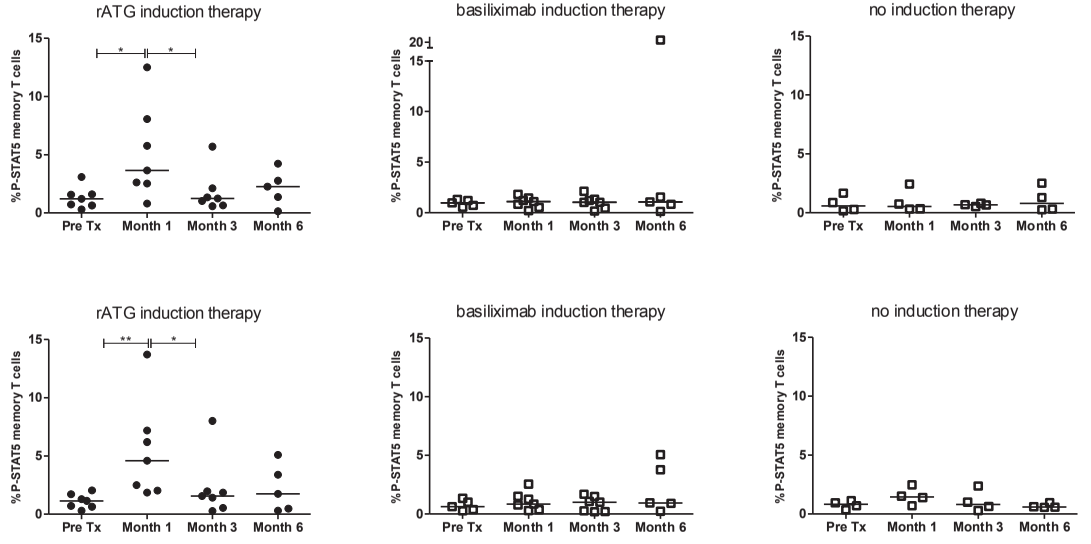


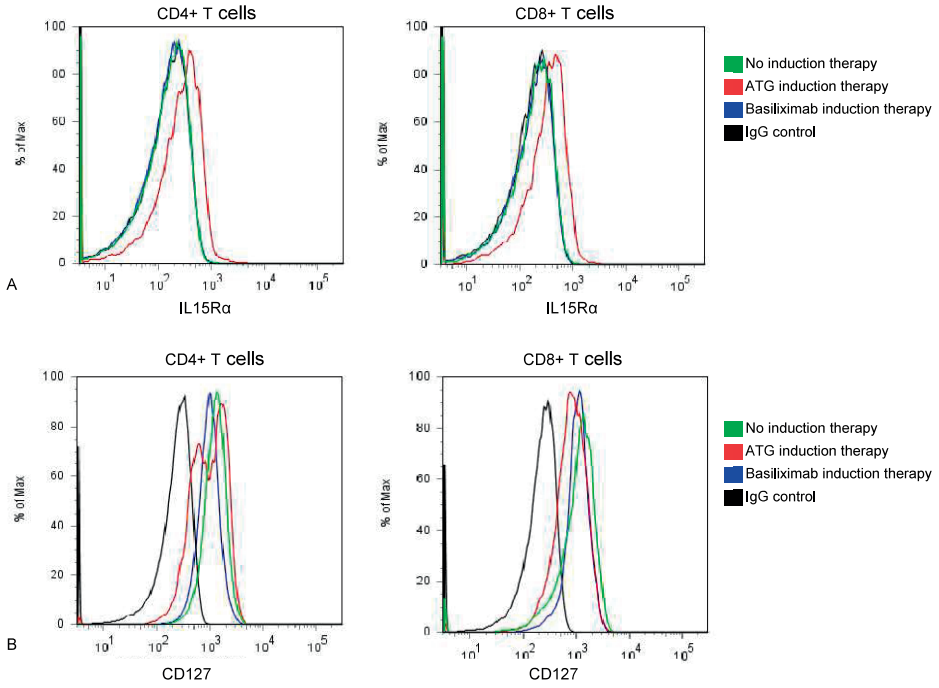
Figure 4. Stimulus for homeostatic proliferation. **(A)** Percentages of Ki-67+CD31+ naive T cells in patients treated with rATG (●), basiliximab (■) or without (▲) induction therapy at 1 month, 3 months and 6 months after transplantation. **(B)** Percentages of Ki-67+CD31- naive T cells in patients treated with rATG (●), basiliximab (■) or without (▲) induction therapy at 1 month, 3 months and 6 months after transplantation. **(C)** Phosphorylation status of STAT5 as measured by flow cytometry. An example of before and after transplantation of a rATG patient is shown. **(D)** percentage of memory CD4+ and CD8+ T cells positive for pSTAT5 in patients treated with rATG, basiliximab or no induction therapy before and 1, 3, and 6 months after transplantation. A minimum of 100 events per T-cell subset was analyzed; consequently sometimes fewer data points are depicted. *P< 0.05;**P<0.01; *** P<0.001.


CD4+ T cells
CD8+ T cells
CD4+ T cells
CD8+ T cells
Comp-FITC-A: CD8
Comp-PerCP-A: CD4
25,71
65,81
Comp-PE-Cy7-A: CCR7
Comp-Pacific Blue-A: CD45RO
51,17
48,28
35,38
65,03
% of Max
Comp-PE-A: Stat5
Pre-transplantation
Month 1 after transplantation

C


rATG induction therapy
basiliximab induction therapy
no induction therapy
%P-STAT5 memory T cells
Pre Tx
Month 1
Month 3
Month 6
CD4+ T cells
CD8+ T cells

D



5 A & B

therapy (6, 13, 14, 17). Earlier studies in humans measured homeostatic proliferation indirectly by an increase in the absolute number of memory T cells (6, 16). However, by solely studying T-cell numbers, a role of proliferating naive T cells adopting a memory phenotype, or a phenotypic shift from naive to memory T cells, and a relative resistance of specific memory T-cell subtypes to depletion cannot be determined (6, 16, 33). We here directly measured proliferation by the marker Ki-67 and observed, at 1 month, the highest percentages of Ki-67-expressing cells in rATG-treated patients, both for the naive and memory CD4+ and CD8+ T-cell subsets. Memory T cells showed the highest proportions of Ki-67+ T cells, indicating a higher proliferation rate of this subset. The high proportion of EM CD4+ T cells, however, could not be explained by higher percentages of Ki-67+ cells in this particular subset than in the other subsets (Fig. 1 and 3).

Surprisingly, the high percentages of Ki-67+ T cells disappeared at month 3 for the CD8+ T cells and at month 6 for CD4+ T cells, whereas these rATG-treated patients were still severely lymphopenic at these time points. The explanation for this phenomenon is not clear yet. We here speculate that T-cell exhaustion might be involved. T-cell exhaustion is found in patients suffering from chronic infections and malignancies and is the gradual loss of T-cell effector functions starting with a decrease in proliferation capacity. It is thought that the underlying mechanisms are prolonged or persistent antigen stimulation and disruption of lymphoid tissue, which also has been found in nonhu-

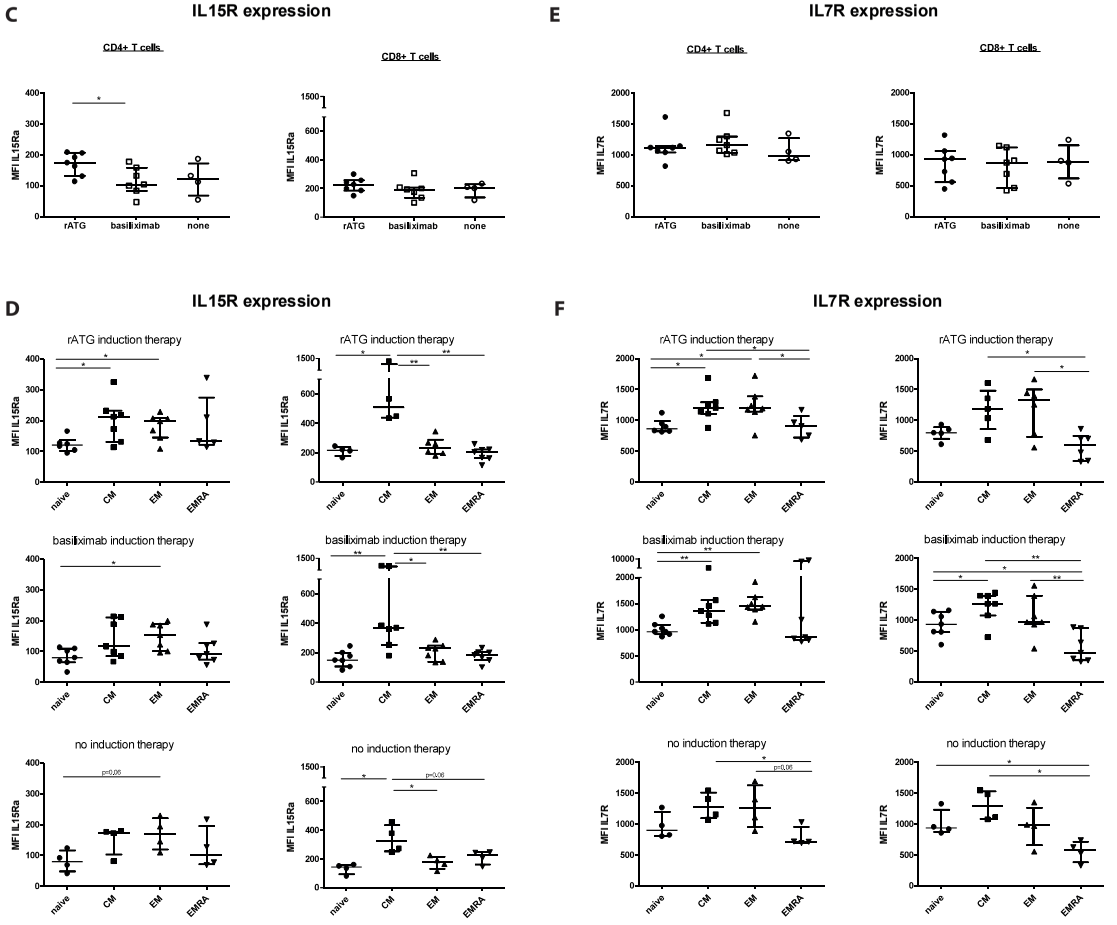


Figure 5. IL-15 and IL-7R expression histogram of the IL-15R α **(A)** and IL-7R α (CD127) **(B)** for the different treatment groups. **(C)** Expression of the IL-15R->, 3 months after transplantation, in the total population of CD4+ and CD8+ T cells for all treatment groups: rATG (●), basiliximab (□) and none (○). **(D)** IL-15Rα expression in the different CD4+ T-cell subset (left) and CD8+ T-cell subset (right) for all treatment groups; rATG, basiliximab, and no induction therapy. **(E)** Expression of the IL-7Rα 3 months after transplantation, in the total population of CD4+ and CD8+ T cells for all treatment groups; rATG (●), basiliximab (□) and none (○). **(F)** IL-7R expression in the different CD4+ T-cell subset (left) and CD8+ T-cell subset (right) for all treatment groups; rATG, basiliximab, and no induction therapy A minimum of 100 events per T-cell subset was analyzed; consequently sometimes fewer data points are depicted. *P<0.05; **P<0.01; *** P<0.001.

man primates after rATG therapy (3, 34, 35). In our rATG treated patients, the persistent stimulation of the immune system by donor antigen and the disruption of the lymphoid architecture by rATG therapy both may contribute to the development of an exhausted T-cell compartment and thus to an incomplete recovery of the T-cell pool.

The mechanisms underlying homeostatic proliferation are studied extensively in immunocompromised mouse models, which demonstrated that proliferation in naive and memory CD4+ and CD8+ T cells depends on both contact of the major histocompatibility complex with self-peptides or non-self-peptides and binding of the STAT5 activating cytokines IL-7 and IL-15 (13, 14, 36). The high percentages of Ki-67+CD31+, in contrast to the Ki-67+CD31-, T cells and in vivo phosphorylation of STAT5 in memory T cells demonstrated that these homeostatic cytokines play an important role in the early phase of immune reconstitution after rATG therapy. However, it is expected that, next to cytokine-induced proliferation, antigen stimulation of T cells also contributes to the total proliferation of memory T cells. For that purpose, TCR signaling studies, for example, measurement of Zap70, should be performed. In the present study, we focused on the contribution of homeostatic cytokines by pSTAT5 studies and IL-7Rα and IL-15Rα measurements.

To conclude, our study demonstrates that, in the first month after rATG therapy, cytokine-induced homeostatic proliferation is involved in T-cell repopulation of both naïve and memory T cells. At later time points, the contribution of homeostatic proliferation diminished, which explains the observed incomplete T-cell recovery.

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Chapter 4

The impact of induction therapy on the homeostasis and function of regulatory T cells in kidney transplant patients

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ABSTRACT

Background

To evaluate the influence of induction therapy on Tregs we investigated their origin, kinetics and function in kidney transplant patients after treatment with T-cell depleting rabbit antithymocyte globulin (rATG) or IL-2 receptor antagonist basiliximab.

Methods

Flow cytometry was used to study thymopoiesis by CD31+ naive Tregs, homeostatic proliferation by Ki-67+ Tregs and Treg origin by the expression of Helios (nTreg-marker). FACSsorted Tregs were analysed for the demethylation status of the Treg-specific demethylated region (TSDR) of the FoxP3 gene, and Treg-suppressive function.

Results

Differential effects of rATG and basiliximab induction therapies were measured on the repopulation kinetics of Tregs. While decreased absolute numbers of Tregs were found in both study arms, increased percentages of Tregs were found in rATG treated patients and decreased percentages in basiliximab treated patients. In both groups, Treg repopulation was the result of homeostatic proliferation and not of thymopoiesis. At 1 month after rATG and 6 months after basiliximab therapy, high percentages of Ki-67+ Treg were measured, which in the rATG group, was accompanied by low percentages of Ki-67+Helios+ Treg, and by cells with a demethylated TSDR in the FoxP3 gene. After both rATG and basiliximab therapy, repopulated Tregs inhibited proliferation of alloantigen activated T effector cells (Teff).

Conclusion

In kidney transplant patients, repopulation of Treg after rATG and basiliximab therapy is the result of homeostatic proliferation and not of thymopoiesis. These repopulated Treg were functional after both induction strategies; however only after rATG therapy, were increased proportions of Helios-methylated FoxP3 Treg found.

INTRODUCTION

Induction therapy is given to kidney transplant patients to prevent acute rejection and to avoid nephrotoxic effects of high dose calcineurin inhibitors (1, 2). Commonly used agents are the T-cell depleting rabbit antithymocyte globulin (rATG; thymoglobulin) and the non-depleting basiliximab, a monoclonal antibody directed against CD25, the α-chain of the IL-2 receptor (IL-2R) (1–3). Apart from the effects of rATG and basiliximab to effector T cells, these agents also influence regulatory T cells (Treg), the immunosuppressive countermechanism, important for immune regulation (4–13).

Kidney transplant patients treated with rATG induction therapy have increased proportions of circulating Treg (4–7). The *in vivo* underlying mechanisms of this phenomenon are not elucidated yet. It is, however, known that Treg, like other T-cell subsets, repopulate after lymphopenia by thymopoiesis, the formation of new T cells called recent thymic emigrants (RTE) and by homeostatic proliferation, the expansion of residual T cells (5, 14, 15). In addition, there is *in vitro* evidence that after rATG therapy, functional Treg expand by conversion of conventional T cells into induced Treg (16–21). Unfortunately, *in vivo*, conversion of Treg is hard to define. Basiliximab is thought to hamper Treg, as blocking of the IL-2R deprives Treg from IL-2 which is important for their homeostasis, survival and function (22–25). It is unclear whether blockade of the IL-2R depletes Treg from the circulation, or modifies the CD25-expression (7–12, 26–29).

Tregs can be divided into two subsets; natural Treg (nTreg) and induced Treg (iTreg). nTreg originates directly from the thymus and constitutively express FoxP3; the transcription factor that plays a critical role in their development and function. iTreg, which also express FoxP3, originates in the periphery by the differentiation of conventional T cells into Treg upon exposure to antigen in the presence of tolerogenic cytokines (30, 31). After rATG induction therapy, it is likely, but still unknown, that *in vivo* iTreg will contribute to the repopulating Treg pool, as thymopoiesis, the generation of nTreg, decreases with age, and conversion of conventional T cells into iTreg may occur in the presence of donor antigen (16–20, 32). Both subtypes of Treg have similar phenotypical and functional characteristics and consequently distinction between nTreg and iTreg by the standard markers, CD25, CD127 and FoxP3, is not possible. Recently, a specific DNA methylation pattern within the Treg-specific demethylated region (TSDR) of the FoxP3 gene has been identified that is highly demethylated in nTregs and methylated in iTregs (33). Moreover, Helios, a member of the Ikaros transcription factor family, has come forward as a marker for nTregs (34).

Here, we assessed how depleting and non-depleting induction therapies influence the mechanism of Treg homeostasis in kidney transplant patients. We determined Treg repopulation mechanisms, thymopoiesis, homeostatic proliferation and Treg conversion along with the analysis of whether nTreg or iTreg are present after induction therapy. These data were completed by functional analysis of the repopulated Treg.

MATERIALS AND METHODS

Patients

Blood samples were obtained from 33 consecutive kidney transplant patients treated with either rATG (n = 15, Thymoglobuline®, Genzyme/Sanofi, Paris, France) in a dose of 2 mg/kg on Days 1, 2 and 3 after transplantation, or basiliximab (n = 18, Simulect®, Novartis Pharma, Basel, Switzerland), a monoclonal antibody targeting the IL-2R, induction therapy in a dose of 20 mg on Days 0 and 4 after transplantation. Patients received triple maintenance therapy consisting of Tacrolimus (Prograf®, Astellas Pharma Inc., Tokyo, Japan), mycophenolate mofetil (MMF; CellCept®, Hoffmann-La Roche Ltd, Basel, Switzerland) and steroids, which were tapered after 3 months. There were no differences in patient age, gender, cytomegalovirus status and the occurrence of acute rejection episodes between the treatment groups (Table 1). In the rATG group, one patient was lost to follow-up due to graft loss after month 6 and two patients in the basiliximab group because of rATG anti-rejection therapy at month 1 and month 6. The medical ethics committee of the Erasmus Medical Centre approved the study (MEC-2010-022). Blood (heparinized and ethylenediaminetetraacetic acid (EDTA)) was drawn before and at 1, 3, 6 and 12 months after transplantation. Heparinized Lithium blood was used to isolate peripheral blood mononuclear cells (PBMCs) by density gradient centrifugation using standard Ficoll-Paque procedures. Due to a shortage of material we were not able to analyse all parameters for each patient.

Flow cytometry

Absolute numbers of CD4+ T cells.

Absolute counts of CD4+ T cells were determined in EDTA blood using BDMultiTest™ 6 color reagent and BD TruCount™ tubes (BD Biosciences, San Jose, CA), according to themanufacturer's instructions. In brief, EDTA blood (50 µL) was added to 20 µL MultiTest reagent in a BD TruCount tube containing a known number of fluorescent beads and incubated for 15 min in the dark at room temperature. After lysation with BD FACSLysing solution, samples were measured on a FACSCantoII™ (BD Bioscience) and analysed by FACSDiva™software.

Percentage and absolute number of CD4+CD25+CD127− T cells

The percentage of CD4+CD25+CD127− T cells was determined in whole blood. Two hundred microlitres of blood was incubated with the following antibodies: CD3-PerCP, CD4-PB, CD25B-PE-Cy7 (BD Bioscience) and CD127-FITC (eBioscience, San Diego, CA); hereafter, cells were washed, red blood cells lysed, and fixated using lyse/Fix buffer (BD Bioscience). Next, samples were washed and measured on a FACSCantoII and analysed by BD FACSDiva Software. The absolute number of Tregs was determined by multiplying the percentage of CD4+CD25+CD127− T cells with the number of CD4+ T cells.

Table 1. Patient characteristics

Induction Therapy	rATG (n=15)	Basiliximab (n=18)
Male	10	13
Recipient age[#] (yrs)	49 (29-69)	51 (18-72)
DR-MM 0/1/2[$]	4/5/6	4/9/5
No. of first transplants	13	14
Living/Deceased donors	13/2	16/2
Pre-emptive/ Dialysis	8/7	14/4
% cPRA[&]	0 (0-89)	0 (0-98)
CMV: donor-recipient:		
pos-pos	6	8
pos-neg	4	3
neg-pos	4	4
neg-neg	1	3
Primary disease:		
DM	1	2
Hypertension	5	7
PKD	3	1
Glomerulopathy	5	5
Refluxnephropaty	0	3
Congenital urological disorder	1	0
Acute Rejection*	2	5

No: number. [#]: age in years; median and (range). [$]: number of patients with 0, 1 or 2 mismatches on DR-locus. [&]: PRA[c]: Current Panel Reactive Antigen: median and (range). CMV: cytomegalo virus IgG status (positive or negative) before transplantation. DM: diabetes mellitus. PKD: polycystic kidney disease. *: biopsy proven acute rejection (defined according to the Banff 07 classification (41)) within the first year after transplantation.

Phenotypical characterization of CD4+CD127−FoxP3+ regulatory T cells

PBMCs were thawed and extracellular stained with the following fluorochrome-conjugated antibodies: CD3-AmCyan, CD4-APC-H7 (BD Bioscience), CD31-PE (Biolegend), CD127-PeCy7 and CD45RO-PerCP-Cy5 (BD Bioscience). For intracellular staining with FoxP3-APC, Helios-PB (Biolegend) and Ki-67-FITC (BD Bioscience), the anti-human FoxP3 Staining Kit (eBioscience) was used. As negative control, Fluorescence Minus One controls were performed. A minimum of 100 events per population was set for analysis.

Cytokine production

PBMCs were stimulated in the presence of Brefeldin A (Golgiplug, BD Bioscience) with phorbol myristate acetate (50 ng/mL, Sigma-Aldrich, St. Louis, MO) and Ionomycine (1 µg/mL, Sigma-Aldrich) for 4 h. For determining the production of intracellular cytokines,

cells were stained with FoxP3-APC, IL-10-AlexaFluor488 (Biolegend) and IFNγ-PE (BD Bioscience) using the anti-human FoxP3 Staining Kit (eBioscience). Unstimulated cells were used as baseline samples.

Suppression assay

PBMCs were stained with CD3-AmCyan, CD4-PB, CD25-PeCy7, CD127-PE, 7AAD (BD Bioscience) and cell-sorted for Treg; CD3+CD4+CD25+CD127− and Teff; CD4+CD25−CD127+, using the BD FACSAriaII (BD Bioscience) cell sorter. Purity of sorted Tregs and Teff was >95%. FACSsorted Treg were co-cultured in a ratio of 1:5 (Treg:Teff) in a mixed lymphocyte reaction (MLR) consisting of Teff cells stimulated in a 1:1 ratio with γ-irradiated (40 Gy) donor or human leucocyte antigen mismatched (2–2–2) third party stimulator cells. After 7 days' incubation, proliferation was measured as counts per minute (CPM), using Wallac1450 MicroBeta TriLux (PerkinElmer, Groningen, The Netherlands), after 16h incubation with [^{3}H]-thymidine incorporation (0.5 mCi/well; PerkinElmer) before harvesting (as described by Hendrikx et al. (36)). Treg function was defined as Treg capable of inhibiting proliferation of donor or third party antigen stimulated Teff cells.

$$\%\ \text{inhibition} = \frac{\text{CPM Teff} - (\text{CPM Teff} + \text{Treg})}{\text{CPM Teff}} \times 100$$

Methylation status of the FOXP3 gene

The methylation status of the TSDR of the FoxP3 gene was determined in FACSsorted CD3+CD4+CD25+CD127−Treg. Cell pellets were digested with proteinase K and treated with bisulfite using the EZ DNA Methylation-Direct™ Kit (Zymo Research, Irvine, CA). During bisulfite treatment unmethylated cytosines were converted into uracils while methylated cytosines remain unmodified. Hereafter, the TSDR of the FoxP3 gene was amplified by quantitative real-time PCR using the StepOnePlus™ Real-Time PCR System and the TaqMan® Genotyping Master Mix (Applied Biosystems, Foster City, CA). Methylation-specific and demethylation-specific amplification primers and probes were chosen as described by Wieczorek et al. (37). The percentage of Treg with a demethylated TSDR was calculated using the ratio of amplified demethylated TSDR copies and the sum of amplified methylated and unmethylated TSDR copies. In female patients, the percentage of demethylated TSDR was multiplied by two to correct for the X-linked nature of the FoxP3 gene.

Statistical analysis

Statistical analyses were performed using Graphpad Prism version 5.01. For comparisons within multiple groups the Kruskal–Wallis with Dunn's multiple comparison test, for comparison between time points or groups, the Mann Whitney U-test was used.

Analysis of contingency tables was conducted using Fisher's exact test. For relations between parameters, a linear regression model was used and Spearman Rho correlation coefficient (rs) was calculated. P values ≤ 0.05 were considered statistically significant.

RESULTS

CD4+ T cells and CD4+CD25+CD127− T cells

The absolute number of CD4+ T cells and CD4+CD25 +CD127− T cells were determined in the blood of kidney transplant patients treated with rATG or basiliximab induction therapy (Fig. 1A–C). As basiliximab hinders the detection of CD25 by certain antibodies, we used clone MA251 detecting CD25epitope B, which does not interfere with basiliximab (38). At 1 month after rATG induction we observed a significant decrease in the absolute number of both CD4+ T cells and CD4+CD25+CD127− T cells (P<0.001 versus pre-transplantation, Fig. 1B). Thereafter the number of CD4+ T cells and CD4+CD25+CD127− T cells slowly increased but did not reach pre-transplant levels (Fig. 1B). Basiliximab treated patients showed no differences in the absolute number of CD4+ T cells while the absolute numbers of CD4+CD25 +CD127− T cells decreased at 1 and 3 months (P<0.01, Fig. 1C). At all post-transplant time points, basiliximab treated patients had higher absolute numbers of CD4+ and CD4+CD25 +CD127− T cells (Fig. 1B, C, P<0.001). Figure 1D depicts the proportion of CD4+CD25+CD127− T cells within the CD4+ T-cell population; at 3 and 6 months after rATG induction therapy the percentage of Treg was higher than before transplantation (Fig. 1D, P<0.05). In contrast, the proportion of Treg after basiliximab induction therapy was significantly lower for the whole study period (P<0.01). At month 3 and month 6, rATG treated patients had a higher percentage of CD4+CD25+CD127− T cells than basiliximab treated patients (Fig. 1D, P<0.01).

Regulatory T cells after induction therapy and repopulation mechanisms

In addition, Tregs were characterized for the Treg marker FoxP3 and the memory marker CD45RO (Fig. 2A). At month 6, higher percentages of CD4+CD127−FoxP3+ Treg were observed in the rATG group and lower percentages in the basiliximab group (P<0.05, Fig. 2B) than in patients before transplantation. In both patient groups, at all time points, the majority of CD127−FoxP3+ Treg expressed CD45RO, though after rATG treatment the CD45RO expression remained constant, while we found a low percentage of CD4+CD45RO +CD127−FoxP3+ Treg, 6 months after basiliximab therapy (P<0.05, Fig.2C).

Thymopoiesis, the formation of CD31 expressing RTEs, is an age related process; the presence of CD31+ naive T cells decreases with age (39, 40). This inverse correlation was also present for CD31+CD45RO− Treg and age, before and after transplantation (Fig.3B;

r=−0.6798, P=0.04 and r=−0.5671, P<0.001). Consequently, we used CD31 as a marker for RTE-Treg. No differences in the percentage of RTE-Treg were observed (Fig. 3C). Homeostatic proliferation of CD4+CD127−FoxP3+ Treg was measured by the expression of Ki-67, an antigen present during the G1–M phase of the cell cycle (Fig.3D) (41). Patients

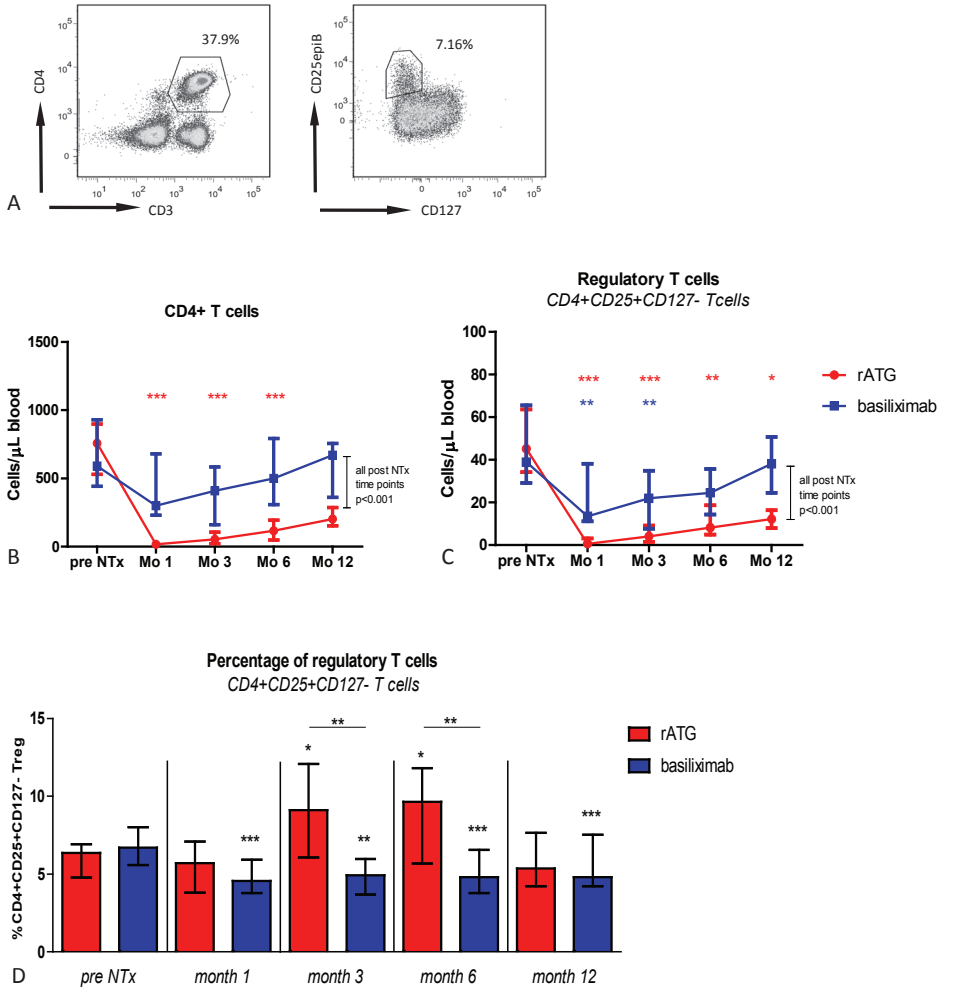


Figure 1. Whole blood analyses of CD4+ and CD4+CD25+CD127- T cells. **(A)** The absolute number of CD4+ and CD4+CD25+CD127- T cells was determined by flow cytometry. **(B)** The absolute number of CD4+ T cells before transplantation (pre NTx) and at 1, 3, 6 and 12 months after transplantation in patients treated with rATG (red) and basiliximab (blue) induction therapy. **(C)** The absolute number of CD4+CD25+CD127- T cells pre NTx and at 1, 3, 6 and 12 months after transplantation in patients treated with rATG (red) and basiliximab (blue) induction therapy. **(D)** The percentage of CD4+CD25+CD127- T cells within the total CD4+ T cell population pre NTx and at 1, 3, 6 and 12 months after rATG (red) and basiliximab (blue) induction therapy, compared to the percentage CD4+CD25+CD127- T cells before transplantation. Data are shown as median ± Inter Quartile Range (IQR), * p<0.05, ** p<0.01, *** p<0.001.

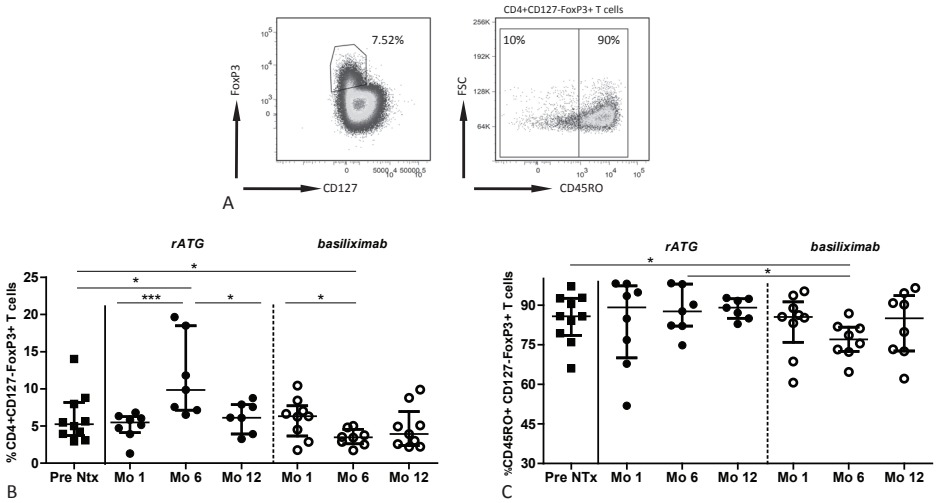
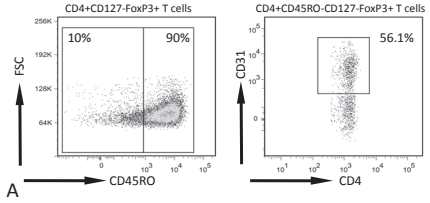


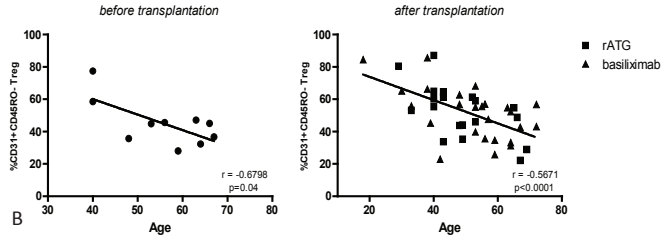
Figure 2. The phenotype of CD4+CD127-FoxP3+ Treg after induction therapy. **(A)** Representative example of CD127 and FoxP3 expression by CD4+ T cells and the expression of the memory marker CD45RO in CD4+CD127-FoxP3+ Treg. **(B)** The percentage CD4+CD127-FoxP3+ Treg in patients before and in rATG and basiliximab treated patients 1, 6 or 12 months after transplantation. **(C)** The percentage of CD45RO expressing CD4+CD127-FoxP3+ Treg in patients before and in rATG and basiliximab treated patients 1, 6 or 12 months after transplantation. Due to a shortage of material we were not able to measure all parameters for all patients. Data are shown as median ± IQR. * p<0.05, ** p<0.01, *** p<0.001.

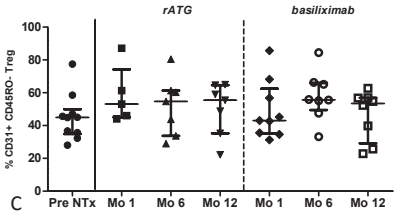
at 1 month after rATG induction therapy had higher percentages of Ki-67+ Tregs than patients before transplantation (Fig. 3E, P<0.001). At 1 month after basiliximab therapy, patients had low percentages of Ki-67+ Tregs. Surprisingly, at 6 months after basiliximab, the percentage of Ki-67+ Tregs was higher than in patients before transplantation (Fig. 3E, P<0.05). A comparable expression pattern of Ki-67 was seen by CD45RO+, memory Treg (Fig. 3F), while for the CD45RO− naïve Treg, only at 6 months after basiliximab therapy, were high percentages of Ki-67+CD4+CD45RO−CD127−FoxP3+ Treg observed (P<0.05, Fig. 3G).

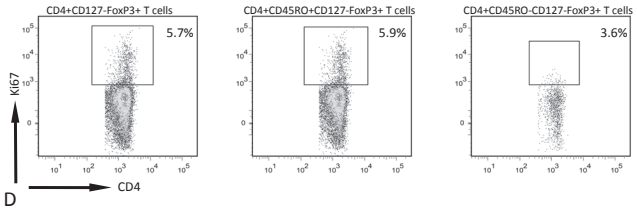
Origin of repopulated Tregs

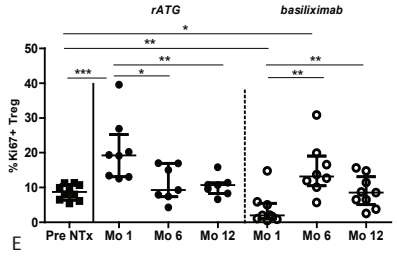
To determine the origin of Tregs present after induction therapy we studied Helios expression by Ki-67+CD4+CD45RO+CD127−FoxP3+ Treg and the FoxP3 demethylation profile of cell-sorted CD4+CD25+CD127− Treg (Fig. 4). At 1 and 12 months after rATG therapy, low percentages of Helios+ Ki-67+CD4+CD45RO+CD127−Foxp3+ Treg were measured (Fig. 4B, P<0.05), suggestive for the generation of iTreg, while after basiliximab therapy the percentage of Helios+ Ki-67+CD4+CD45RO+CD127−Foxp3+ Treg was comparable (Fig. 4B). The relation between the expression of Helios and Ki-67 in CD45RO+Treg is shown in Figure 4C. From a subset of patients, we had samples at 6–12

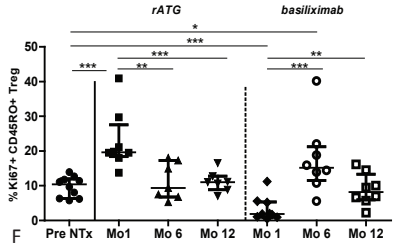

CD4+CD127-FoxP3+ T cells
10%
90%
FSC
CD45RO
CD4+CD45RO-CD127-FoxP3+ T cells
56.1%
CD31
CD4
A


before transplantation
after transplantation
%CD31+CD45RO- Treg
r = -0.6798
p=0.04
r = -0.5671
p<0.0001
Age
rATG
basiliximab
B


rATG
basiliximab
% CD31+ CD45RO- Treg
Pre NTx
Mo 1
Mo 6
Mo 12
C


CD4+CD127-FoxP3+ T cells
5.7%
CD4+CD45RO+CD127-FoxP3+ T cells
5.9%
CD4+CD45RO-CD127-FoxP3+ T cells
3.6%
Ki67
CD4
D


rATG
basiliximab
% Ki67+ Treg
Pre NTx
Mo 1
Mo 6
Mo 12
E


rATG
basiliximab
% Ki67+ CD45RO+ Treg
Pre NTx
Mo1
Mo 6
Mo 12
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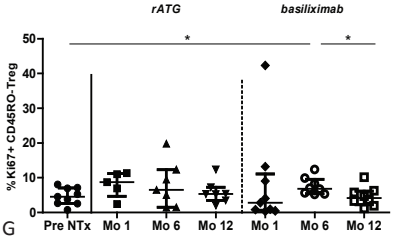

rATG
basiliximab
% Ki67+ CD45RO- Treg
Pre NTx
Mo 1
Mo 6
Mo 12
G

Figure 3. Thymopoiesis and homeostatic proliferation after induction therapy. **(A)** Representative example of the gating strategy of RTE-Treg; CD4+CD31+CD45RO-CD127-FoxP3+ T cells. **(B)** Correlation between age and CD4+CD31+CD45RO-CD127-FoxP3+ Treg before and after transplantation; left: before transplantation, r=-0.6798, p<0.05, right: after transplantation, r=-0.5671, p<0.001. **(C)** The percentage CD4+CD31+CD45RO-CD127-FoxP3+ Treg in patients before transplantation (Pre NTx) and in patients at 1, 6 or 12 months after rATG or basiliximab induction therapy. **(D)** Representative example of the gating strategy of the expression of the proliferation marker Ki-67 by CD4+CD127-FoxP3+ Treg and in the naïve, CD45RO-, and memory, CD45RO+, subset. **(E)** The percentage of Ki-67+ CD4+CD127-FoxP3+ Treg in patients before and in patients at 1, 6 or 12 months after rATG or basiliximab induction therapy. **(F)** The percentage of Ki-67+ CD4+CD45RO+CD127-FoxP3+ Treg in patients before transplantation and in patients at 1, 6 or 12 months after rATG or basiliximab induction therapy. **(G)** The percentage of Ki-67+ CD4+CD45RO-CD127-FoxP3+ Treg in patients before transplantation and in patients at 1, 6 or 12 months after rATG or basiliximab induction therapy. Due to a shortage of material we were not able to measure all parameters for all patients. Data are shown as median ± IQR. * p<0.05, ** p<0.01, *** p<0.001.

months after induction therapy available to study the methylation of TSDR in the FoxP3 gene, where a demethylated TSDR represents an nTreg. Figure 4D shows that after rATG induction therapy, the percentage of cells with a demethylated TSDR in the FOXP3 gene was lower than after basiliximab therapy, suggestive for the induction of iTreg after rATG therapy (P<0.05).

Functional capacities of regulatory T cells

We next studied the functional capacities of repopulated Treg. There was no difference in the IFNγ production capacity by Treg in patients before and after induction therapy (Fig. 5A, B). For the IL-10 production capacity of Tregs, we observed a different pattern. At 1 and 12 months after rATG therapy, Tregs had a higher IL-10 production capacity than Tregs from patients before transplantation, while only at month 1 after basiliximab a higher IL-10 production was observed (Fig. 5C).

The suppressive capacity of the repopulated Tregs was studied by the classical MLR suppression assay. Teff cells from patients before and at 6–12 months after transplantation were stimulated with donor or third party antigen, in the absence or presence of FACSsorted CD4+CD25+CD127− Treg (Treg:Teff = 1:5). Before transplantation, there was no difference in the capacity of Treg to inhibit donor or third party stimulated proliferation of Teff cells; respectively, 6 and 5 out of 11 patients inhibited the proliferative response to donor and third party stimulated cells (Fig. 5D). After rATG therapy, Treg from four out of five patients inhibited donor stimulated proliferation but could not control proliferation of third party stimulated Teff cells (Fig. 5D, E, P<0.05). After basiliximab therapy,

Tregs comparably inhibited donor and third party stimulated Teff cells in both situations Treg from 4 out 7 patients inhibited Teff proliferation. Due to a large variation in the suppressive capacity of Treg, no statistical differences were found in the percentage of inhibition (Fig. 5E).

Rejectors versus non-rejectors

We observed no difference in the occurrence of acute rejection episodes (biopsy proven and defined according to the Banff 07 classification (35) between rATG and basiliximab treated patients (Table 1). At baseline, no differences were found between rejectors and non-rejectors. No associations were found between rejection and the studied parameters.

DISCUSSION

To define the impact of induction therapy on the immunesuppressive counter mechanism, we here studied different Treg features after rATG and basiliximab induction therapy. In absolute numbers, rATG therapy depleted CD4+CD25+CD127− T cells with an incomplete recovery at 12 months. During repopulation, particularly the first 6 months, high percentages of CD4+CD25+CD127− T cells and CD4+CD127−FoxP3+ T cells were observed. Basiliximab treated patients showed decreased absolute numbers of CD4+CD25+CD127− T cells, which was accompanied by a decreased percentage of CD4+CD25+CD127− T cells, for at least 1 year. For CD4+CD127−FoxP3+ T cells a different observation was made; only at 6 months low percentages of CD4+CD127−FoxP3 T cells were found. After both induction therapies, the Treg pool reconstituted by homeostatic proliferation of Treg with the memory phenotype. However, rATG treated patients had increased percentages of Helios-iTreg, with a demethylated FoxP3 gene, while after basiliximab no changes in Treg phenotype were found. After T-cell depletion, repopulated Treg were able to inhibit donor, but not third party antigen, stimulated Teff cells.

After T-cell depletion, in adult patients, homeostatic proliferation largely contributes to T-cell reconstitution as thymopoiesis is inversely correlated with age (5, 14, 15, 42–44). However, in patients suffering from T-cell depletion due to chemotherapy, human immunodeficiency virus infection or rATG therapy, increased thymopoiesis has been described (5, 45–47). We could not confirm these data. An explanation for these findings may be the use of CD31 as RTE-marker, as CD31+ naïve CD4+ T cells in human adults can also be formed by peripheral proliferation (48). However, as shown earlier by our group, the percentages of Ki-67+CD4+CD31+CD45RO− T cells after rATG therapy are small. Therefore, we speculate that the contribution of proliferating CD31+ naive Treg is limited. Furthermore, differences in timing (5) or a difference in the extent of end stage renal disease and consequently the duration of uraemia, which influences thymic output (49–51), may underlie the different findings.

As high percentages of memory T cells are found in the circulation after T-cell depletion therapy, Treg repopulation is thought to be the result of homeostatic proliferation of memory FoxP3+ T cells (5, 41, 44). In addition, in vitro experiments showed that rATG converts CD25− effector T cells into CD25+FoxP3+ suppressor cells (8, 16, 18, 20).

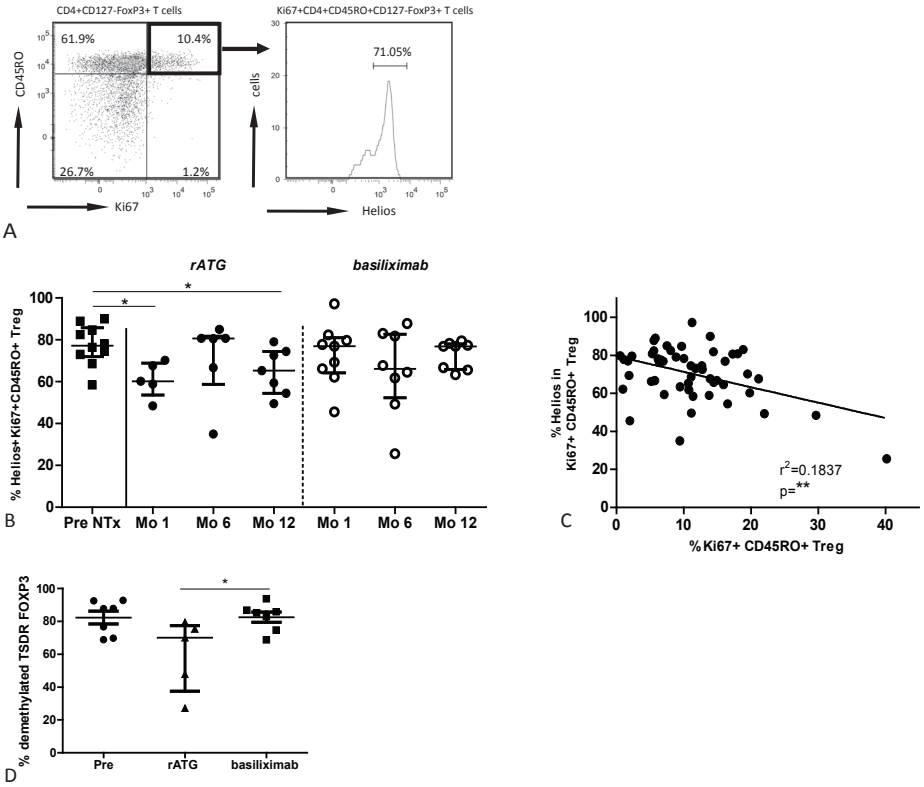
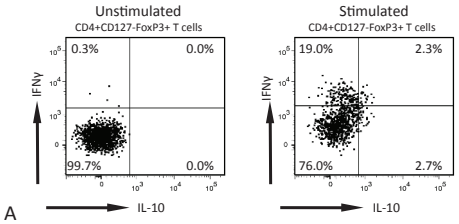


Figure 4. Helios expression and the demethylation status of the *FoxP3* gene. **(A)** Helios expression was measured in Ki67+CD4+CD45RO+CD127-FoxP3+ Treg by flow cytometry. **(B)** The percentage Helios+ Ki-67+CD4+CD45RO+CD127-FoxP3+ Treg in patients before transplantation and in patients at 1, 6 or 12 months after rATG or basiliximab induction therapy. **(C)** The relation between the percentage of Ki-67+CD45RO+ T cells and the expression of Helios in Ki-67+CD45RO+T cells and **(D)** The percentage of cells with a demethylated TSDR in the *FoxP3* gene in cell sorted CD4+CD25-CD127+ Treg of patients before transplantation and in patients 6-12 months after rATG or basiliximab induction therapy. Due to a shortage of material we were not able to measure all parameters for all patients. Data are shown as median ± IQR. * $p<0.05$

However, Broady et al. (52) reported that these cells were just activated FoxP3+ cells. By analysing the proliferation marker Ki-67, we found, shortly after rATG therapy, indeed high percentages of especially Ki-67+CD45RO+, memory, Treg. In addition, our findings of the nTreg-marker Helios and the methylation status of the FoxP3 gene, as well as the suppressive capacities of Treg, showed that rATG induces iTreg in vivo and that these cells were not activated cells. Though, the Treg repopulation mechanisms do not completely restore the Treg pool (Fig. 1). Phenomena like T-cell exhaustion and deprivation of the cytokine milieu may explain this incomplete recovery (15, 53).

After basiliximab therapy, low numbers of CD4+CD25+CD127− T cells were also observed. This cannot be explained by competition between basiliximab and anti-CD25 antibodies (38). Some reports state that after basiliximab therapy, CD25+ and CD25+FoxP3+ T cells are depleted, while others state that functional, suppressive,



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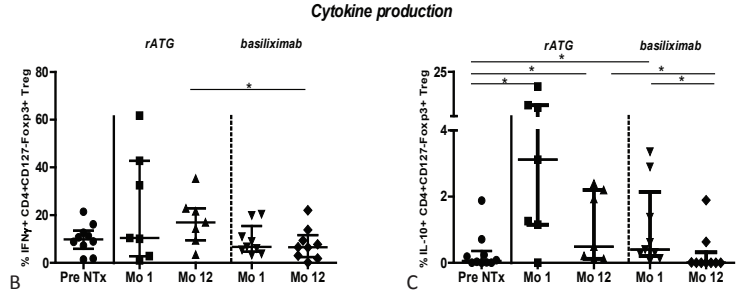


B C

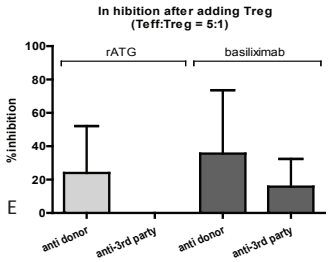
	Functional CD4+CD25+CD127- T cells (n$) *		
	Donor	3rd party	p value
Pre NTx			
all (n=11)#	6	5	ns
Post NTx			
rATG (n=5)	4	0	p<0.05
basiliximab (n=7)	4	4	ns

D

$ n= number

*Suppressive Treg were defined as cells that are able to inhibit the proliferative response of donor or third party antigen stimulated T effector cells, as describedby Hendrikx et al (35)

1 patients sample was lost as a result of a technical problem



E

Figure 5. Functional capacities of repopulated Treg. **(A)** The capacity of repopulated Treg to produce IFNγ and IL-10 was measured by flow cytometry. **(B)** The capacity of Treg to produce IFNγ and **(C)** IL-10 was measured in patients before transplantation and in patients at 1 or 12 months after rATG or basiliximab induction therapy **(D-E)** The suppressive capacity of FACSsorted CD4+CD25+CD127- T cells from patients before transplantation and patients 6-12 months after rATG or basiliximab induction therapy, was measured in a classic suppression assay where effector T cells were stimulated with donor or third party antigen. Suppressive capacity was defined as **(D)** a decrease in the incorporation of 3H after 7 days of incubation of Teff cells in the presence of Treg (Treg:Teff=1:5), and **(E)** by the percentage of inhibition of proliferation of Teff cells after adding Treg in a ratio of Treg:Teff = 1:5 for 7 days. Due to a shortage of material we were not able to measure all parameters for all patients. Data are shown as median ± IQR. * p<0.05.

FoxP3+ cells remain in the circulation, albeit with low CD25 expression, caused by modulation or shedding of the CD25 molecule (7, 9, 12, 27–29). Our findings point to an effect on the CD25 molecule as well, as at 1 month after basiliximab therapy low percentages of CD4+CD25+CD127− T cells, but not of CD4+CD127−FoxP3+ T cells, were found. At 6 months after basiliximab, CD4+CD127−FoxP3+ T cells were affected. This is most likely the net-result of the given immunosuppressive medication, or late apoptotic events triggered by the earlier blockade of the IL-2–IL-2R pathway.

To determinate the origin of Ki-67+Treg, we analysed the expression of Helios, a marker for nTreg (34). In the rATG group, relatively low percentages of Helios+ proliferating Treg were measured, suggesting a contribution of Helios-iTreg. After basiliximab therapy, we did not observe this phenomenon. Currently, there is some debate about the value of Helios as a marker for nTreg as recently published studies showed that this marker is also up regulated by Treg and non-Treg after stimulation (54). In addition, it was found that Helios− and Helios+ Treg, co-exist in the nTreg pool (55–58). Therefore, we complemented our Helios data with the analysis of the demethylation status of the FoxP3 gene. After rATG therapy, the percentage of CD4+CD25+CD127− Tregs with a demethylated TSDR of the Foxp3 gene, i.e. nTreg, decreased confirming our Helios findings.

Among the various mechanisms by which Treg suppress immune responses, the immunoregulatory cytokine IL-10 plays a prominent role (13). After rATG and basiliximab therapy, the IL-10 production capacity was higher than in patients before transplantation. When we studied the suppressive capacities of Treg, differences between groups were observed. After rATG therapy the FACSsorted CD4+CD25+CD127− Tregs suppressed donor antigen-specific proliferation of Teff cells, whereas proliferation in response to third party antigen was insufficiently inhibited. Tregs of basiliximab treated patients comparably inhibited the proliferation of donor and third party stimulated Teff cells. This difference in suppression between the two treatment groups might be related to the difference found in Helios expression and the demethylation status of the FoxP3 gene; the relative increase in iTreg favoured by rATG treatment. However, as in our study no significant differences were found in the percentage of inhibition between treatment groups and between donor and third party stimulated effector T cells, we can only speculate that these newly formed iTreg, after rATG therapy, have donor-specific immunoregulatory properties.

In conclusion, in kidney transplant patients, repopulation of Treg after rATG and basiliximab therapy is the result of homeostatic proliferation and not of thymopoiesis. These repopulated Treg were functional after both induction strategies; however, only after rATG therapy, were increased proportions of Helios− methylated FoxP3 Treg found.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest. For laboratory consumables the study was partially financially supported by Sanofi/Genzyme (Paris, France). The authors declare that the results presented in this paper have not been published previously in whole or part, except in abstract form.

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Chapter 5

T cells exhibit reduced signal transducer and activator of transcription 5 phosphorylation and upregulated coinhibitory molecule expression after kidney transplantation.

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ABSTRACT

Background

T-cell depletion therapy is associated with diminished interleukin (IL)-7/IL-15-dependent homeostatic proliferation resulting in incomplete T-cell repopulation. Furthermore, it is associated with impaired T-cell functions. We hypothesized that this is the result of impaired cytokine responsiveness of T cells, through affected signal transducer and activator of transcription (STAT)5 phosphorylation and upregulation of coinhibitory molecules.

Materials and Methods

Patients were treated with T-cell depleting rabbit antithymocyte globulin (rATG) (6 mg/kg, n = 17) or nondepleting, anti-CD25 antibody (basiliximab, 2 x 40 mg, n = 25) induction therapy, in combination with tacrolimus, mycophenolate mofetil, and steroids. Before and the first year after transplantation, IL-7 and IL-2 induced STAT5 phosphorylation, and the expression of the coinhibitory molecules programmed cell death protein 1 (PD-1), T cell immunoglobulin mucin-3 (TIM-3), lymphocyte activation gene-3 (LAG-3), cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), cluster of differentiation (CD) 160, and CD244 was measured by flow cytometry.

Results

The first year after rATG, CD4+, and CD8+ T cells were affected in their IL-7–dependent phosphorylation of STAT5 (pSTAT5) which was most outspoken in the CD8+ memory population. The capacity of CD4+ and CD8+ T cells to pSTAT5 in response to IL-2 decreased after both rATG and basiliximab therapy. After kidney transplantation, the percentage of TIM-3+, PD-1+, and CD160+CD4+ T cells and the percentage of CD160+ and CD244+CD8+ T cells increased, with no differences in expression between rATG- and basiliximab-treated patients. The decrease in pSTAT5 capacity CD8+ T cells and the increase in coinhibitory molecules were correlated.

Conclusion

We show that memory T cells in kidney transplant patients, in particular after rATG treatment, have decreased cytokine responsiveness by impaired phosphorylation of STAT5 and have increased expression of coinhibitory molecules, processes which were correlated in CD8+ T cells.

INTRODUCTION

Commonly used induction agents in kidney transplant patients are T-cell depleting rabbit antithymocyte globulin (rATG), consisting of multiple antibodies directed toward T cells and non–T cells and alemtuzumab, a humanized monoclonal antibody directed against CD52. Also, the nondepleting basiliximab, a monoclonal antibody directed against CD25, the α-chain of the interleukin (IL)-2 receptor (IL-2R) is often used (1-3). After T-cell depletion therapy, immune reconstitution is associated with increased proportions of memory T cells. In general, memory T cells rapidly and vigorously respond to antigens (4-6). The predominance of these cells after immune reconstitution does, however, not result in increased necessity of immunosuppressive therapy. In contrast, these patients can be treated with reduced dosages of immunosuppressive drugs (7-11).

After T-cell depletion therapy, decreased T-cell reactivity to donor, third party and recall antigens, was measured by mixed lymphocyte reactions and interferon-γ enzyme-linked ImmunoSpot assays in kidney transplant patients (12-15). Increased percentages of suppressive, regulatory T cells may explain, at least in part, these findings (5,14,16,17). However, we here postulate that other mechanisms are also involved; immunosuppressive drugs intrinsically affect memory T-cell function by affecting cytokine signaling via the Janus kinase (JAK)–signal transducer and activator of transcription (STAT) pathway. This hypothesis is supported by our finding that after rATG T-cell depletion therapy, IL-7/15R expressing T-cells numbers do not recover to pre-transplant level as a result of diminished homeostatic proliferation, a process dependent on the homeostatic cytokines IL-7 and IL-15 (18,19).

The homeostatic cytokines IL-7 and IL-15 are part of the common cytokine receptor γ-chain family and signal via the JAK-STAT pathway. On binding of IL-7 and IL-15 to their receptors, JAK molecules associate with the intracellular domain of the receptor, are activated and phosphorylate the receptor that subsequently act as docking place for STAT 5a and 5b. After recruitment, these STAT molecules become phosphorylated (p), form heterodimers, and interact with specific DNA sequences to modify the expression of genes involved in T-cell activation, differentiation, survival, and proliferation (20-23). Impaired STAT signaling was observed in T cells of patients with chronic hepatitis C virus (HCV) and human immunodeficiency virus (HIV) infections. These cells also had poor effector functions and high expression of the coinhibitory molecules PD-1 and/or TIM-3 (24-26). Increased expression of coinhibitory molecules was also observed in T cells of patients with cytomegalovirus (CMV) and Epstein-Barr virus (EBV) infection (27,28). These cells, called exhausted T cells, are induced by persistent antigen exposure and have altered memory maintenance. They poorly respond to IL-7 and IL-15 and need binding with their cognate antigen for homeostasis and survival (29,30). Apart from PD-1 and TIM-3, also the sustained high expression of the coinhibitory molecules LAG-3, CTLA-4, CD160, and CD244 is associated with poor T-cell effector functions (29).

In the present study, we examined the hypothesis that kidney transplantation impairs cytokine responsiveness of memory T cells by affecting STAT phosphorylation and up-regulating coinhibitory molecules. For that purpose, we determined in patients treated with rATG or basiliximab induction therapy, and in patients with or without CMV infection, the STAT5 phosphorylation capacity and the expression of various coinhibitory receptors in naive and memory CD4+ and CD8+ T cells.

Table 1. Patient characteristics

Induction therapy	**rATG (n=17)**	**Basiliximab (n=25)**	
Male	11	17	
Recipient age (yrs)[#] (median + range)	49 (28-69)	56 (18-77)	
DR-MM 0/1/2[$]	4/5/8	4/14/7	
No. of first transplants	14	21	
Living/Deceased donors	15/2	22/3	
Pre-emptive/ Dialysis	9/8	8/17	
% cPRA (median + range)	0 (0-89)	0 (0-98)	
Primary disease:			
DM	2	5	
Hypertension	5	8	
PKD	3	2	
Glomerulopathy	5	6	
Refluxnephropaty	0	3	
Congenital urological disorder	2	0	
other	0	1	
CMV: donor-recipient:			
pos-pos	7	13	
pos-neg	5	5	
neg-pos	4	3	
neg-neg	1	4	
CMV: primo infection	2	1	
CMV: reactivation	6	1	*p<0.01*
Rejection*	3	6[‡]	

$: number of patients with 0, 1 or 2 mismatches on DR-locus. cPRA: current panel reactive antigen. DM: diabetes mellitus. PKD: polycystic kidney disease. CMV donor-recipient: cytomegalo virus donor-recipient combination, pos: IgG positive (seropositive), neg: IgG negative (seronegative). CMV primo infection: positive CMV Polymerase Chain Reaction-test in CMV-seronegative patients within the first year after transplantation. CMV reactivation: positive CMV Polymerase Chain Reaction-test in CMV-seropositive patients within the first year after transplantation. *: Biopsy proven acute rejection within the first year after transplantation defined according to the Banff 07 classification. ‡: 6 rejection episodes in 5 patients.

MATERIALS AND METHODS

Patients

Blood samples were obtained from 42 consecutive kidney transplant patients treated with either rATG, 2 mg/kg at days 1, 2, and 3 after transplantation (n = 17, Thymoglobuline; Genzyme/Sanofi, Paris, France), or basiliximab, 20 mg on days 0 and 4 after transplantation (n = 25, Simulect; Novartis Pharma, Basel, Switzerland) induction therapy. Patients received maintenance therapy consisting of Tacrolimus (Prograf; Astellas Pharma Inc, Tokyo, Japan), mycophenolate mofetil (CellCept; Hoffmann-La Roche Ltd., Basel, Switzerland), and steroids, the last tapered to 0 within approximately 3 months. Patients received CMV prophylaxis with valganciclovir (Hoffmann-La Roche Ltd.) for 4 months, except for the combination of CMV seronegative donor and recipient. Before transplantation, there were no differences in patient characteristics between the treatment groups (Table 1). The medical ethics committee of the Erasmus Medical Centre approved the study (MEC-2010-022). Blood samples were drawn before and at 1, 3, 6, and 12 months after transplantation.

Isolation of peripheral blood mononuclear cells

Heparinized blood was used to isolate peripheral blood mononuclear cells (PBMC) by density gradient centrifugation (18). The PBMC were frozen in 10% dimethylsulfoxide-enriched medium (RPMI 1640-GlutaMAX-I) and stored at −140°C.

Flow cytometry

Absolute counts of CD4+ and CD8+ T cells were determined in ethylenediamine tetraacetic acid blood using BD MultiTest 6 color reagent and BD TruCount tubes (BD Biosciences, San Jose, CA), according to the manufacturer's instructions. In brief, ethylenediamine tetraacetic acid blood (50 µL) was added to 20 µL MultiTest reagent in a BD TruCount tube containing a known number of fluorescent beads and incubated for 15 minutes in the dark at room temperature. After lysation with BD FACSLysing solution, samples were measured on a FACSCantoII flow cytometer (BD Bioscience) and analyzed by FACSDiva software.

STAT5 phosphorylation capacity was determined in CD4+ and CD8+ naive (CCR7+CD45RO−), central memory (CCR7+CD45RO+), effector memory (CCR7−CD45RO+), and effector memory re-expressing CD45RA (EMRA) (CCR7−CD45RO−) T cells, by phosphospecific flow cytometry. Heparinized blood, 200 µL, was stained using; CD3-AmCyan (BD Bioscience), CD8-FITC and CCR7-PE-Cy7 (both BD Biosciences Pharmingen) antibodies. Hereafter, samples were stimulated with 50 µL IL-7 (100 ng/mL; PeproTech, London, UK), IL-2 (Proleukin 2000 IU/mL; Novartis Pharma GmbH, Nürnberg,Germany), or medium alone (unstimulated sample) for 30minutes at 37°C. Next, cellswere lysed and fixed using Lyse/Fix Buffer (BD Biosciences). For intracellular staining, cells were

permeabilized with cold 70% methanol for 30 minutes at −20 °C, washed in staining buffer with 0.5% bovine serum albumin, and stained with CD4-PerCP (BD Bioscience), CD45RO-PB (Biolegend, San Diego, CA) and pSTAT5-PE (BD Bioscience) antibodies. CD4 and CD45RO were stained after permeabilization with 70% methanol as this affects the CD4-PerCP and CD45RO-PB staining. Cells were measured and analyzed as described above. A minimum of 100 events per T-cell subset was set for analysis. The effect of IL-2 and IL-7 on STAT5 phosphorylation was calculated by the percentage pSTAT5-positive cells after stimulation minus the unstimulated sample.

Expression of coinhibitory receptors was determined on defrosted PBMCs. Cells were stained for 30 minutes in the dark at room temperature with CD3-AmCyan (BD Bioscience), CD8-APC, CD4-FITC, CCR7-PeCy7, CD45ROPerCP-Cy5.5, CD160-PE, CTLA4-APC, PD-1-PE (all BD Bioscience Pharmingen), TIM-3-APC, CD244-APC (both, eBioscience, San Diego, CA) and LAG-3-PE (R&D Systems, Minneapolis, MN) antibodies. Cells were measured and analyzed as described above.

Statistical analysis

Statistical analyses were performed using Graphpad Prism version 5.01. For comparisons within time points, the Kruskal-Wallis with Dunn multiple comparison test was used and for comparison with pretransplant levels, the Wilcoxon signed rank test was used. Contingency tables were analyzed using Fisher exact test. Correlations between parameters were analyzed with Spearman rank correlation coefficient. P values of 0.05 or less were considered statistically significant.

RESULTS

The T-cell compartment after kidney transplantation

We studied the T-cell compartment in 17 patients treated with rATG and 25 patients treated with basiliximab therapy. Before transplantation, patient characteristics were comparable between groups. After transplantation, 1 patient died to a cardiovascular event after month 1 and 1 patient lost the graft due to chronic transplant glomerulopathy after month 6 in the rATG treatment group. In the basiliximab group, 2 patients received rATG antirejection therapy after months 3 and 6; 1 patient suffered from graft loss due to thrombosis of the iliac vein after month 3; 1 patient died of respiratory failure during urosepsis after month 3; and 2 patients discontinued follow-up after months 3 and 6. After transplantation, the incidence of rejection and CMV primo infections were comparable, the incidence of CMV reactivation was significantly higher in the rATG than in the basiliximab group ($P<0.01$; Table 1).

Induction therapy with rATG resulted, at 1 month, in low numbers of CD4+ and CD8+ T cells, followed by a slow recovery of CD4+ and a much faster recovery of CD8+ T cells

(Fig. 1B). Relatively few CD4+ and CD8+ naive T cells were present after rATG. In the CD4+ population, especially effector memory T cells repopulated, while for the CD8+ T cells this were mainly EMRA cells (Fig. 1D, E). After basiliximab induction therapy, no statistically significant alterations in the absolute number of CD4+ and CD8+ T cells and their subsets were found (Fig. 1C); however, the proportion of cells did alter. The first 6 months, a higher proportion CD4+ naive T cells was present, where for the CD8+ population, a decrease in naive and central memory and an increase in EMRA T cells was observed at 12 months (P<0.05, Fig. 1F-G).

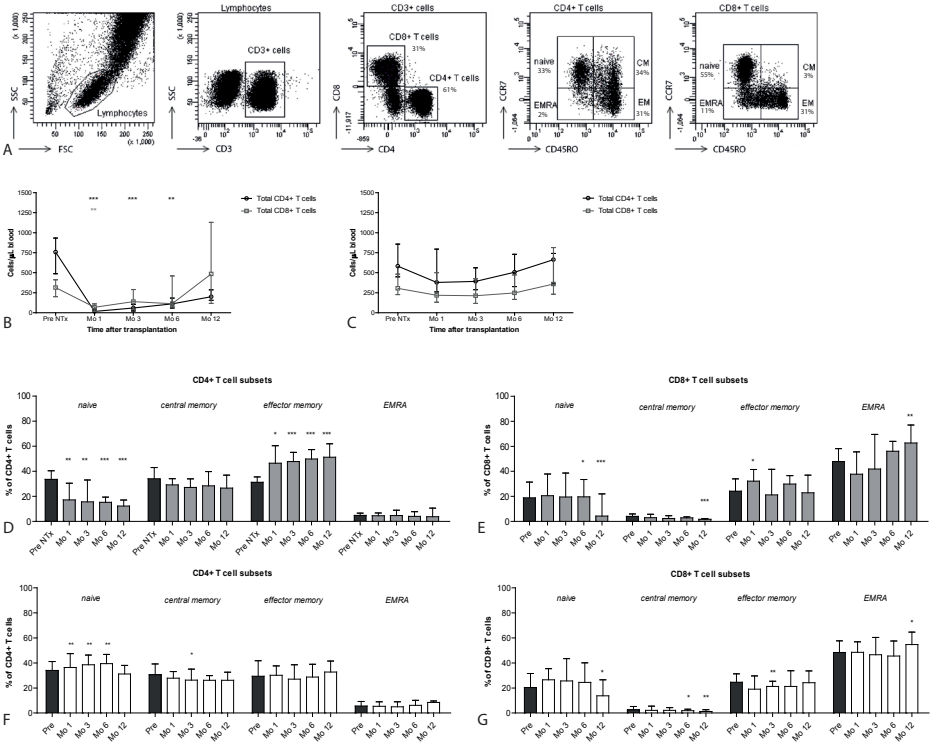


Figure 1. The T-cell compartment after kidney transplantation. **(A)** Typical example of the gating strategy; CD4+ and CD8+ naive (CCR7+CD45RO–), central memory (CCR7+CD45RO+), effector memory (CCR7-CD45RO+) and EMRA (CCR7-CD45RO–) T cells. **(B)** Absolute number of CD4+ (black) and CD8+ (grey) T cells before and at months 1, 3, 6, and 12 after rATG therapy. **(C)** Absolute number of CD4+ (black) and CD8+ (grey) T cells before and at months 1, 3, 6, and 12 after basiliximab therapy. **(D-E)** Percentage CD4+ (D) and CD8+ (E) naïve, central memory, effector memory, and EMRA T cells before and at months 1, 3, 6, 12 after rATG therapy. **(F-G)** Percentage CD4+ (F) and CD8+ (G) naive, central memory, effector memory, and EMRA T cells before and at months 1, 3, 6, and 12 after basiliximab therapy. Data are shown as median ± IQR, *P<0.05, **P<0.01, ***P<0.001; significant differences comparing pretransplant values to posttransplant values using the Kruskal-Wallis or the Wilcoxon Signed-Rank test. IQR, interquartile range.

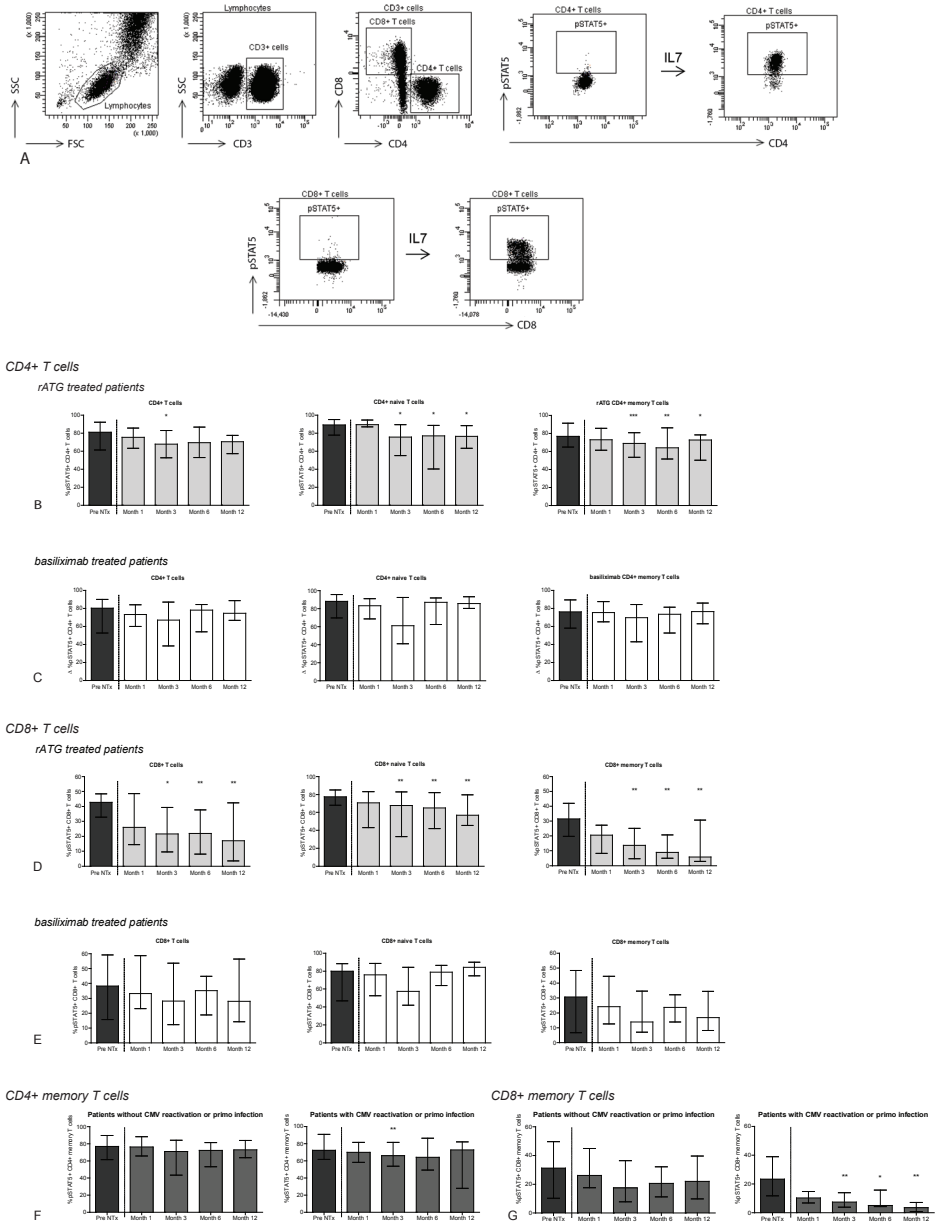


Figure 2. IL-7–induced STAT5 phosphorylation capacity, IL-7–stimulated percentage pSTAT5 minus unstimulated percentage pSTAT5. **(A)** Typical example of the pSTAT5 gating unstimulated and after IL-7 stimulation. **(B-C)** STAT5 phosphorylation capacity of total, naive, and total memory CD4+ T cells; before (black) and at 1, 3, 6, and 12 months after rATG (grey, B) and basiliximab (white, C) therapy. **(D-E)** STAT5 phosphorylation capacity of total, naïve, and total memory CD8+ T cells; before (black) and at 1, 3, 6, and 12 months after rATG (grey, D) and basiliximab (white, E) therapy. **(F-G)** STAT5 phosphorylation capacity of total memory CD4+ (F) and total memory CD8+ (G) T cells in patients without and with CMV reactivation

or primo infection. Data are shown as median ± IQR, *P<0.05, **P<0.01, ***P<0.001, significant differences comparing pretransplant values to posttransplant values using the Wilcoxon Signed-Rank test.

Phosphorylation of STAT5 in response to IL-7 and IL-2

The capacity of CD4+ and CD8+ T cells to phosphorylate STAT5 in response to the homeostatic cytokine IL-7 was determined before (black) and after rATG (gray bars) or basiliximab (white bars) induction therapy. From 3 months after rATG, but not basiliximab therapy, CD4+ and CD8+ naive and memory T cells had a decreased responsiveness to IL-7 (Fig. 2B-E). These impaired responses were observed in various memory T-cell subsets (Suppl. Fig. 1A–D; P<0.05). Figure 3 shows the responsiveness of T cells to the T-cell growth factor IL-2, which also phosphorylates STAT5 (22). After rATG therapy, we found a decreased pSTAT5 capacity of CD4+ and CD8+ T cells, present in the different memory subsets (Fig. 3B,D and Suppl. Fig. 2; P<0.05). In basiliximab-treated patients, all T-cell populations responded, as expected, poorly to IL-2 during IL-2Rα blockade by basiliximab, the first month after transplantation (Fig.3C,E, P<0.001). CD4+ memory, in particular effector memory T cells, remained unresponsive to IL-2 at months 3 and 6 (P<0.05). CD8+ T cells recovered however the EMRA cells lost responsiveness to IL-2 again at month 12 (P<0.05; Fig. 3E, Suppl. Fig. 2D).

Apart from induction therapy, viral infections may also influence STAT signaling (24-26). In our study population, the incidence of CMV infections (CMV primo infection and reactivation) was significantly higher in the rATG (n = 8) than that in the basiliximab (n = 2) group (P < 0.01; Table 1). We therefore addressed whether CMV infection had an effect on pSTAT5 capacity and found that in response to IL-7, pSTAT5 capacity in CD8+ T cells was affected in patients with CMV, but not in patients without CMV infection (Fig. 2F,G). There was no additional effect of CMV infection to pSTAT5 capacity in CD8+ T cells in response to IL-2 (Fig. 3F,G).

Expression of Coinhibitory molecules

We determined in a subset of patients, due to the availability of samples, rATG (n = 9) and basiliximab (n = 9), the expression of the coinhibitory receptors, PD-1, TIM-3, LAG-3, CTLA-4, CD160, and CD244 (2B4). For CD4+ T cells, we observed increased percentages of TIM-3+, PD-1+, and CD160+ cells (Fig. 4B-D; P<0.05). The increase in TIM-3+ cells at months 3 to 6 was the result of both naive and memory, in particular, effector memory and EMRA, T cells, whereas for PD-1, increased proportions were found in the central memory and effector memory subset (Fig. 4B, C; Suppl. Fig. 3). A high frequency of CD4+CD160+ T cells was found at month 12 in the effector memory subset (Fig. 4D; see Suppl. Fig. 3). The other studied coinhibitory molecules, CTLA-4, LAG-3, and CD244, and did not show changed expression profiles in CD4+ T cells after transplantation.

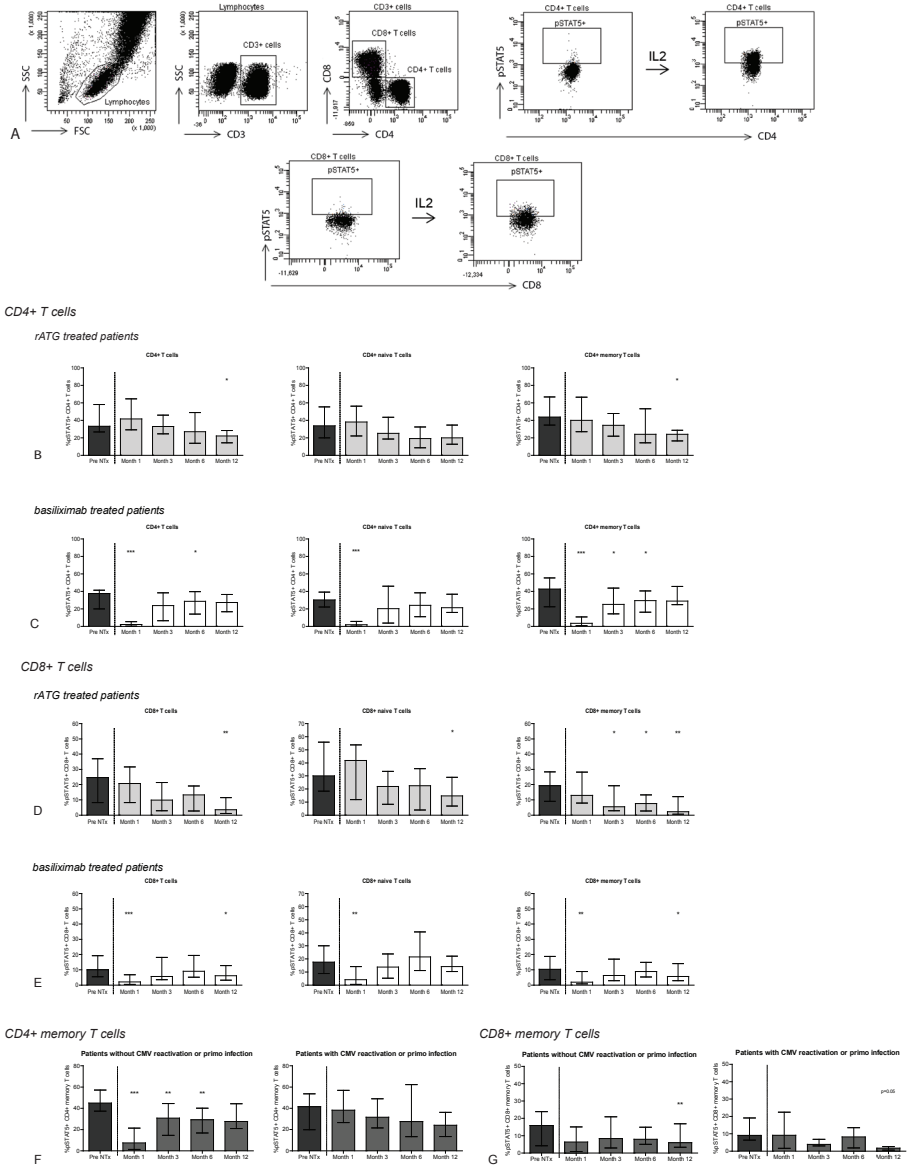


Figure 3. IL-2–induced STAT5 phosphorylation capacity, IL-2–stimulated percentage pSTAT5 minus unstimulated percentage pSTAT5. **(A)** Typical example of the pSTAT5 gating unstimulated and after IL-2 stimulation. **(B-C)** STAT5 phosphorylation capacity of total, naive, and total memory CD4+ T cells; before (black) and at 1, 3, 6, and 12 months after rATG (grey, B) and basiliximab (white, C) therapy. **(D-E)** STAT5 phosphorylation capacity of total, naive, and total memory CD8+ T cells; before (black) and at 1, 3, 6, and 12 months after rATG (grey, D) and basiliximab (white, E) therapy. **(F-G)** STAT5 phosphorylation capacity of total memory CD4+ (F) and totalmemory CD8+ (G) Tcells in patients without and with CMV reactivation or primo infection. Data are shown as median ± IQR, *P<0.05, **P<0.01, ***P<0.001, represent significant differences comparing pretransplant values to posttransplant values using the Wilcoxon Signed-Rank test.

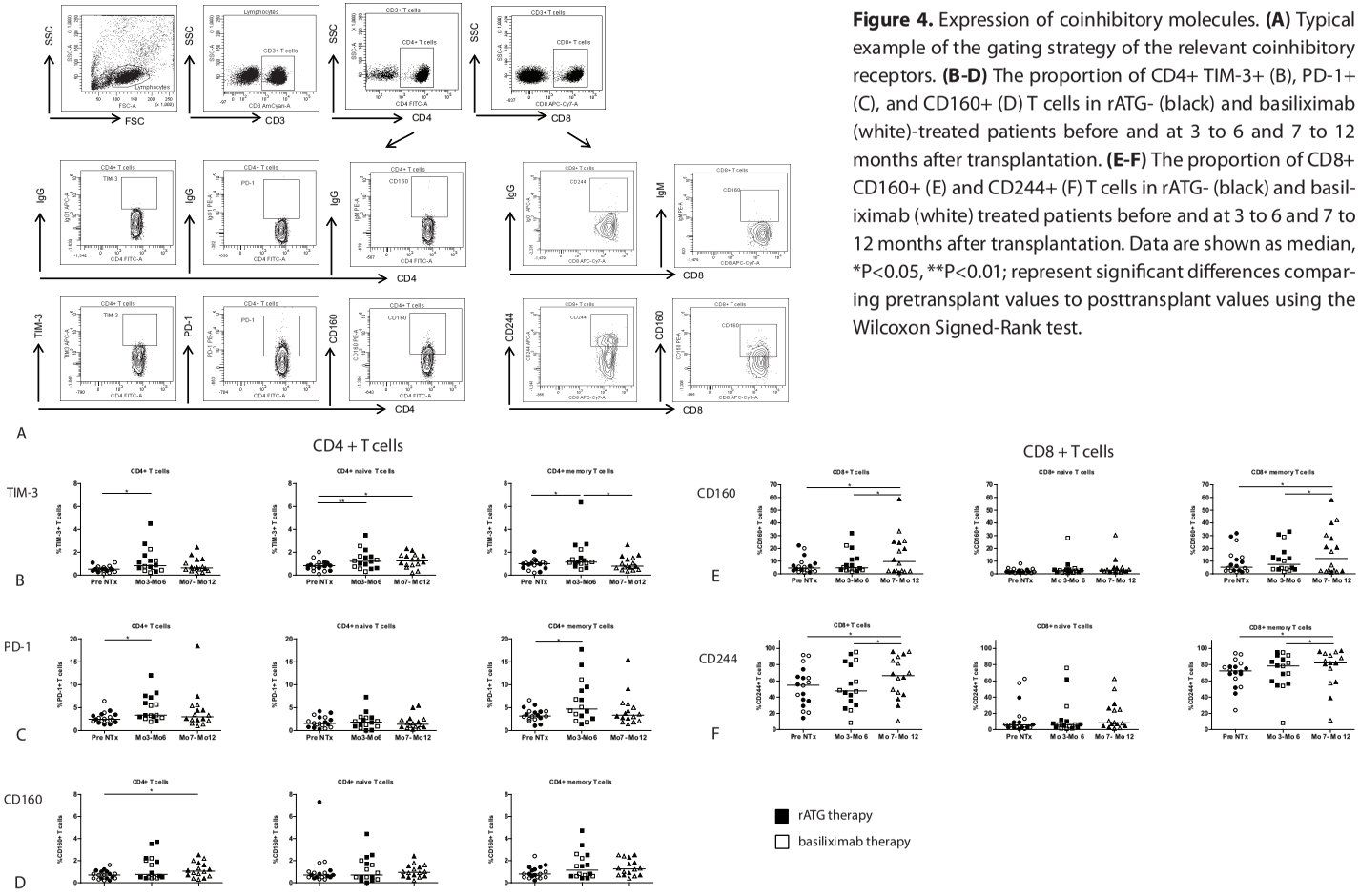


Figure 4. Expression of coinhibitory molecules. **(A)** Typical example of the gating strategy of the relevant coinhibitory receptors. **(B-D)** The proportion of CD4+ TIM-3+ (B), PD-1+ (C), and CD160+ (D) T cells in rATG- (black) and basiliximab (white)-treated patients before and at 3 to 6 and 7 to 12 months after transplantation. **(E-F)** The proportion of CD8+ CD160+ (E) and CD244+ (F) T cells in rATG- (black) and basiliximab (white) treated patients before and at 3 to 6 and 7 to 12 months after transplantation. Data are shown as median, *P<0.05, **P<0.01; represent significant differences comparing pretransplant values to posttransplant values using the Wilcoxon Signed-Rank test.

For CD8+ T cells, an increase in the percentages of CD160+ and CD244+ cells was found after transplantation (Figure 4E, F). The expression of TIM-3, PD-1, LAG-3, and CTLA-4 was comparable with baseline. Increased CD160 expression was seen in the total memory, and particularly in the central memory population, increased CD244 expression was observed in the total memory population (Fig. 4E, F; Suppl. Fig. 3). Between rATG and basiliximab, no differences in the expression of coinhibitory molecules were measured. In this subset of patients, there were only 4 patients who suffered from CMV infection. Therefore, we were not able to study the correlations between CMV and expression of coinhibitory molecules.

Correlation between pSTAT5 capacity and the expression of coinhibitory molecules

To assess whether the observed changes in STAT5 phosphorylation capacities were associated with alterations in the expression of coinhibitory molecules, we determined their correlation. In contrast to CD4+ T cells, a correlation was found for CD8+ T cells. At 12 months after transplantation, the decrease in pSTAT5 capacity after IL-7 stimulation inversely correlated with the increased proportion of CD8+ T cells expressing CD160 and CD244. This relationship was found for the total (data not shown) and memory but not

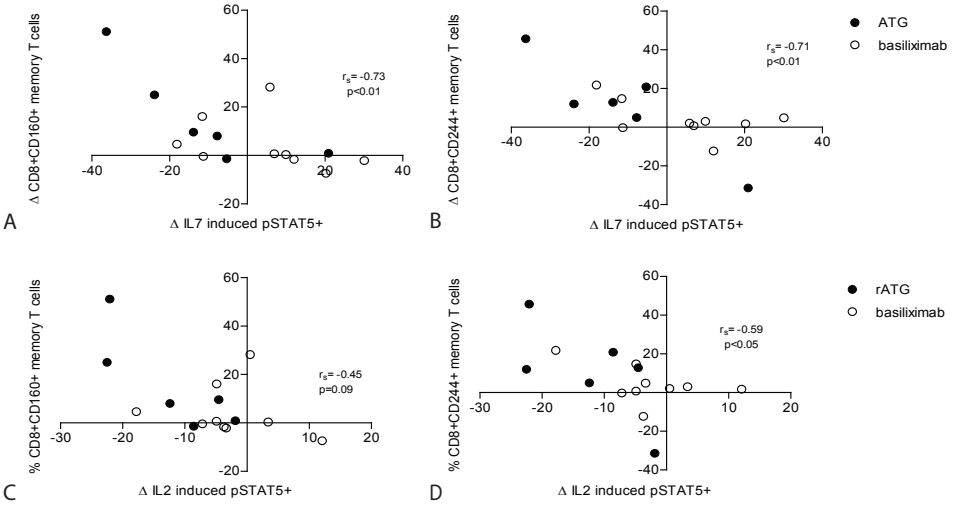


Figure 5. Correlation between pSTAT5 capacity and the upregulation of coinhibitory molecules. The Δ percent in pSTAT5 and the expression of coinhibitory molecules were determined by subtracting the pretransplant percentage from the month 12 percentage. **(A-B)** Correlation between alterations in phosphorylation capacity of STAT5 in response to IL-7 and changes in the expression of CD160 (A) and CD244 (B). **(C-D)** Correlation between alterations in phosphorylation capacity of STAT5 in response to IL-2 and changes in the expression of CD160 (C) and CD244 (D). Statistical significance was determined using Spearman rank correlation coefficient (r_s).

the naive CD8+ T-cell population (Fig. 5A, B). A similar relationship was measured between changes in IL-2 dependent STAT5 phosphorylation and the expression of CD244, but not CD160 (Fig. 5C, D). To determine a difference in rATG- or basiliximab-treated patients, we studied correlations within both treatment arms. Only for CD160- and IL-7–dependent pSTAT5, a correlation was found for both treatment arms (rATG: r_s=−0.94, P<0.05 and basiliximab r_s=−0.78, P<0.05).

DISCUSSION

We here studied the hypothesis that kidney transplantation impairs cytokine responsiveness of memory T cells by affecting STAT phosphorylation and the upregulation of coinhibitory receptors. The principal finding of our study is that after transplantation, a decreased T-cell responsiveness to IL-2 and IL-7 was observed, which was associated with increased expression of coinhibitory receptors with different kinetics after rATG than after basiliximab induction therapy. This report demonstrates, in kidney transplant patients, that these 2 processes are not self-contained but intertwined.

T-cell depletion is followed by immune reconstitution, characterized by an increase in the proportion of memory T cells (Fig. 1) (4, 5, 18) As thymopoiesis decreases with age, homeostatic proliferation is the main contributor to T-cell reconstitution (19). Apart from sensing "empty space" in the immune compartment, multiple signals are required for homeostatic proliferation, antigen contact with the T-cell receptor, and binding of the homeostatic cytokines IL-7 and IL-15 (19). We earlier reported that despite empty space, homeostatic proliferation of naive and memory T cells was incomplete in T-cell depleted transplant patients. Because no difference in cytokine receptor expression in T-cell depleted versus nondepleted patients was found, downregulation of the IL-7Rα induced by high cytokine levels cannot explain these findings (18). In basiliximab-treated patients, homeostatic proliferation to maintain sufficient T-cell numbers is not often found (18). The observed shift from naive to EMRA T cells in the CD8+ T-cell compartment, therefore, cannot be explained by this process. We speculate that foreign donor antigen drives T-cell differentiation. Exposure of naive T cells to antigen results in memory T-cell differentiation followed by their differentiation into EMRA T cells. The increased EMRA T-cell numbers might also explain the decreased IL-2–induced pSTAT5 at 12 months because these cells are known to be less responsive than their effector memory counterparts.

To understand incomplete T-cell recovery after rATG therapy, we tested the postulation that cytokine signaling, at the level of STAT, changes after transplantation and found, after rATG and not after basiliximab therapy, a decreased STAT5 phosphorylation capacity in response to IL-7. Decreased IL-2 responsiveness was observed after both rATG and basiliximab therapies, although with different kinetics. Decreased IL-7 responsiveness is

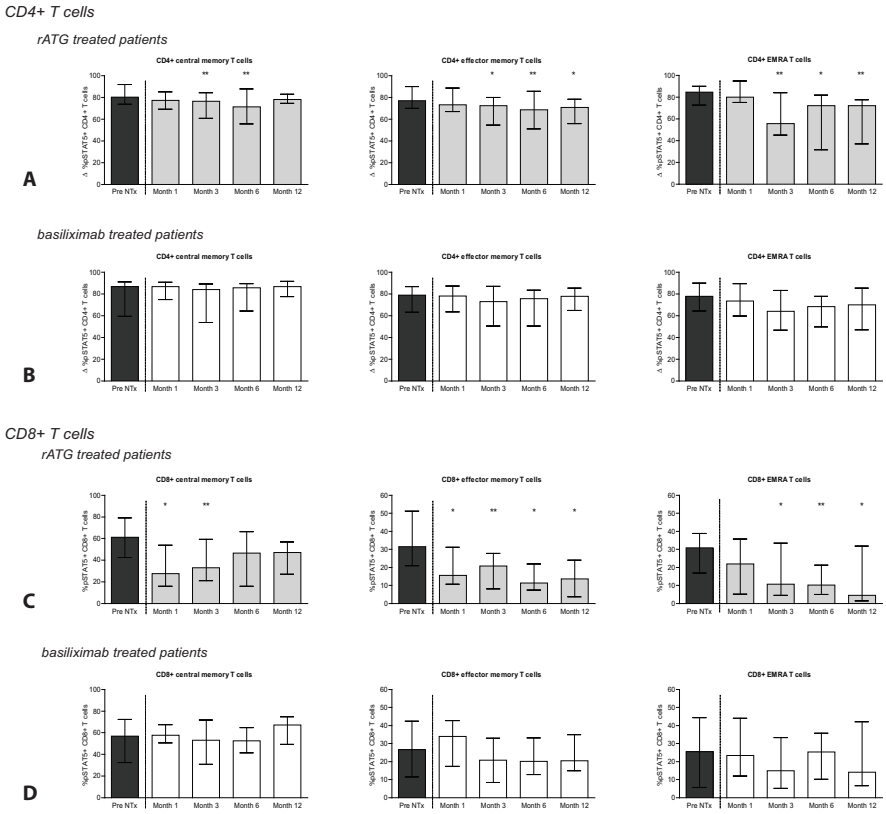
one of the characteristics of T-cell exhaustion, induced in patients with persistent antigen exposure due to chronic infections or malignancies (29-31). A possible explanation here might be the decreased expression of CD127, the IL-7Rα, and CD122, the β chain of the IL-2 and IL-15 receptor, although in our earlier report, we did not find altered receptor expression (18,32-34). Furthermore, impaired access of STAT5 to the nuclear compartment might contribute to decreased IL-7 responses as this was found in T-cells of HIV-infected patients (35, 36) The authors speculate that this defect likely results in decreased STAT5 DNA binding and/or transcriptional activity, explaining the defective induction of several STAT5 target genes, such as Bcl-2, FoxP3, and CD25 (35-37).

Virus-specific T cells expressing coinhibitory molecules, like TIM-3 or PD-1, exhibit impaired STAT5 phosphorylation (24, 25). In our study, a correlation between the expression of coinhibitory markers and pSTAT5 activation was observed. In HCV infection, a link between STAT and PD-1 was found as PD-1 and SOCS-1, a key molecule controlling the JAK-STAT pathway, were both upregulated in HCV infection. Blocking experiments proved that when PD-1 was blocked, SOCS-1 was inhibited, thereby enhancing phosphorylation of STAT1 (26). Also, other reports describe a relation between coinhibitory receptors and altered STAT5 signaling with roles for SOCS and other signaling molecules supporting our findings (26, 38, 39). More studies are required to further explore the molecular relationship between pSTAT5 and coinhibitory molecules in transplant patients. In addition, it is reported that high basal levels of pSTAT5, indicating chronic immune activation, alters STAT signaling (24, 36, 40). Increased percentages pSTAT5+ memory T cells were also found after rATG therapy (18). It might be that this activation is the first incentive for impaired STAT signaling (18). Maintenance immunosuppression with calcineurin inhibitor tacrolimus, mycophenolate mofetil, and steroids did not affect pSTAT5 capacity (41).

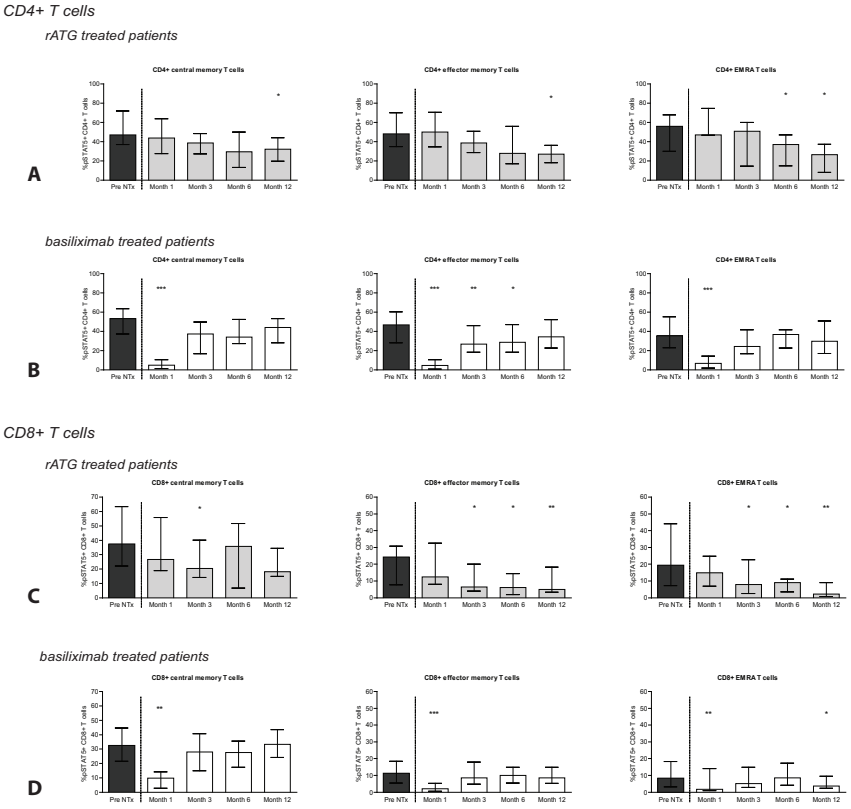
In organ transplant recipients, T-cell exhaustion might be an underlying mechanism by which donor-specific immune responses vanish over time (42). T-cell depletion therapy might have an extra effect because it results in disruption of lymphoid structures, immune reconstitution of few CD4+ T cells, and competition among T-cell clones, all associated with T-cell exhaustion (5, 18, 29 42, 43). In our study, rATG T-cell depletion therapy indeed more severely affected the capacity to phosphorylate STAT5, suggestive for an exhausted T-cell compartment. Mouse, bone marrow transplant models showed that CD4+ T cells chronically stimulated with antigen adapt to this situation by negative costimulation, that is, upregulation of PD-1, and that blocking of this pathway recovered T-cell effector functions (44). In addition, immunosuppressed transplant recipients are prone to infections and may therefore develop virus-specific exhausted T cells. Dysfunctional, antigen-specific, PD-1+ T cells were observed in transplant patients with CMV and Epstein-Barr virus infections (27, 28, 45). Because of the availability of patient materials, the effect of viral infections on coinhibitory molecules in our cohort could not be

studied, whereas an effect on pSTAT5 was observed, for CD8+ memory T cells. Also other factors such as recipient age and dialysis may influence the outcome of cytokine and antigen-mediated responses, that is, pSTAT5 and coinhibitory molecules. Our pilot data suggest that age has an influence on the expression of coinhibitory molecules. As in our study, both patient groups were comparable for age, this could not have influenced our observed effects of transplantation and ATG therapy. Renal replacement therapy was not associated with expression of either coinhibitory molecules or pSTAT5. It is unknown whether chronic infections, chronic alloantigen exposure, immunosuppressive medication, or a combination of these results in dysfunctional memory T cells, which are unable to regulate rejection responses, thereby explaining the reduced need of immunosuppressive therapy and low numbers of rejection in kidney transplant patients treated with T-cell depletion induction therapy. By using the study design with characterization of T-cell function and phenotype on fixed time points, we were not able to study T-cell features during rejection.

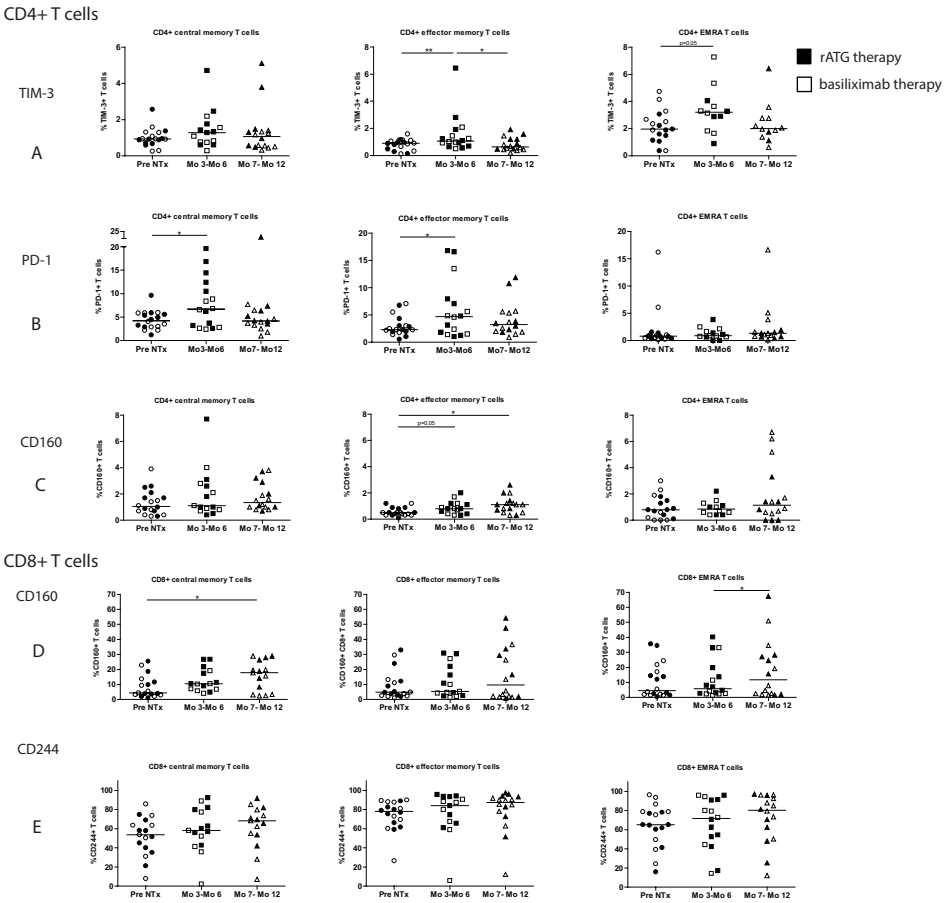
In conclusion, we here show that memory T cells in kidney transplant patients, and in particular after rATG treatment, have decreased cytokine responsiveness by impaired phosphorylation of STAT5 and have increased expression of coinhibitory molecules, processes which were correlated in CD8+ T cells.



Supplemental Figure 1. IL-7 induced STAT5 phosphorylation capacity, IL-7 stimulated percentage pSTAT5 minus unstimulated percentage pSTAT5 **(A-B)** STAT5 phosphorylation capacity of central memory, effector memory and EMRA CD4+ T cells; before (black) and at 1, 3, 6 and 12 months after rATG therapy (grey, A) and basiliximab (white, B) therapy **(C-D)** STAT5 phosphorylation capacity of central memory, effector memory and EMRA CD8+ T cells; before (black) and at 1, 3, 6 and 12 months after rATG (grey, C) and basiliximab (white, D) therapy. Data are shown as median ± Inter Quartile Range (IQR), * $p<0.05$, ** $p<0.01$, *** $p<0.001$ represent significant differences comparing pre-transplant values to post transplant values using the Wilcoxon Signed-Rank test.



Supplemental Figure 2. IL-2 induced STAT5 phosphorylation capacity, IL-2 stimulated percentage pSTAT5 minus unstimulated percentage pSTAT5 **(A-B)** STAT5 phosphorylation capacity of central memory, effector memory and EMRA CD4+ T cells; before (black) and at 1, 3, 6 and 12 months after rATG (grey, A) and basiliximab (white, B) therapy. **(C-D)** STAT5 phosphorylation capacity of central memory, effector memory and EMRA CD8+ T cells; before (black) and at 1, 3, 6 and 12 months after rATG therapy (grey, C) and basiliximab (white, D) therapy. Data are shown as median ± Inter Quartile Range (IQR), * $p<0.05$, ** $p<0.01$, *** $p<0.001$, represent significant differences comparing pre-transplant values to post transplant values using the Wilcoxon Signed-Rank test.



Supplemental Figure 3. The expression of co-inhibitory molecules in the CD4+ and CD8+ memory subsets **(A-C)** CD4+ TIM-3+ (A) PD-1+ (B) and CD160+ (C) T cells in central memory, effector memory and EMRA T-cells before and at 3-6 and 7-12 months after rATG (black) and basiliximab (white) induction therapy **(D-E)** CD8+ CD160+ (D) and CD244 (E) T cells in central memory, effector memory and EMRA T-cells before and at 3-6 and 7-12 months after rATG (black) and basiliximab (white) induction therapy. * $p<0.05$, ** $p<0.01$ represent significant differences comparing pre-transplant values to post transplant values using the Wilcoxon Signed-Rank test.

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Chapter 6

Alemtuzumab as anti-rejection therapy: T-cell repopulation and cytokine-responsiveness

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ABSTRACT

Background

Alemtuzumab induction therapy in kidney transplant patients results in T-cell depletion followed by slow immune-reconstitution of memory T cells with reduced immune functions. The kinetics and functional characteristics of T-cell reconstitution when alemtuzumab is given during immune activation, i.e. as anti-rejection therapy are unknown.

Methods

Patients (n=12) with glucocorticoid-resistant or severe vascular kidney transplant rejection were treated with alemtuzumab. Flow cytometric analysis was performed on whole blood to measure cell division by the marker Ki-67, and cytokine responsiveness by IL-2 and IL-7 mediated phosphorylation of STAT5 of T-cells, before and during the first year after rejection therapy.

Results

At 1 year after alemtuzumab anti-rejection therapy, the total T-cell population recovered to baseline level. Repopulation of CD4+ and CD8+ T cells was associated with increased percentages of Ki-67+ proliferating T cells ($p<0.05$). In addition, both populations showed a phenotypic shift towards relatively more memory T cells ($p<0.01$). At the functional level, IL-7 reactivity of CD4+ memory T cells was diminished, reflected by a decreased capacity to phosphorylate STAT5 during the first six months after alemtuzumab treatment ($p<0.05$), while reactivity to IL-2 was preserved. CD8+ T cells were affected in terms of both IL-2 and IL-7 responses (both $p<0.05$). After reconstitution, relatively more Tregs were present, and a relatively high proportion of Ki-67+ T cells was observed.

Conclusions

Preliminary data from this small series suggests that alemtuzumab anti-rejection therapy induces homeostatic proliferation of memory and regulatory T cells with diminished responsiveness to the homeostatic cytokine IL-7. IL-2 responsiveness was affected in repopulated CD8+ T cells.

INTRODUCTION

T-cell depleting antibody therapy is the treatment of choice for severe or glucocorticoid-resistant kidney transplant rejection (1). The most commonly used T-cell depleting agent is rabbit anti-thymocyte globulin (rATG), but in recent years the use of alemtuzumab to treat rejection has gained popularity (2-6).

Alemtuzumab (Campath-1H) is a humanized monoclonal antibody directed against the cell surface antigen CD52, which is expressed not only by T cells but also by B cells, NK cells, monocytes, macrophages and dendritic cells. Ligation of alemtuzumab with CD52 induces apoptosis and lysis of immune cells through antibody- and complement-dependent cytotoxicity, which leads to profound and long-lasting lymphocyte depletion. Studies in kidney transplant patients given alemtuzumab as induction therapy have shown that low T-cell numbers persisted for more than 1 year and that CD8+ T cells reach baseline levels earlier than CD4+ T cells (7).

After T-cell depletion therapy, T-cell repopulation results from two processes: (i) thymopoiesis, the formation of new, naive T cells called recent thymic emigrants (RTE) and (ii) homeostatic proliferation, the expansion of residual naïve but mainly memory T cells. Naïve RTE can be identified by the expression of CD31, which is lost upon antigen binding and proliferation of the naïve cell (8, 9). Homeostatic proliferation of both naive and memory cells is the result of antigen binding to the T-cell receptor (TCR) and/or binding of the Signal Transducer and Activator of Transcription (STAT5) activating cytokines IL-7 and IL-15 to their cytokine receptor (9-13). As thymopoiesis decreases with age, homeostatic proliferation is the main contributor to T-cell reconstitution in T-cell depleted adults. Furthermore, memory cells are relatively resistant to depletion and proliferating naive cells can also adapt a memory phenotype, resulting in a T-cell pool which mainly comprises memory T cells after T-cell depletion therapy (6, 14-17). In addition to higher numbers of memory cells, higher percentages of regulatory T cells have also been found after T-cell depletion therapy (18-20). Homeostatic proliferation, in an activated immune environment, i.e, high levels IL-2 might play a role in the induction of Treg (19, 21).

Memory T cells can rapidly and vigorously respond to donor antigen, a response difficult to inhibit by immunosuppressive drugs. Therefore, memory cells are thought to endanger transplant survival (22, 23). However, several studies reported that patients treated with T-cell depletion therapy can be treated with reduced doses of maintenance immunosuppression, suggesting reduced immune functions of the repopulated T cells (24-28). *In vitro,* this impaired T-cell function is reflected by hampered T-cell responses to donor, third-party and recall antigens (7, 16, 20, 29). Furthermore, after T-cell depletion, T cells showed diminished homeostatic proliferation despite incomplete T-cell reconstitution, and the phosphorylation capacity of STAT5 of recovered cells in response to IL-2 and IL-7 is affected (9, 30). These recovered T cells also have increased expression of coinhibitory molecules (30). Impaired STAT signaling, as well as increased expression

of coinhibitory molecules, are features of T-cell exhaustion, a phenomenon induced by persistent antigen exposure resulting in dysfunctional T cells that is thought to contribute to donor hypo-responsiveness after kidney transplantation (30, 31).

During rejection, the immune system is highly activated, resulting in high concentrations of cytokines and antigen presentation, processes capable of influencing T-cell reconstitution and function of the T-cell pool by affecting the formation of Treg and the induction of T-cell exhaustion. We therefore speculated that after alemtuzumab anti-rejection therapy, T-cell reconstitution and T-cell functions are altered and impaired due to the activated environment. In the present study, we monitored the kinetics of T-cell repopulation and their cytokine responsiveness in kidney transplant patients before and after T-cell depletion therapy during rejection.

MATERIAL AND METHODS

Patients

Blood samples were collected from 12 consecutive kidney transplant patients (Table 1) with biopsy-proven acute rejection (BPAR; graded according to the Banff criteria (32)) which was resistant to glucocorticoid treatment (i.e., 1000 mg of methylprednisolone intravenously for 3 consecutive days) or very severe. All patients were treated with alemtuzumab (Campath-1H, Sanofi, Paris, France) at a dose of 30 mg subcutaneously on two consecutive days. Blood samples were drawn before alemtuzumab treatment was initiated (after glucocorticoid treatment), and again at week 1 and months 1, 3, 6 and 12 after alemtuzumab therapy. The maintenance immunosuppressive regimen following alemtuzumab was left to the discretion of the attending physician but typically consisted of a calcineurin inhibitor and mycophenolate mofetil, with or without prednisolone (Table 2). Following alemtuzumab treatment, patients received *pneumocystis jirovecii* pneumonia and cytomegalovirus prophylaxis when T-cell numbers were below 200 cells/ µL blood. CMV-PCR status was determined before transplantation, and subsequently if there was a clinical suspicion of CMV infection. The medical ethic committee of the Erasmus MC approved the study (MEC-2010-022).

Flow cytometry

Absolute counts of CD4+ and CD8+ T cells were determined in freshly drawn EDTA blood using BD MultiTest™ 6-color reagent and BD TruCount™ tubes (BD Biosciences, San Jose, CA), according to the manufacturer's instructions. In brief, EDTA blood (50 µL) was added to 20 µL MultiTest reagent in a BD TruCount tube containing a known number of fluorescent beads and incubated for 15 minutes in the dark at room temperature. After lysis with BD FACSLysing solution, samples were measured on a FACSCantoII™ flow cytometer and analyzed by FACSDiva™ software (BD Bioscience).

Table 1. Patient characteristics

Patient	Gender	Age at time of KT (years)	Primary kidney disease	Pre-emptive	Transplant No.	Donor type	HLA mismatch A/B/DR	CMV D/R	CMV infection (< 1yr after treatment)
1	Male	19	Alport syndrome	yes	1	Living-related	0/1/1	neg/neg	no
2	Male	39	Focal segmental glomerulosclerosis	yes	2	Living-unrelated	1/2/1	pos/neg	no
3	Female	72	Polycystic kidney disease	yes	1	Living-unrelated	1/2/1	neg/pos	yes
4	Male	38	Focal segmental glomerulosclerosis	no	2	Living-unrelated	1/2/1	pos/pos	yes
5	Male	48	Polycystic kidney disease	no	2	Non-heart beating postmortal	1/2/0	pos/neg	no
6	Male	59	Polycystic kidney disease	yes	1	Living-unrelated	2/2/0	neg/pos	no
7	Male	51	Reflux nephropathy / chronic pyelonephritis	no	1	Living-related	1/1/1	pos/neg	no
8	Male	62	Diabetic nephropathy	yes	1	Living-unrelated	1/2/0	pos/neg	no
9	Female	40	Hypertensive nephropathy	no	3	Heart-beating postmortal	1/1/2	pos/pos	yes
10	Male	14	Reflux nephropathy / chronic pyelonephritis	no	1	Heart-beating postmortal	1/2/0	neg/neg	no
11	Male	32	Hypertensive nephropathy	no	1	Heart-beating postmortal	1/2/2	neg/pos	yes
12	Female	37	Reflux nephropathy / chronic pyelonephritis	yes	1	Living-related	1/1/1	pos/neg	no

KT: kidney transplantation, HLA mismatch at DR: 0, 1 or 2 mismatches on DR-locus, CMV D/R: cytomegalo virus donor-recipient combination, CMV infection within 1 year after alemtuzumab treatment; defined by a positive CMV Polymerase Chain Reaction-test.

CD4+ and CD8+ T-cell subsets were studied; naive (CCR7+CD45RO-), central memory (CM; CCR7+CD45RO+), effector memory (EM; CCR7-CD45RO+), terminally differentiated effector memory (EMRA; CCR7-CD45RO-) and regulatory (Treg; CD4+CD25+CD127-) T cells. Phosphorylation (p) of STAT5 was determined by phospho-specific-flow-cytometry. In brief, heparinized blood, 200 µL, was stained using; CD3-AmCyan, CD8-FITC, CCR7-PE-Cy7, or with CD25-PE-Cy7 and CD127-FITC (all BD Bioscience). Samples were stimulated with 100 ng/mL IL-7 (PeproTech, London, UK), IL-2 (Proleukin® 2000IU/mL, Novartis Pharma GmbH, Nürnberg, Germany) or medium (unstimulated sample) for 30 minutes at 37°C. Next, cells were lysed and fixed using Lyse/Fix Buffer. For intracellular staining, cells were permeabilised with cold 70% methanol for 30 minutes at -20°C, washed in staining buffer with 0.5% BSA and stained with CD4-PerCP (BD Bioscience), CD45RO-PB (Biolegend) and pSTAT5-PE (BD Bioscience) antibodies. Staining with PerCP and PB conjugated antibodies was performed after permeabilisation with 70% methanol as this affects their staining.Thymopoiesis and homeostatic proliferation were determined in the previously described naive and memory CD4+ and CD8+ T cells and in regulatory T cells which were here, due to the staining protocol, characterized as CD4+CD127-FoxP3+ T cells (Treg). Heparinized blood (100 µL) was stained for 30 minutes with CD3-AmCyan (BD Bioscience), CD4-PacBlue (BD Bioscience), CD8-APC-Cy7 (BD Bioscience), CD45RO-APC (BD Bioscience), CCR7-PE-Cy7 (BD Bioscience) and CD31-PE (Biolegend) antibodies. Treg samples were stained with CD3-AmCyan, CD4-APC-Cy7 (BD Bioscience), CD127-PE-Cy7 (BD Bioscience), CD45RO-PerCP-Cy5 (BD Bioscience) and CD31-PE. Hereafter red blood cells were lysed using RBC lysis Buffer (BD Bioscience), washed with staining buffer with 0.5% bovine serum albumin (BSA) and then incubated for 60 minutes at 4°C with fixation and permeabilisation solution (BD Bioscience). After washing the cells with staining buffer with 0.5% BSA, cells were intracellular stained for 60 minutes in the dark using Ki-67-FITC (BD Bioscience) and FoxP3-APC (eBioscience).

Cells were measured and analysed as described above. A minimum of 100 events per T-cell subset was required for analysis.The effect of IL-2 and IL-7 on STAT5 phosphorylation was calculated by the percentage pSTAT5 positive cells after stimulation minus the unstimulated sample.

Statistical analysis

Statistical analyses were performed using Graphpad Prism version 5.01. For comparisons between time points the Wilcoxon signed rank test was used. Data are presented as median plus interquartile range (IQR). P values ≤0.05 were considered statistically significant.

Table 2. Rejection therapy

Patient	Time of rejection episode (days)	Methyl-prednisolone	IVIg	Immunosuppressive therapy at time of rejection						Time of graft loss (days)
				Tac	CsA	MMF	Aza	Pred	ERL	
1	260	yes	yes	x	-	x	-	x	-	-
2	745	yes	no	x	-	-	x	x	-	-
3	68	yes	no	x	-	x	-	x	-	-
4	33	yes	yes	x	-	x	-	x	-	-
5	10	no	no	x	-	x	-	x	-	239
6	55	yes	no	x	-	x	-	x	-	-
7	253	yes	no	-	-	x	-	-	x	-
8	156	yes	yes	-	x	x	-	-	-	-
9	2	yes	no	x	-	x	-	x	-	-
10	1740	yes	yes	x	-	x	-	-	-	189
11	514	yes	yes	x	-	x	-	x	-	250
12	104	yes	no	x	-	x	-	x	-	-

Methylprednisolon; 1000mg methylprednisolone for 3 days, IVIg: intravenous Immunoglobuline, Tac: tacrolimus, CsA: cyclosporine A, MMF: mycophenolate mofetil (Cellcept), Aza: azathioprine, Pred: prednisolone, ERL: Everolimus; Time of graft loss: days after alemtuzumab therapy.

RESULTS

Clinical data

A total of 12 kidney transplant recipients received alemtuzumab for glucocorticoid-resistant or severe BPAR, which occurred in 5/7 patients within the first 90 days after transplantation and in 7/12 patients after this period. After anti-rejection therapy, kidney function as assessed by serum creatinine levels recovered or stabilized in 9 of the 12 patients, while 3 patients lost their graft.

The clinical characteristics of the patients are shown in Tables 1 and 2. Patient and rejection characteristics and type of immunosuppression were not associated with immunological outcomes.

T-cell compartment & immune reconstitution

Alemtuzumab therapy resulted in depletion of both CD4+ and CD8+ T cells, which was followed by T-cell repopulation (Fig. 1A, B). T-cell repopulation was more marked in CD8+ than in CD4+ T-cells. Six months after alemtuzumab treatment there were clear signs of CD8+ T-cell recovery in the peripheral blood ($p<0.05$), while the numbers of CD4+ T cells were barely measurable. For both populations, repopulation following alemtuzumab treatment was accompanied by a shift towards more memory T cells (Fig. 1C, $p<0.05$). In the CD4+ T-cell pool, the increase in memory T-cells was the result of an increased frequency of EM T-cells, while the repopulated CD8+ T cells largely consisted

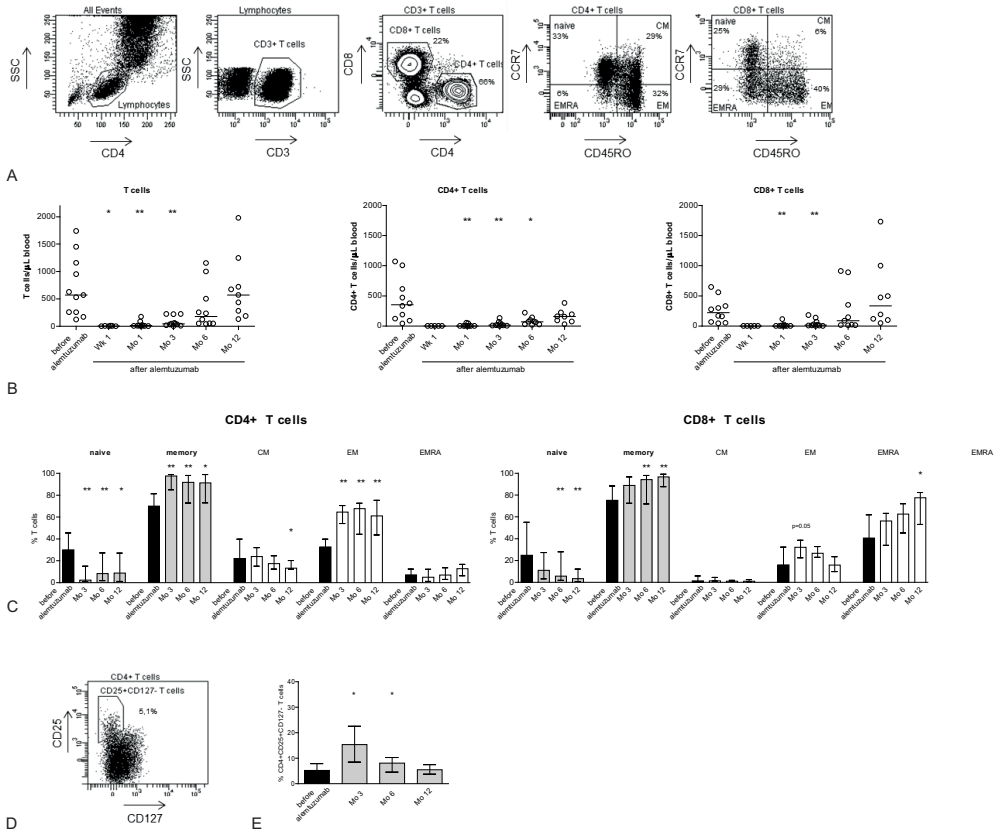


Figure 1. T-cell repopulation after alemtuzumab therapy. **(A)** Typical example of the gating strategy; CD4+ and CD8+ naive (CCR7+CD45RO-), central memory (CCR7+CD45RO+), effector memory (CCR7-CD45RO+) and EMRA (CCR7-CD45RO-) T cells. **(B)** Absolute number of the total (left), CD4+ (middle) and CD8+ (right) T-cell population before and at month 1, 3, 6 and 12 after alemtuzumab treatment. **(C)** Percentage CD4+ (left) and CD8+ (right) naive total memory, central memory, effector memory and EMRA T-cells before and at month 1, 3, 6, 12 after alemtuzumab therapy. **(D)** Typical example of the gating strategy of CD4+CD25+CD127- regulatory T cells. **(E)** The percentage of CD4+CD25+CD127- regulatory T cells before and after alemtuzumab therapy. Data are shown as median ± Inter Quartile Range (IQR), *p<0.05, ** p<0.01; significant differences comparing pre-transplant values to post transplant values the Wilcoxon Signed-Rank test. Due to low cell numbers not all samples could be analysed reliable and are therefore not depicted.

of EMRA T cells. In addition to the naive and memory subsets, the presence of Treg after alemtuzumab anti-rejection therapy was analyzed (Fig. 1D). As shown in Figure 1E, an increased percentage of these Treg cells was observed at months 3 and 6 after therapy, which recovered to baseline levels at 12 months ($p<0.05$).

Thymopoiesis & homeostatic proliferation of T cells

In a limited number of samples (due to low naive T-cell counts after treatment) we analysed CD31 expression and observed no changes in the percentage of CD31+ T-cells,

suggesting that thymopoiesis and peripheral repopulation contributed equally to T-cell repopulation after alemtuzumab therapy (Fig. 2B).

Homeostatic proliferation was measured by the expression of the proliferation marker Ki-67, a nuclear antigen selectively expressed in dividing cells (Fig. 2C) (33). At 3 months, the percentages of Ki-67+ CD4+ and of CD8+ T cells were higher than before alemtuzumab treatment, demonstrating high rates of cell division (Fig. 2D, 2E, $p<0.05$). For both CD4+ and CD8+ T-cells, the highest percentages of proliferating cells were measured in memory cells, with no differences in the median percentage Ki-67+ cells between the various memory subsets (month 3; CD4+ CM; median 22.6%, CD4+ EM median 20.9%, CD8+ EM median 11.7%, CD8+ EMRA median 7.9%). Increased proliferation continued until 6 months after therapy only for CD8+ memory T cells the (Fig.2E).

At 3 months after alemtuzumab therapy, high percentages of Ki67+ Treg cells were also measured (Fig. 2F). Only one patient received everolimus as an immunosuppressant, in whom no high percentage of Treg cells was found.

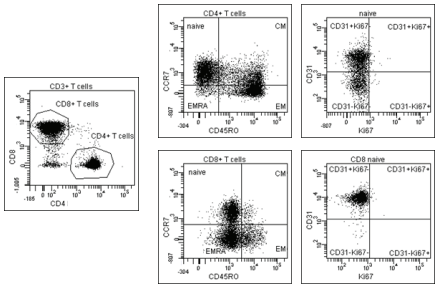
Cytokine responsiveness of repopulated T-cells

Cytokine responsiveness of T cells was measured by pSTAT5 (for gating strategy and STAT5 expression; see Fig. 3A). First, pSTAT5 was measured *ex vivo* in freshly drawn blood samples. Very low percentages of pSTAT5+ T-cells were observed during rejection, *i.e.* before anti-rejection treatment was given (Fig. 3B). Three months after T-cell depletion treatment, increased percentages of pSTAT5+ in CD4+ and CD8+ T cells were found, indicating T-cell activation by members of the common-γ chain cytokine family (*i.e.*, IL-2, IL-7 and IL-15).

Second, to assess whether the repopulated T cells had functional potential, their responsive capacities to IL-2 and IL-7 was determined. At months 3 and 6 after alemtuzumab therapy, CD4+ memory T cells showed decreased responsiveness to IL-7 (Fig. 3D, $p<0.05$), which returned to baseline at month 12. Decreased responsiveness was also observed in CD8+ T cells, albeit with different kinetics: the responses decreased with time and minimal responsiveness was present at month 12 (Fig. 3E). The kinetics of the different memory subsets followed the same pattern (Suppl. Fig. 1).

With regard to IL-2 response, CD4+ memory T cells showed a trend towards decreased percentages of pSTAT5 in response to IL2 (Fig. 3F and Suppl. Fig. 1). Memory CD8+ T cells were affected in functionality as 12 months after anti-rejection therapy their STAT5 phosphorylation capacity was still diminished (Fig. 3G and Suppl. Fig. 1).

The pSTAT5 capacity in the Treg population in response to IL-7 and IL-2 was also determined (Fig. 3I, H). No difference was found in the expression of pSTAT5 by Treg cells before or after transplantation. After anti-rejection therapy, Treg showed poor responsiveness to IL-7 which partly recovered at month 12 (Fig. 3I). As shown in Figure 3I, IL-2 responses of Treg were unaffected.


CD3+ T cells
CD8+ T cells
CD4+ T cells
CD4
CD8
CD4+ T cells
naive
CM
EMRA
EM
CCR7
CD45RO
CD8+ T cells
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CM
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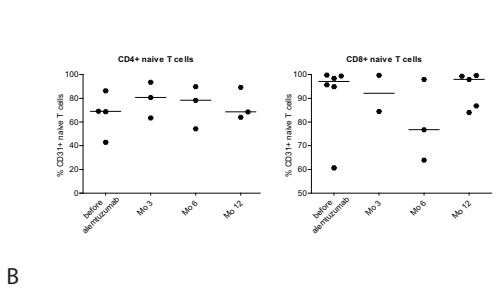
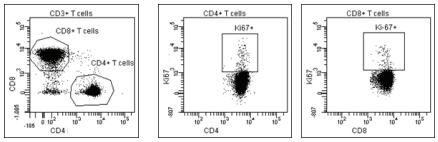
CD45RO

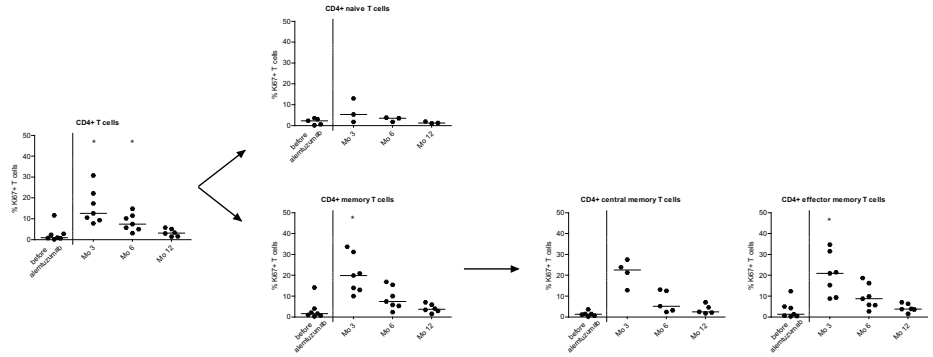
A


CD4+ naive T cells
% CD31+ naive T cells
CD8+ naive T cells
% CD31+ naive T cells
before alemtuzumab
Mo 3
Mo 6
Mo 12

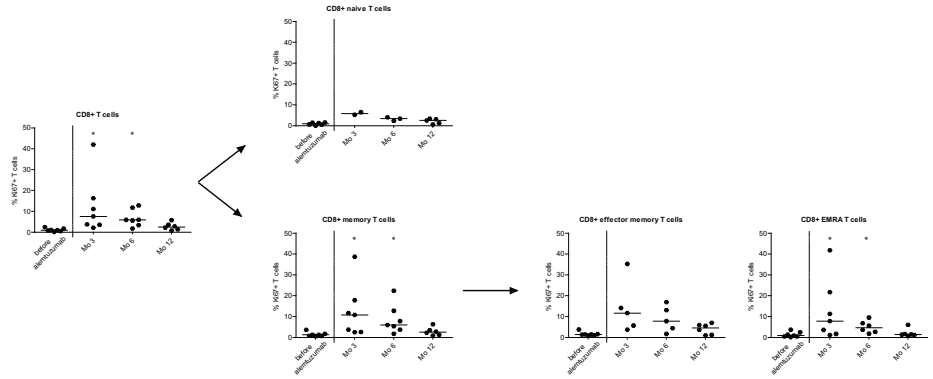
B


CD3+ T cells
CD8+ T cells
CD4+ T cells
CD4
CD8
CD4+ T cells
Ki67+
Ki67
CD4
CD8+ T cells
Ki-67+
Ki67
CD8

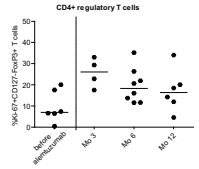
C


CD4+ T cells
% Ki67+ T cells
CD4+ naive T cells
CD4+ memory T cells
CD4+ central memory T cells
CD4+ effector memory T cells
before alemtuzumab
Mo 3
Mo 6
Mo 12

D


CD8+ T cells
% Ki67+ T cells
CD8+ naive T cells
CD8+ memory T cells
CD8+ effector memory T cells
CD8+ EMRA T cells
before alemtuzumab
Mo 3
Mo 6
Mo 12

E


CD4+ regulatory T cells
%Ki-67+CD127-FoxP3+ T cells
before alemtuzumab
Mo 3
Mo 6
Mo 12

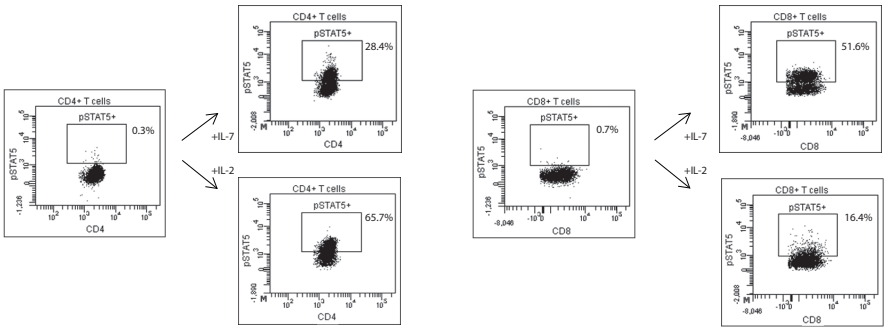
F

Figure 2. Thymopoiesis and homeostatic proliferation. **(A)** Typical example of the gating strategy: expression of CD31 within CD4+ and CD8+ naive (CCR7+CD45RO-) T cells. **(B)** Percentage of recent thymic emigrants (RTE); naive T cells expressing CD31, before and after alemtuzumab therapy. **(C)** Typical example of the gating strategy of Ki-67+ CD4+ and CD8+ T cells. **(D)** The percentage Ki-67 expressing T cells within the total CD4+, CD4+ naive, CD4+ total memory and CD4+ central memory and effector memory T cells before and after alemtuzumab therapy. **(E)** The percentage Ki-67 expressing T cells within the total CD8+, CD8+ naive, CD8+ total memory and CD8+ effector memory and EMRA T cells before and after alemtuzumab therapy. **(F)** The percentage of Ki-67+ Treg. Because of low number of cells, we could not determine the percentage RTE and Ki-67+ cells for all patients. Data are shown as median, *$p<0.05$; significant differences comparing pre-transplant values to post transplant values using the Wilcoxon Signed-Rank test.

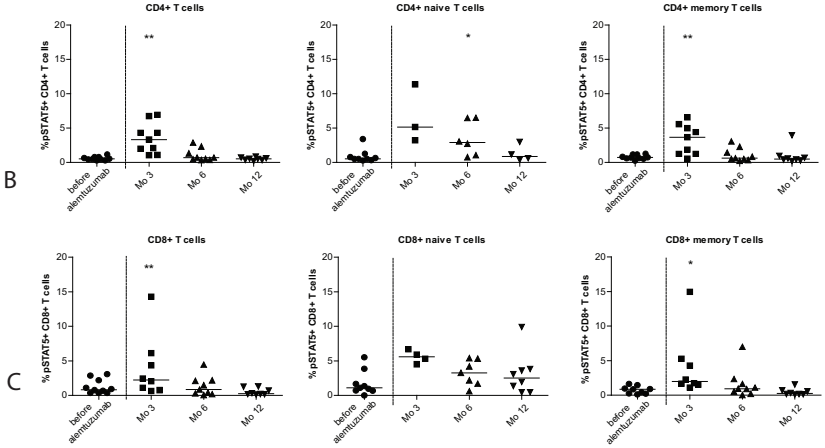
DISCUSSION

Results from this preliminary study suggest that use of alemtuzumab as to treat early or late acute rejection after kidney transplantation results in a profound depletion of both CD4+ and CD8+ T cells from the circulation, followed by slow reconstitution, which for CD4+ T cells is incomplete at one year after treatment. Furthermore, both CD4+ and CD8+ T-cells appear to be repopulated by means of homeostatic proliferation rather than thymopoiesis. The reconstituted T-cell pool consisted predominantly of CD4+ effector memory and CD8+ effector memory and EMRA T-cells which showed a decreased capacity for IL-7 and IL-2 dependent STAT5 phosphorylation. Finally, after reconstitution relatively more Tregs were present, which again seemed to result from homeostatic proliferation. These repopulated Treg responded poorly to IL-7, while their response to IL-2 was largely unaffected.

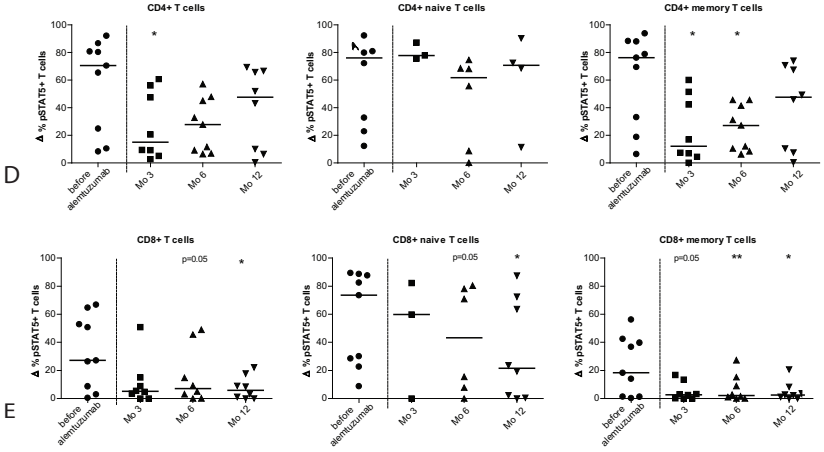
The suggestion that reconstitution after alemtuzumab results from homeostatic proliferation, as defined here by the proliferation marker Ki-67, is in line with our previous findings regarding T-cell reconstitution after rATG induction therapy (9, 19). In the current study, a higher frequency of Ki67+ memory T cells was also measured. Firm conclusions about the kinetics and function of naïve T cells from the current study are hard to draw since their numbers were extremely low after treatment. From the limited data collected, it appeared that thymopoiesis before and after T-cell depletion therapy was comparable, but we cannot rule out the possibility that increased thymopoiesis also contributed to a certain degree. In contrast to studies of T-cell reconstitution after alemtuzumab induction therapy, the patients in this study were pretreated with high-dose corticosteroids. Corticosteroids exert pleiotropic immunosuppressive effects by preventing protein synthesis, which ultimately results in decreased chemokine and cytokine synthesis and affected lymphocyte activation, migration and recruitment. Lymphopenia after corticosteroid therapy is induced by lysation of cells but mainly by redistribution of T cells towards the bone marrow, spleen, thoracic duct and lymphocytes (34-36). Because of these effects, corticosteroids could have modified T-cell numbers before alemtuzumab treatment and the reconstitution process. In our study we have not monitored T-cell numbers and characteristics before and after corticosteroid therapy



Ex-vivo



IL-7 stimulated



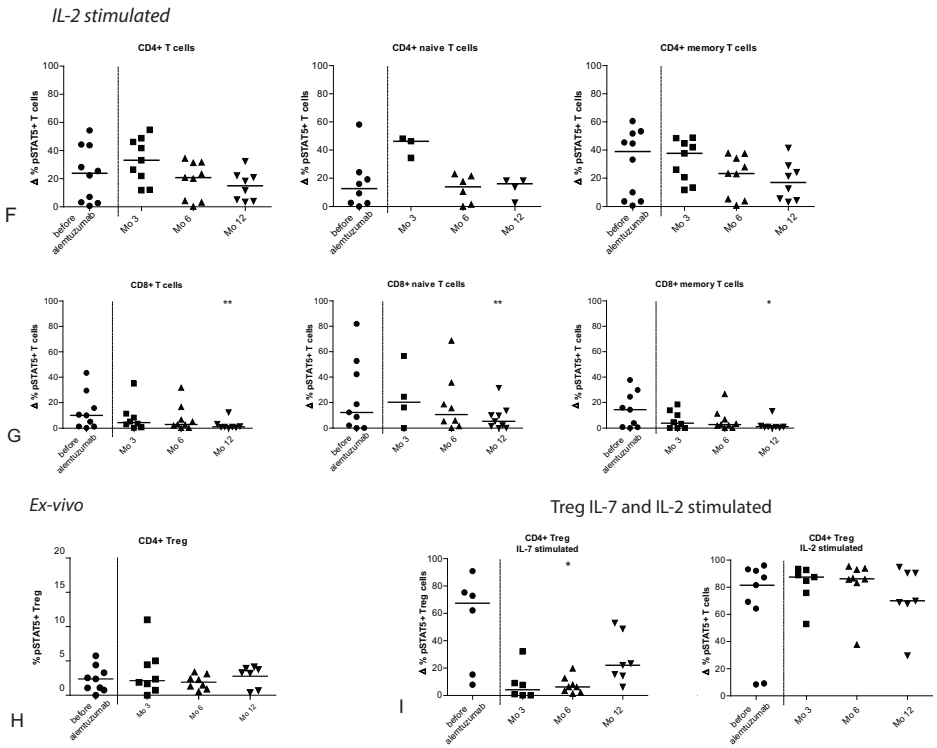


Figure 3. IL-7 and IL-2 induced STAT5 phosphorylation capacity; IL-7/IL-2 stimulated percentage pSTAT5 + cells minus unstimulated percentage pSTAT5. **(A)** Typical example of the pSTAT5 gating, unstimulated and after stimulation **(B)** Percentage of pSTAT5+ total, naive and memory CD4+ cells before and after alemtuzumab therapy in unstimulated blood (*ex vivo*). **(C)** Percentage of pSTAT5+ total, naive and memory CD8+ cells before and after alemtuzumab therapy in unstimulated blood (*ex vivo*). **(D)** STAT5 phosphorylation capacity in response to IL-7 of total, naive, total memory CD4+ T cells; before and at 3, 6 and 12 months after alemtuzumab therapy. **(E)** STAT5 phosphorylation capacity in response to IL-7 of total, naive, total memory CD8+ T cells; before and at 3, 6 and 12 months after alemtuzumab therapy. **(F)** STAT5 phosphorylation capacity in response to IL-2 of total, naive, total memory CD4+ T cells; before and at 3, 6 and 12 months after alemtuzumab therapy. **(G)** STAT5 phosphorylation capacity in response to IL-2 of total, naive, total memory CD8+ T cells; before and at 3, 6 and 12 months after alemtuzumab therapy. **(H)** Percentage of pSTAT5+ CD4+ Treg cells before and after alemtuzumab therapy in unstimulated blood (*ex vivo*). **(I)** IL-7 and IL-2 induced STAT5 phosphorylation capacity in Treg. Left: The percentage pSTAT5+ Treg after IL-7 stimulation. Right: The percentage pSTAT5+ Treg after IL-2 stimulation. Data are shown as median, * $p<0.05$, ** $p<0.01$, significant differences comparing pre-transplant values to post transplant values using the Wilcoxon Signed-Rank test.

and can therefore not rule out the possibility that steroid influenced the repopulation of T cells. However, the data presented here do not suggest different kinetics and dynamics of T-cell repopulation when compared to repopulation after T-cell induction therapy (9).

The higher proportion of memory T cells which was observed during immune reconstitution can be explained by two mechanisms. First, memory T cells are relatively

resistant to depletion, and are thus the proliferating subtype during immune reconstitution. Second, proliferating naive cells develop a memory phenotype due to proliferation in a lymphopenic environment (9-11, 17, 22, 37). A higher proliferation capacity of a certain memory subset within CD4+ of CD8+ T cells was excluded in this study, since at 3 months the percentages of Ki67+ CD4+ EM and CM were comparable, as were the percentages of CD8+ EM and EMRA T cells. Although the percentage of proliferating CD8+ T cells was lower than that of CD4+ cells, at 12 months the absolute number of CD8+ cells exceeded the number of CD4+ T cells. A possible explanation is a relative resistance of CD8 T cells to depletion.

It is of interest to understand whether the T cells that escape depletion by alemtuzumab are alloreactive cells, especially in patients suffering from graft rejection. Published reports have shown that T-cell depletion therapy in kidney transplant patients resulted in impaired T-cell function of the repopulated cells, e.g. decreased T-cell reactivity to donor, third party and recall antigen, impaired cytokine responsiveness and low numbers of interferon-γ producing cells using ELISPOT assays (7, 16, 20, 29, 30).

The here observed increase in *ex vivo* STAT5 phosphorylation reflects the response of T cells to cytokine stimulation observed *in vivo*. This may have resulted from any pSTAT5 activating cytokine (e.g., IL-21, IL-15, TSLP (Thymic stromal lymphopoietin)), including the cytokines IL-2 and IL-7 studied *in vitro*. It is well known that also other proteins might activate STAT5, such as growth hormone and erythropoietin. After *in vitro* stimulation, the repopulated T cells exhibited reduced pSTAT5 capacity in response to IL-7 and, to a lesser extent, to IL-2. These findings indicate that the repopulating cells after anti-rejection depletion therapy are also functionally hampered in cytokine-driven T-cell responses. This can be explained by several mechanisms. First, high basal levels of pSTAT5 are reported to alter STAT signaling. These pSTAT5 levels indicate chronic immune activation that might be the first incentive for impaired STAT signaling (30, 43). Second, alemtuzumab treatment resulted in increased percentages of functionally active Treg controlling T-cell responses (19, 38-40). A third mechanism could be the predominant expansion of terminally differentiated, functionally impaired immunosenescent CD28- CD8+ T cells (15). These functionally impaired T cells compete for "immune space" with CD4+ T cells, suppressing their proliferation and may therefore also explain their incomplete repopulation. Additionally, we observed increased percentages of terminally differentiated CD8+ EMRA cells. Forth, T-cell exhaustion, the loss of T-cell effector functions induced by persistent donor antigen stimulation, may explain our findings (31). As well as the loss of proliferative capacity, cytokine production and killing capacity, exhausted cells have altered memory maintenance since in contrast to "normal" memory T cells, they poorly respond to IL-7 and IL-15 and only survive by contact with their cognate antigen (41). Impaired access of STAT5 to the nuclear compartment and decreased receptor expression, although not found earlier in our study, might contribute to these decreased

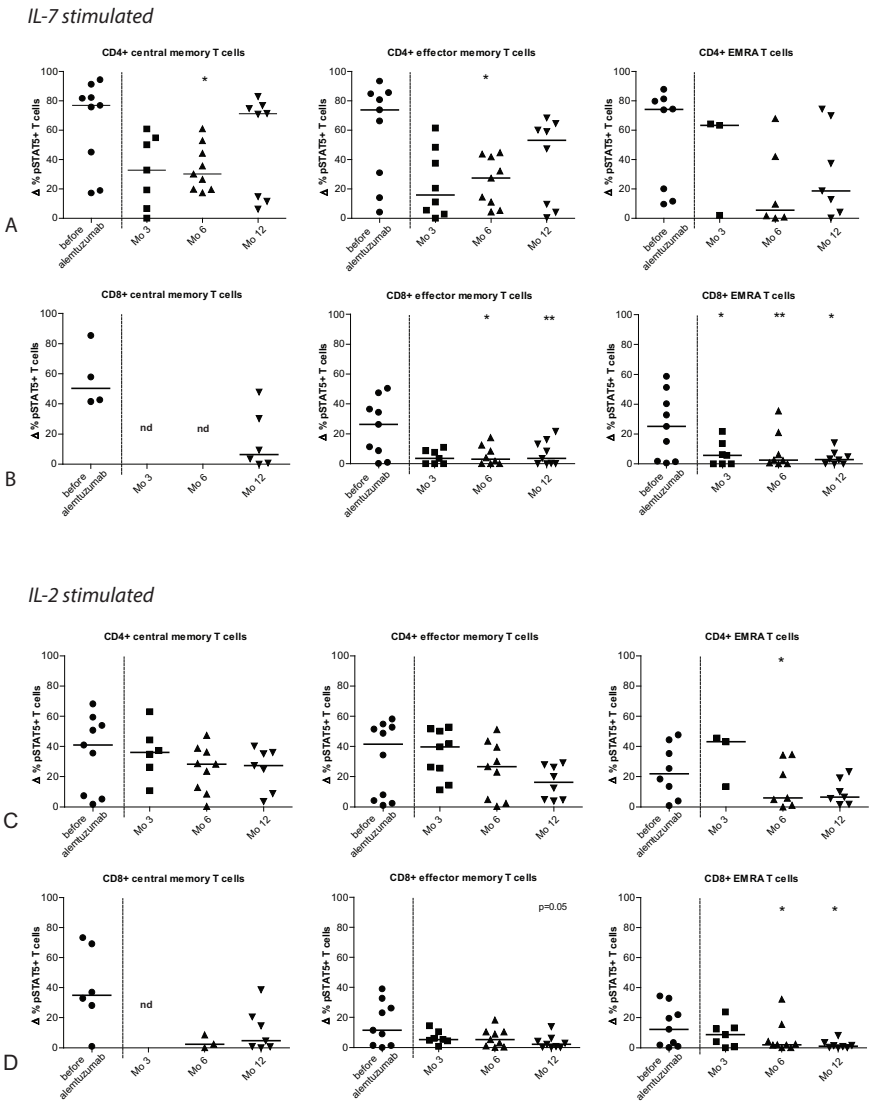
IL-7 responses (42-45). Phenotypically, exhausted T-cells overexpress multiple inhibitory receptors and exhibit poor cytokine responsiveness. Recently, we demonstrated that T cells from rATG-treated kidney transplant patients have affected STAT5 phosphorylation and high levels of cell surface expression of inhibitory receptors. The latter observation strengthened our hypothesis that the impaired T-cell function after T-cell depletion therapy may be explained by T-cell exhaustion phenomena. Clinical evidence that alemtuzumab treatment results in impaired T-cell function is based on data showing that patients are prone to developing infections and malignancies (5, 41, 50). A study by Peleg *et al.* showed that when alemtuzumab was given as anti-rejection therapy more opportunistic infections were observed than when given as induction therapy, indicating an even more dysfunctional T-cell system (46). Although CMV infection is associated with increased percentages of CD8+ T cells and T-cell exhaustion, in this study CMV reactivation was not associated with decreased function (data not shown).

With a population of only 12 patients, this study can only be regarded as hypothesis-generating, and by no means conclusive. Other factors should also be taken into account. In particular, all patients were not given the same maintenance immunosuppression. Most of the patients were treated with the calcineurin inhibitors tacrolimus or cyclosporine, and one patient was treated with the mTOR inhibitor everolimus. Although we know that maintenance tacrolimus immunosuppression does not affect pSTAT5 expression, it is unknown whether the other agents have an effect on T-cell exhaustion parameters (47). The retransplanted patients were treated with rATG for rejection of their previous transplant. We do not know whether this has an effect on our study parameters. Due to very low cell numbers, the co-inhibitory molecules and antigen-specificity of the depletion-resistant cells were not studied. Previously published studies on the effects of T-cell depletion induction therapy report less alloreactivity. It would be interesting to determine whether this is the result of depletion of alloreactive cells or of alloreactive cells becoming functionally impaired. A possible effect of IVIg use should also be considered.

Since we speculate that T-cell exhaustion plays an important role in decreased T-cell reactivity, it would be helpful to know whether inhibitory molecules are upregulated on repopulated T cells after alemtuzumab anti-rejection therapy. Patients who can be treated with reduced-dose immunosuppression could potentially be identified by means of an exhaustion profile, reduced pSTAT5 capacity and upregulation of inhibitory molecules. In addition to T-cell exhaustion, decreased IL-7 induced pSTAT5 is also clinically important for monitoring and perhaps influencing T-cell reconstitution. A study by Mai *et al.* showed an allograft survival advantage in mice IL-7 signaling was blocked (48). Phosphorylation of STAT5 is a key step in IL-7 signaling and therefore a potential monitoring tool. The whole blood assay used here is of interest as this assesses the functionality of T cells under full immunosuppression. For our studies we chose high

concentrations of IL-2 and IL-7 to stimulate T cells because *in vivo* these cytokines are highly present as a result of decreased IL-7 consumption and cell lysis (49, 50)

In conclusion, the profound T-cell depletion after alemtuzumab anti-rejection treatment observed in this small series suggests that the drug's effects are not affected by a pro-inflammatory environment. Repopulation of T cells appeared to arise from increased homeostatic proliferation measured by Ki-67. The reconstituted population predominantly consisted of CD8+ T-cells and T-cells with a memory and regulatory phenotype. Both CD4+ and CD8+ T-cells, as well as Treg cells, showed diminished cytokine responsiveness which was more pronounced for IL-7 than for IL-2. The hypotheses generated by these preliminary findings require exploration in a controlled trial using protocol-specified immunosuppression with a larger study population.



Supplemental figure 1. IL-7 and IL-2 induced STAT5 phosphorylation capacity, IL-7/IL-2 stimulated percentage pSTAT5 minus unstimulated percentage pSTAT5. **(A)** STAT5 phosphorylation capacity in response to IL-7 of central memory, effector memory and EMRA CD4+ T cells; before and at 3, 6 and 12 months after alemtuzumab therapy. **(B)** STAT5 phosphorylation capacity in response to IL-7 of central memory, effector memory and EMRA CD8+ T cells; before and at 3, 6 and 12 months after alemtuzumab therapy. **(C)** STAT5 phosphorylation capacity in response to IL-2 of central memory, effector memory and EMRA CD4+ T cells; before and at 3, 6 and 12 months after alemtuzumab therapy. **(D)** STAT5 phosphorylation capacity in response to IL-2 of central memory, effector memory and EMRA CD8+ T cells; before and at 3, 6 and 12 months after alemtuzumab therapy. ND, not determined due to low numbers of CM cells. Data are shown as median, * $p<0.05$, ** $p<0.01$, significant differences comparing pre-transplant values to post transplant values using the Wilcoxon Signed-Rank test.

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Chapter 7

Phospho-specific flow cytometry for pharmacodynamic monitoring of immunosuppressive therapy in transplantation.

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ABSTRACT

Organ transplant recipients frequently suffer from toxicity or from lack of efficacy of immunosuppressive drugs, which can be attributed to individual variations in drug sensitivity. This problem can be resolved by applying pharmacodynamic monitoring that focuses on measuring the biological effects of drugs. Here we discuss the new technique called phospho-specific flow cytometry to monitor the activity of intracellular immune signaling pathways at the single-cell level in whole blood samples. Through this tool the efficacy of immunosuppressive medication can be assessed, novel targets can be identified, and differences in drug sensitivity between cells and patients can be clarified.

REVIEW

Background

To prevent and to treat alloreactivity, transplant recipients are on immunosuppressive medication for life. These drugs target the immune system in a non-specific manner by affecting immune cell activation, clonal expansion and differentiation. Standard immune suppressing regimens consists of a calcineurin inhibitor (CNI, e.g., tacrolimus/cyclosporine), an inosine monophosphate dehydrogenase inhibitor (mycophenolate mofetil, MMF) and corticosteroids. The therapeutic window of CNIs is small, which places patients at risk of toxicity in case of over-dosing and rejection when under-dosed. Examples of debilitating side effects of immunosuppressive medication are infections, diabetes, nephrotoxicity and malignancies, all influencing patient and graft survival and quality of life. Because of these debilitating side effects, there is a great need for (1) safer and more selective immunosuppressive agents and (2) better monitoring tools and parameters.

In the recent years, new pharmacologic agents have been introduced in the transplantation clinic to more specifically target the molecules of the T-cell activation cascade. Novel agents are drugs that target T-cell receptor (TCR) signaling (e.g., sotrastaurin), co-stimulation pathways (belatacept) and cytokine signaling pathways (tofacitinib). Based on the results found in experimental models, it is expected that these novel immunosuppressants are more specific than CNI.

Here we will touch briefly on the mechanisms of action of these immunosuppressants and discuss the developments in pharmacodynamic monitoring in transplantation patients.

T-cell activation and intracellular signaling pathways: targeting signal 1

After T-cell activation, several intracellular signaling pathways are used for proliferation, differentiation and death. This involves a cascade of phosphorylation and dephosphorylation of intracellular molecules by phosphatases (enzymes that remove phosphate groups from other proteins) and kinases (enzymes that modify proteins by adding phosphate groups). In brief, TCR activation results in the activation of tyrosine kinases of the Syk/Zap-70 family, Tec and Scr families. Upon ligand binding, Src-family kinases phosphorylate tyrosine residues located within immunoreceptor tyrosine-based activation motifs on the cytosolic side of the TCR/CD3 complex by lymphocyte protein tyrosine kinase (Lck) followed by its phosphorylation and activation by CD45 receptor tyrosine phosphatase. Zap-70 is recruited to the TCR/CD3 complex where it becomes activated, promoting recruitment and phosphorylation of downstream adaptor or scaffold proteins. Zap-70 promotes recruitment of the molecule named Vav and phosphorylates the linker for activated T cells (LAT), a transmembrane and adaptor that links the TCR signal to many downstream events, resulting in the activation of the transcription factors, e.g.,

JNK, MAPK, NF-κB, AP-1 and NF-AT (1-3). For example, NF-κB activation is promoted by protein kinase C (PKC)θ, which acts as an intermediate in the transduction of activation by TCR receptor signaling and CD28 co-stimulation (4). NF-κB in concert with the transcription factors NF-AT and AP-1 contributes to interleukin (IL)-2 messenger RNA transcription, a key molecule in the response towards the allograft (Fig. 1A).

Searching for selective immunosuppressive agents to prevent and to treat allograft rejection, phase I and II trials are running with immunosuppressive drugs that target the NF-κB pathway. Particularly, the efficacy and specificity of sotrastaurin (formerly named AEB071), a low molecular mass synthetic compound that potently inhibits all PKC isoforms is currently being studied in kidney transplant patients (Table 1). The phase II trial of sotrastaurin showed little benefit to renal graft function with an excess of acute rejection episodes, gastrointestinal disorders and serious infections when used as maintenance therapy in combination with mycophenolate mofetil and steroids (5,6).

Table 1. Complications after organ transplantation and therapeutic options

Complications & immune responses	Identified cell type	Key Signalling Molecule(s) of	Therapeutic options
Ischemia reperfusion injury (IRI)	NK and NKT cells, neutrophils, macrophages	NF-AT, NF-κB, STATs, MAPK-p38, ERK, JNK NF-κB, AP-1	T cell depletion (rATG/Alemtuzumab)
Hyper acute rejection	B cells & plasma cells	NF-AT, NF-κB, AKT MAPK-p38, ERK, JNK, AP-1	IvIG, T cell & B cell depletion (rATG, Alemtuzumab, Rituximab), Bortizomib
Acute rejection	Cells from the innate & adaptive immune system	NF-AT PKC, NF-κB JAK1/3 NF-κB mTOR	CNI Sotrastaurin/AEB071, Tofacitinib/CP-690,550 Belatacept Everolimus/Sirolimus T cell depletion (rATG/Alemtuzumab)
Chronic rejection	Memory T cells, B cells	NF-AT, NF-κB, AKT, PI3K, MAPK-p38, ERK, JNK, AP-1	Anti-CD2 fusion protein (Alefacept) Depletion of memory T cells (Efalizumab)
Tolerance	FoxP3+ regulatory T cells	mTOR	Everolimus/Sirolimus

CNI: Calcineurin inhibitor, IvIG: Intravenous immune globulin, rATG : rabbit anti thymocyte globulin

Co-stimulation: targeting signal 2

The co-stimulatory signal augments T-cell activation by interactions with cell surface molecules expressed by antigen-presenting cells (APC). In this respect, augmenting interactions are: CD40/CD40ligand, CD28/CD80 and/or CD28/CD86. Inversely, T-cell activation is reduced by the co-stimulation molecule cytotoxic T lymphocyte antigen (CTLA)-4, which transmits a T-cell inhibitory signal after binding to CD80 and/or CD86. The humanized receptor conjugate of CTLA-4 and IgG1, CTLA-4 Ig (belatacept), which

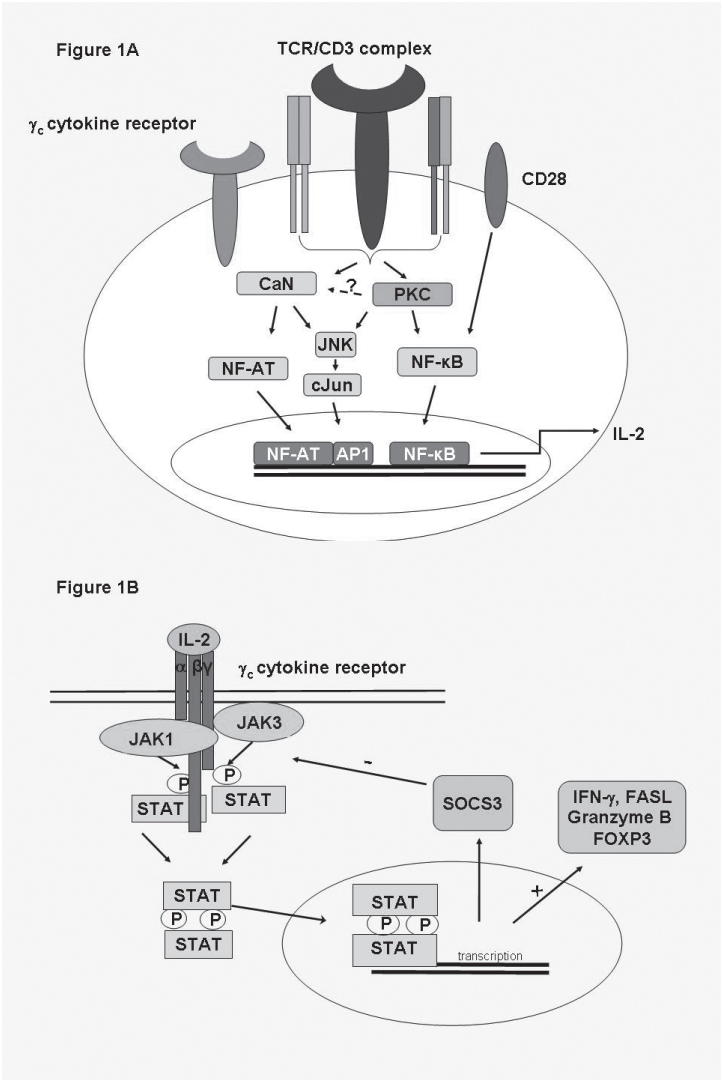

Figure 1. (A) The role of PKC in TCR–CD28-induced signaling in T cells. In response to TCR–CD28 stimulation, PKC is recruited and activated that enables JNK and NF-κB activation. The PKC mediated activation of NFAT is controversial. Taken together, PKC plays the central role in the TCR–CD28-mediated induction of gene transcription leading to proliferation and cytokine secretion in T cells. **(B)** The IL-2R consists of three subunits: α-chain (IL-2R ; i.e., CD25), β-chain (IL-2Rß; CD122) and the common cytokine-receptor γ-chain (γc; CD132). Binding of IL-2 to the heterotrimer IL-2R initiates the activation of janus activated kinase 3 (JAK3) which associates with the γc, while JAK1 associates with IL-2Rβ. Both phosphorylate tyrosine residues in the cytoplasmic part of IL-2Rβ and the γc. Subsequently the JAK molecules are activated which amplifies the association of these tyrosine kinases and the signal transducer and activator of transcription 5 (STAT5) or STAT3, with the cytoplasmic tail of IL-2Rβ. Recruited STATs are phosphorylated by activated JAKs. Activated STATs translocate to the nucleus and activate gene transcription of interferons e.g. (IFN)-γ, granzyme B, FasLigand (FasL), and the transcription factor for regulatory T cells: FOXP3 binding to DNA promoter sequences.

indirectly blocks CD28 signaling, is approved for use in organ transplantation. Blockade of CD28 activation prevents the activation of NF-κB activation, the transcriptional target for co-stimulatory activity (7).

Two recently completed phase 3 trials of belatacept (nulojixW) in kidney transplantation, the so-called BENEFIT and BENEFIT-EXT studies, indicate that this agent is a safe and effective immunosuppressant leading to significantly better renal function (GFR, glomerular filtration rate) as compared to a cyclosporine-based regimen in kidney transplant recipients (8-10). Acute rejection rates were higher but clinically acceptable among belatacept-treated patients (17-22% at 12 months in the belatacept groups versus 7-14% in the cyclosporine groups). While posttransplant lymphoproliferative disease was also more common in those on belatacept, this was largely limited to previously EBV seronegative recipients.

Cytokine signaling: targeting signal 3

The third option studied to treat organ transplant patients with more selective immunosuppressive medication is targeting signal 3: the IL-2 signaling pathway. Blockade of the IL-2 pathway prevents T-cell differentiation and production of effector molecules. This can now be achieved with the immunosuppressant named tofacitinib, formerly known as CP-690,550 and tasocitinib (Table 1). The Janus kinase (JAK)/signal transducer and activator of the transcription (STAT) signal transduction pathway is essential in transmitting cytokine-mediated signals of the IL-2 family members (IL-2, IL-4, IL-7, IL-15, IL-21) to the nucleus in order to alter gene expression programs (11). JAKs possess two near-identical phosphate-transferring domains ((12), Fig. 1B). One domain exhibits the kinase activity, while the other negatively regulates the kinase activity of the first. The four enzymes JAK1, JAK2, JAK3 and tyrosine kinase (TYK) 2 transduce cytokine-mediated signals. After recruitment, STATs are phosphorylated by the JAKs at a specific activating tyrosine residue. This results in dissociation from the receptor, formation of STAT homo- or heterodimers, and translocation to the nucleus. Seven STATs have been identified that after activation interact with specific DNA sequences in target promoters to modify gene expression (12). For instance IL-2 receptor signaling is mediated through activation of JAK1 and JAK3 with subsequent phosphorylation and activation of STAT3 and STAT5, which are key molecules for T-cell development and activation.

Treatment with the JAK1/3 antagonist tofacitinib in kidney transplant recipients demonstrated acceptable safety and tolerability in combination with mycophenolate mofetil (13). Tofacitinib-based treatment resulted in relatively low acute rejection rates but was unfortunately accompanied by high infection rates suggestive of over-immunosuppression (14).

Rational for pharmacodynamic immunomonitoring

Overall the novel immunosuppressants currently being tested in phase II and III trials (sotrastaurin, tofacitinib) and the FDA-approved belatacept failed to show potent, selective immunosuppression. To gain insight into the mechanisms behind these unexpected side effects and the sometimes observed lack of efficacy, it is useful to perform immune monitoring. This will give answers to the questions about the selectivity and efficacy of these novel immunosuppressive compounds. By immune monitoring, questions like the following will be unraveled: "Do the agents indeed block their target(s) in vivo?" ,"How do naive and antigen-experienced memory T cells behave?" ,"Do immunosuppressive agents influence the function of the suppressor regulatory T cells (Tregs) that control immune reactivity?", "Does redundancy in the cytokine network affect the efficacy of the studied immunosuppressant?" Recent developments in phospho-specific flow cytometry now provide the opportunity for routine measurements of intracellular signaling molecules in different T-cell subsets. In the next part of the article, we will discuss this tool with its recent developments and applications to better determine the balance between drug efficacy and side effects for the individual patient.

Immune monitoring by classical flow cytometry

Flow cytometry is the standard immune monitoring method to measure T-, B- and NK-cell numbers in the circulation of rATG/alemtuzumab-treated patients and to verify CD25 blockade on T cells during basiliximab therapy. Reports on these studies showed correlations between cell numbers and the occurrence of opportunistic infections and the malignancies without providing insight on the function of the repopulated T cells and of T cells covered with anti-CD25 mAb (15-18). For instance the infections in rATG-treated patients are explained by the low T-cell and NK-cell numbers. However, there is now evidence that the function of the repopulated T cells is impaired, which may affect the immunity of rATG-treated patients (17-19). Also the role of regulatory T cells (Tregs) in clinical organ transplantation is often studied by their phenotype. One of the first papers describing a role for these cells in drugfree tolerant kidney transplant patients was by Louis et al., who reported high numbers of peripheral Tregs compared to patients with chronic rejection (20). In line with effector T cells, also the numbers and function of Tregs are influenced by immunosuppressive drugs whereby both Treg-favoring and -hampering effects were reported (21-25). Also, the first studies analyzing the mechanisms of action of tofacitinib and belatacept report an effect on peripheral Treg numbers. A significant decrease in Treg numbers with unexpected potent regulatory capacities of tofacitinib-treated patients was found (26). Additional information on why the suppressive function was not influenced by tofacitinib or how rATG triggers the induction of Tregs in vivo would have been helpful to understand these findings.

Accordingly, there is a clear-cut need for methods that provide information at the molecular level of immune competent cells. A method that recently became available for clinical research and diagnostics and offers these applications is phospho-specific flow cytometry. By this method intracellular signaling pathways at the single-cell level can be measured. It is a quick and reliable method that combines the extracellular cell surface characteristics with intracellular molecular activities and thus will provide the additional information required for to better understand T-cell functions during immune responses in immunosuppressed transplantation patients.

Phospho-specific flow cytometry: a novel tool to measure intracellular signaling pathways

With the recent advances in flow cytometry, the number of parameters that can be measured has been largely expanded. These new parameters now allow us to monitor immune responses functionally at rest and following activation at the single-cell level. This includes parameters such as ligand-induced activation, intracellular cytokine production, cytotoxic and proliferative activities, and most recently intracellular signaling pathways. The availability of phospho-specific antibodies makes it possible to study protein phosphorylation at the posttranslation level, thereby influencing the activity of intracellular proteins. Protocols have been developed that can be used to study, in whole blood, cellular activation cascades both in the presence and absence of agents that stimulate or inhibit cellular functions. This approach will provide information on the dynamics of the immune responses seen in organ transplant patients. But one should be aware that this is only the beginning of this new research area as more than 80,000 unique phosphorylations have been described in mammalian cells (www.phosphosite.org).

Key complications that occur after transplantation are ischemia reperfusion injury and acute- and chronic rejection (Table 1). The underlying mechanisms are complex, and different immune competent cells are involved that interact with each other and with the grafted tissue. Consequently, the therapeutic approach differs between these clinically relevant complications. As summarized in the table, many molecules participate in transmitting signals. The immunosuppressive agents tofacitinib and sotrastaurin were designed to specifically target these molecules, JAK1/3 and PKCθ, respectively. Others like the calcineurin inhibitors and mTOR inhibitors, both isolated from fungi, also target intracellular signaling molecules. One of the first papers showing the potential of pharmacodynamic monitoring of mTOR inhibitor molecules was recently reported (27). By whole blood phospho-specific flow cytometry, rapamycin was found to inhibit a downstream target of mTOR: the phosphorylated S6 ribosomal protein. This assay may have advantages over the existing mTOR activity tests, which are far from reliable in predicting the efficacy of sirolimus and everolimus. Proof that indeed the analysis of

the phosphorylated S6 ribosomal protein is useful for clinical purposes has not been provided yet, but the paper by Barten's group using clinically relevant concentrations of sirolimus shows its potential for therapeutic drug monitoring. A nice example demonstrating the power of phospho-specific flow cytometry was given by Wu et al., who reported STAT5 activation in NK cells and T cells of melanoma patients who received a bolus infusion of IL-2 (28). It was speculated that the responders and non-responders to IL-2 treatment can be identified by STAT5 measurements of peripheral blood cells. Further defective STAT1 phosphorylation was reported in lymphocytes from the majority of melanoma patients vs. healthy individuals after treatment with type I interferon (29). And basal activation of STAT signaling and reduced response to type I and II interferons, IL-2, IL-6 and IL-10, may be helpful to identify the activity and severity of systemic lupus erythematosus (30). In kidney transplant patients, we analyzed the effects of the JAK1/3 antagonist tofacitinib on IL-2-, IL-7- and IL-15 triggered activation of STAT5 and found a dose dependent inhibition (Fig. 2A, B). The whole blood analysis of tofacitinib-treated patients nicely showed inhibition of the IL-2-, IL-7- and IL-15-activated P-STAT5. An example of P-STAT5 measured in CD4+ T cells from a kidney transplant patient during tofacitinib treatment is depicted in Figure 3. Phospho-specific flow cytometry can also be used to study cross reactivity of an immunosuppressive compound. For instance, tofacitinib inhibits JAK2 activation, a pathway that plays a role in hematopoiesis and may explain the mild anemia observed in tofacitinib-treated patients (25,31). Apart from the analysis of typical target signaling molecules, e.g., NF-κB in sotrastaurin-treated patients, mTOR in sirolimus and p38 MAPK signaling in CNI-treated kidney transplant patients, this phospho-specific flow cytometry technique can also be used to study T-cell function before and after rATG induction treatment and to further unravel the mechanism of action of immunosuppressive agents like CNI (26,32). For instance, we used this technique to study the function of T cells after rATG induction therapy and found impaired signal 3 responses by the memory T-cell population. Particularly the CD8+ memory T cells responded poorly upon IL-2 activation by P-STAT5 (Fig. 4). This finding may help to explain why rATG-treated patients often suffer from severe viral infections. It is a combination of different immune phenomena: (1) low T-cell numbers and (2) impaired T-cell function. The latter finding shows the strength of the phospho-flow technology: detection of abnormal signaling signatures as an indicator for impaired cellular function.

Challenges to clinical practice and limitations of flow cytometry

The clinical applications for phospho-protein analysis by flow cytometry are clear: the efficacy and side effects of immunosuppressive drugs, pharmodynamic profiling, cellular functions and disease activity. However, like any other laboratory technique, also this method has its typical difficulties. A limitation is the availability of antibodies of interest. Yet it is expected that more and more phospho- and epitopic-specific antibodies will

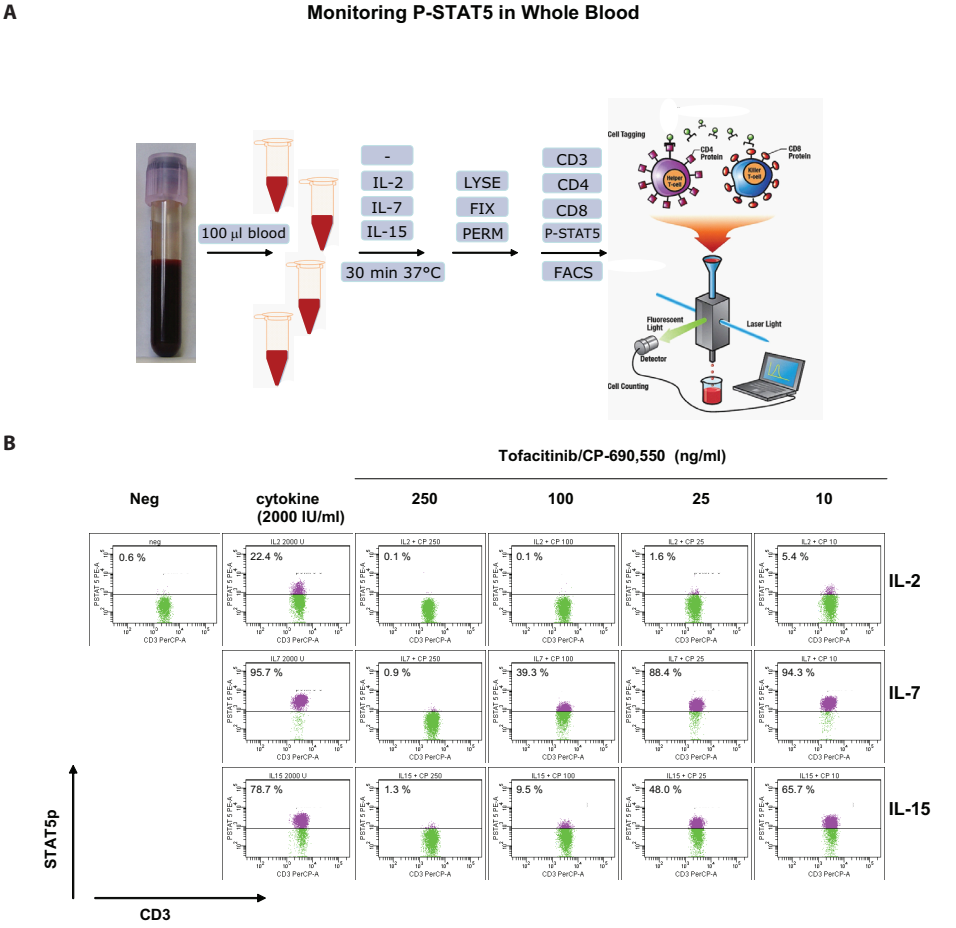

Figure 2. (A) Staining procedure of STAT5. Whole blood (100 µl) is stimulated by IL-2, IL-7 or IL-15 for 30 min at 37°C. Red blood cells are lysed and white blood cells are fixed for 10 min at 37°C with Lyse/Fix Buffer (BD Biosciences, San Jose, CA). Next, the cells are washed in FacsFlow buffer (BD Biosciences) and permeabilized with cold 70% methanol for 30 min at −20°C, washed twice in FacsFlow buffer supplemented with 0.5% bovine serum albumin followed by standard staining procedures for P-STAT5 (clone Y694), CD3, CD4 and CD8. **(B)** Whole blood stimulated for 30 min with IL-2, IL-7 and IL-15 (2,000 IU/ml) in the absence and presence of different concentrations of tofacitinib/CP-690,550. P-STAT5 expression by CD3 T cells.

become available soon. For instance, antibodies that detect TCR induced T-cell activation would be highly valued. These antibodies can be used to determine whether or not T cells are present in the peripheral blood of organ transplant patients that respond to donor antigen. Analysis of activated Zap-70 will provide this key information and will at the same time be the functional alternative that overcomes the difficulties and limitations of TCR tetramer flow cytometry (33). Further, simultaneous measurements of phosphorylated proteins together with cytokines are far from optimal, and reproducibility from one experiment to the next remains a problem. In this respect, standardiza-

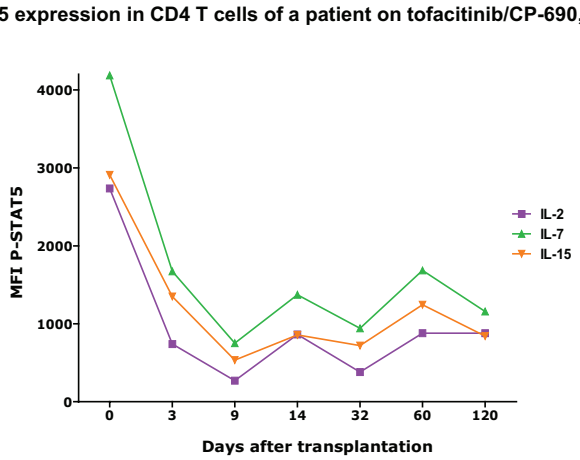

Figure 3. STAT5 phosphorylation by CD4+ T cells after whole blood activation with IL-2, IL-7 and IL-15 was inhibited in this patient on tofacitinib/CP-690,550 therapy. Cytokine activated P-STAT5 is plotted at the Y-axis as the median fluorescence intensity (MFI) of cytokine stimulated P-STAT5 minus the MFI of the unstimulated sample.

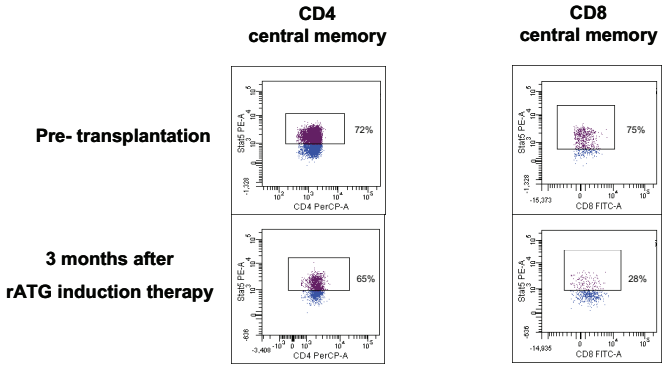

Figure 4. Example of decreased IL-2 induced STAT5 phosphorylation after rATG therapy in CD8 central memory T cells (CD45RO+CCR7+) and not in CD4 central memory T cells.

tion of the instrument using multicolor bead samples is helpful. Data interpretation is also a question to address: should percentages or mean fluoresce intensities (MFI) be used. The relative MFI values are based on control samples like the unstained control samples, isotype control or fluorescent minus one control to set thresholds and are used to measure a shift in fluorescence intensity of the entire cell populations. Consequently, it is not clear how many cells of the entire cell population have changed expression of the molecule of interest.

As stated in the paper by Herzenberg et al., interpreting flow cytometry data is the 'Tower of Babel': negative, unstained populations can seem to be positive and vice versa (34). Therefore, optimization and standardization are key for the success of (phospho-specific) flow cytometry for measuring immune responses in healthy and diseased individuals. Addressing these limitations will further improve the utility of phospho-specific flow cytometry for clinical applications.

CONCLUSIONS

Recently, phospho-specific flow cytometry came forward as a new and powerful tool to analyze the activity of intracellular signaling pathways by rapid and sensitive detection of intracellular phosphorylated proteins at the single-cell level. It is a technique that can be used to study (pathological) conditions in immune cells, can be used as a screening tool to identify novel targets of immunosuppressive drugs and for pharmacodynamic monitoring of patients on immunosuppressive medication. In particular patients treated with drugs that target these intracellular signaling molecules: the inhibitors of calcineurin, mTOR and kinase activity should be monitored by phospho-specific flow cytometry. Analysis of signaling pathways in large patient populations will show patient-specific differences in immune reactivity, drug susceptibility and drug-related side effects.

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Chapter 8

Summary and general discussion

SUMMARY

T-cell depletion therapy is an effective immunosuppressive strategy for the prevention and treatment of acute rejection early after organ transplantation. Its immunosuppressive potency is also reflected by the increased risk of infections and malignancies after treatment (1-4). Moreover, the opportunity to treat patients with a CNI sparing regimen or early steroid withdrawal points to long lasting effects of this type of immunosuppression (5-14). In the search for immunosuppressive minimization strategies and opportunities to avoid unwanted side effects, the mechanisms responsible for these long-lasting immunosuppressive effects of T-cell depleting agents are of interest. The current thesis describes studies concerning immune reconstitution after T-cell depletion therapy. Furthermore, it aimed to provide more knowledge about the immunomodulatory mechanisms underlying the immunosuppressive effects of T-cell depletion therapy.

After the general introduction (**chapter 1**), describing the mechanisms of action of divers immunosuppressive drugs, including T-cell depletion therapy by rATG and alemtuzumab, **chapter 2** studied the effects of different dosages of rATG induction therapy, ultra-low (3 x 0.5 mg/kg), low (3 x 1.0 mg/kg) and normal dosing (3 x 2.0 mg/kg) on the number of peripheral leucocytes. All rATG dosages depleted T and NK cells, while B-cell counts were hardly affected. NK cells repopulated, in all groups, within week 1. In contrast, T-cell recovery was dose-dependent; recovery of T-cell numbers varied from 1 month in the ultra-low dose group to over a year in the standard treatment group. This study showed that significantly lower dosages rATG can be given when T-cell depletion is wanted for several months.

T-cell repopulation is the result of thymopoiesis and homeostatic proliferation. In **chapter 3** we studied both mechanisms in patients treated with rATG, basiliximab or no induction therapy. We found that thymopoiesis, measured by the percentage CD31+ naive T cells, was not different in patients treated with rATG than in patients treated with basiliximab or no induction therapy. In contrast, the first month after therapy, homeostatic proliferation, defined by the percentage of Ki-67+ naive and memory cells, was highest after treatment with rATG. At three months, levels of increased homeostatic proliferation in the rATG group were diminished despite low T-cell numbers. Proliferation of naive but also memory cells was at least partly the result of cytokine induced proliferation, measured by the expression of CD31 and pSTAT5. In the repopulated T-cell pool we found a predominance of CD8+ and memory T cells.

A repopulated T-cell pool is not only associated with a predominance of memory T cells, also increased percentages of Treg are described. This cell population is an important immunosuppressive counter mechanism controlling immune activation Therefore, in **chapter 4**, we described the impact of rATG and basiliximab induction therapy on the number, the repopulation mechanisms and the function of these cells. We found, after rATG, increased percentages of Treg, characterized as CD4+CD25+CD127- and

CD4+CD127-FoxP3+ cells, which were not the result of increased thymopoiesis but of increased homeostatic proliferation. After basiliximab decreased percentages of CD4+CD25+CD127- Treg were found which might be explained by blockade of CD25, the α-chain of the IL-2 receptor, which is highly expressed on Treg. In addition we found that the observed high proportion of Treg after rATG was not solely caused by homeostatic proliferation of residual cells but was also the result of the induction of induced Treg (iTreg), determined by the absence of the nTreg marker Helios in combination with a predominance of a methylated FoxP3 gene. After basiliximab no increased percentages of iTreg were found. At the functional level we measured an increased production of IL-10 after both induction strategies. When we studied the suppressive capacities of Treg, only after rATG therapy, signs of selective suppression of donor-specific immune responses by Treg were observed, which was in contrast to Treg of patients treated with basiliximab.

In **chapter 5** we studied the hypothesis that cytokine responsiveness of T cells is affected after repopulation as we earlier observed that T-cell proliferation diminishes despite low T-cell numbers after rATG therapy. For that purpose the capacity of T cells to phosphorylate STAT5, a master regulator in T-cell activation and proliferation in response to cytokines, was determined. After rATG, the phosphorylation capacity of T cells in response to IL-7 and IL-2 showed decreased responses, especially CD8+ memory cells, which deteriorated over time. T cells from basiliximab-treated patients showed different kinetics, they were not affected in their IL-7 responsiveness but IL-2 responses were affected, especially the first month after therapy. Next to decreased STAT5 phosphorylation, CD4+ and CD8+ T cells showed upregulated expression of co-inhibitor receptors which, for CD8+ cells but not for CD4+ cells, were correlated with decreased percentages pSTAT5 in response to IL-7. Both decreased pSTAT5 and upregulation of co-inhibitory molecules are characteristics of exhausted T cells, a dysfunctional state of T cells induced by persistent antigen expression.

Because of a favorable side effect profile, alemtuzumab is currently used for T-cell depletion as anti-rejection treatment. In **chapter 6** we evaluated the effects of alemtuzumab depletion therapy on the mechanisms of T-cell repopulation and cytokine responsiveness in an activated immune system, during and after rejection. After T-cell depletion T cells slowly repopulated by homeostatic proliferation. Also after anti-rejection therapy a predominance of memory T cells was observed that had a decreased IL-7 and IL-2 dependent STAT5 phosphorylation capacity. Furthermore, an increased percentage of Treg with preserved responsiveness to IL-2 was found.

After transplantation it is important to treat patients with an immunosuppression regimen that is minimal enough to prevent toxic side effects but sufficient to prevent rejection. This requires tools that better determine the balance between drug efficacy and side effects. By phospho-specific flow cytometry the biological effects of immuno-

suppressive agents can be measured at the single cell level in whole blood, so under full immunosuppression. This monitoring tool is described in **chapter 7**.

DISCUSSION

The overall aim of this thesis is to better understand the process of T-cell reconstitution and by which mechanisms T-cell depletion therapy modulates the immune system after kidney transplantation. Therefore, the first aim was to determine whether different dosages of rATG induce a different duration and degree of T-, B- and NK cell lymphopenia. Currently, a dose of 6 mg/kg is generally accepted for induction therapy (15). However, despite the numerous years of clinical rATG use there is no consensus about its optimal dose, balancing safety and efficacy. With growing experience, it became clear that high doses of rATG induction therapy, more than 10.0 mg/kg, were not necessary to achieve sustained lymphocyte depletion (15). For example, Agha et al. showed similar efficacy of 6 mg/kg rATG (16). Multiple trials explored lower dose regimens with less than 6 mg/kg rATG; some reported comparable outcomes, whereas others observed a higher incidence of early acute rejection episodes (17-22). In our study we evaluated the effects on cells involved in the rejection process and determined the number of peripheral T, B and NK cells after treatment with different dosages rATG; i.e., 1.5, 3.0 and 6.0 mg/kg. Our findings demonstrated that B cells were not depleted in contrast to NK and T cells. NK cells repopulated within one week and T-cell reconstitution was dose-dependent. The current standard protocol of 6.0 mg/kg depletes the recipient's T- cell system over 12 months. Because the goal of induction therapy is to suppress the immune system in the first few months post-transplantation we here speculate that current rATG regimen can be adapted to lower dosages for the individual patient. The efficacy will be preserved, whereas safety profiles will clearly improve. Whether a shorter duration of T-cell depletion has consequences for the function of repopulated immune cells is unknown.

We and others described that the duration of T-cell depletion after rATG is dose-dependent (18, 23). A definite explanation for this phenomenon has not been found. From the literature it is known that apart from the cumulative rATG dose also other co-variables influence T-cell recovery. Peak concentration, the prolongation of serum concentrations of rATG as well as patient characteristics such as age, are important contributing factors for T-cell reconstitution (24, 25). In non-human primates, Preville et al. showed dose dependent lymphocyte depletion in blood and, to a lesser extent, in lymph nodes and spleen but not in the thymus (25). The extent of T-cell depletion in the lymph nodes and spleen was suggested to be related to the peak doses rather than to the cumulative dose. In kidney transplant patients pharmacodynamics and pharmacokinetics of rATG were studied by Büchler et al. (26). It was demonstrated that over a longer period, total rATG, as well as the fraction of rATG antibodies that bind human cells (therapeutic, active

rATG), were significantly higher in the high dose group (24, 26, 27). This difference only resulted in a prolonged decrease of naïve CD4+ T cells, reconstitution of other T-cell subsets did not differ between treatment arms. As the thymus appeared to be unaffected by rATG in non-human primates, the persistence of active rATG in plasma, capable of triggering processes like antibody dependent cellular toxicity and apoptosis might contribute to this finding (24-26). Similar finding have been reported for alemtuzumab treatment. In a human CD52 transgenic mouse model it was shown for that high doses of alemtuzumab are needed to achieve T-cell depletion in lymph nodes and spleen, and even than a maximum depletion of 50% of single and double positive thymocytes was achievable in the thymus (28). In contrast to the study of Büchler et al, others report that the proportion of reconstituted Treg dependents on rATG dosing, as only after high dosages of rATG increased percentages of Treg were found (26, 29-31)

The second aim of the thesis was to unravel the mechanisms responsible for T-cell reconstitution. After depletion therapy no increased thymopoiesis was measured (chapter 3, 6). The latter contrasts with earlier studies performed in T-cell depleted patients, both kidney transplant and others, like HIV infected and bone marrow transplant patients. In those patient populations thymic renewal did occur (31-35). Possible explanations for this discrepancy might be differences in study design, used control groups and patient characteristics like age, underlying disease and given immunosuppression. For example it is known that end-stage renal disease independent of the duration of dialysis and underlying kidney disease results in loss of thymic function caused by a lack of IL-7, susceptibility of T cells for apoptosis and involution of the thymus and other lymphoid organs (36-40). Kidney transplantation cannot reverse these effects. It has been hypothesized that DNA modifications by epigenetic changes, an important cellular control mechanism, persist after kidney transplantation (41). In addition, maintenance immunosuppression by glucocorticoids, MMF and CNI's, decrease the number of thymocytes by affecting different developmental stages of thymocytes (42-45).

It is well known that the production of newly formed recent thymic emigrants decreases with age due to thymic involution. Therefore, the most important mechanism for T-cell reconstitution in adults is homeostatic proliferation of residual T cells (46). Our studies showed, both after rATG induction and alemtuzumab anti-rejection therapy, increased homeostatic proliferation of both memory and naive T cells. The role of homeostatic proliferation of residual memory T cells was earlier concluded from the finding that especially memory cells are present in the circulation after T-cell reconstitution (31, 46, 47). However, by solely studying T-cell numbers, a role for proliferating naivecells adopting a memory phenotype is neglected. In a mouse model, Sener et al. demonstrated that naive T cells exhibited the greatest increase in homeostatic proliferation, after which they were converted into a memory phenotype (48). Furthermore, CD8+ naive T cells can acquire characteristics of memory cells by homeostatic proliferation

under lymphopenic conditions (49). In our study, we analyzed proliferation directly by the Ki-67 expression and measured proliferation of both naive and memory T-cells. Both CD4+ and CD8+ memory T cells had the largest percentages of dividing cells, with no significant differences between memory subpopulations. The latter implicates that the predominance of CD4+ effector memory and CD8+ EMRA T cells after depletion therapy is not the result of enhanced proliferation of these subsets but of their relative resistance to depletion. Surprisingly, the increased percentages of proliferating T cells disappeared after 3 and 6 moths for CD8+ and CD4+ T cells, respectively. At this time point, T-cell numbers were still below baseline levels. Homeostatic proliferation is dependent upon the homeostatic cytokines IL-7 and IL-15 and on antigen-driven interactions. Especially, the latter is associated with a shift from a naive to a memory phenotype of T cells, resulting in a skewed, oligoclonal T-cell population, making hosts more susceptible for de novo infections (46). The underlying mechanisms of lymphopenia induced homeostatic proliferation are extensively studied in mouse models, which revealed that IL-7 is an important cytokine for the homeostatic proliferation of both naive and memory T cells and IL-15 especially for memory T cells (50). The mechanisms in humans are less well studied but knowledge might be valuable for manipulation of these processes in order to improve immune reconstitution. In our study we found indirect evidence for antigen driven stimulation by the predominance of memory cells. More clear evidence for cytokine driven proliferation was found. The presence of Ki-67+CD31+ naive cells and high percentages of cytokine triggered pSTAT5 suggest that homeostatic proliferation of naive T cells occur, at least partly, via cytokines after T cell depletion therapy. This cytokine dependent stimulation may be a possibility to modulate the immune system.

Despite the predominance of memory cells, patients who were given T-cell depletion therapy do not need higher dosages of maintenance immunosuppression. In contrast, these patients can be treated with reduced dosages of immunosuppressive drugs (5-14). The decreased need for immunosuppressive therapy reflects immunomodulatory mechanisms by T-cell depletion therapy. The finding that despite low peripheral T-cell numbers T-cell proliferation was not different between kidney transplant patients who were treated with a T-cell depleting and non-depleting regimen strengthened us in the idea that reconstituted T cells have impaired function. Hence the third aim of this thesis was to determine the mechanisms that explain these impaired T-cell functions. The first mechanism studied was the presence and function of suppressive Treg (chapter 4). After both rATG induction and alemtuzumab anti-rejection therapy increased percentages of these suppressive T cells were present in the peripheral blood. These Treg resulted from homeostatic proliferation and from the conversion of CD4+ T cells into induced (i) Helios- iTreg. We speculate that these iTreg play an important role in the suppression of donor antigen specific proliferation as these cells are formed during lymphopenia in the presence of donor antigen. Although the increased percentages

of Treg in the peripheral blood normalized 6 months after therapy, their donor specific immunomodulatory function remained intact. This implicates that these cells may reside at the graft or secondary lymph nodes, from which activation, proliferation and release in the bloodstream takes place during immune activation. The second studied mechanism explaining impaired immune functions after T-cell depletion therapy was decreased cytokine responsiveness of reconstituted T cells. The diminished homeostatic proliferation despite a far from complete recovery of T-cell numbers in combination with the observation that cytokines play a pivotal role in T-cell reconstitution in rATG treated kidney recipients, let us hypothesize that T cells exhibit impaired responsiveness towards cytokines after T-cell depletion therapy. Cytokines are soluble factors that activate cells after binding to their cognate receptors after which transcription factors of the JAK-STAT pathway are activated that eventually leads to T-cell activation and differentiation (51, 52). In our studies, CD8+ T cells showed impaired responses to IL-7 after T-cell reconstitution. Also IL-2 responsiveness was affected by depletion and anti-CD25 therapy. In addition to impaired pSTAT5 responses, rATG therapy resulted in upregulation of co-inhibitory receptors. These observations are also made in patients suffering from viral infections and malignancies also known as the concept of T-cell exhaustion (53). Multiple reports in virus infected patients describe a relationship between altered STAT signaling and co-inhibitory molecules with roles for SOCS (a key molecule in controlling the JAK-STAT pathway) and other signaling molecules (54-57). We conclude from our data that continuous pressure by donor antigen in combination with the disruption of lymphoid structures, CD4+ T cell lymphopenia and competition among T-cell clones contribute to an exhausted T-cell system in kidney transplant patients. Next to donor specific exhausted T cells also virus specific exhausted T cells may be present in kidney transplant patients, due to the increased prevalence of infections after T-cell depletion therapy. A third mechanism is the presence of terminally differentiated immunosenescent CD8+CD28- T cells (58). These functionally impaired T cells compete for "immune space" with immunocompetent CD4+ and CD8+ T cells, preventing their proliferation and therefore explaining decreased responsiveness of the repopulated T-cell pool. In contrast to exhausted T cells, these cells cannot be reinvigorated by blocking co-inhibitory receptors (53). It has been reported that rapid recovery of CD8+ cells and low percentages of CD4+ T cells may be associated with improved graft outcome (18, 47, 58, 59) The forth underlying mechanism might be hyporesponsiveness by T-cell anergy. Anergic T-cells are unresponsive cells induced when T-cell are stimulated by antigen in the absence of co-stimulatory signals (60-62). RATG blocks these co-stimulation and adhesion molecules in an environment with a high antigenic load. Consequently, T-cell anergy might be a plausible mechanism of hyporesponsiveness. Overall, we state that after T-cell reconstitution, memory T cells are not simply hazardous cells endangering the graft. These cells have duality in function exhibiting both negative and positive features

after repopulation. On the one hand they can quickly and vigorously respond to donor antigen, contributing to transplant rejection thereby endangering transplant survival. On the other hand memory cells are indispensable for protection against pathogens and malignancies. Moreover, after T-cell reconstitution in the presence of donor antigen these cells are functionally impaired, inducing donor-hyporesponsiveness and therefore a more tolerable state towards the graft.

The overall aim of this thesis was to gain knowledge about the process of T-cell repopulation and immunomodulatory mechanisms after T-cell depletion therapy. Uncovering these mechanisms can then be used to manipulate the immune system, thereby improve clinical practice in order to avoid over-immunosuppression and its associated complications. One of the therapeutic options might be enlargement of the naive T-cell pool by promoting repopulation of naive cells, thereby broadening the TCR repertoire, by IL-7 therapy (63). Studies performed in HIV infected or bone marrow transplant patients showed that IL-7 administration increases the naive T-cell compartment with broadening of the TCR repertoire (64-66). In addition, in a study performed in alemtuzumab treated Multiple Sclerosis patients, in vitro treatment with IL-7 induced the expansion of Treg and inhibited Th17 and Th1 cells (67). Treatment with IL-7 unfortunately also has a downside, as proliferation of memory cells depends on IL-7 as well (63, 68). The study of Mai et al. showed, in a mouse model, that blocking the IL-7 receptor promoted allograft survival (69). Therefore, we speculate that IL-7 therapy is only suitable for low-risk patients or patients with an exhausted or hyporesponsive memory T-cell system. Moreover, IL-7 therapy should be given shortly after transplantation as, shown in this thesis, immune responses diminish during reconstitution. Another clinical implication based on findings described in this thesis is the use of Treg to allow reduction of the current used immunosuppression. Currently, the first clinical trials to test the safety and feasibility of Treg therapy are ongoing (70). We and others showed that after depletion therapy increased percentages of Treg are present the peripheral blood (71-75). Moreover, we found that after rATG Treg have an iTreg phenotype and suppress donor immune responses on the expense of third party responses (chapter 4). Hence we speculate that T-cell depletion therapy could be given to transplant patients to induce donor-specific iTreg. Based on studies described in this thesis, the presence of immune modulation by donor specific iTreg and exhausted T cells contributing to impaired memory responses suggest that maintenance immunosuppressive regimen might be given at lower dosages in T-cell depleted transplant patients. Multiple studies support this assumption (5-14). Impaired T-cell functions can be monitored by phospho-specific flow cytometry. Knowledge obtained by this technology may enables us to tailor immunosuppressive regimen based on the functionality of T-cell reactivity of the individual patient. By this tool we are able to measure T-cell responses under full immunosuppression at the molecular level of immune competent cells. The power of

this assay was shown in patients with melanoma or systemic lupus erythematosus to identify responders and non-responders to IL-2 treatment and disease activity (76, 77).

Although the evidence for immunomodulatory mechanisms by Treg and exhausted T cells brings us closer unraveling the mechanisms underlying the long-lasting effects of T-cell depletion therapy more research is required. For example how can we promote the induction of donor-specific iTreg? Are antigen specific iTreg still present at 6 months after transplantation and control these cell the anti-donor response? What are the additional characteristics of exhausted T cell in kidney transplant patients and how can we use these to identify and detect these cells in order to obtain information about T-cell reactivity in these patients? Are exhaustion markers new biomarkers? Furthermore, are memory T cells unresponsive in general or predominantly towards donor antigen after T-cell depletion therapy? How can we join the characteristics of immune modulating mechanisms which result in an unresponsive memory compartment in order to define a fingerprint of T-cell reactivity use for clinical practice?

CONCLUSION

The described research in this thesis aimed to provide further knowledge about T-cell repopulation after depletion therapy and its immunomodulatory mechanisms underlying the long-lasting immunosuppressive effects. It was concluded that T-cell repopulation is a dose dependent process that is predominantly the result of, at least partly cytokine driven homeostatic proliferation of both naïve and memory T cells, which diminishes before T-cell numbers are on baseline level. Within the repopulated T-cell pool we found a predominance of CD8+ memory cells. Moreover immune modulation was studied. We found increased percentages of regulatory T cells, able to suppress donor-specific T-cell responses, were detected. The impaired T-cell functions were further explained by up-regulation of co-inhibitory molecules and a decreased capacity to phosphorylate STAT5; both features of T-cell exhaustion. With these findings we conclude that the presence of donor specific Treg and T-cell exhaustion are underlying mechanisms of observed long-lasting immunomodulatory effects of depletion therapy. These observations can be monitored with phospho-specific flow cytometry and can be used to optimize individualize immunosuppressive therapy; improving the balance between under and over immunosuppression.

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Chapter 9

Dutch summary (samenvatting)

SAMENVATTING

De meest effectieve behandeling voor patiënten met eindstadium nierfalen is vervanging van de nierfunctie door transplantatie. Om afstoting van het transplantaat te voorkomen worden deze patiënten levenslang behandeld met immunosuppressieve, oftewel afweeronderdrukkende therapie. In de loop der jaren zijn de immunosuppressieve medicijnen sterk verbeterd waarmee een steeds betere overleving van het transplantaat werd behaald. Helaas hebben deze medicijnen ook ongewenste bijwerkingen zoals een verhoogde kans op infecties en maligniteiten.

Bij de afstotingsreactie van een niertransplantaat spelen verschillende afweercellen zoals macrofagen, B-cellen, NK-cellen en T-cellen een belangrijke rol. Dit proefschrift gaat over immuunsuppressiva die de T-cellen verwijdert uit het bloed; T-cel depletie therapie. T-cel depletie therapie wordt gegeven rondom de operatie als inductietherapie en, wanneer een transplantaat wordt afgestoten, als anti-rejectie therapie. Naast het verwijderen van T-cellen, maar ook NK-cellen en soms zelfs B-cellen, geeft deze therapie ook andere langdurige immuunsuppressieve effecten. In dit proefschrift hebben we deze processen bestudeerd. We keken naar het aantal T-, B- en NK-cellen en meer uitgebreid naar de repopulatiemechanismen en functies van T-cellen. Tevens hebben we gekeken naar immuunmodulatie; mechanismen die het langdurige immuunsuppressieve effect van T-cel depletie therapie kunnen verklaren.

De meest gebruikte medicijnen voor T-cel depletie zijn rabbit antithymocyte globulin (rATG) en alemtuzumab. Ondanks dat rATG al jaren gebruikt wordt in de kliniek is er geen consensus over de optimale dosering; de dosis waarbij effectiviteit en risico op bijwerkingen in de juiste balans zijn. In **hoofdstuk 2** hebben wij het effect van verschillende doses rATG op het aantal T-, B- en NK-cellen bestudeerd; ultra-lage (3 x 0.5 mg/kg), lage (3 x 1.0 mg/kg) en een standaard (3 x 2.0 mg/kg) dosering. Het bleek dat alle rATG doseringen T- en NK-cellen depleteerden, het aantal B-cellen daarentegen was niet aangedaan. De terugkomst van NK-cellen en T-cellen verschilde; na 1 week was het aantal NK-cellen in alle groepen weer op het oude niveau terwijl het terugkomen van de T-cellen verschilde van 1 maand in de ultra laag gedoseerde groep tot meer dan een jaar in de groep met de standaard dosering rATG. Deze resultaten laten zien dat voor T-cel depletie voor een aantal maanden minder hoge doseringen rATG gebruikt kunnen worden dan op dit moment standaard zijn in de kliniek.

T-cellen komen weer terug in het bloed door thymopoiese; de nieuwvorming van T-cellen in de thymus en homeostatische proliferatie; het delen van aanwezige naïeve en geheugen T-cellen in de circulatie. In **hoofdstuk 3** hebben we deze processen bestudeerd nadat patiënten behandeld waren met rATG, basiliximab (niet T-cel depleterend maar T-cel remmend), of zonder aanvullende inductie therapie. Het fenotype van de gerepopuleerde cellen bestond voornamelijk uit CD8+ geheugencellen. Daarnaast vonden we dat de mate van thymopoiese niet verschilde tussen de genoemde patiënten-

groepen. Wel was er een verschil in de mate van homeostatische proliferatie, de eerste maanden na transplantatie was deze hoger na rATG behandeling. Dit effect doofde na 3 tot 6 maanden uit, interessant omdat op dit moment het aantal cellen nog niet op het oude niveau terug was. Proliferatie van T-cellen in het bloed is het gevolg van stimulatie met antigeen of stimulatie met cytokinen, oplosbare factoren die belangrijk zijn voor de activatie, proliferatie en delingsactiviteit van cellen. Onze studie toonde aan dat voor zowel naïeve als geheugen T-cellen stimulatie met cytokinen belangrijk is.

De gerepopuleerde T-cel populatie bestaat voornamelijk uit CD8+ geheugen T-cellen. Naast deze toename in effector cellen wordt na T-cel depletie therapie ook een toename gezien in het aantal regulatoire T-cellen (Treg) zoals beschreven in **hoofdstuk 4**. Treg, hier gedefinieerd als CD4+CD25+CD127- en CD4+CD127-FoxP3+ T-cellen, zijn belangrijk in het reguleren van immuunresponsen; ze onderdrukken effector cellen en voorkomen daarmee onder andere auto-immuunziekten, overbodige immuunreacties tegen lichaamseigen weefsel. In onze studie vonden we dat de toename van Treg na rATG behandeling niet het resultaat was van een verhoogde thymopoiese maar van een toename aan homeostatische proliferatie. Ook basiliximab beïnvloedt het voorkomen van Treg; lagere percentages van CD4+CD25+CD127- Treg werden gevonden. Dit wordt mogelijk verklaard door de blokkade van CD25 door basiliximab. Naast toegenomen homeostatische proliferatie van Treg vonden we ook meer geïnduceerde Treg (induced Treg; iTreg) in het bloed van rATG behandelde patiënten. Deze cellen werden gedefinieerd door de afwezigheid van Helios; de marker voor natural Treg (nTreg), in combinatie met een hoog percentage Treg met een gemethyleerd *FoxP3* gen. Deze iTreg werden niet gevonden na basiliximab therapie. Treg werden ook op functioneel niveau bekeken. Na zowel rATG als basiliximab therapie werd een toename van het cytokine IL-10 waargenomen. Wanneer we de immunosuppressieve capaciteiten van Treg bekeken zagen we tekenen van donor selectieve suppressie na rATG maar niet na basiliximab therapie. Deze bevinding kan ten minste gedeeltelijk de geobserveerde verminderde effector T-cel reactiviteit na T-cel depletie therapie verklaren.

Vanwege de uitgedoofde homeostatische proliferatie bij een nog niet hersteld aantal T-cellen hebben we in **hoofdstuk 5** de hypothese onderzocht dat T-cellen minder goed kunnen reageren op cytokines na T-cel depletie therapie. Hiervoor hebben we de capaciteit van T-cellen om STAT5, een regulator in T-cel activiteit en proliferatie, te kunnen fosforyleren bestudeerd. Na rATG bleek deze capaciteit in respons op IL-2 en IL-7 verminderd te zijn, vooral in CD8+ T-cellen. Ook na basiliximab inductie therapie vonden we dit effect dan wel met een andere kinetiek. Naast verminderde STAT5 fosforylatie capaciteit lieten CD4+ en CD8+ T-cellen ook een verhoogde expressie zien van co-inhibitoire receptoren. Voor CD8+ T-cellen was deze verhoogde expressie gecorreleerd met de verlaagde STAT5 fosforylatie capaciteit in respons op IL-7. Zowel verminderde STAT5 fosforylatie als de verhoogde expressie van co-inhibitoire receptoren zijn kenmerken

van uitgeputte T-cellen; "exhausted T cells", een status van de cel waarin deze niet meer goed functioneert, veroorzaakt door continue stimulatie met antigeen.

Depletie therapie met rATG wordt ook gegeven als anti-rejectie therapie maar door het gunstigere bijwerkingsprofiel wordt tegenwoordig alemtuzumab veelvuldig gebruikt. In **hoofdstuk 6** hebben we de effecten van alemtuzumab depletie therapie in een door afstoting geactiveerd immuunsysteem geëvalueerd. We zagen dat ook hier de T-cellen terugkwamen in de circulatie door homeostatische proliferatie. De gerepopuleerde T-cellen hadden voornamelijk het CD8+ geheugen fenotype en ook hier was de fosforylatie capaciteit van STAT5 in respons op IL-2 en IL-7 gestoord. Ook na alemtuzumab werden meer Treg in de circulatie gemeten. Deze cellen toonden goede responsen op hun belangrijkste cytokine IL-2.

Niertransplantatie patiënten hebben levenslange behandeling met immuunsupressiva nodig. Om toxische bijwerkingen te voorkomen is een minimale hoeveelheid medicatie gewenst. Deze hoeveelheid moet hoog genoeg zijn om het afweersysteem te onderdrukken maar laag genoeg om overmatige immunosuppressie te voorkomen. Om deze juiste afstemming te monitoren zijn nieuwe, betere methodes nodig dan de nu voor handen zijnde. Een mogelijke nieuwe methode is fosfo-specifieke flowcytometrie zoals beschreven in **hoofdstuk 7**.

Samenvattend tonen de resultaten beschreven in dit proefschrift aan dat T-cel depletie dosis afhankelijk is en dat de repopulatie van T-cellen het gevolg is van homeostatische proliferatie van zowel naïeve als geheugen T-cellen. Cytokines spelen hierbij een belangrijke rol. In het gerepopuleerde T-cel compartiment komen relatief veel CD8+ geheugen cellen voor. Daarnaast vonden we veel regulatoire T-cellen en waren er aanwijzingen dat deze cellen donor specifieke reacties kunnen onderdrukken. De geheugen cellen vertoonden verminderde delingscapaciteit alsook een verminderde fosforylatiecapaciteit van STAT5. Dit laatste was gecorreleerd aan een verhoogde expressie van co-inhibitoire receptoren, beide eigenschappen van uitgeputte T-cellen. Hieruit concluderen we dat regulatoire T-cellen en uitgeputte cellen beide onderliggende mechanismen zijn van de langdurige immunomodulatoire effecten van T-cel depletie therapie. Een mogelijke methode om zowel reactiviteit van het immuunsysteem alsook het effect van immunosuppressieve therapie te kunnen meten is fosfo-specifieke flow cytometrie. In de toekomst kunnen we hiermee wellicht de therapie op het individu afstemmen en zodoende de balans tussen effectiviteit en bijwerkingen verbeteren.

Appendices

PhD PORTFOLIO

Name PhD Student	Anne Pauline Bouvy
Erasmus MC department	Internal Medicine, section Nephrology and Transplantation
Research school	Postgraduate School Molecular Medicine
PhD period	2010-2016
Promotors	Prof.dr. C.C. Baan
	Prof.dr. W Weimar

Courses and workshops

2010 Irradiation Hygiene Expertise 5A/B*

2010 Short introductory Course on statistics & survival Analysis for MD's*

2011 Course of Molecular Immunology for PhD students*

2011 European Transplant Fellow Workshop; European Society of Organ Transplantation (ESOT)

2011 Biostatistical Methods I: Basic Principles*

2012 Course of (neuro)-immunology*

2013 Biomedical English Writing and Communication*

* Erasmus MC, Rotterdam

Participation and presentations at (inter)national conferences

2010	Science days, dept. of Internal Medicine Erasmus MC, Antwerp, Belgium	participation
2010	Annual meeting Dutch Transplant Society (NTV) (Bootcongres), Rotterdam, The Netherlands	participation
2010	American Society of Transplantation (AST): Annual Scientific Exchange, Orlando, USA	participation
2011	Science days, dept. of Internal Medicine Erasmus MC, Antwerp, Belgium	poster
2011	MolMed Day, Rotterdam, The Netherlands	poster
2011	Annual meeting NTV (Bootcongres), Amsterdam, The Netherlands	oral
2011	American Transplant Congress, Philadelphia, USA	poster
2011	TTS Basic Science Symposium and ESOT Basic Science Meeting, Boston, USA	poster
2011	Congress of ESOT, Glasgow, UK	oral
2012	Science days, dept. of Internal Medicine Erasmus MC, Antwerp, Belgium	oral
2012	Annual meeting NTV (Bootcongres), Maastricht, The Netherlands	oral (2x)
2012	American Transplant Congress, Boston, USA	oral/poster (2x)
2012	International congress of the Transplantation Society (TTS), Berlin, Germany	oral/poster
2012	ESOT and AST Joint Meeting, Nice, France	poster
2012	Waddensymposium, Texel, The Netherlands	oral
2013	Annual meeting NTV (Bootcongres), Duiven, The Netherlands	oral (2x)
2013	American Transplant Congress, Seattle, USA	oral/poster

2013	Congress of ESOT, Vienna, Austria	oral (2x)
2013	ESOT Basic Science Meeting and TTS Basic Science Symposium Paris, France	poster
2014	Annual meeting NTV (Bootcongres), Leiden, The Netherlands	oral
2014	World Transplant Congress, San Francisco, USA	poster
2014	Congress of ESOT, Brussels, Belgium	oral

Travel grants and awards

2011 Bootbeurs NTV

2012 Poster with distinction, ATC, Boston, USA

2013 Poster with distinction, ATC, Seattle, USA

2013 Travel grant Vereniging Trustfonds Erasmus Universiteit Rotterdam

2013 ESOT/TTS Travel Grant Award for the ESOT Basic Science Meeting and TTS Basic Science Symposium, Paris, France

2013 Travel Grant Astellas for the -ESOT Basic Science Meeting and TTS Basic Science Symposium, Paris, France

2014 Poster with distinction, WTC, San Francisco, USA

2014 Bootbeurs NTV

Teaching activities

2011-2013 Lectures for first and second year medical students

Memberships

2015-present Jonge Nederlandse Internisten Vereniging (JNIV)

2010-present Nederlandse Transplantatie Vereniging

2010-2015 The Transplantation Society

2010-2015 The European Society of Organ Transplantation

CURRICULUM VITAE AUCTORIS

Anne Pauline Bouvy was born on February 2nd 1983 in Vlaardingen, the Netherlands. In 2000 she completed secondary school at Scholengemeenschap Spieringshoek in Schiedam and started studying Life Science and Technology at the Delft University of Technology and the University of Leiden. After a year Anne switched to medical school at the Erasmus University in Rotterdam. She obtained her medical degree in October 2009 and subsequently worked as a resident at the Department of Internal Medicine at the Erasmus Medical Center. In January 2010 she started her PhD project at the Transplantation Laboratory of the Internal Medicine Department; Nephrology and Transplantation at the Erasmus MC, under the supervision of prof.dr. Carla Baan and prof.dr. Willem Weimar. This research resulted in this thesis. In 2014 Anne began her internal medicine residency at the IJsselland Ziekenhuis in Capelle aan den IJssel, the Netherlands, under supervision of dr. H.E. van der Wiel. On May 23rd 2015 Anne married Ralph Aarts with whom she lives in Rotterdam.

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Anne

ABBREVIATIONS

APC	antigen presenting cell
APC	allophycocyanin
ATN	acute tubular necrosis
BPAR	biopsy proven acute rejection
CCR	CC chemokine receptor
CD	cluster of differentiation
CM	central memory
CMV	cytomegalovirus
CNI	calcineurin inhibitor
CTLA-4	cytotoxic T-lymphocyte antigen-4
DC	dendritic cell
DM	diabetes mellitus
EBV	epstein-barrvirus
EDTA	ethylenediamine tetraacetic acid
ELISPOT	enzyme-linked immunospot
EM	effector memory
EMRA	effector memory cells expressing CD45RA
ESRD	end stage renal disease
FACS	fluorescence-activated cell sorting
FITC	fluorescein isothiocyanate
FoxP3	forkhead box P3
GFR	glomerular filtration rate
GITR	glucocorticoid-induced TNFR-related protein
GPI	glycosylphosphatidylinositol
HCV	hepatitis C virus
HIV	human immunodeficiency virus
HLA	human leucocyte antigen
ICAM	intracellular adhesion molecule
IFNγ	interferon gamma
IgG	immunoglobulin G
IL	interleukin
iTreg	induced regulatory T cell
JAK	Janus kinase
KDIGO	kidney disease improving global outcomes
LAG-3	lymphocyte-activation gene 3
mAb	monoclonal antibody
MAP	mitogen activated protein

MHC	major histocompatibility complex
MLR	mixed lymphocyte reaction
MMF	mycophenolate mofetil
mTOR	mammalian target of rapamycin
NFAT	nuclear factor of activated cells
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NK	natural killer
nTreg	natural regulatory T cell
PB	pacific blue
PBMC	peripheral blood mononuclear cell
PCR	polymerase chain reaction
PD-1	programmed cell death-1
PE	phycoerythrin
PerCP	peridinin chlorophyll protein
PI3-K	phosphoinositide 3-kinase
PKC	protein kinase C
PKD	polycystic kidney disease
PMA	phorbol 12-myristate 13-acetate
PRA	panel reactive antibody
rATG	rabbit antithymocyte globulin
RTE	recent thymic emigrant
SOCS	suppressor of cytokine signaling
STAT	signal transducer and activator of transcription
Tc-cell	cytotoxic T cell
TCR	T-cell receptor
Tfh-cell	follicular T helper cell
TGFβ	transforming growth factor beta
Th-cell	T helper cell
TIM-3	T cell immunoglobulin mucin-3
TNFα	tumor necrosis factor alpha
TREC	T cell receptor excision circle
Treg	regulatory T cell
TSDR	Treg-specific demethylated region
TSLP	Thymic stromal lymphopoietin
TYK2	tyrosine kinase 2