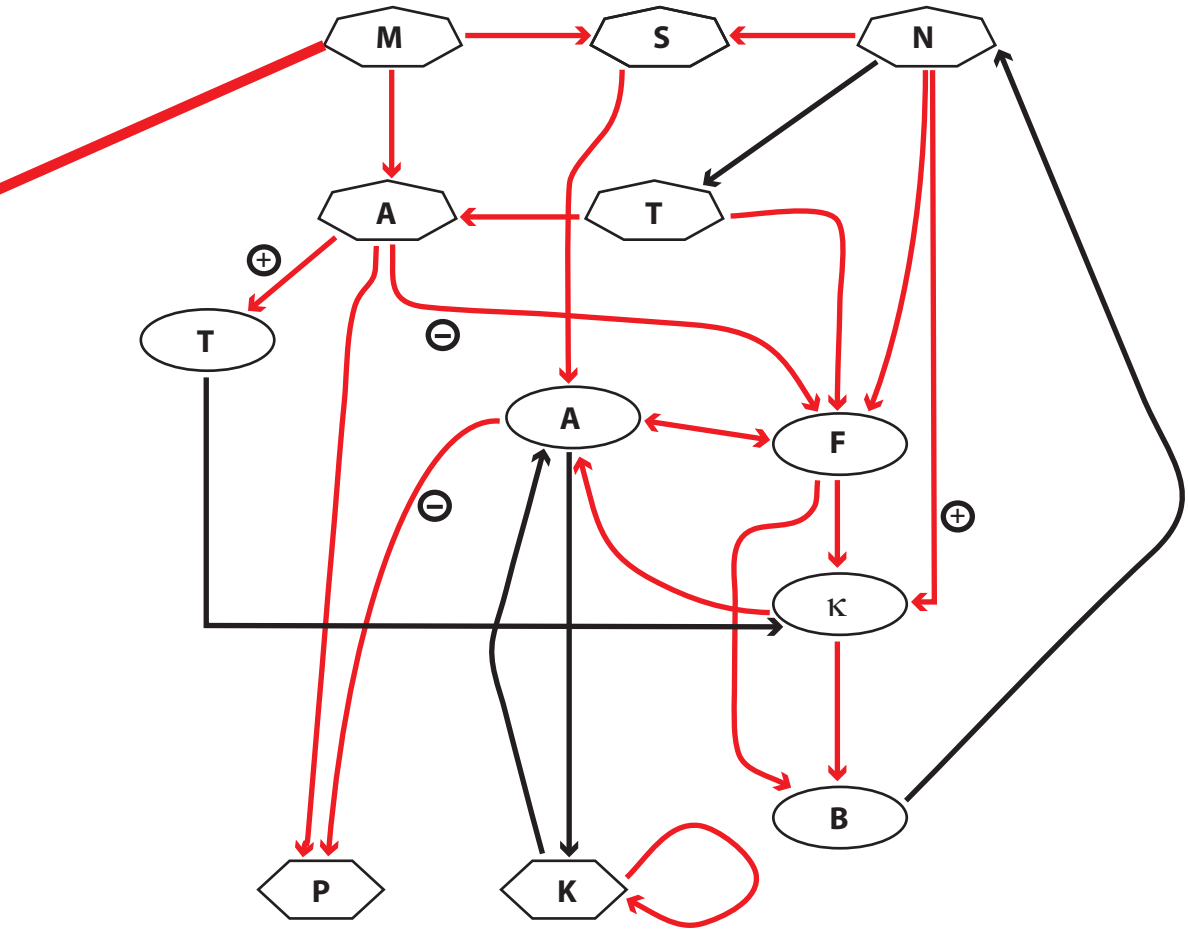
Exploring Pharmacodynamics of Immunosuppressive Agents in Transplantation

Ramin Vafadari



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Exploring Pharmacodynamics of Immunosuppressive Agents in Transplantation

Meten van de biologische effecten van immunosuppressieve medicijnen bij transplantatiepatiënten

Proefschrift

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بر لوح نشان بودنیها بوده است

پیوسته قلم ز نیک و بد فرسوده است

در روز ازل هرآنچه بایست بداد

غم خوردن و کوشیدن ما بیهوده است

The Moving Finger writes; and, having writ,

Moves on: nor all thy Piety nor Wit

Shall lure it back to cancel half a Line,

Nor all thy Tears wash out a Word of it.

Omar Khayam

(Translation E. Fitzgerald)

Contents

Chapter 1

General Introduction

Background

Supported by advancements in technology, surgical techniques and immunosuppressive drugs, solid organ transplantation has become the preferred solution to end stage organ failure (1). The first solid organ transplantation was performed in 1954 under supervision of Joseph Murray in the Peter Bent Brigham Hospital in Boston. A kidney from a healthy donor was transplanted into his identical twin, who suffered from chronic glomerulonephritis (2). The transplanted organ functioned immediately and the recipient survived for 25 years while the donor lived for another 56 years. Since the twins were genetically identical, no suppression of the immune system was needed.

Transplantation between individuals other than identical twins was made possible a decade later by the use of the combination of azathioprine and corticosteroids, achieving a 1 year allograft[1] survival rate of 40 to 50 percent (3). The introduction of the calcineurin inhibitor (CNI), cyclosporine A in the early 1980s (4) and tacrolimus in 1989 (5), greatly improved 1 year allograft survival rates to more than 80 percent (6). Thereafter a substantial number of immunosuppressive agents have entered the transplantation arena (7-8). Nevertheless, due to a less toxic and favorable immunosuppressive profile CNI are still the cornerstone of the drug regimen after transplantation of the lung, heart, liver, pancreas, hand, and kidney. Therefore, the current regimen after kidney transplantation comprises induction therapy (monoclonal or polyclonal anti-T cell antibodies) at the time of transplantation followed by chronic medication: a combination of CNI, corticosteroids and an anti-proliferative agent such as mycophenolate mofetil. Tacrolimus has proven to be superior to cyclosporine in terms of acute rejection and graft survival (6, 9-10) and is consequently the most commonly prescribed CNI after renal transplantation.

Over the last decade, the short-term allograft survival rates have improved to over 90% (1 year survival rate), while the long term organ survival rates still have much room for improvement (6, 11). In the United States for example 29% of kidney allografts are lost within five years after transplantation; and 53% are lost after ten years (12). Paradoxically, the chronic immunosuppressive regimen is associated to graft loss due to (nephro-)toxicity or ineffectiveness of the drugs leading to immunologic injury (11, 13-14). At the same time, over 40% of recipients develop severe drug related short and long term side effects, such as diabetes, infections, malignancies and cardiovascular morbidity and mortality (15-16). Better medical care and measurements of drug blood concentrations (pharmacokinetics) might have improved long term outcomes (15-17).

[1]Allografts are tissues or cells transplanted from a donor to a recipient of the same species.

Pharmacodynamics & Pharmacogenomics

In a substantial number of transplant recipients however, pharmacokinetic monitoring does not provide sufficient information on drug effectiveness and toxicity (17). Hence further individualization of the immunosuppressive therapy using pharmacodynamic parameters and pharmacogenomics is necessary and currently investigated in most transplant centers (18-20). Pharmacodynamic studies hold the promise to explain and predict the variation in drug susceptibility of patients, by investigating the relationship between drug concentrations and their biological effects (21). Pharmagenomics studies the influence of genetic variation on drug response in patients, by correlating gene expression or single-nucleotide polymorphisms (SNP) with drug efficacy and toxicity (22). In this regard the influence of polymorphisms in *ABCB1* (ATP-binding cassette sub-family B member 1) on the pharmacokinetics of CNI is under investigation. The *ABCB1* gene encodes for the para-glycoprotein, an efflux pump which is expressed on the cell surface of many cell types including T and B cells (23) and transports exogenous and endogenous substrates, including CNI, from the inside of the cells to the outside (23-24). The ABCB1 3435CC genotype has been associated with a higher pump function compared to the 3435CT and TT genotypes (25) and this variance in drug-efflux from immune cells might explain the therapeutic resistance seen in part of the patient population.

Allograft Rejection and T Cell-Drug Interactions

Transplantation of an organ from a donor to a genetically non-identical recipient induces an immune response in the recipient against the foreign antigens of the donor allograft. T cells are important mediators of acute rejection and a major risk factor for chronic allograft dysfunction (15). In recipients with progressive allograft dysfunction, these cells are a common cause of graft loss later than one year after transplantation. Other contributors are natural killer cells, macrophages, complement and B cells. T cells, the studied cell population in this thesis, are primed in the recipient's draining lymph nodes to donor human leukocyte antigen (HLA) peptides either directly by donor antigen presenting cells (APC) or indirectly by recipient APC (26). Once activated, these cells differentiate into various subgroups, migrate to and infiltrate the allograft (15) where they attack the transplanted organ.

T cells are activated by three consecutive signals. The first signal is initiated by binding of the T cell receptor (TCR) to HLA molecules on the surface

of professional APC, like dendritic cells and non-professional APC, e.g. B cells and macrophages. For T cell activation a second signal is required and provided by co-stimulation via triggering of the CD28 molecule on the surface of T cells by CD80/86 from APC (27).

Activation of signal 1 and 2 initiates three intracellular signaling pathways which induce transcription of target genes for production of cytokines (e.g. interleukin (IL)-2, tumor necrosis factor (TNF)-α and interferon (IFN)-γ), chemokines (e.g. granulocyte-macrophage colony-stimulating factor) and surface proteins (e.g. CD25, CD40L). The three signaling pathways initiate and control the immune response. They comprise the calcineurin-nuclear factor of activated T cells (NFAT) signaling pathway, the mitogen activated protein kinase (MAPK) pathway and the nuclear factor κB (NF-κB) signaling pathway. The calcineurin pathway is initiated by calcium influx which activates calcineurin, a serine/threonine protein phosphatase. Activated calcineurin phosphorylates the cytosolic nuclear factor of activated T cells (NFAT) and allows its nuclear translocation, which promotes the transcription of lymphokine genes, production of pro-inflammatory cytokines (e.g. IL-2 and IFN-γ) and T cell activation (28) (Fig. 1). The calcineurin inhibitors tacrolimus and cyclosporine, form a complex with the immunophilins cyclophilin A and FKBP-12 respectively to inhibit calcineurin phosphatase activity.

The MAPK pathway is a conserved eukaryotic signaling module in which MAPKK kinase (MAPKKK or MAP3K) phosphorylates MAPK kinase (MAPKK). The latter activates cytosolar p38 MAPK, allowing its nucleur translocation and activation of many transcription factors resulting in immediate gene transcription of important cellular proteins and cytokines (29) (Fig. 1). P38 MAPK activity is negatively regulated by MAPK phosphatases that remove phosphate from its serine/threonine or tyrosine residues (30-31). The NF-κB pathway is activated downstream of the TCR by PKC which controls the formation of the Carma1, BCL10, MALT1 (CBM) complex . This complex activates IκB kinase (IKK), the enzyme that degrades the inhibitor ofκB (IκB) protein. In resting cells NF -κB hetero-dimers are held in an inactive state by IκB proteins. After activation by the CBM-complex, NF-κB dimers are liberated to translocate to the nucleus and start transcription of genes which encode for cytokines, chemokines and adhesion molecules (32-33) (Fig. 1). These activation pathways are not independent but interact with each other. For example p38 MAPK is needed for the transcriptional activation of NF-κB-dependent gene expression (34). The signaling pathways and their interactions are further discussed in the following chapters, where we investigate how intracellular signaling molecules are influenced by the CNI tacrolimus.

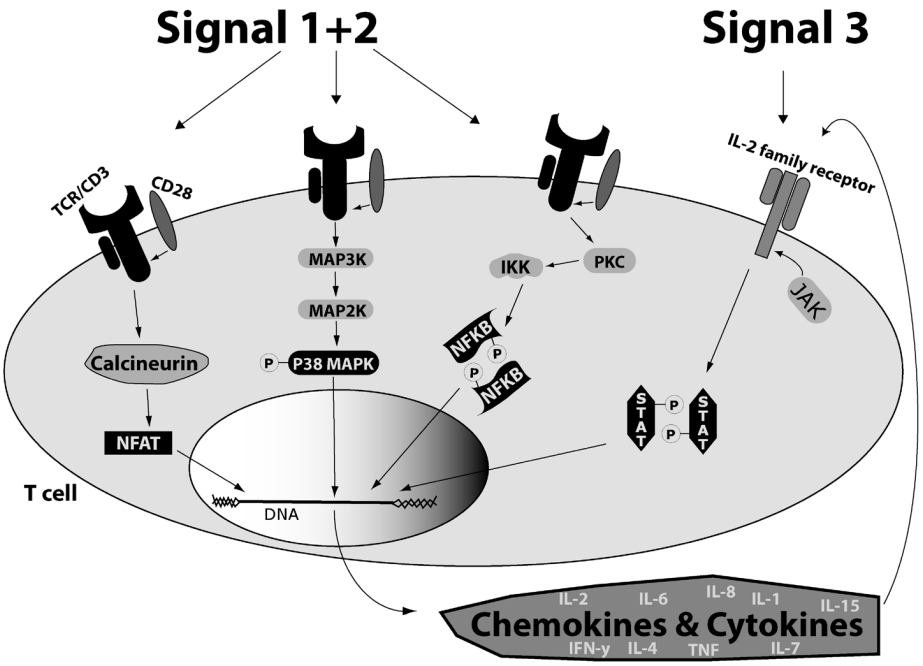

Figure 1: Intracellular Signaling Pathways examined in this thesis.
Cytokine production is initiated by stimulation of the T cell receptor-CD3 complex (signal 1) in conjunction with CD28 engagement (signal 2).Three main transcription factors are involved: p38 mitogen activated protein Kinase (MAPK), nuclear factor κB (NF-κB) and nuclear factor of activated T cells (NFAT). Signal 3 in T cells is activated via their cytokine-receptors through the Janus activated kinase (JAK)-signal transducer and activator of transcription (STAT) signaling pathway in which JAK-molecules are recruited to the IL-2-family cytokine-receptor. The JAK-molecules phosphorylate the receptor facilitating STAT-phosphorylation and translocation to the nucleus.

Pro-inflammatory cytokines produced by APC together with the cytokine-end-products of signal 1 and 2 in T cells trigger signal 3 in T cells. Signal 3 is amongst others activated by the interleukin (IL)-2 family cytokines (i.e. IL-2, IL-4, IL-7, IL-9, IL-15 and IL-21). It stimulates the intracellular Janus activated kinase (JAK)-signal transducer and activator of transcription (STAT) signaling pathway and drives CD4+ T helper cells and CD8+ T cells differentiation into effector and regulatory cells (35). Tofacitinib is a small drug molecule that interferes with signal 3. It is a novel orally-administered JAK-inhibitor currently successfully investigated in a phase 2b trial for the prevention of acute rejection in 331 renal allograft recipients (36). Clinical efficacy of tofacitinib was comparable to the CNI CsA, while renal function was better at the expense of a higher incidence of side effects (37).

Scope and Outline of this Thesis

The scope of this thesis is to unravel and quantify the biological effects of immunosuppressive drugs used after renal transplantation, within the ongoing quest to optimize individual treatment.

Tacrolimus in combination with mycophenolate mofetil are the most often used immunosuppressive drugs after kidney transplantation. These drugs are included in over 80% of kidney transplant recipients initial maintenance immunosuppression (12). In chapter 2 and 3 the biological effects of tacrolimus on human T cells and their potential use for pharmacodynamic monitoring will be described while in chapter 4 variations in the biological effects of tacrolimus by genetic causes is investigated. Chapter 5 and 6 involve the new promising JAK inhibitor tofacitinib which is currently tested in clinical trials of CNI sparing therapies after renal transplantation.

In **Chapter 2** we examined the effect of tacrolimus on mitogen-induced p38 MAPK activation and the potential of p38 MAPK as a biomarker. The effect of tacrolimus on the p38 MAPK signaling pathway in T cells of healthy volunteers and renal transplant recipients is described. Most of the pharmacodynamic monitoring strategies for the calcineurin inhibitor tacrolimus analyze downstream molecules of NFAT activation, e.g. IL2. The MAPK activation pathway on the other hand is a master regulator of the calcineurin-NFAT pathway in T cells and therefore arguably a more relevant signaling pathway. In **Chapter 3** the possible effect of tacrolimus on the NF-κB signaling pathway in peripheral human T cells is studied. Here for the first time the effect of tacrolimus on this activation pathway is measured at clinically relevant concentrations in peripheral human T cells to further unravel the mechanism of action of the drug. This study is a precedent for the development of new and clinically relevant targets for pharmacodynamic monitoring. **Chapter 4** describes the genetic variability affecting the function of the ABCB1 efflux pump on T cells. The association between the 3435 SNP in *ABCB1* and inter-individual differences in tacrolimus efficacy is studied here *in vitro* and in renal transplant recipients.

Chapter 5 serves as a preface to the following chapter, and discusses the development of the phosphospecific flow cytometry technique for monitoring the JAK-STAT activation pathway. In **chapter 6** the pharmaco-dynamic monitoring results obtained by this technique, in a tofacitinib phase 2b trial of renal transplant recipients, are presented.

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Chapter 2

Inhibitory Effect of Tacrolimus on p38 Mitogen Activated Protein Kinase Signaling in Kidney Transplant Recipients Measured by Whole Blood Phosphospecific Flow Cytometry

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Willem Weimar, Carla C. Baan

Abstract

Introduction: Tacrolimus (TAC), the cornerstone of immunosuppressive therapy after solid organ transplantation, inhibits calcineurin activation. Despite pharmacokinetic monitoring, patients frequently suffer from toxicity or lack of efficacy, which could be prevented by pharmacodynamic monitoring. In Jurkat T cell lines it has been shown that TAC, in addition to calcineurin, inhibits the p38 mitogen-activated protein kinase (MAPK) pathway, which is important in T cell activation and therefore a potential drug-specific biomarker. We studied whether TAC inhibits p38 MAPK signaling in primary human T cells and ex vivo in healthy volunteers and kidney transplant recipients.

Methods: PMA/ionomycin induced MAPK signaling was measured by whole blood phosphospecific flow cytometry.

Results: In vitro, TAC 10 ng/mL inhibited p38 MAPK phosphorylation by 27% in CD3+ T cells, 26% in CD4+ T cells and, 34% in CD8+ T cells (mean, $p<0.01$ compared to baseline). In healthy adults (n=4), 2 hours after a single oral dose of 10 mg TAC, the p38 MAPK activation was inhibited by 35% in CD3+, CD4+ and CD8+ T cells ($p<0.05$ compared to baseline). In kidney transplant recipients (n=24) TAC predose concentrations (range 3.2 – 10.5 ng/mL) were inversely correlated with p38 MAPK activation in CD3+, CD4+ and CD8+ T cells ($r^2 = 0.51$, 0.34 and 0.37, respectively; $p<0.01$).

Conclusions: TAC inhibits activation of the MAPK pathway in a dose dependent manner in kidney transplant patients and this may be a potential marker for immune-monitoring.

Introduction

After kidney transplantation patients are routinely treated with the calcineurin inhibitors (CNI), cyclosporin A (CsA) and tacrolimus (TAC), to prevent rejection [1-2]. CNI have a narrow therapeutic range and therapeutic drug monitoring (TDM) is required [3-5]. While the area under the concentration-time curve (AUC) is the most reliable predictor of drug exposure, most transplant centers use the trough concentration for therapeutic drug monitoring which is a poor predictor of drug-related complications [6]. Despite intensive pharmacokinetic monitoring, a substantial number of organ transplant patients experience acute rejection episodes or may even lose their allograft through lack of efficacy [7-10]. At the same time, over 40% of patients develop severe drug-related short and long term side effects [9] such as diabetes, hypertension, dislipidemia and nephrotoxicity [11]. These drug-related complications are not explained by interindividual differences in drug exposure and therefore pharmacodynamic monitoring which measures the biological effects of immunosuppressive drugs may be a better means of performing TDM [12-13]. Pharmacodynamic monitoring is a promising alternative. Nonetheless, the strategy to be used for drug-effect monitoring is under development and far from crystallized [14]. In the current study the feasibility of a new biomarker for TAC-monitoring is investigated.

For pharmacodynamic monitoring, insight in the mechanism of action of the drug is necessary. Two mechanisms of action have been described for CNI. The first and best-known mechanism is inhibition of the protein phosphatase activity of calcineurin: an important component in T cell activation which induces nuclear translocation of the transcription factor: nuclear factor of activated T cells (NFAT). By blocking calcineurin, CNI inhibit NFAT activation and production of the cytokines interleukin 2 (IL-2) and interferon γ (IFNγ) [15-17]. Apart from this, CNI also affect the activation of the MAPK (mitogen activated protein kinase) signaling pathway by inhibiting p38 MAPK [18-22]. In T cells p38 MAPK is activated upon T cell receptor-CD28 ligation as well as in response to inflammatory cytokines. It mediates the production of major cytokines (*e.g.* IL-2, IL-10, TNFα and IFNγ) [23-26] and is an activator of NFAT [27-30] (Fig. 1). P38 MAPK is involved in the development, activation and apoptosis of T cells [31-32], which mediate alloreactivity [33].

While the inhibition of MAPK signaling in the Jurkat T cell-line by CNI was conclusively shown by the group of Matsuda et. al. [21], the effect of CNI on p38 MAPK signaling in primary human T cells has not been studied previously. Here we report on two complementary studies in which p38 MAPK activation was quantified by measuring phosphorylation of the molecule at specific

activation sites by the sensitive phosphospecific flow cytometry technique, a suitable tool for routine monitoring which allows analysis of intracellular signaling pathways at the single cell level in small amounts of whole blood [34]. First we analyzed whether TAC inhibits p38 MAPK phosphorylation in vitro in primary human T cells and second we investigated ex vivo whether p38 MAPK phosphorylation levels in T cells are associated with TAC whole blood concentrations in healthy volunteers and in kidney transplant recipients.

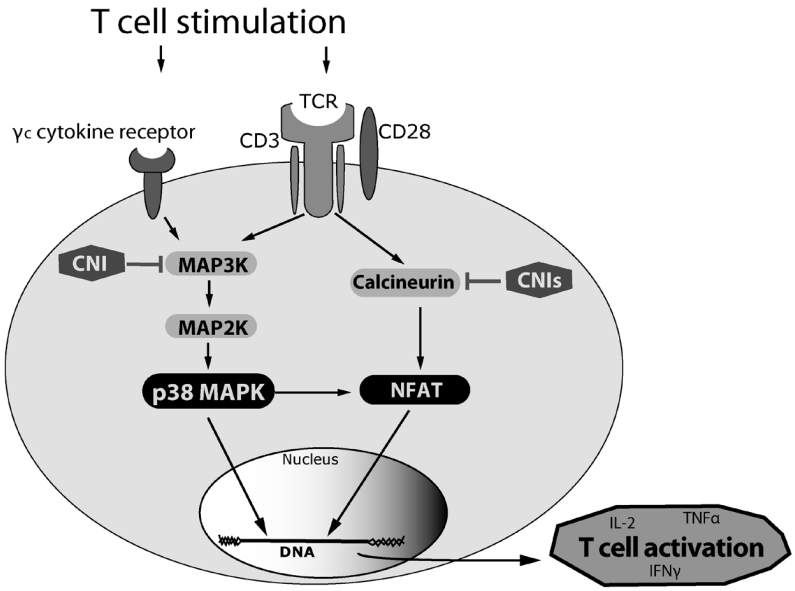

Figure 1: Calcineurin Inhibitors (CNI) and T cell signaling.
CNI inhibit both calcineurin and p38 MAPK, two related T cell activation pathways. P38 MAPK is activated upon T cell receptor-CD28 stimulation and in response to Y chain (Yc) cytokines. It activates T cells directly by mediating cytokine transcription and indirectly by activating nuclear factor of activated T cells (NFAT), the signalling molecule downstream of calcineurin.

Material and Methods

In Vitro Study

To measure the IC50, drug concentration required for 50% inhibition, p38 MAPK phosphorylation was measured in freshly drawn, heparinized human whole blood from healthy adults (n=5). Before activation, whole blood was spiked for one hour at 37°C with either vehicle, CsA (Sigma-Aldrich, Steinheim, Germany) in

concentrations of 100 to 800 ng/mL or TAC (Prograf® injection, Astellas Pharma Inc., Tokyo, Japan) in concentrations of 10 to 200 ng/mL. Whole blood (200 uL) was activated for 30 min at 37 °C with either vehicle or phorbol-12-myristate-13-acetate (1.6 uM) and ionomycin (10 ug/mL) (PMA/iono; Sigma-Aldrich, Steinheim, Germany). The vehicle used was IMDM medium (Gibco BRL, Carlsbad, CA) supplemented with 10 % heat-inactivated fetal bovine serum (BioWhittaker, Verviers, Belgium). P38 MAPK activation was measured by phosphospecific flow cytometry.

To measure cytokine production heparinized whole blood from healthy adults (n=4) was spiked for one hour at 37°C with either vehicle or TAC 10 ng/mL (Astellas Pharma Inc.) and activated with PMA/iono (1.6 uM / 10 ug/mL, Sigma-Aldrich) for 4 hours. To retain cytokines within the cells for analysis, Golgiplug (BD Biosciences, San Jose, CA, USA) was added to the samples during the activation period. Cells were fixed for 10 min with facs lysing solution (BD Biosciences), treated with permeabilization buffer II (BD Biosciences) for 10 min and stained for 30 min with fluorochrome-conjugated monoclonal antibodies (mAb) for intracellular cytokines and cell surface markers. For intracellular cytokines, IL-2-allophycocyanin (APC) and TNFα-phycoerythrin (PE; BD Biosciences) were used and for surface staining the following lineage-specific mAb were used: CD3-peridinin chlorophyll protein complex (PERCP), CD4-pacific blue (PB) and CD8-PE-cyanin7 (PE-CY7; BD Biosciences). Samples were analyzed on a FACS Canto II flow cytometer (BD Biosciences). Ten thousand gated cell events were acquired from each tube. Fluorescence-minus-one control tubes were included.

Ex Vivo Study in Healthy Volunteers and Kidney Transplant Recipients

Four healthy adults took a single oral dose of 10 mg TAC (Prograf® capsules, Astellas Pharma Inc., Tokyo, Japan). Blood samples were collected before and at 2, 4 and 24 hours after drug intake.

Twenty-four stable kidney transplant recipients at least one year after transplantation, on an immunosuppressive regimen with TAC (Astellas Pharma Inc) either with or without mycophenolate mofetil (MMF, Cellcept®, Roche Laboratories, Nutley, NJ) and without concomitant corticosteroid treatment, were enrolled. Patients were treated with oral TAC ranging from 1 to 3 mg twice daily and oral MMF 500 to 1000 mg twice daily. Blood samples from patients were collected in heparin and EDTA-containing vacutainers before drug intake during routine visits to the outpatient clinic. TAC concentrations were determined in EDTA whole blood using the ACMIA-Flex immunoassay on a Dimension XPand analyzer (Siemens HealthCare Diagnostics Inc, Newark, DE) in

accordance with the manufacturers' instructions. For measurement of p38 MAPK phosphorylation heparinized whole blood samples were stimulated with PMA/iono as described for the in vitro study. The study protocols were approved by the local ethics committee (MEC 2010-022), and written informed consent was obtained from each individual.

Whole Blood Phosphospecific Flow Cytometry

Whole blood phosphospecific flow cytometry was performed according to the manucfacturer's instructions for phosphoprotein analysis (Application Handbook, BD biosciences). After activation, with or without prior incubation with TAC, cells were fixed for 10 min with Lyse/fix buffer (BD Biosciences). Fixed cells were treated with permeabilization buffer III (BD Biosciences) and immediately cooled at -20 °C. Within 24 hours the permeabilized samples were stained with fluorochrome conjugated mAb (BD Biosciences). To measure the intracellular signaling pathway, a PE-conjugated mAb (clone 36/p38; BD Biosciences), which recognizes the conserved dual phosphorylated site pT180/pY182 on α, β, γ, and δ p38 MAPK was used. For cell surface staining the following lineage-specific mAb were used simultaneously: CD3-APC, CD4-PB and CD8-PE-Cy7 (BD Biosciences). Cells were incubated with mAb mixtures for 30 min at room temperature and analyzed on a FACS Canto II flow cytometer (BD Biosciences). Ten thousand gated cell events were acquired from each tube. Isotype control IgG1-PE (clone X40, BD Biosciences) and FMO control tubes were included. To exclude inter-day variability of the flow cytometer, Quantibrite PE beads (BD Biosciences) were measured and analyzed according to the manufacturer's instructions.

Analysis of Data

Median fluorescence intensity (MFI) values were generated by data-analysis with diva version 6.0 software (BD Biosciences) and statistical analysis was performed using Graph Pad Prism 5.0 (Graph Pad Software Inc., La Jolla, CA, USA). For the in vitro experiments, inhibition of activated p38 MAPK by TAC was calculated as percentage of the sample without TAC.

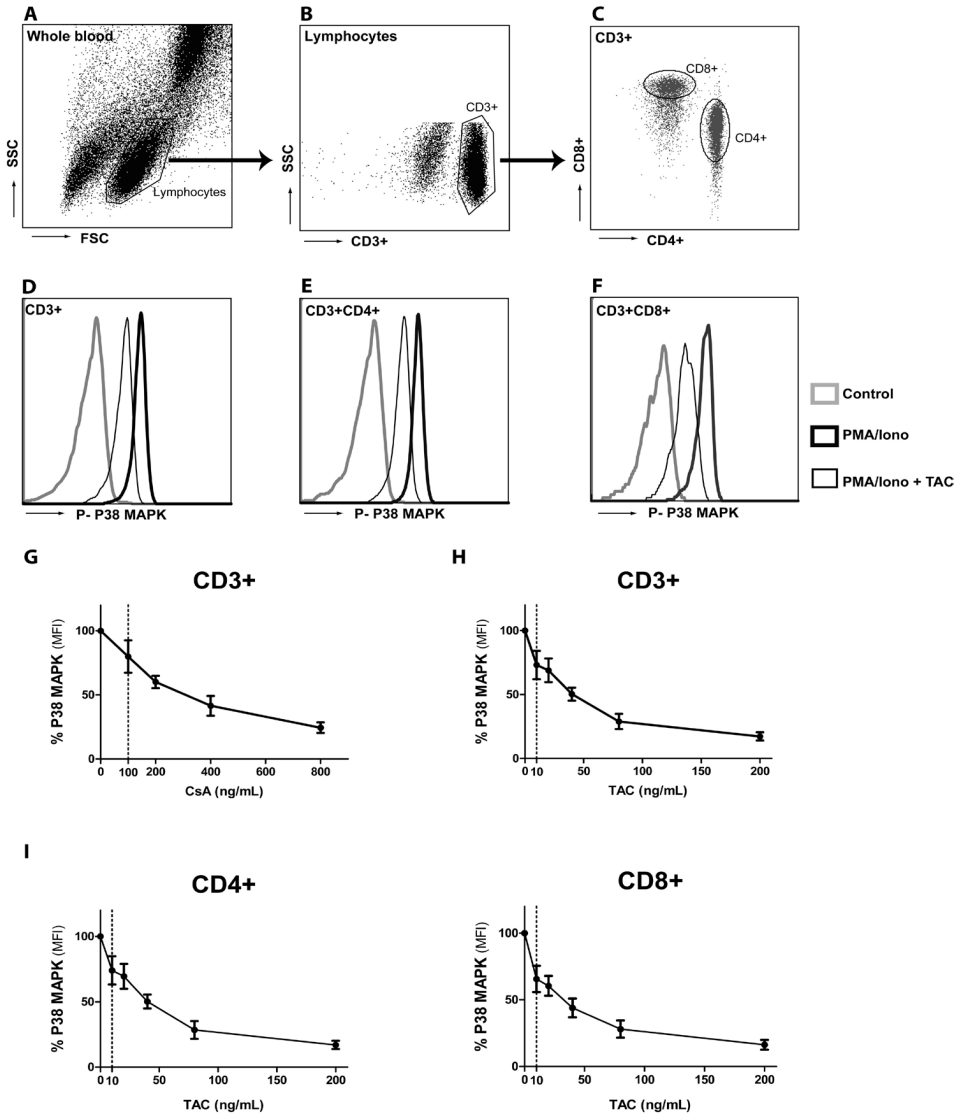

Figure 2: In vitro: CNI inhibit p38 MAPK phosphorylation
Scatter dot plots for the selection of lymphocytes (A), CD3+ T cells (B), CD4+ and CD8+ T cells (C) from whole blood samples. Histograms (D-F) illustrating p38 MAPK phosphorylation after PMA/iono activation of whole blood for 30 min. The phosphorylation is inhibited by the addition of TAC (10 ng/mL) 1 hr before PMA/iono activation. Inhibition of PMA/iono induced phospho-p38 MAPK by cyclosporine A (CsA) in CD3+ T cells (G) and by TAC in CD3+ T cells (H) and CD4+ T cells and CD8+ T cells (I), is shown as the percentage decrease of median fluorescence intensity (MFI; $p < 0.01$ for all subsets; plotted are the mean [+SD] from five different whole blood donors). FSC, forward scatter; SSC, side scatter.

Results

Phosphorylation of p38 MAPK in Whole Blood: In Vitro Study

After 1 hour incubation with vehicle or CNI, whole blood from five healthy adults was activated with PMA/Iono and the induced p38 MAPK phosphorylation was measured by phosphospecific flow cytometry. Figure 2 shows an example of p38 MAPK activation and the inhibition by TAC in CD3+, CD4+ and CD8+ T cells. Vehicle-stimulated samples had a low MFI (range, CD3+: 13-108, CD4+: 100-134 and CD8+: 13-113) while PMA/iono activation for 30 min. markedly increased the phospho-p38 MAPK MFI in T cells (range, CD3+: 616-1116, CD4+: 653-1144 and CD8+: 507-986). Both CsA and TAC inhibited the induced p38 MAPK activation in a dose dependent manner (Fig. 2). The IC50 for CsA was 299 ng/mL (95% CI; 262-341 ng/mL) in CD3+ T cells; and at 100 ng/mL CsA inhibited p38 MAPK signaling by 20% (95% CI 7 - 33%, $p<0.05$) in CD3+ T cells. The IC50 for TAC was 37 ng/mL (95% CI; 32 - 44 ng/mL) in CD3+ T cells, 38 ng/mL (95% CI; 32 - 45 ng/ml) in CD4+ T cells and 28 ng/mL (95% CI; 23 – 33 ng/mL) in CD8+ T cells. At 10 ng/mL TAC inhibited p38 MAPK activation by 27% (95% CI; 13 – 41, $p<0.01$) in CD3+ T cells, by 26% (95% CI; 13 - 39%, $p<0.01$) in CD4+ T cells and by 34% (95% CI 22 - 47%, $p<0.01$) in CD8+ T cells, compared to the PMA/iono activated samples without TAC. To determine the downstream effect of p38 MAPK inhibition, intracellular cytokine production by T cells was measured after PMA/iono activation of whole blood. TAC 10 ng/mL inhibited production of the MAPK dependant cytokines, IL-2 and TNFα, in CD3+ T cells, CD4+ T cells, and CD8+ T cells ($p<0.05$ for all subsets; Fig. 3).

Phosphorylation of p38 MAPK: Ex Vivo Study

Healthy volunteers

For the ex vivo measurement of p38 MAPK signaling, four healthy adults (2 male, 2 female, age range 27-48 years) took a single oral dose of 10 mg TAC. Two hours after drug intake, blood tacrolimus levels were highest at a median 23.7 ng/mL (range, 17.8 – 37.6 ng/mL). At the same time-point p38 MAPK activation compared to baseline was inhibited by a median of 35.4% (range, 16.4 – 60.3%; $p<0.05$) in CD3+ T cells, by 35.2% (range, 16.0 - 59.7%; $p<0.05$) in CD4+ T cells and by 35.4% (range, 18.2 - 66.2%; $p<0.05$) in CD8+ T cells. P38 MAPK activation gradually recovered to baseline after 24 hours (Fig. 4).

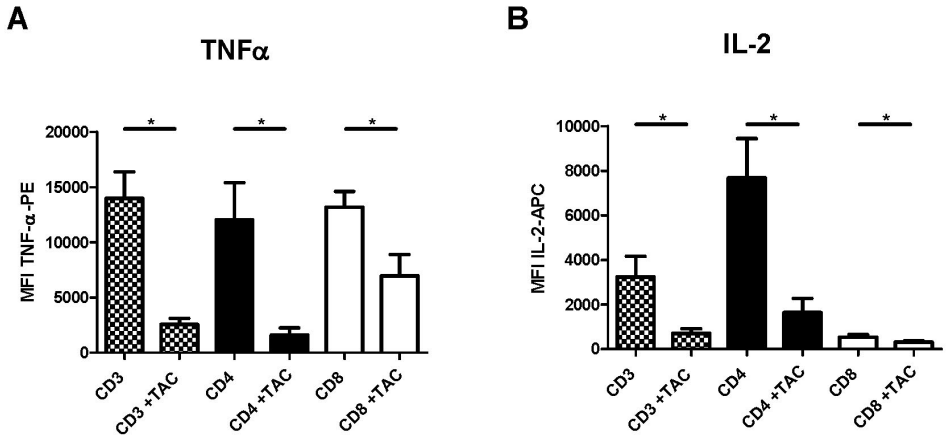

Figure 3: In vitro: CNI inhibit the downstream targets of MAPK signaling
After PMA/iono activation of whole blood from healthy volunteers (n=4) for 4 hr intracellular production of cytokines by CD3+ T cells, CD4+ T cells and CD8+ T cells was measured. TAC inhibited TNFα MFI by 81% (mean) in CD3+ T cells, by 87% in CD4+ T cells and by 49% in CD8+ T cells, compared to the PMA/iono activated samples without TAC (A). At the same time TAC inhibited IL-2 MFI by a mean 77% in CD3+ T cells, 80% in CD4+ T cells, and 37% in CD8+ T cells (B). (data plotted are mean ± SEM). * $p<0.05$. MFI, median fluorescence intensity; PE, phycoerythrin; APC, allophycocyanin)

Kidney transplant recipients

For the measurement of p38 MAPK activation in patients on TAC-based immunosuppressive treatment, whole blood from 24 stable kidney transplant recipients more than 1 year after transplantation was analyzed. The mean age was 55 years; mean time after transplantation was 4.7 yrs; the ratio of men to women was 16:8 of whom 86% received TAC treatment in combination with MMF. Whole blood TAC predose-concentrations varied between 3.2 to 10.5 ng/mL. Patients were enrolled over a 6- month period and to correct for variation of the flow cytometer in time, PE-labeled microbeads were measured as an external standard. The median fluorescence intensity of PE-labeled phospho-p38 MAPK was converted to "number of phospho-p38 MAPK molecules per cell" by using the microbeads' fluorescence. An inverse relationship between TAC predose-concentrations and PMA/iono induced p38 MAPK phosphorylation in CD3+, CD4+ and CD8+ T cells ($p < 0.01$) was found (Fig. 5). Fifty-one percent of the variation in p38 MAPK activation in CD3+ T cells could be explained by TAC predose-concentrations.

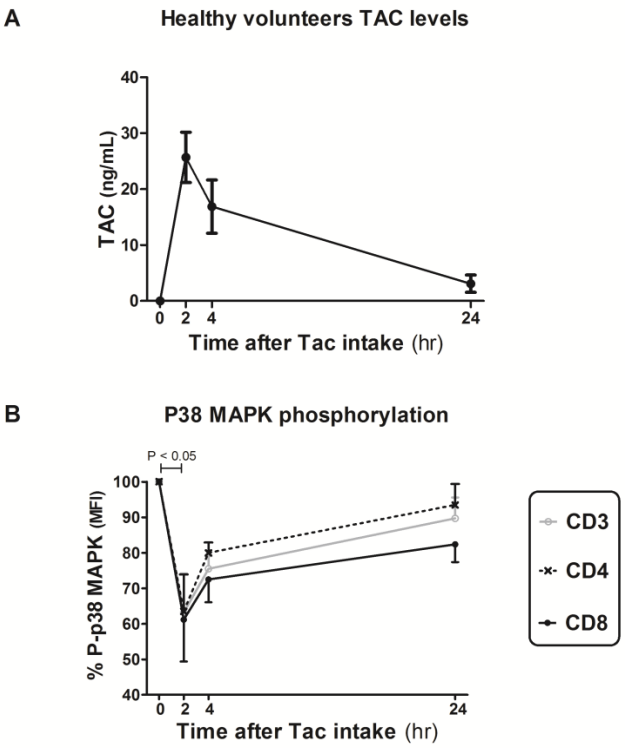

Figure 4: Ex vivo: p38 MAPK inhibition after TAC intake by healthy volunteers.
Whole blood TAC levels of healthy volunteers (n=4) after a single oral dose of 10 mg TAC (A). PMA/iono-induced phosphorylated-p38 MAPK decreased two hrs after intake of TAC, in CD3+ T cells, CD4+ T cells and CD8+ T cells ($p<0.05$) and gradually recovered within 24 hrs (B). At two hrs after drug intake p38 MAPK phosphorylation decreased 37 % in CD3+ T cells, 36 % in CD4+ T cells and 39% in CD8+ T cells. (Data plotted are mean ± SEM)

Discussion

Previous studies have shown in T cell lines that CNI block MAPK signaling. The current study is the first to show the inhibitory effect of CNI on p38 MAPK in vitro in primary human T cells, ex vivo in healthy volunteers and in TAC-treated kidney transplant recipients. MAPK signaling is key in T cell development and activation [32] and acts as an upstream activator of NFAT [27-30]. Thus, by inhibition of the MAPK pathway, CNI suppress the proliferation and function T cells [35-36], which are prominent players in the cascade of alloreactivity leading to graft rejection [37-38]. The proof that MAPK monitoring has clinical utility should come from a study that correlates this biomarker with the occurrence of rejection. We propose the following study setup: prior to transplantation, measurement of MAPK activation before and two hours after intake of a single CNI-dose, in line with the study in healthy volunteers described in figure 4. This

will provide information on the susceptibility of the individual patient for CNI-induced MAPK inhibition and excludes inter-individual variation as the biomarker is compared within the same patient before and after drug-intake. Additionally, by measuring prior to transplantation confounding factors such as alloreactivity and other given immunosuppressants are excluded.

Next to the efficacy of CNI, p38 MAPK activity has also been associated with CNI-related side effects like nephrotoxicitiy [39-40], hypertension [41] and dislipidaemia [42-44]. Therefore monitoring of the MAPK signaling pathway can also be helpful in our understanding of CNI related side effects. In that case MAPK activation measurements of cells derived from the inflicted compartments, instead of in T cells, is warranted.

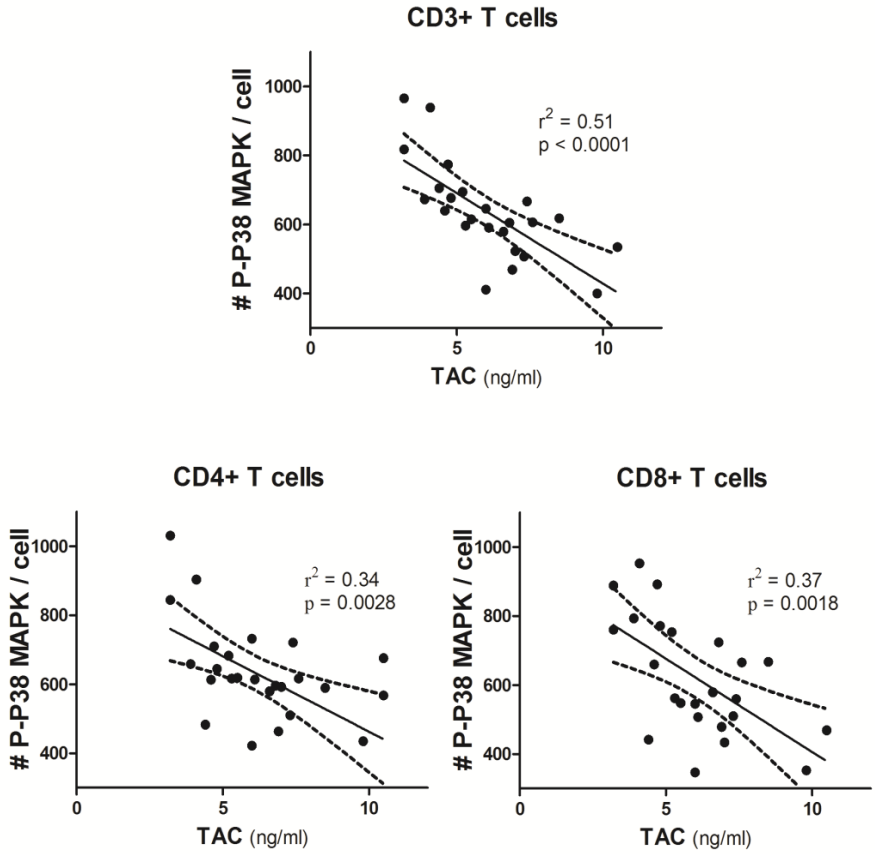

Figure 5: p38 MAPK inhibition in kidney transplant patients.
Activated p38 MAPK in CD3+ T cells, CD4+ T cells and CD8+ T cells inversely correlated with tacrolimus (TAC) predose whole blood concentrations of stable kidney transplant patients. The slope was significantly different from zero ($P < 0.01$, F-test).

Immune monitoring of signaling molecules, possible by phosphospecific flow cytometry, provides a new perspective and additional information on the biological effects of CNI. Measuring signaling molecules can give drug-specific information on the effect of CNI because these molecules act upstream in the activation of immune cells. The currently available tools to determine the drug-specific biological effects of CNI are analyis of I. calcineurin phosphatase activity and II. NFAT-regulated cytokine genes, IL-2, IFNγ and GM-CSF. CsA treated patients with chronic graft versus host disease (GVHD) express high calcineurin activity compared to patients who do not develop GVHD [12] and in liver transplant patients a relation between rejection, nephrotoxicity and calcineurin activity was found [12]. Nonetheless routine monitoring of calcineurin activity is not implemented at large due to insufficient evidence for its clinical usefulness [14], although the tool has been studied for more than a decade. The other pharmacodymic monitoring tool, measuring NFAT-regulated cytokine genes, is now an available and standardized tool [45]. Gene expression is measured by real-time PCR after stimulation of whole blood with PMA/iono. Data showing a relationship between NFAT-regulated gene expression and acute rejection are lacking. Nevertheless, the assay has clinical potential in preventing over-immunosuppression, *e.g.* recurrent infections and malignancies [45].

There is a need for additional reliable and practical tools to study the consequences of immunosuppressive medication on cells that are important in allo-immune responses. Here we studied whether phosphospecific flow cytometry can be used as such a tool. The studied biomarker, p38 MAPK, was inhibited by TAC and inversely correlated with TAC concentrations in renal transplant recipients. In contrast to reports on the measurements of calcineurin activity [14, 46], p38 MAPK activation correlated with TAC predose-concentrations. This finding suggests that phosphospecific flow cytometry is a relevant tool because it is a whole blood assay without the need for drug diluting cell isolation steps. Moreover, it is fast, robust [47], does not require radioactive reagents and can be performed in small amounts of blood. These features make phosphospecific flow cytometry an interesting tool for routine pharmacodynamic monitoring. In renal transplant patients, we have already applied phosphospecific flow cytometry to monitor the immunomodulatory effect of the JAK-inhibitor tofacitinib (CP-690,550) [48].

Another application for phosphospecific flow cytometry is the analysis of intracellular signaling pathways and their function. For example, it can be used to study the signaling pathway by which naïve T cells convert into memory T cells, to unravel the interactions between immunosuppressive medication and

regulatory T cells and to determine the underlying mechanisms of T cell exhaustion [49].

In conclusion, analysis of p38 MAPK activation levels by phosphospecific flow cytometry can be used as a functional assay to determine the immunomodulatory effect of immunosuppressive medication, for example tacrolimus, and be suitable for monitoring the immunosuppressive load in patients.

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Chapter 3

Tacrolimus Inhibits NF-κB Activation in Peripheral Human T Cells

PlosOne. Submitted

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Willem Weimar, Carla C. Baan

Abstract

Background: The calcineurin inhibitor, tacrolimus (TAC), inhibits the protein phosphatase activity of calcineurin, leading to suppression of the nuclear translocation of NFAT and inhibition of T cell activation. Apart from NFAT also the transcription factor NF-κB plays a key functional role in T cell activation. Therefore, blockade of the NF-κB activation cascade by immunosuppressive drugs prevents immune activation. Here we studied whether TAC blocks NF-κB activation in peripheral human T cells.

Methods: After anti-CD3/CD28-activation of T cells from healthy volunteers, NF-κB (p65) phosphorylation was measured by flow cytometry in CD3+ T cells, CD4+ helper T cells and CD8+ cytotoxic T cells in the absence and presence of TAC 10 ng/mL, sotrastaurin 500nM (positive control) and mycophenolic acid 10 μg/mL (negative control; n=6). NF-κB transcriptional activity was measured by ELISA and intracellular TNFα protein, a downstream target, was measured by flow cytometry to assess the functional consequences of NF-κB blockade.

Results: Anti-CD3/28-activation induced NF-κB phosphorylation in CD3+ T cells, CD4+ T cells and CD8+ T cells by 34% (mean), 38% and 30% resp. ($p<0.01$). Sotrastaurin inhibited NF-κB activation in the respective T cell subsets by 93%, 95% and 86% ($p<0.01$ vs. no drug), while mycophenolic acid did not affect this activation pathway. Surprisingly, TAC also inhibited NF-κB phosphorylation, by 55% ($p<0.01$) in CD3+ T cells, by 56% ($p<0.01$) in CD4+ T cells and by 51% in CD8+ T cells ($p<0.01$). In addition, TAC suppressed NF-κB DNA binding capacity by 55% ($p<0.05$) in CD3+ T cells and TNFα protein expression was inhibited in CD3+ T cells, CD4+ T cells and CD8+ T cells by 76%, 71% and 93% resp. ($p<0.01$ vs. no drug), confirming impaired NF-κB signaling.

Conclusion: This study shows the suppressive effect of TAC on NF-κB signaling in peripheral human T cell subsets, measured at three specific positions in the NF-κB activation cascade.

Introduction

Tacrolimus (TAC) is a commonly used drug in the immunosuppressive regimen following solid organ transplantation [1]. It is highly effective in the prevention of graft rejection [2,3,4,5], an immunological process where T cells are often involved [6]. In these cells TAC binds to and inhibits calcineurin after forming a complex with the immunophilin FKBP12 (FK506 binding protein) [7]. Calcineurin is activated after engagement of the T cell receptor (TCR) and co-stimulation (i.e. CD28-receptor stimulation). Activation of these receptors leads to depletion of endoplasmatic reticulum calcium-stores into the cytosolar cell compartment and following calcium influx via CRAC (calcium release activated calcium current) channels to slowly replenish the calcium levels in the endoplasmic reticulum [8]. Activation of the calcium-dependent calcineurin leads to phosphorylation and translocation of nuclear factor of activated T cells (NFAT) into the nucleus. NFAT binds in conjunction with AP-1 to DNA binding sites and initiates transcription of pro-inflammatory cytokines (e.g. IL-2 and IFNγ) [8].

Since its introduction over two decades ago, textbooks without exception have attributed the immunosuppressive effect of TAC to inhibition of the calcineurin/NFAT pathway. Interestingly some studies have linked inhibition of the calcineurin pathway to suppression of NF-κB signaling in the Jurkat T cell line [9,10,11]. The interaction between these two major activation pathways is still under investigation [12] and suggests that TAC also suppresses T cell activation via the NF-κB pathway. In resting T cells IκB (inhibitor of κB) proteins keep NF-κB inactive by masking its nuclear localization sequence. After TCR-engagement and co-stimulation, the inhibitory IκB proteins are degraded by IKK (IκB kinase) complexes via the 26S proteasome. This enables the release of NF-κB hetero-dimers primarily consisting of NF-κB1 (p50) and RelA (p65) to enter the nucleus and bind to the κB sites within promoters and enhancers thereby initiating transcription of adhesion molecules, chemokines, regulators of apoptosis and pro-inflammatory cytokines, e.g. tumor necrosis factor (TNF) α [13,14]. Hence, the NF-κB signaling pathway is in-dispensable in T cell biology and plays an important role in their development, activation and survival [15,16]. Consequently NF-κB is an important mediator of rejection processes after solid organ transplantation [17,18].

The current study is the first to report on the effect of TAC on the NF-κB activation pathway by quantitative analysis of NF-κB phosphorylation in human primary T cell subsets. In addition, the effects of TAC on NF-κB DNA binding activity and NF-κB dependent cytokine production were analyzed. The analysis was conducted in anti-CD3/CD28 activated peripheral T cells and the effects were compared to those of sotrastaurin which inhibits protein kinase C (PKC),

the enzyme that mediates NF-κB-activation after TNFα receptor engagement and TCR activation [19,20]. The immunosuppressant mycophenolic acid (MPA) which blocks *de novo* biosynthesis of purine nucleotides and lymphocyte proliferation by suppressing the enzyme inosine monophosphate dehydrogenase, was used as negative control [21].

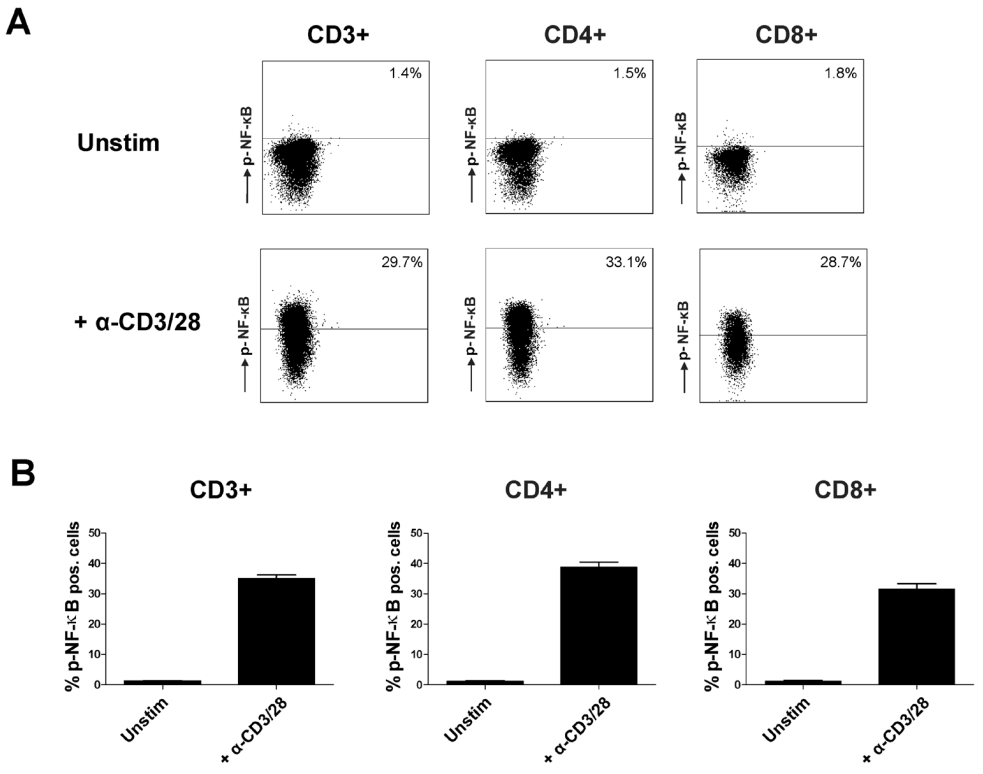

Figure 1: Facs analysis of NF-κB phosphorylation in T cells.
The total CD3+ T cell population was acquired by MACS isolation of PBMC from healthy volunteers. A) Example scatter dot plots of an unstimulated sample (above) and an anti-CD3/CD28 stimulated sample to illustrate the gating strategy for analysis of percentages CD3+, CD4+ and CD8+ T cells which express phosphorylated NF-κB. Both samples were stained with a monoclonal antibody against phosphorylated NF-κB p65. B) The average percentages of cells with elevated NF-κB phosphorylation in unstimulated and stimulated samples without drug are depicted as mean ± SEM of 6 independent experiments.

Material and Methods

Isolation and Stimulation of Human Peripheral T Cells

The study protocol was approved by the local ethics committee of the Erasmus medical center and written informed consent was obtained from each individual. Peripheral blood mononuclear cells (PBMC) were isolated from freshly drawn, heparinized normal human whole blood by density-gradient centrifugation over Ficoll-paque (Amersham Pharmacia Biotech, Uppsala, Sweden) and frozen at -135°C in RPMI 1640-DM (Gibco BRL, Scotland, UK) supplemented with 2 mM/L L-glutamine (Gibco BRL), 100 IU/mL penicillin (Gibco BRL), 100 ug/mL streptomycin (Gibco BRL), 10% pooled human serum (Blood Bank, Rotterdam, the Netherlands) and 10% dimethylsulfoxide (Merck, Schuchardt, Germany). Defrosted PBMC were washed twice with RPMI 1640 (Gibco BRL, Scotland, UK), supplemented with 100IU/mL penicillin (Gibco BRL) and 100 ug/mL streptomycin (Gibco BRL). Purified T cells were isolated by negative selection using MACS magnetic cell separation with a pan T cell isolation kit II (Miltenyi Biotech, Auburn, CA) according to manufacturer's instructions. Negative selection was used to avoid possible T cell activation by binding to beads. Purity of the isolated T cells was determined by flow cytometry using a CD3-AmCyan monoclonal antibody (mAb; BD Biosciences, San Jose, CA), on a FACS Canto II flow cytometer (BD Biosciences) along with facs Diva, version 6.0 software (BD Biosciences). For each experiment the purity of the magnetically isolated CD3+ T cells was at least 98%. Cells were rested overnight in IMDM medium supplemented with 0.5 nM β-mercaptopurin (Sigma-Aldrich, Steinheim, Germany) and 10 % heat inactivated fetal bovine serum (FBS) (BioWhittaker, Verviers, Belgium).

Phosphospecific Flow Cytometry

MACS isolated T cells (10^6) were spiked for one hour at 37°C with drugs at the following final concentrations: TAC 0, 5, 10 and 50 ng/mL (Astellas Pharma Inc., Tokyo, Japan), sotrastaurin 50, 100 and 500 nM (Novartis Pharmaceuticals, Basel, Switzerland) and mycophenolic acid 10 µg/mL (Sigma-Aldrich). Cells were activated for ten minutes with mouse anti-human CD3 (BD Pharmingen, San Diego, CA) and mouse anti-human CD28 (BD Pharmingen) 1 µg each, according to manufacturer's instructions at 37°C. After activation cells were fixed for 10 min with Lyse/fix buffer (BD Biosciences). Fixed cells were permeabilized using a 70% aqueous ethanol solution and immediately cooled at -20 °C. The permeabilized samples were stained in the dark with fluorochrome conjugated mAb mixtures for 30 min at room temperature. To measure the intracellular signaling pathway,

we used a PE-conjugated mAb which recognizes phosphorylation at serine 529 in the activation domain of human NF-κB p65 (BD Biosciences). The p65-isoform is abundantly expressed in all mammal cells, including T cells [22,23]. Simultaneously with intracellular staining the following mAb's were used for cell surface staining: CD4-PB and CD8-PE-Cy7 (BD Biosciences). Samples were analyzed on a FACS Canto II flow cytometer (BD Biosciences). Ten thousand gated cell events were acquired from each tube and FMO negative control tubes were included.

Intracellular Cytokine Production

MACS isolated T cells ($0.5 \cdot 10^6$) were spiked for one hour with: TAC 0, 10 ng/mL or sotrastaurin 500 nM. Samples were activated with 12.5 µL human T-activator Dynabeads (Invitrogen Dynal AS, Oslo, Norway) for 24 hours at 37°C. Golgistop (BD Biosciences) was added during stimulation, which was stopped with EDTA, final concentration 2µM. Beads were removed using a magnet and cells were fixed and lysed for 10 minutes with 4mL FACS lysing solution (BD Biosciences). After washing, PBMC were permeabilized for 10 minutes with FACS perm solution II (BD Biosciences). Cells were stained and measured on the flow cytometer as described above. The following mAbs were used: CD4-PB, CD8-PE-CY7 (BD Biosciences) and PE conjugated anti-TNFα (BD Pharmingen).

ELISA

Enzyme-Linked Immunosorbent Assay (ELISA) was used to analyze the effect of TAC on NF-κB DNA binding activity. MACS isolated T cells ($3 \cdot 10^6$) were spiked with: TAC 0, 10, 50 ng/mL ng/mL or sotrastaurin 500 nM for 1 hour. Next samples were activated for 40 minutes at 37°C with mouse anti-human CD3 (1 µg; BD Pharmingen) and mouse anti-human CD28 (1 µg; BD Pharmingen). Nuclear extracts were prepared using a nuclear extraction kit (Cayman Chemical, Ann Arbor, MN). NF-κB p65 DNA binding activity was determined by an ELISA kit (Cayman Chemical), according to the manufacturer's instructions. A bicinchoninic acid (BCA; Pierce, Rockford, IL) assay was used to determine the total protein concentration of nuclear extracts, using acetylated Bovine serum albumin (Promega, Madison, WI, USA) as standard.

Analysis of Data

Statistical analysis was performed using Graph Pad Prism 5.0 (Graph Pad Software Inc., La Jolla, CA, USA).

Results

NF-κB Phosphorylation

In the first set of experiments signaling activation was studied via phyoshorylation of NF-κB p65 at serine 529. The latter is only phosphorylated when the inhibitory IκB protein is released from the NF-κB dimer complex [24,25]. NF-κB activation was measured by phosphospecific flow cytometry as the percentage of cells in which NF-κB phosphorylation was induced compared to the unstimulated sample. Figure 1A shows an example of the analysis of an unstimulated sample and an anti-CD3/CD28 stimulated sample, both stained with the monoclonal NF-κB antibody. Stimulation increased the percentage of phospho-NF-κB expressing CD3+ T cells from 1,3% in unstimulated samples to 35.0% in stimulated samples (mean, n=6; $p<0.01$; figure 1B). In the CD4+ and CD8+ T cell subsets NF-κB phosphorylation was induced from 1.2% to 38.8% and from 1.2% to 31.6% respectively ($p<0.01$).

The influence of different TAC concentrations on NF-κB phosphorylation was measured and the immunosuppressive drug inhibited NF-κB phosphorylation in a dose dependent manner in CD3+ T cells, CD4+ T cells and CD8+ T cells, as did the positive control sotrastaurin (Fig. 2). The negative control, mycophenolic acid, at a clinically high concentration of 10 µg/mL, did not affect NF-κB activation ($p>0.05$ compared to no drug; figure 3). TAC 10 ng/mL inhibited NF-κB phosphorylation by 55.0% in CD3+ T cells, by 55.7% in CD4+ T cells and by 51.2% in CD8+ T cells ($p<0.01$ for all T cell subsets, figure 3). The positive control, sotrastaurin 500 nM, strongly inhibited phosphorylation, by 93.9% in CD3+ T cells, by 94.0% in CD4+ T cells and by 89.7% in CD8+ T cells ($p<0.01$ for all T cell subsets; figure 3).

TAC also inhibited NF-κB phosphorylation in regulatory CD4+25+127- T cells (Treg; supplemental figure S1). These cells are essential for immune homeostasis by suppressing effector T cell responses [26]. NF-κB p65 is associated with induction of Foxp3 transcription [27,28] which marks thymic Treg generation [29]. Further studies are necessary to validate whether the reduction of Treg numbers by calcineurin inhibitors [30,31,32] is NF-κB mediated.

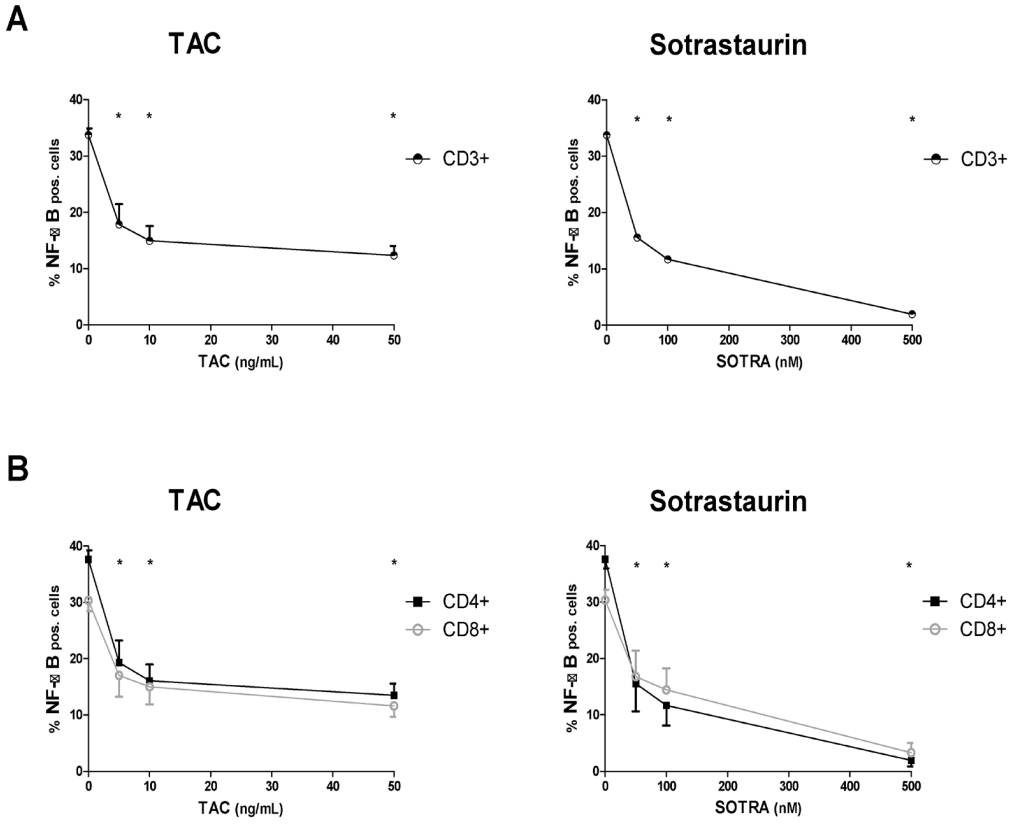

Figure 2: NF-κB phosphorylation inhibited at different TAC-concentrations.
MACS isolated CD3+ T cells from healthy volunteers were stimulated by anti-CD3/CD28 in the presence of TAC 0, 5, 10, 50 ng/mL or sotrastaurin 50, 100 and 500 nM. Inhibition of NF-κB phosphorylation is shown for CD3+ T cells (above), CD4+ T cells and CD8+ T cells (below; shown are the mean ± SEM of 6 independent experiments).

NF-κB Binding to Target Genes

After activation, phosphorylated NF-κB binds to DNA sequences called κB sites and regulates their transcription. Here we studied the downstream targets of NF-κB phosphorylation by measuring the effects of immunosuppressive drugs on the DNA binding activity of NF-κB p65 to its target-genes in CD3+ T cells. For each sample the nuclear extract of purified CD3+ T cells (3.10^6) from healthy volunteers was prepared for analysis by ELISA. Figure 4A shows the mean

amount of nuclear extract expressed in µg protein for each tested condition. This was similar at each of the tested conditions. Regarding NF-κB DNA binding activity, unstimulated samples had a mean absorbance of 1.0 and stimulated samples without drug had a mean absorbance of 2.1 ($p<0.01$ compared to unstimulated samples) with all individual measurements within the linear range of the assay: smaller than 3 at OD450nM. The positive control drug sotrastaurin at 500 nM inhibited the anti-CD3/28 induced DNA binding capacity of NF-κB in peripheral T cells by 84.4% ($p<0.05$ compared to no drug), while TAC 10 ng/mL inhibited the DNA binding capacity by 54.5% and TAC 50 ng/mL by 74.3% (both $p<0.05$).

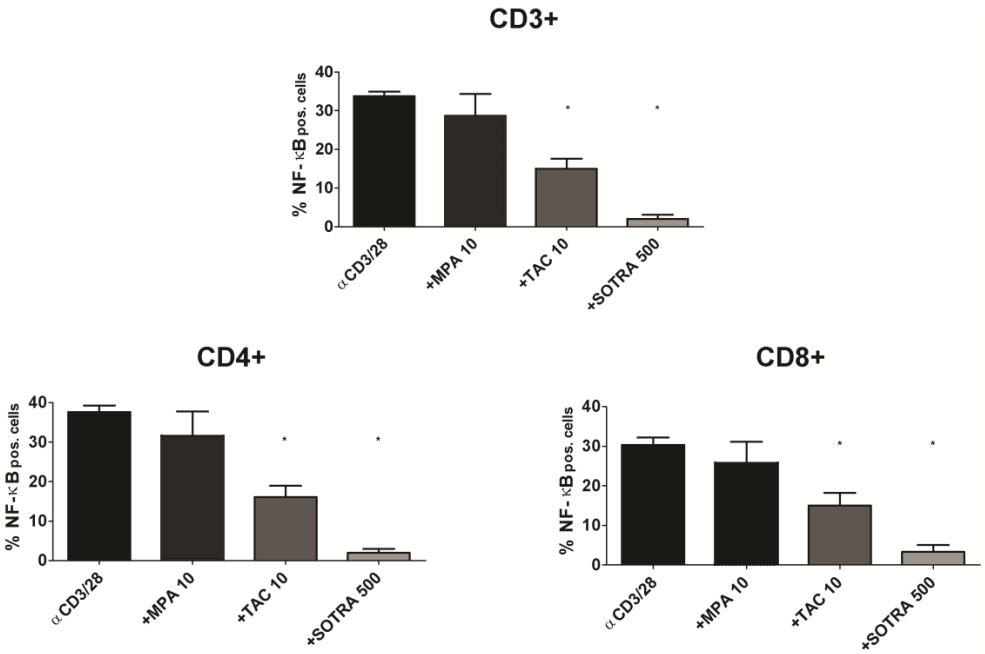

Figure 3: NF-κB phosphorylation inhibited by immunosuppressive drugs.
The effect of the immunosuppressive drugs mycophenolic acid 10 µg/mL, TAC 10 ng/mL and sotrastaurin 500 nM on NF-κB p65 phosphorylation in peripheral T cells from healthy volunteers is depicted. Mycophenolic acid did not influence NF-κB phosphorylation ($p>0.05$), while the positive control sotrastaurin inhibited phosphorylation in the CD3+, CD4+ and CD8+ T cell subsets ($p<0.01$ compared to no drug). TAC 10 ng/mL also suppressed phosphorylation in the T cell subsets ($p<0.01$; shown are mean ± SEM of 6 independent experiments).

Intracellular Cytokine Production

Translocation of NF-κB into the nucleus and subsequent binding of NF-κB to its target-genes results in downstream expression of inflammatory cytokines, chemokines and immune receptors. To examine TAC-induced effects on the expression of inflammatory cytokines, intracellular TNFα production was measured in T cell subsets. Purified CD3+ T cells were stimulated for 24 hours by the engagement of both TCR and CD28. Examples of the flow cytometric dot plots for unstimulated and stimulated samples are given in figure 5A. The mean percentages of TNFα-producing T cell subsets of four experiments are depicted in figure 5B. Anti-CD3-CD28 stimulation induced TNFα production in 15.0% of CD3+ T cells, 24.0% of CD4+ T cells and 5.1% of CD8+ T cells (mean; $p<0.01$ unstimulated vs stimulated samples). TAC 10 ng/mL inhibited this TNFα production in CD3+, CD4+ and CD8+ T cells by 75.9%, 71.4% and 93.1% respectively ($p<0.01$ compared to no drug; figure 5C). Sotrastaurin inhibited the NF-κB dependent cytokine production in the T cell subsets by 88.5%, 85.7%; and 88.0% respectively ($p<0.01$ compared to no drug; figure 5C).

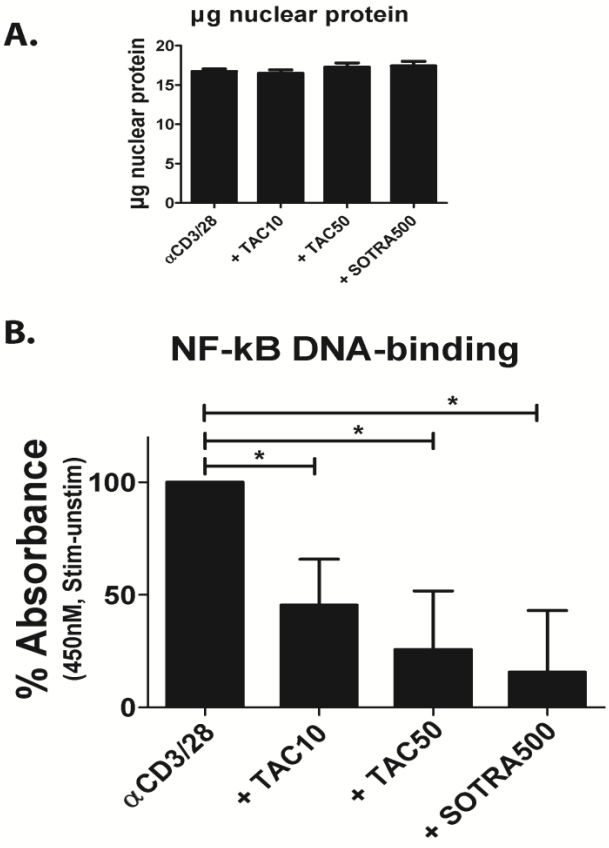

Figure 4: TAC inhibits NF-κB DNA-binding capacity in human T cells.
MACS isolated CD3+ T cells (3.106) from three healthy volunteers were stimulated in the presence of TAC 0, 10, 50 ng/mL or sotrastaurin 500 nM. A) The amount of nuclear protein extracted from the cells was comparable in the samples , at the different tested conditions. B) The DNA binding capacity of NF-κB in the nuclear fractions was inhibited by both TAC and the positive control sotrastaurin. (Data shown are mean ± SEM of 3 independent experiments).

Discussion

The transcription factor NF-κB plays an important role in T cell development, activation and survival [15,16]. These cells are an important contributor to alloreactivity in organ transplant patients [6]. TAC suppresses T cell activation and an effect on NF-κB in T cells is eminent in unraveling its precise immunosuppressive working mechanism. Previous studies have shown that very high TAC concentrations interfere with κB degradation [33] and κB kinase activity in T cell lines [12,34], without answering whether the pathway and NF-κB activation are affected in peripheral human T cells at low drug doses as used after allograft transplantation.

The current study for the first time describes the effect of clinically relevant TAC concentrations on the NF-κB signaling pathway in peripheral human T cells. We quantitatively analyzed distinct steps in the intracellular activation cascade, i.e. phosphorylation of the NF-κB molecule (Fig. 2) and the downstream NF-κB DNA binding activity (Fig. 4). Both parameters showed the inhibitory effect of TAC on NF-κB activation, including the inhibition of NF-κB phosphorylation in CD4+ helper T cells and CD8+ cytotoxic T cells. Further downstream in the activation cascade, production of the NF-κB-dependent cytokine TNFα was also suppressed by TAC in the T cell subsets (Fig. 5).

The traditional view on the working mechanism of TAC is that it binds a specific intracellular immunophilin FKBP12, to inhibit calcineurin phosphatase activity and subsequently the production of pro-inflammatory cytokines. Our finding that TAC also interferes with T cell activation via the NF-κB pathway can be explained by at least two theories. First, TAC can influence the NF-κB pathway via calcineurin [10,11,12,35]. Biswas *et al.* [35] showed that calcineurin binds to and inactivates the cytoplasmic IκB in a mouse myoblast cell line, leading to activation of the transcription factor NF-κB and its nuclear translocation. A different mechanism of calcineurin mediated NF-κB activation was proposed by Palkowitsch *et al.* [12] who showed that downstream of TCR-CD28 engagement calcineurin controls dephosphorylation of BCL10 to facilitate formation of the Carma1, BCL10, MALT1 (CBM) complex, a pivotal step in the activation of IκB kinase. Via the CBM-complex, calcineurin phosphorylates IκB, leading to its proteosomal degradation and NF-κB activation [12,36].

PKC phosphorylates Carma1 and is therefore also essential for the formation of the CBM complex [37]. TAC did not inhibit NF-κB activation to the same extent as the PKC inhibitor sotrastaurin (Fig. 3 and 4). This might be explained by partial TAC-induced calcineurin inhibition: even at 100 µg/mL this agent suppresses calcineurin activity by only 50 to 70% [38,39]. Nevertheless, TAC-induced NF-κB suppression cannot be trivialized, since in T cells calcineurin

activation is a pre-requisite for maximum IKK activity and *in vivo* IκB phosphorylation. Hence, both PKC and calcineurin are required for effective NF-κB activation [34]. Partial inhibition by TAC suggests that the affected activation pathways are not paralyzed and can still function in other essential NF-κB-dependent cell types, which is beneficial for the immunosuppressant's efficacy and safety profile [40].

The second theory to explain how TAC influences NF-KB activation involves the MAPK (mitogen activated protein kinase) signaling pathway. Even though the precise mechanism is still under investigation, we and others have shown that TAC *in vitro* and *in vivo* inhibits the p38 MAPK activation pathway in human T cells [41,42]. This finding can also explain NF-κB suppression by TAC as the transcription factor p38 MAPK regulates both the nuclear recruitment of NF-κB and its transcriptional activity [43,44,45,46]. The signaling pathways downstream of the TCR, like the calcineurin-NFAT pathway, the PKC/NF-κB pathway and the MAPK pathway are highly interlinked and therefore it is apprehensible that TAC inhibits activation of these pathways. This is for example illustrated by studies which showed that p38 MAPK induces transcription and translation of NFAT mRNA in T cells [47] and controls NFAT transcriptional activity [48,49].

Besides investigating the mechanism of action, the current study gives a new lead in the search for biomarkers of TAC-treatment in transplant patients. While there is a need to study intracellular activation pathways until now mostly the calcineurin-NFAT pathway has been utilized [50]. The results of our study may also influence planned clinical studies to combine sotrastaurin and TAC [51,52]. Combining two drugs with overlapping mechanism of action is not warranted and based on the assumption that sotrastaurin selectively inhibits the PKC/NF-κB pathway, while TAC has a selective effect on the calcineurin/NFAT pathway [51,52]. Our results show otherwise. Moreover Evenou *et al.* [53] reported that sotrastaurin also suppresses NFAT nuclear translocation and DNA binding.

In conclusion, our study shows the suppressive effect of TAC on NF-κB signaling in peripheral human T cell subsets, measured at three specific positions in the NF-κB activation cascade. Hence, TAC is more than just a calcineurin inhibitor and a more balanced view on its immunosuppressive working mechanism is required.

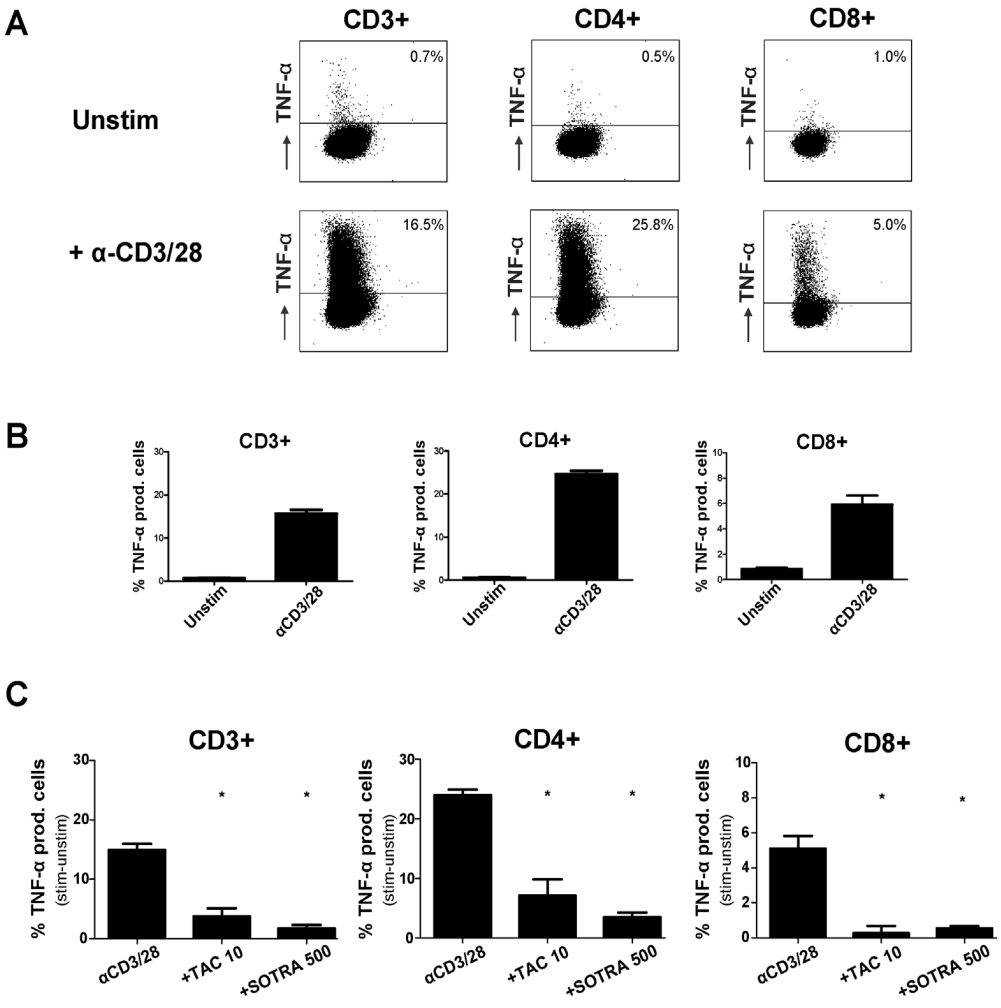

Figure 5: TAC induces inhibition of NF-κB dependent cytokine production.
CD3+ T cells were acquired by MACS isolation of PBMC from healthy volunteers. Cells were anti-CD3/CD28 stimulated for 24 hours in the presence of TAC 0 and 10 ng/mL; and sotrastaurin 500nM. A) Dot plots showing CD3+, CD4+ and CD8+ T cells with intracellular TNFα production in an unstimulated and a stimulated sample. Both sample types were stained with a monoclonal antibody against TNFα. B) The average percentages of TNFα producing T cells in unstimulated and stimulated samples without drug are depicted (shown are mean + SEM of 4 independent experiments). C) TAC 10 ng/mL and sotrastaurin 500nM both inhibited the induced TNFα expression in the T cell subsets ($p<0.01$; shown are mean ± SEM of four independent experiments).

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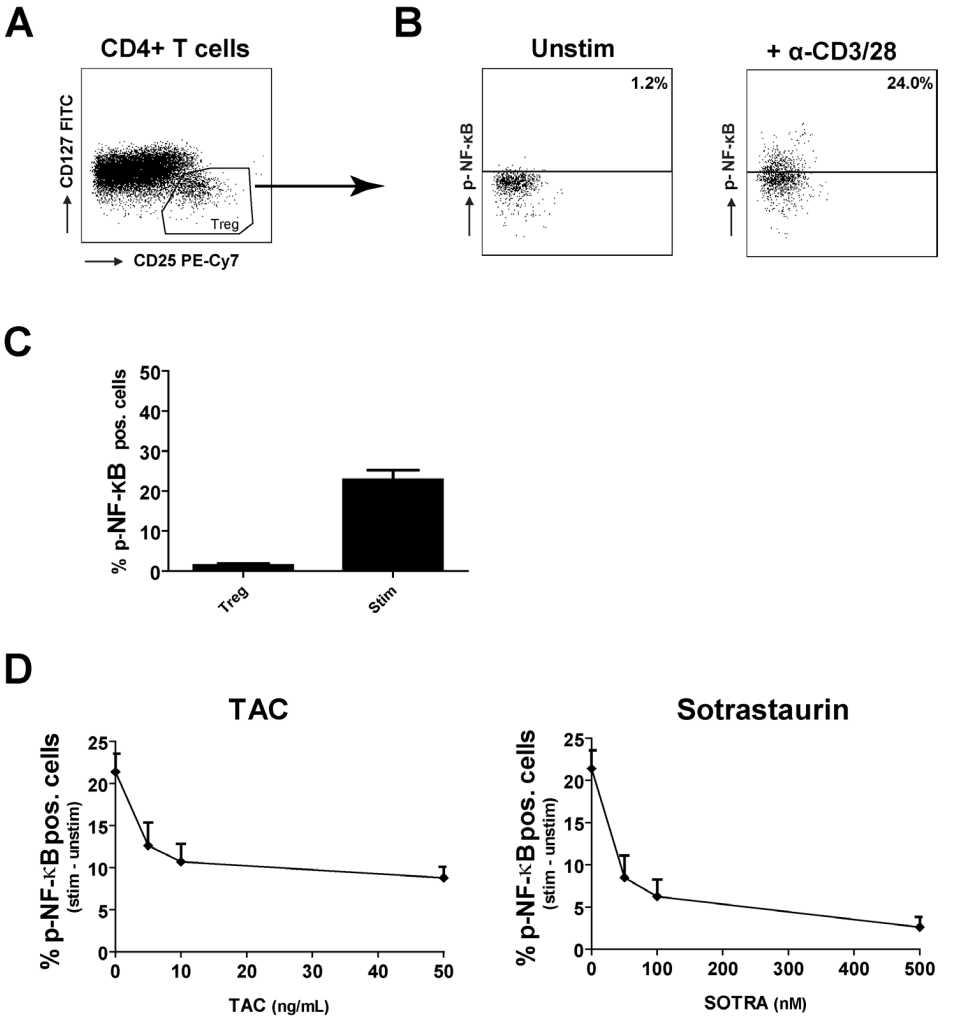

Supplemental figure S1: TAC inhibits NF-κB phosphorylation in regulatory CD4+25+127- T cells. CD3+ T cells were acquired by MACS isolation of PBMC from healthy volunteers. A) Example scatter dot plots to illustrate the gating strategy for selection of CD4+CD25+127- regulatory T cells (Tregs) from the total CD4+ T cell population. B) Example scatter dot plots to illustrate the percentage of Tregs expressing phosphorylated NF-κB in an unstimulated sample and an anti-CD3/28 stimulated sample. Both stimulated and unstimulated samples were stained with a monoclonal antibody against NF-κB p65 phosphorylation. B) The average percentage of Tregs expressing NF-κB phosphorylation in unstimulated and stimulated samples are depicted as mean + SEM of six independent experiments. C) Inhibition of phosphorylated NF-κB is shown for Tregs. TAC 10 ng/mL inhibited NF-κB phosphorylation by 44.5 % in Tregs ($p<0.05$; shown are mean ± SEM of 6 independent experiments).

Chapter 4

Genetic Polymorphisms in ABCB1 Influence the Pharmacodynamics of Tacrolimus

Therapeutic Drug Monitoring. Submitted

Ramin Vafadari, Rachida Bouamar, Dennis A. Hesselink, Rens Kraaijeveld, Ron H.N. van Schaik, Willem Weimar, Carla C. Baan, Teun van Gelder

R. Vafadari and R. Bouamar contributed equally.

Chapter 5

Phosphospecific Flow Cytometry for Pharmacodynamic Drug Monitoring: Analysis of the JAK-STAT Signaling Pathway

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Ramin Vafadari, Willem Weimar, Carla C. Baan

Abstract

Cytokines of the IL-2 receptor family act via activation of the JAK-STAT (janus tyrosine kinase-signal transducer and activator of transcription) signaling pathway. These cytokines are pivotal for the development and function of lymphocyte subsets involved in immune response after organ transplantation including T, B and natural killer cells. The new small drug molecule and JAK1/3 inhibitor, tofacitinib, is currently being tested in phase II and III clinical trials for rheumatoid arthritis, psoriasis and in organ transplantation. This agent specifically targets the JAK-STAT signaling pathway. Here we discuss phosphospecific flow cytometry as a novel tool to monitor the JAK-STAT signaling pathway in kidney transplant patients and speculate that through the use of this pharmacodynamic tool the efficacy of immunosuppressive drugs can be assessed enabling optimization of the immunosuppressive therapy for individual transplant patients.

1. Background

Organ transplantation has become common practice for end-stage renal disease patients. While the success of these procedures is increasing, patients are faced with life-long non-specific immunosuppression. Unfortunately, this places patients at risk of developing diabetes, hyperlipidemia, hypertension, infections, malignancies and renal insufficiency [1-2]. Life long use of the currently approved medication like CNI (calcineurin inhibitors) and m-TOR (mammalian target of rapamycin) inhibitors is accompanied by debilitating side effects influencing the quality of life, and patient and graft survival [3]. Therefore, there is a strong clinical need for safer and more selective immunosuppressive agents that specifically target a particular molecule or pathway. Interference in the interleukin (IL)-2 signaling pathway provides this opportunity. IL-2 is a prominent player in anti-donor responses and exerts its function after binding to its specific IL-2 receptor (IL-2R) α-chain, i.e. CD25. Subsequently, treatment with selective anti-CD25 monoclonal antibodies i.e. daclizumab and basiliximab are given to organ transplant patients to block the interaction of IL-2 with its receptor [4]. IL-2 exhibits broad functional pleiotropy by binding to the hetero-trimeric IL-2R, consisting of IL-2Rα, IL-2Rß, and the common γ-chain (γ_c). After binding, several intracellular signaling pathways are activated. Key elements in cytokine signaling are the kinases named Janus tyrosine kinases (JAKs). This name is taken from the two-faced Roman god of gates, Janus, because the JAKs possess two near-identical phosphate-transferring domains. 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. On activation, JAKs phosphorylate the γ_c receptor at specific tyrosine residues that subsequently serve as docking sites for the signaling molecules named signal transducer and activator of transcription (STAT). The γ_c receptor is a shared component of the IL-2, IL-4, IL-7, IL-9, IL-15 and IL-21 receptors which are collectively named the IL-2 receptor family [5]. 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. At present, seven STATs have been identified. Active STAT molecules interact with specific DNA sequences in target-promoters to modify gene expression [6] (Fig. 1). The JAK-STAT pathway is negatively regulated at multiple levels. For instance the suppressors of cytokine signaling (SOCS) inhibit STAT phosphorylation by binding and inhibiting JAKs or compete with STATs for phosphotyrosine binding sites on cytokine receptors [7].

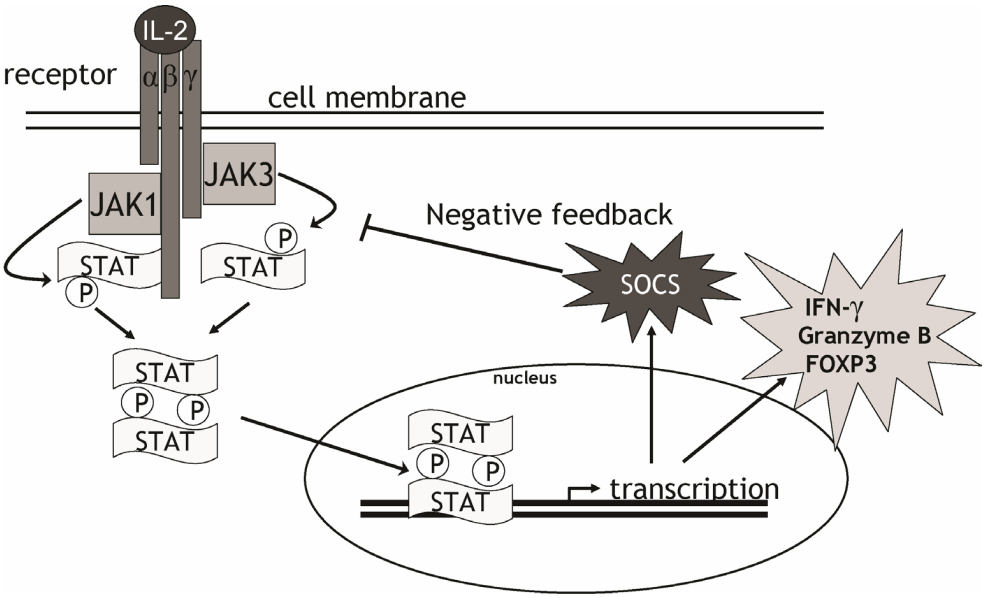

Figure 1: Downstream signaling of the IL-2 receptor.
The high-affinity IL-2R consists of three subunits: an ∝-chain (IL-2R∝; i.e., CD25), a β-chain (IL-2Rß; CD122) and the common cytokine-receptor γ-chain (γ_c; CD132). Binding of IL-2 to the IL-2Rα drives the association of the α -chain with IL-2Rβ and the γ_c to form a heterotrimer. This leads to initiation of signal transduction. Janus activated kinase 3 (JAK3) associates with the γ_c, and JAK1 associates with IL-2Rβ and both phosphorylate tyrosine residues in the cytoplasmic part of IL-2Rβ and the γ_c. Subsequently the JAK molecules themselves are activated. This 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 cytokines, growth factors and interferons, e.g. IFN-γ, the transcription factor key for regulatory T cells: FOXP3 and the suppressor of cytokine signalling (SOCS), by binding to DNA promoter sequences.

Of the four known JAK molecules, JAK3 is unique in its expression and association. JAK3 is predominantly expressed in hematopoetic cells [8]. Each of the γ_c cytokines activates a certain type or a combination of different JAKs. For instance IL-2 receptor signaling is primarily mediated through activation of JAK1 and JAK3 with subsequent phosphorylation and activation of STAT3 and STAT5, which is essential for lymphocyte development and differentiation, while IL-4 activates STAT6 (Fig. 2). Apart from the IL-2 receptor family, JAK-STAT activation is also critical for signaling of other cytokines e.g. IL-6, IL-10, IL-17 and interferons.

Cytokines of the IL-2 receptor family are involved in cellular events such as proliferation and differentiation; and they are involved in almost all T cell functions (e.g., cytotoxicity, help, and immune regulation) [9]. The importance of the JAK-STAT signaling pathway is shown in patients with severe combined immunodeficiency (SCID) syndrome. Deficiencies in JAK3 cause SCID syndrome that mirrors γ_c receptor deficiency, a condition characterized by the complete lack of T cells and natural killer (NK) cells, whereas B cells are present with impaired function [10]. SCID causes severe recurrent and/or opportunistic infections in early life and if left untreated is life-limiting. At present, hematopoietic stem-cell transplantation is the only lifesaving therapy.

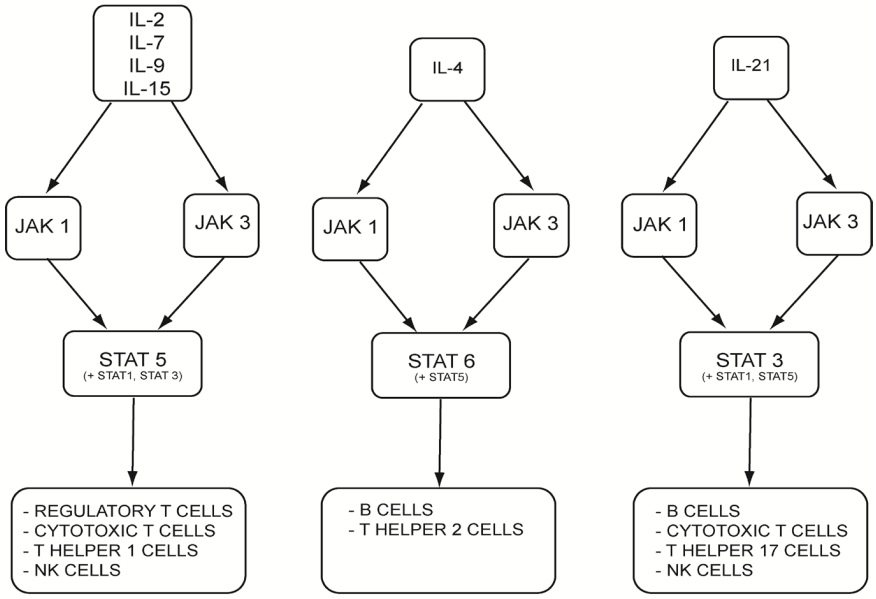

Figure 2: The JAK-STAT signaling pathway.
The IL-2 family of cytokines, all sharing a common receptor subunit γ-chain (γ_c), play a major role in promoting and maintaining T, B and natural killer (NK) cell populations. The IL-2 receptor family cytokines, after binding their receptor, activate the JAK/STAT signalling pathway. At the cytoplasmatic side of the receptor, either JAK1 or JAK3 is phospohorylated. Activation of JAKs (~ 130kDa) leads to attraction of a specific combination of STATs (70 – 94 kDa). IL-2, IL-7 and IL-15 are essential for T cell memory homeostasis, proliferation and survival, while IL-2 and IL-15 are important for NK cell development [5, 35]. IL-2 additionally regulates the homeostatic maintenance of Tregs and their function, and stimulates IFN-γ production by T-cells upregulating the cytolytic properties of CD8+ cytotoxic T cells. IL-4 activates STAT6 and T helper 2 (Th2) development [36-37]. IL-21, by activating STAT1 and STAT3, induces differentiation of B cells into plasma cells and stimulates cytotoxic T cell proliferation. IL-21 also has regulatory effects. It inhibits the antigen-presenting function of dendritic cells (DC) and is a pro-apoptotic factor for NK cells and incompletely activated B cells [38].

2. New Therapeutic Approaches using Specific Kinase Inhibitors

Defective JAK phosphorylation is associated with a number of diseases, including chronic myeloproliferative disorders, certain hematologic and solid malignancies and immune system disorders including rheumatoid arthritis and psoriasis. These findings encouraged pharmaceutical companies to develop drugs targeting protein kinases, which resulted in a new class of immunosuppressive agents: the protein kinase inhibitors. These agents inhibit the effect of one or more of the JAKs and interfere with the JAK-STAT signaling pathway. Table 1 shows some examples of this novel class of immunosuppressive agents, their targets and clinical use.

JAK1, JAK2, and TYK2 are expressed in many tissues, while JAK3 is restricted to haematopoietic cells. The limited expression of JAK3 provides the opportunity for the development of selective immunosuppressive compounds. In organ transplantation, the association of JAK3 inhibition with immunosuppression was first demonstrated by Behbod et al. who showed that inhibition of JAK3 signaling pathways by tyrphostin AG490 prolonged the survival of heart allografts [11]. However, the selectivity of AG490 has not been well established. Another JAK-inhibitor named tofacitinib previously called tasocitinib or CP-690,550 received much more attention and has progressed furthest in clinical studies. Tofacitinib is highly potent for JAK3 inhibition with some cross reactivity for JAK1 and JAK2 [12]. In a murine model of heart transplantation and in cynomolgus monkeys receiving kidney transplants the efficacy for preventing rejection was shown, which was associated with a decrease in total peripheral blood lymphocytes [12-15]. The phase 1 dose-escalation study of tofacitinib in 22 stable kidney allograft recipients reported no graft loss [16]. Also treatment with tofacitinib demonstrated acceptable safety and tolerability when administered at dosages up to 30 mg BID in combination with mycophenolate mofetil (and with CNI in some patients) over a treatment course of 28 days. The immunosuppressive potency was further demonstrated in a pilot study in de novo kidney transplant recipients on tofacitinib-based CNI-free immunosuppression [17]. Tofacitinib treatment resulted in relatively low acute rejection rates, with evidence of over-immunosuppression when tofacitinib 30 mg BID was combined with mycophenolate mofetil (MMF). The incidence of BK virus nephropathy in patients treated with 30 mg BID of tofacitinib in combination with MMF was unacceptably high. Tofacitinib at a lower dose of 15 mg BID coadministered with MMF resulted in similar outcomes to the CNI (tacrolimus (TAC))-treated control group but was associated with modest lipid elevations and a higher rate of viral infections. The efficacy of tofacitinib was also demonstrated in patients with active rheumatoid arthritis

[18]. In a large double-blind, placebo-controlled phase IIa trial (N=264) tofacitinib was effective in the treatment of rheumatoid arthritis, resulting in rapid, statistically significant, and clinically meaningful reductions in signs and symptoms. These promising results in auto- and allo-immunity initiated several new phase I and phase II trials. Currently, fifteen studies recruit patients that suffer from chronic plaque psoriasis or rheumatoid arthritis, or patients after kidney transplantation. Details of these studies can be found at www.clinicaltrials.gov.

Table 1. Examples of JAK-STAT inhibitors

Compound	**Kinase Target**	**Pre-clinical**
NVP-BSK805	JAK2	Myelofibrosis [35]
HSP90	JAK2	Thrombocytosis [36]
AG490	JAK3	Organ transplantation [11]
Compound	**Kinase Target**	**Clinical use**
Pimozide	STAT5	Chronic myelogenous leukemia [37]
Ruxolitinib	JAK1/JAK2	Psoriasis, myelofibrosis, rheumatoid arthritis [38]
LY3009104	JAK1/JAK2	Rheumatoid arthritis [39]
TG101348	JAK2	Myelofibrosis [40]
SB1518	JAK2	Myelofibrosis and relapsed/ refractory lymphoma [39]
Lestaurtinib	JAK2	Acute myelogenous leukemia [41]
Tofacitinib	JAK1/JAK3	Psoriasis, rheumatoid arthritis [18] and organ transplantation [17,29]

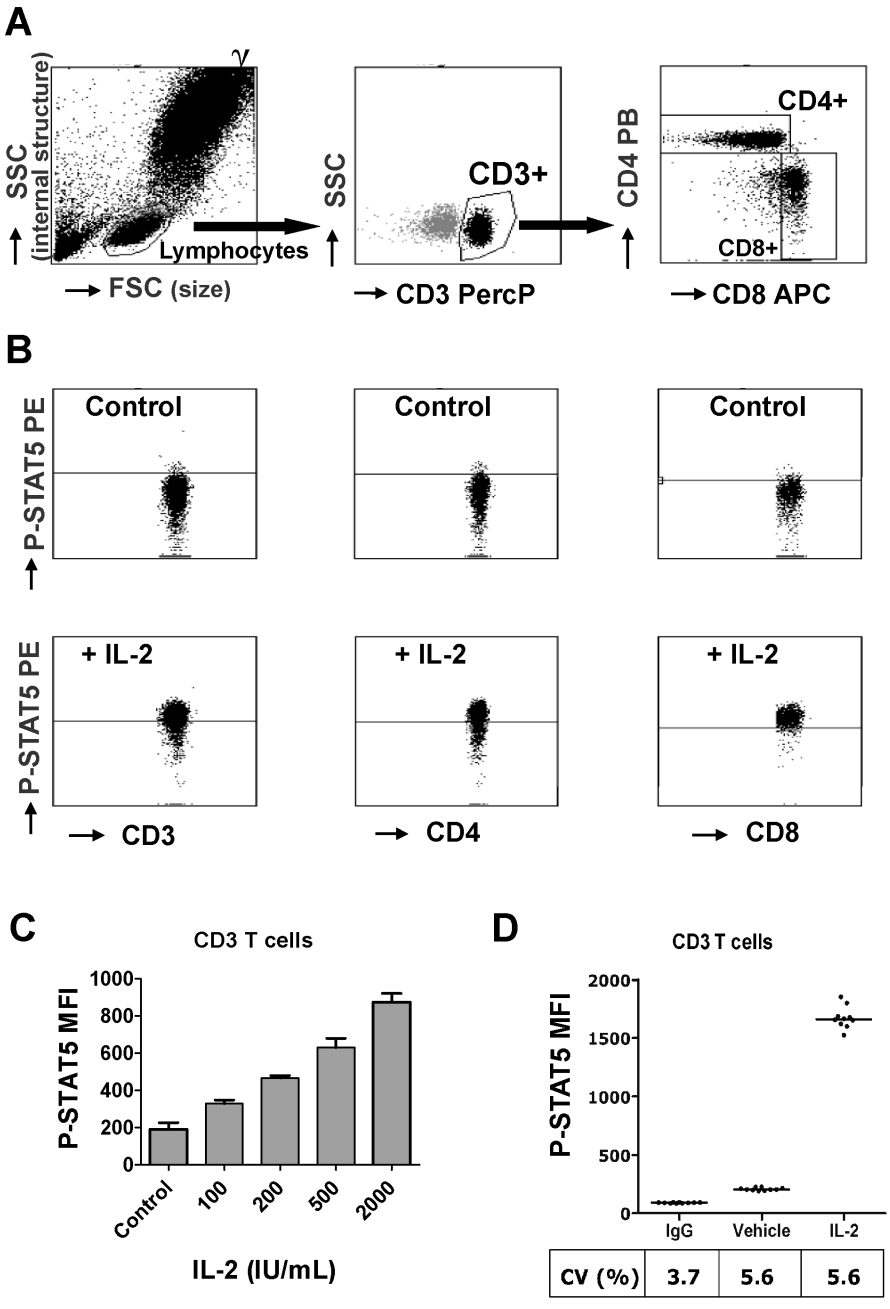
A
SSC (internal structure)
Lymphocytes
FSC (size)
SSC
CD3+
CD3 PercP
CD4 PB
CD4+
CD8+
CD8 APC
B
P-STAT5 PE
Control
Control
Control
+ IL-2
+ IL-2
+ IL-2
CD3
CD4
CD8
C
CD3 T cells
P-STAT5 MFI
1000
800
600
400
200
0
Control
100
200
500
2000
IL-2 (IU/mL)
D
CD3 T cells
P-STAT5 MFI
2000
1500
1000
500
0
IgG
Vehicle
IL-2
CV (%)
3.7
5.6
5.6

Figure 3 Analysis of STAT5 phosphorylation by flow cytometry.
Dot plots of flow cytometric analysis of STAT5 phosphorylation where each dot represents a single cell. (A) The gating strategy for T cell subsets: CD3+ T cells are labeled with a Peridinin chlorophyll protein (Percp) conjugated monoclonal antibody (mAb), CD4+ T cells with a pacific blue (PB) conjugated mAb and CD8+ T cell to a allophycocyanin (APC) conjugated mAb. In the illustrated example 208,718 cells were measured of which 20.000 lymphocytes were gated based on size and internal structure (left plot). The gated lymphocytes on the left plot were separated in CD3- and CD3+ cells (middle plot) and CD3+ T cells were further divided in CD4+ and CD8+ T cell subsets (right plot). (B) Dot plots showing STAT5 phosphorylation after activation of whole blood with either vehicle or 2000 IU/mL IL-2 for 30 minutes. (C) Whole blood samples were stimulated with medium (control) or IL-2 concentrations from 100 to 2000 IU/mL and phosphorylated STAT5 per cell was quantified with a phycoerythrin (PE)-labeled mAb. On the Y-axis median IL-2-induced STAT5 phosphorylation is quantified in MFI (median fluorescence intensity). Plotted are median+range of 2 independent experiments. (D) Intra-assay reproducibility of the assay was analyzed by stimulation of whole blood samples (100 uL) of the same healthy volunteer in ten independent experiments. MFI of P-STAT5 is plotted on the y-axis (CV: coefficient of variation)

3. Monitoring Immunologic Responses

Effective manipulation of T cell responses by immunosuppressive agents requires immune monitoring to help us understand the mechanisms that result in auto- and allo-reactivity and to define surrogate markers for clinical efficacy of the medication. Analysis of the immune parameters of tofacitinib-treated kidney transplant patients revealed: a decrease in the absolute numbers of NK cells, a decrease in regulatory CD4+CD25^{high+} regulatory T cells (Tregs), an increase in circulating B cells; and inhibition of IL-2 triggered IFN-γ production of peripheral blood mononuclear cells (PBMC) [19]. The observed impaired IFN-γ production results from blocked STAT5 phosphorylation by tofacitinib, as IL-2-induced binding sites of STAT5 are present in the IFN-γ gene (Fig. 1). Immune monitoring further revealed that the regulatory function of the residual CD4+CD25^{high+} T cells remained intact. A next interest is to determine the biological effect of tofacitinib at the molecular level in cells of immune-suppressed patients. The novel phosphospecific flow cytometry technology provides this opportunity. By this technique intracellular signalling molecules in the immune system can be studied at the single-cell level. Particularly, the studies by the group of Nolan et. al. showed the power of this technique [20-22]. We used phosphospecific flow cytometry to study the effect and efficacy of tofacitinib on cytokine-induced phosphorylation of STAT5 in T cells and found that flow cytometry is a reliable, sensitive and specific tool to determine the inhibitory effect of this agent on the JAK/STAT pathway in different lymphocyte populations [23] (Fig. 3). In the following sections this technique will be discussed in detail together with the first findings using phosphospecific flow cytometry as a tool for pharmacodynamic monitoring in the clinical setting.

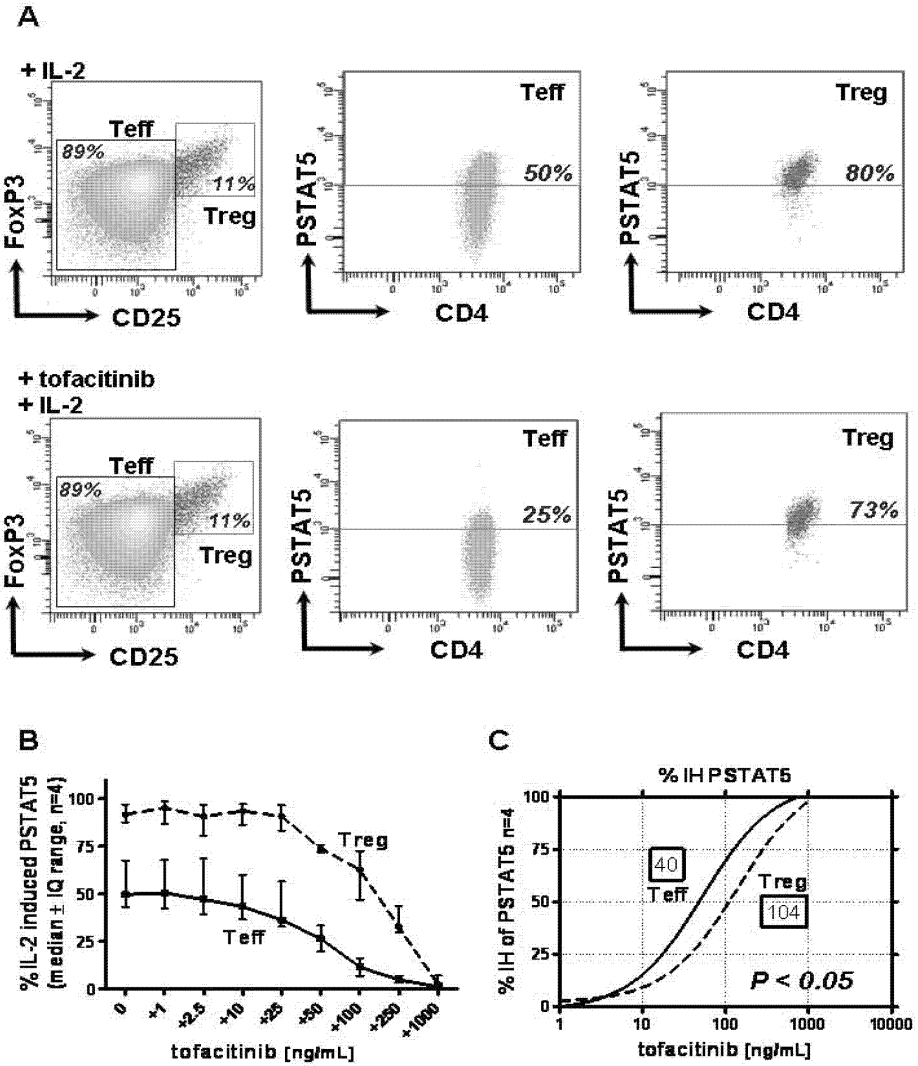

Figure 4: Difference in drug sensitivity between effector T cells (Teff) and regulatory T cells (Tregs).
(A) Flow cytometry example of IL-2-induced (2000 IU/mL for 30 minutes) STAT5 activation in CD4+CD25-FoxP3- T cells and CD4+CD25^{high+}FoxP3+ regulatory T cells in the presence and absence of 50 ng/mL tofacitinib. (B) Percentage positive cells of phosphorylated STAT5 at increasing doses of tofacitinib are shown within CD4+CD25-FoxP3- effector T cells (Teff, black line) and CD4+CD25^{high+}FoxP3+ regulatory T cells (Treg, dotted line) for tofacitinib treated patients (n=4). (C) Sigmoid curves with percentage inhibition of phosphorylated STAT5 by tofacitinib calculated on the percentage STAT5 positive cells. The IC_{50} for the CD4+CD25- FoxP3- effector T cells and CD4+CD25^{high}FoxP3+ regulatory T cells was significantly different ($p < 0.05$).

4. Phosphospecific Flow Cytometry to Monitor the JAK-STAT Pathway

Historically, phosphoprotein detection has been performed using techniques such as immunoprecipitation, immunofluorescence microscopy and Western blotting. Major draw backs of these techniques are the lack of sensitivity, number of cells required and quantification difficulties. Consequently, Western blot analysis was often performed on Jurkat T cell lines, which was useful to unravel the signaling pathways involved in T cell activation but not for routine phosphoprotein studies in patient materials [24].

A novel technique that has the potential for routine monitoring of these proteins in patients is phosphospecific flow cytometry. This technique provides the opportunity to monitor intracellular signaling pathways in less than 3 hours using small amounts (100 µL) of whole blood. Phosphospecific flow cytometry can be performed on every cytometer and measures activation-induced changes of signaling molecules inside the cell relative to unstimulated populations of identical cells (Fig. 3). For example STAT5 phosphorylation in T cells can be measured after activation of whole blood by IL-2, IL-7 or IL-15 [25] while for monocytes activation with GM-CSF (granulocyte macrophage-colony stimulating factor) is required. STAT3 phosphorylation in T cells can be measured after activation with IL-6 and IL-21 [26]. Phosphospecific flow cytometry provides the opportunity for a rapid, quantitative, multiparameter analysis of single cells and distinct cell subpopulations within a sample, while by the classical Western blot technique only phosphorylation of the total sample is measured.

The disadvantages of phosphospecific flow cytometry are obvious. Setting up simultaneous analysis of the multiple parameters, like surface markers in combination with intracellular signaling molecules requires a time consuming optimization step. Also the range of commercially available antibodies is not as extensive as for Western blot. Nonetheless, Krutzik et al. [21] have developed and standardized a robust phosphospecific flow cytometry assay for STAT-phosphorylation. They give a good description and introduction on how to get started with the technique. A detailed protocol is also available via the Application Handbook: "Techniques for phosphoprotein analysis" from BD biosciences (San Jose, CA, first edition 2005), which we have used to set up phosphospecific flow cytometry assays.

Phosphospecific flow cytometry can be used I. to determine the normal signaling responses of immune cells in response to antigen challenge II. to unravel the signal transduction networks that drive functional cellular changes in diseased individuals III. to perform drug screening in cell lines and primary

cells; and IV. to monitor the specificity and efficacy of immunosuppressive agents [20-23, 27]. Thus, the biologic effects of immunosuppressive agents on intracellular signaling pathways at the single cell level and how these molecules are activated in response to stimuli can now be easily studied. We used this technique to monitor the biological effect of immunosuppressive treatment in immune cells of kidney transplant patients. Tofacitinib significantly reduced IL-2-induced STAT5 activation in CD4+ and CD8+ T cell populations [23]. The relevance of blocked STAT5 activation was confirmed by low expression of STAT5 downstream target genes like interferon-γ, SOCS3 and the transcription factor of regulatory T cells, FoxP3. By applying the phosphoflow technique we also showed that it can be used to analyse the difference in drug susceptibilities between lymphocyte subsets. A significant difference in drug sensitivity between effector T cells and Tregs [28] was found as illustrated in figure 4. At a clinically relevant concentration of 50 ng/mL tofacitinib, IL-2-induced STAT5 phosphorylation was significantly inhibited in effector CD4+CD25-FoxP3- T cells, while only partially blocked in the CD4+CD25^{high+}FoxP3+ regulatory T cells. The IC_{50} was 2 times higher for Tregs than for effector T cells (Fig. 4C). These differences in sensitivity for tofacitinib can be explained by a different distribution of the IL-2 receptor α, β and γ_c chains or a variation in the recruitment of JAK1 and JAK3 molecules. Tregs might have more JAK3 and/or JAK1 molecules and need more tofacitinib to inhibit all molecules [29]. Furthermore, it is also unclear how many configurations of STAT homo- and heterodimeric complexes are present in Tregs and effector T cells before, during and after cytokine stimulation [30]. It has been shown that different regions of the cytoplasmic domain of the IL-2Rβ interact and couple with distinct signaling pathways and cellular responses in Tregs and effector T cells [31]. Overall this example nicely shows that by phosphospecific flow cytometry a difference in drug susceptibility between different T cell subsets that are both fundamental in allo-reactivity can be detected. This knowledge not only contributes in our understanding of T cell responses in immunosuppressed patients. It also gives new insights in how immunosuppressive agents interact with immune cells. Furthermore, phosphospecific flow cytometry can be used to find abnormalities in signaling pathways that drive functional cellular changes in diseased individuals. For example in patients with systemic lupus erythematosus, an autoimmune disease, STAT3 activation in T cells was increased, a finding that has been shown by two independent groups [32-33].

5. Activation of STATs by Ex Vivo Whole Blood Analysis

To draw conclusions on how drugs interact with immune cells of patients in vivo, analysis on whole blood of patients is preferred above analysis on PBMC. Analysing whole blood can give more reliable answers to questions like "how do immune cells of immunosuppressed patients behave?", "is there redundancy in the cytokine signaling network?", "what is the specificity and efficacy of a compound", "are there differences in drug susceptibility between T cell subsets?", and "is STAT5 phosphorylation blocked in tofacitinib treated patients?"

Whole blood analysis of activation markers will give more reliable answers to these questions compared to analysis in PBMC, because I. the whole blood matrix is a better reflection of the in vivo situation, e.g. red blood cells and plasma are present during the procedure, and II. to acquire PBMC an extraction technique is needed. During the PBMC isolation procedure the intracellular medication may be partially or even completely washed out of the cells, which influences the result of the subsequent pharmacodynamic analysis. One of the first papers demonstrating the power of whole blood phosphospecific flow cytometry was published by Wu and colleagues [34] who showed STAT5 activation in T cells and NK cells of melanoma patients after bolus infusion of IL-2. The authors speculate that the assay can be used to identify patients who do and do not respond to IL-2 treatment. This is of great importance as only a small percentage (15-16%) of the IL-2 treated patients exhibit a clinical response i.e. tumor regression. In melanoma patients also impaired STAT1 activation by type I interferons was found, giving important insight into immune dysfunction in cancer and new targets for treatment.

Other papers reporting on the efficacy and specificity of immunosuppressive agents and using whole blood phosphospecific flow cytometry, primarily focus on tofacitinib, the compound that inhibits JAK1/JAK3 activation. IL-6 and IL-15 stimulation of mouse and human whole blood demonstrated that JAK1 and JAK3 kinase activity in T cells is inhibited by tofacitinib while JAK2 activity is spared at therapeutic doses [25-26]. Thus, the phosphospecific flow cytometry technique can also be used to determine the specificity of JAK kinase inhibitors.

By applying whole blood analysis in our laboratory we are currently measuring cytokine-induced STAT5 activation in peripheral blood samples of kidney transplant patients who are treated with basiliximab induction therapy on the day of transplantation and at day 4 after transplantation in combination with tofacitinib or TAC maintenance therapy. First we set up the experiments by activating whole blood of healthy volunteers with different concentrations of

recombinant IL-2, at 37°C for 30 min, according to the phosphospecific flow cytometry protocol of BD Biosciences (San Jose, CA) (Fig. 3). The gating strategy and dot plots for STAT5 phosphorylation are shown in figure 3. Whole blood activated by recombinant IL-2 induced STAT5 phosphorylation in T cells in a dose-dependent manner (Fig. 3C). The intra-assay variability of the assay was tested and the coefficient of variation was 5.6% (Fig. 3D).

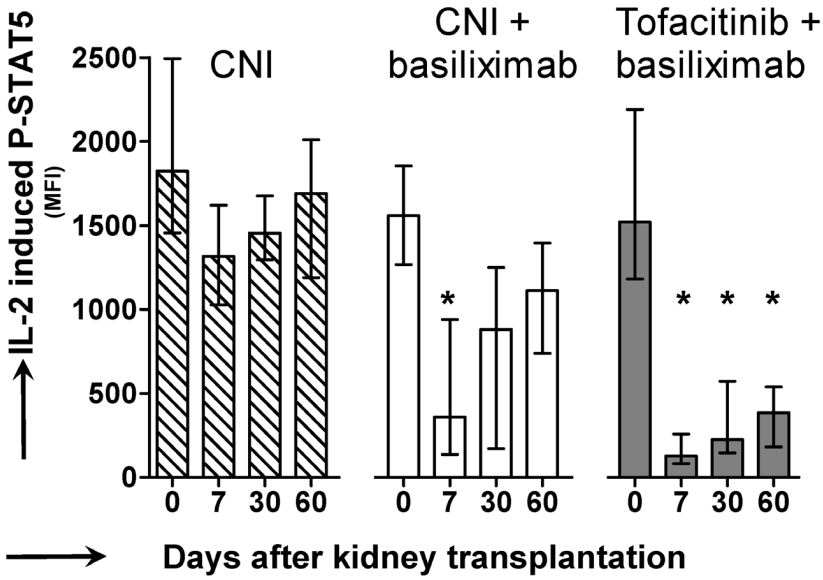

Figure 5: STAT5 activation in renal transplant recipients.
Whole blood phosphospecific flow cytometric analysis of STAT5 phosphorylation in CD4+ T lymphocytes after stimulation with 2000 IU/mL IL-2 at 37 °C for 30 minutes. Phosphorylated STAT5 was determined in samples obtained from kidney transplant patients before and after transplantation. As part of a randomized phase II clinical trial with tofacitinib, patients were allocated to one of two treatment groups: I. CNI (Cysclosporin A) + basiliximab (n = 4) or II. tofacitinib + basiliximab (n = 5). Patients on CNI (TAC) (n = 6) without basiliximab served as controls. All patients additionally received corticosteroids maintenance treatment. Basiliximab induction therapy was given at the day of transplantation and 4 days after transplantation. CNI's and tofacitinib were given as maintenance therapy. IL-2-induced STAT5 phosphorylation for each patient was calculated as MFI of the IL-2-induced sample minus the unstimulated control sample. Asterisks indicate p-value < 0.05 by the Mann Whitney (non-parametric) test for the time point compared to pre-transplantation.

Of the kidney transplant patients whole blood was drawn and activated by 2000 IU/mL human recombinant IL-2 for 30 minutes. In basiliximab/CNI (TAC) treated patients, IL-2-induced STAT5 activation was strongly inhibited at one week after transplantation, due to blockade of the IL-2Rα. In this group, the induced STAT5 activation recovered gradually over time and returned to pre-transplant levels at two month after transplantation (Fig. 5). In tofacitinib/basiliximab treated patients, STAT5 activation was also inhibited and in contrast to the basiliximab/CNI (TAC) group, remained suppressed during the course of tofacitinib therapy. In control patients treated with neither basiliximab nor tofacitinib, STAT5 phosphorylation was not blocked (Fig. 5).

6. Challenges in Organ Transplantation

Phosphospecific flow cytometry provides a rapid and sensitive tool to monitor the efficacy and specificity of immunosuppressive medication on immune cell subsets key in allo-reactivity after organ transplantation. Specifically its power to quickly monitor immune responses in different populations of cells and its application to establish the efficacy and specificity of immunosuppressive agents on cytokine signalling pathway inhibitors makes this technique highly interesting for routine monitoring purposes. By using this technique it has now been shown that tofacitinib not only interferes in JAK1/JAK3-dependent STAT5 responses but also blocks STAT1 dependent reactions. So, phosphospecific flow cytometry shows the specificity of a certain compound and at the same time helps to unravel how immunosuppressive medication interacts with intracellular signaling networks. This information may contribute to explain some of the non-specific side effects of immunosuppressive medication. For instance, the JAK/STAT pathway is not only pivotal for immune cells but also for a vast number of other cell types including tubular epithelial cells and cardiac muscle cells. Furthermore, the narrow therapeutic window of immunosuppressive agents in general puts patients at risk of rejection in case of under dosing or of toxicity when overdosed. Due to the inter-individual variability it appears difficult to determine the optimal therapeutic dosage for each individual patient. This problem can now be solved by applying pharmacodynamic monitoring using phosphospecific flow cytometry. This technique provides a new functional tool for determining patient specific cellular drug efficacy en specificity. More research is necessary to show the relation between JAK-STAT activation and clinical parameters, e.g. rejection and toxicity, in kidney transplant recipients and in other solid organ transplant recipients.

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Chapter 6

Pharmacodynamic Analysis of Tofacitinib and Basiliximab in Kidney Allograft Recipients

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Abstract

Introduction: The common-γ-chain (γ_c) cytokines signal through the JAK-STAT-pathway and play pivotal roles in lymphocyte activation. We investigated the effect of immunosuppressive drugs targeting this pathway, e.g. the JAK inhibitor tofacitinib (CP-690,550) and the anti-IL-2R-antibody basiliximab, as part of a phase 2 study.

Methods: After whole blood activation with the γ_c cytokines IL-2, IL-7 and IL-15, STAT5-phosphorylation was determined in T cells of de novo kidney transplant patients treated with tofacitinib/basiliximab (n=5), CNI (cyclosporine A)/basiliximab (n=4) or CNI (tacrolimus)-based immunosuppression (n=6). The IC_{50} for P-STAT5 inhibition by tofacitinib was determined in cytokine-activated CD4+ and CD8+ T-cells from healthy individuals (n=4).

Results: IC_{50} was 26, 72, and 37 ng/ml tofacitinib for IL-2, IL-7 and IL-15-activation in CD4+ T cells, respectively; and 35, 61, and 76 ng/ml for IL-2, IL-7 and IL-15 activation in CD8+ T cells, respectively. In kidney transplant patients, 7 days after starting tofacitinib/basiliximab treatment, cytokine-induced P-STAT5 was inhibited in CD4+ T cells (92% for IL-2 activation, 60% for IL-7, and 75% for IL-15), which persisted for the 2-month study period. In contrast, CNI/basiliximab treatment did not affect IL-7 or IL-15-activated P-STAT5; only IL-2-activated P-STAT5 was reduced by 77% on day 7, and recovered to pre-treatment levels within 2 months. CD8+ T cells showed a comparable profile to CD4+ T cells. P-STAT5 was not inhibited in CNI-treated control patients.

Conclusions: Tofacitinib therapy strongly inhibits γ_c cytokine-induced JAK/STAT5 activation, while basiliximab suppresses IL-2-stimulated activation only. Pharmacodynamic monitoring offers a unique tool to evaluate the biological effects of immunosuppressive drugs.

Introduction

In organ transplantation, treatment with currently available drugs, such as calcineurin inhibitors (CNI) and mammalian target of rapamycin (mTOR) inhibitors, is accompanied by debilitating side effects, such as infections, diabetes, nephrotoxicity and malignancies all influencing the quality of life, and patient/graft survival [1]. These immunosuppressive drugs have a narrow therapeutic window which places patients at risk of rejection in case of under-dosing or toxicity when overdosed [2-4]. There are no reliable tests to assess the amount or type of immunosuppressive drugs each recipient needs. Development of such a test to optimize drug efficacy will have great impact on patient management [5]. The novel phosphospecific flow cytometry (PFC) technology provides this opportunity. By PFC, intracellular signaling molecules in immune cells can be studied at the single-cell level [6]. Furthermore, it requires small amounts of blood and is cost- and time-efficient.

The need for safer and more specific agents that target immune cells only, resulted in the identification of Janus kinase 3 (JAK3) as a potential target for immunosuppression [7]. JAK3, a member of the JAK family of tyrosine kinases, is involved in cytokine receptor-mediated intracellular signaling. Its expression is limited to hematopoietic cells where it associates with the common γ-chain (γ_c) of the IL-2 receptor family (i.e., IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21). The IL-2 receptor family cytokines are involved in cellular events, for example, proliferation, differentiation and immune regulation, and are indispensible for acquired and innate immunity [8-9]. Following cytokine receptor-JAK activation, signal transducers and activators of transcription (STAT) are phosphorylated and function as nuclear transcription factors. The small molecule compound tofacitinib, previously known as CP-690,550 or tasocitinib, inhibits JAK3 and JAK1 activation leading to anti-inflammatory and immunosuppressive activities [10-11]. For instance, the IL-2-dependent interferon-γ production capacity is largely inhibited in T cells of tofacitinib-treated kidney transplant patients [2, 11]. Importantly, in de novo kidney transplant patients on a tofacitinib-based CNI-free immunosuppressive regimen, the incidence of acute rejection was similar to the CNI control group and tofacitinib-treated patients had better kidney function with less histological damage at 1 year after transplantation. Unfortunately, tofacitinib was associated with more infections and malignancies reflecting over-immunosuppression [12-13]. In these patients, pharmacodynamic monitoring could be useful to optimize immunosuppressive treatment.

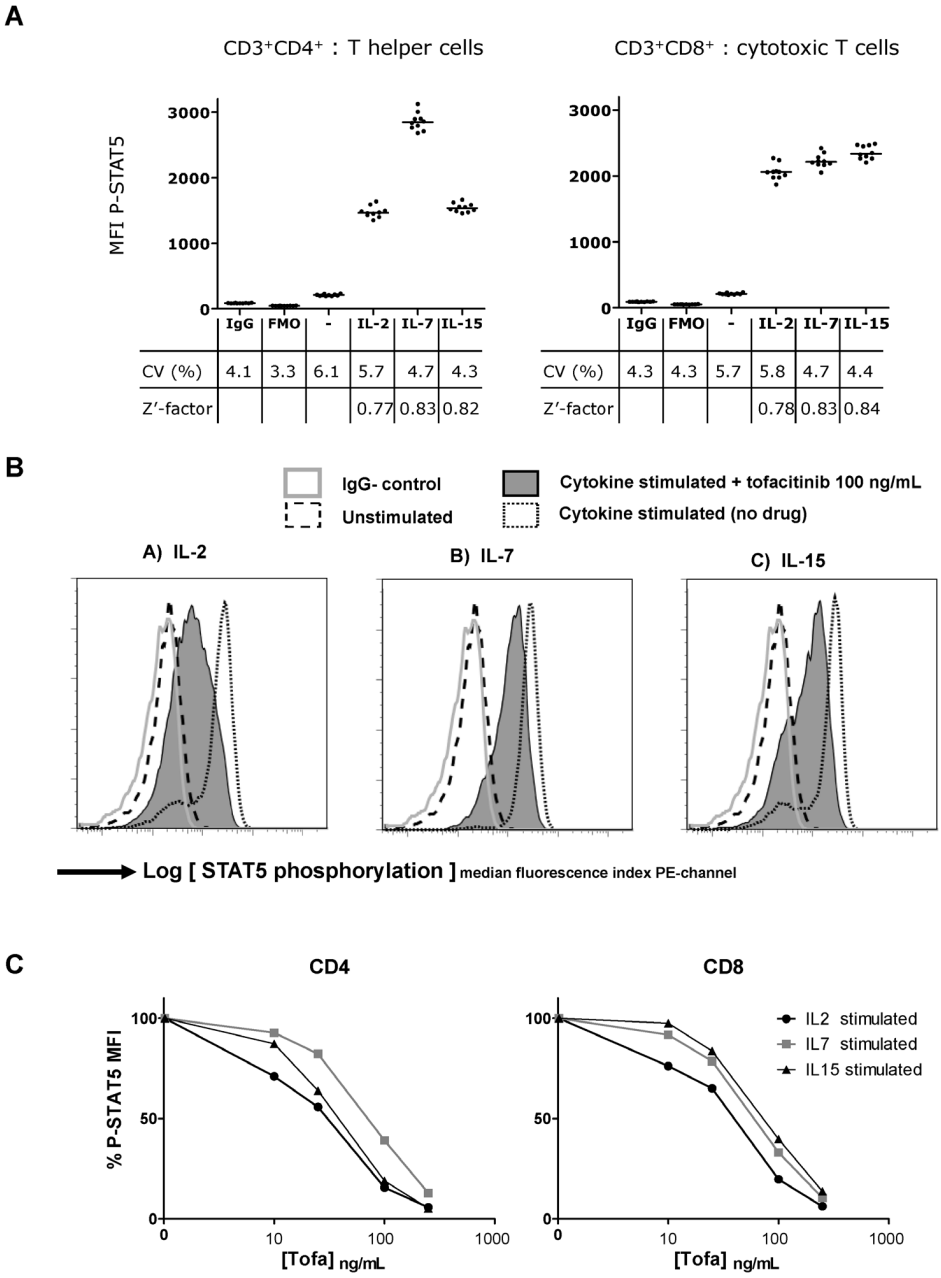

A
CD3+CD4+ : T helper cells
CD3+CD8+ : cytotoxic T cells
MFI P-STAT5
3000
2000
1000
0
IgG
FMO
-
IL-2
IL-7
IL-15
CV (%) 4.1 3.3 6.1 5.7 4.7 4.3
Z'-factor 0.77 0.83 0.82
CV (%) 4.3 4.3 5.7 5.8 4.7 4.4
Z'-factor 0.78 0.83 0.84
B
IgG- control
Unstimulated
Cytokine stimulated + tofacitinib 100 ng/mL
Cytokine stimulated (no drug)
A) IL-2
B) IL-7
C) IL-15
Log [STAT5 phosphorylation] median fluorescence index PE-channel
C
CD4
CD8
% P-STAT5 MFI
100
50
0
10
100
1000
[Tofa] ng/mL
IL2 stimulated
IL7 stimulated
IL15 stimulated

Figure 1: Whole blood phosphospecific flow cytometry to measure the effect of tofacitinib on signal transducer and activator of transcription (STAT)-5 activation.
A) To measure the intra assay reproducibility whole blood samples (100 uL) were analyzed in ten independent experiments. Median fluorescence intensity of phosphorylated STAT (P-STAT) 5 is plotted on the y-axis. B) Representative example of the in vitro effect of tofacitinib on cytokine-induced STAT5 phosphorylation in CD3+ T lymphocytes of healthy volunteers. Phosphorylated STAT5 levels were upregulated after whole blood activation for 30 minutes with IL-2, IL-7 or IL-15 (respective final concentrations of 2000U/mL, 100 ng/mL and 100 ng/mL) and inhibited by Tofacitinib (100 ng/ml). C) IC_{50} measurements after activation of whole blood from healthy individuals with the cytokines, IL-2, IL-7 or IL-15 (final concentrations respectively 2000U/mL, 100 ng/mL and 100 ng/mL). The effect of Tofacitinib on STAT5 phosphorylation was measured in CD4+ and CD8+ T cells (n = 8). Points shown on the graph are mean percentage residual P-STAT5 compared to the sample without tofacitinib. FMO, fluorescence minus one.

In this study, we used whole blood PFC to investigate the activation of STAT5, an event downstream of JAK1/3 phosporylation, in response to IL-2, IL-7 and IL-15-activation in tofacitinib/basiliximab- and CNI/basiliximab-treated de novo kidney transplant patients. Patients on CNI-based maintenance immunosuppression without basiliximab or tofacitinib served as control. Cellular drug sensitivity over time were shown for the drugs targeting the IL-2 family cytokine pathway, tofacitinib and basiliximab. The latter agent blocks the interaction of IL-2 with the α-chain of the IL-2 high affinity receptor [14].

Material and Methods

Renal Transplant Patients

We studied nine de novo kidney transplant patients in a phase 2, multicentre, randomized, partially blinded, active comparator-controlled, parallel-group trial (*12-13*). The local medical ethical committee approved the study and informed consent was obtained from each patient. Patients (n=5) in the study group received 15 mg tofacitinib (Pfizer Inc, Groton, CT) twice daily (B.I.D) for at least 60 days after transplantation and maintenance therapy consisting of mycophenolate mofetil (MMF, Cellcept, Roche Ltd., Basel Switzerland) 500 to 1000 mg B.I.D and prednisolone 5 to 7.5 mg daily. Patients in the comparative group (n=4) received cyclosporine (CsA, Novartis International AG, Basel, Switzerland), MMF and prednisolone. Patients in both aforementioned groups received 20 mg basiliximab (Novartis), anti-CD25 monoclonal antibody induction therapy, at the day of transplantation and day 4 after transplantation. A third group of de novo kidney transplant patients (n=6) received CNI

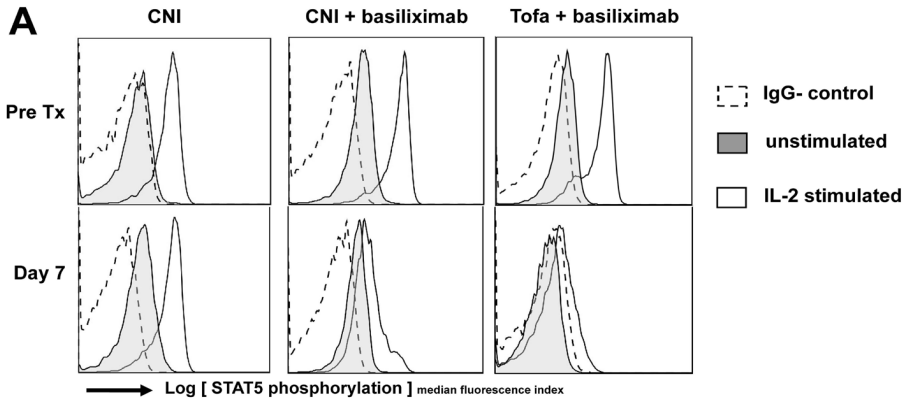
(tacrolimus)-based maintenance immunosuppression in combination with MMF and prednisolone, without basiliximab and served as controls. Blood samples were obtained on day -1 before transplantation, on days 7, and 14, and at months 1 and 2 after transplantation. From one patient blood samples were also drawn at day 85 and 100 post-transplantation. All blood samples were collected at the end of 12-hour dosing intervals of tofacitinib, CsA or tacrolimus.

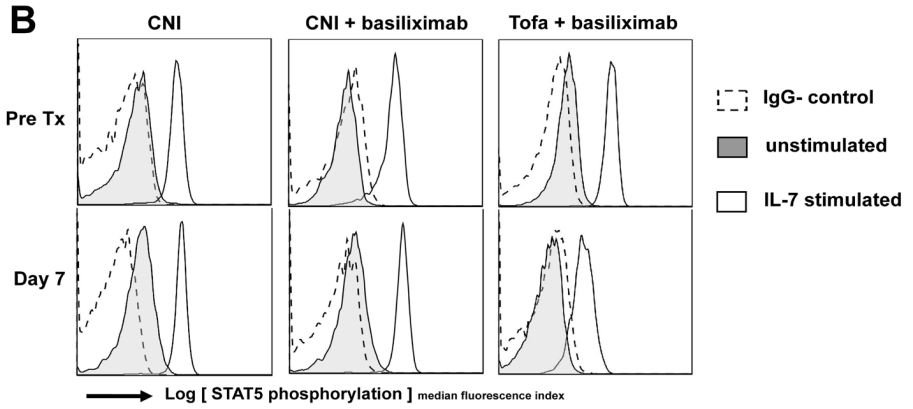
Blood samples were analysed for the number of circulating T cells (CD3+), NK cells (CD3-CD16+CD56+), and B cells (CD19+) by flow cytometry. The following dual monoclonal antibodies (mAb) combinations were added to 100 µL whole blood: CD45-FITC/CD14-PE; IgG_1-FITC/IgG_{2b}-PE as isotype control; CD3-FITC/CD19-PE; and the combinations CD3-FITC/CD16CD56-PE. The antibodies were purchased from BD Biosciences (San Jose, CA). Cells were analysed on a flow cytometer (FACSCanto II, BD Biosciences). To establish an analysis gate that included at least 90% of the lymphocytes, the CD45/CD14 reagent was used. Twenty thousand gated lymphocyte events/cells were acquired from each tube.

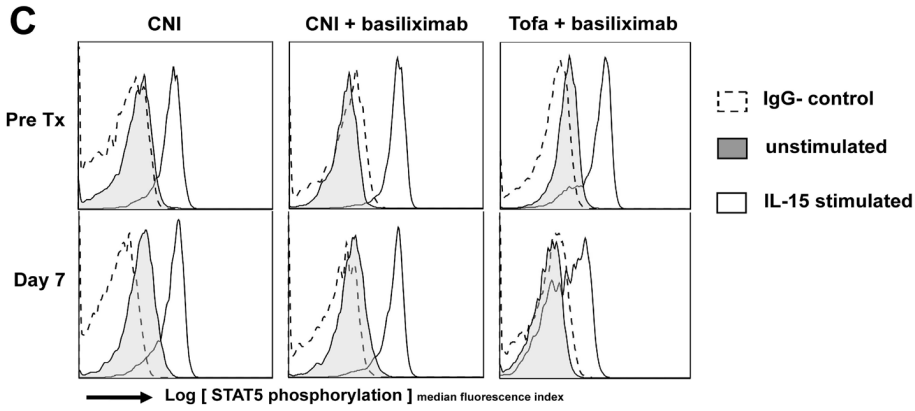
Whole Blood Staining for Phosphorylated STAT5 (P-STAT5)

Whole blood samples (100 µL) were activated with IL-2 (2000 U/mL), IL-7 (100 ng/mL), or IL-15 (100 ng/mL) (PeproTech, Rocky Hill, NJ) for 30 minutes at 37 ºC. Red blood cells were lysed and fixed for 10 minutes at 37 ºC with Lyse/Fix Buffer (BD Biosciences). Next, cells were washed in facsflow buffer (BD Biosciences) and permeabilized with cold 70% methanol for 30 minutes at -20 °C. Cells were washed twice in facsflow buffer (BD Biosciences) supplemented with 0.5% bovine serum albumin.

Figure 2: Interleukin (IL)-2-activated signal transducer and activator of transcription (STAT)-5 phosphorylation: inhibited in both calcineurin inhibitor (CNI)/basiliximab and tofacitinib/basiliximab treated patients.
A) From each of three treatment groups, I. CNI (tacrolimus), II. CNI (cyclosporine A)/basiliximab and III. Tofacitinib/basiliximab, an example of IL-2-activated STAT5 phosphorylation in CD4+ T cells from one patient is shown, before and 7 days after transplantation. At day 7 after transplantation, IL-2-induced STAT5 phosphorylation is unchanged for the control patient, whereas it was inhibited in the CNI/basiliximab and tofacitinib/basiliximab-treated patients. B) IL-7-activated phosphorylated STAT5 was unchanged at day 7 compared to before transplantation for the CNI and the CNI/basiliximab treated patients, whereas it was inhibited for a tofacitinib/basiliximab treated patient. C) An example of IL-15-activated phosphorylated STAT5 inhibited at day 7 after transplantation in a tofacitinib/basiliximab-treated patient, whereas no change was seen in the CNI and CNI/basiliximab-treated patients.


A
CNI
CNI + basiliximab
Tofa + basiliximab
Pre Tx
Day 7
IgG- control
unstimulated
IL-2 stimulated
Log [STAT5 phosphorylation] median fluorescence index


B
CNI
CNI + basiliximab
Tofa + basiliximab
Pre Tx
Day 7
IgG- control
unstimulated
IL-7 stimulated
Log [STAT5 phosphorylation] median fluorescence index


C
CNI
CNI + basiliximab
Tofa + basiliximab
Pre Tx
Day 7
IgG- control
unstimulated
IL-15 stimulated
Log [STAT5 phosphorylation] median fluorescence index

Cytokine-induced phosphorylation of STAT5 was studied in different T cell subsets. Cells were simultaneously incubated for 30 minutes at room temperature with the following antibodies according to manufacturer's specifications: P-STAT5 (Y694)-PE, CD3-PERCP, CD4-PB and CD8-APC, washed in facsflow buffer and analyzed on the FACSCanto II flow cytometer (BD Biosciences). Twenty thousand gated lymphocyte events/cells were acquired from each tube. Median fluorescence intensity (MFI) values were generated by analyzing the data using BD FACSDiva 6.0 software. The effect of IL-2, IL-7 and IL-15-activation on P-STAT5 was calculated as the P-STAT5-PE MFI of the cytokine stimulated sample minus the unstimulated sample (background). Fluorescence Minus One (FMO) controls and IgG controls were included as negative controls.

Tofacitinib IC_{50}-measurement for Cytokine-induced STAT5 Phosphorylation: In Vitro Study

The reproducibility of the assay was determined in freshly obtained normal human whole blood (100 uL) in ten independent experiments. Samples were activated with IL-2 (2000 U/mL), IL-7 (100 ng/mL), or IL-15 (100 ng/mL) for 30 minutes and analyzed for STAT5 phosphorylation as described above. For IC_{50} measurements 100 µl whole blood of healthy individuals (n=8) was pre-incubated at 37° C with tofacitinib (CP-690,550, Pfizer Inc) concentrations 10, 25, 100, or 250 ng/mL for 30 minutes followed by IL-2, IL-7 or IL-15 activation and P-STAT5 measurement. P-STAT5 inhibition was calculated for each tofacitinib concentration compared to the cytokines induced P-STAT5-MFI in the sample without tofacitinib. Sigmoid curves were drawn and IC_{50} values were calculated using Graphpad Prism software (v.5.02, GraphPad Software Inc., La Jolla, CA).

Statistical Analysis

For statistical analysis of patient data, the assumption of normality of distribution of the variables was tested with the Kolmogorov-Smirnov test. When the assumption of normality was rejected, the variables were analyzed using a nonparametric test (Mann Whitney). Statistical significance was defined as $P \leq 0.05$ (2-tailed). Linear regression analysis was performed using Graphpad Prism software (GraphPad Software Inc) and to calculate whether the regression model fitted the data we used the F-test.

Results

Clinical Results

The clinical results of 331 de novo renal transplant patients randomized in the tofacitinib phase 2b study are reported by Vincenti et.al. [12-13]. Here we report the clinical data only for the patients included in the current study. In summary, there were no life-threatening infections, polyomavirus-associated nephropathy, cytomegalovirus disease, neurotoxicity, bone marrow depression, hypertension or malignancies in the first three months after transplantation. Two out of five tofacitinib treated patients suffered from myopathy and discontinued study medication. In the same treatment arm one patient discontinued study medication due to anemia. Two of six patients on CNI-based immunosuppression without basiliximab induction therapy, developed new-onset diabetes mellitus. One patient suffered from an urinary and wound infection and another from a bleeding. There were two urinary infections and one case of systemic infection with hospitalization. In the CNI/basiliximab treatment arm, one of four patients suffered from pulmonary embolism, one patient had a bleeding and another had urinary retention.

Two tofacitinib-treated patients and one patient on CNI-based immunosuppression without basiliximab had an acute allograft rejection episode, Banff cellular grade II.

Immune Cell Counts of Patients treated with Tofacitinib

One month after kidney transplantation, the absolute numbers of CD3-CD19+ B cells, CD3-CD16+CD56+ NK cells and CD3+ T cells were measured. In tofacitinib/basiliximab-treated patients, the number of NK cells decreased from 166 cells/uL blood (interquartile range, 137 – 235 cells/µL) to 38 cells/uL (interquartile range, 26 – 53 cells/µL; $p<0.01$), which is in line with previous findings of the phase I study (*2*). In the CNI/basiliximab-treated patients and in the control CNI-treated patients the number of NK cells was not decreased. In all three treatment arms, the numbers of CD19+ B cells and CD3+ T cells were not significantly influenced by the immunosuppressive medication ($p>0.05$).

Inhibition of Cytokine Signaling in Whole Blood of Healthy Volunteers: In Vitro

Measurement of STAT5 phosphorylation by PFC showed that whole blood activation with IL-2, IL-7, and IL-15 increased STAT5 phosphorylation in both CD4+ and CD8+ T cells. Reproducibility of the assay was tested with a coefficient of variation 6.1% or less for both stimulated and unstimulated samples and a Z'-factor of 0.8 (Fig.1a). The latter is a measure for the robustness of high-

throughput assays with a Z'-factor between 0.5 and 1 indicative for a reproducible assay.

This assay was used to measure the in vitro sensitivity of STAT5 phoshorylation for tofacitinib. Figure 1b shows a representative example of cytokine-induced STAT5-phosphorylation in CD3+ T cells, in the absence and presence of tofacitinib (100 ng/mL). Cytokine-induced STAT5 phosphorylation was inhibited by tofacitinib in CD4+ and CD8+ T cells, in a dose dependant manner (Fig. 1c). The IC_{50} in IL-2-activated CD4+ T cells was significantly lower than in CD8+ T cells; 26 ng/mL vs 35 ng/mL ($p<0.05$, table 1). The IC_{50} after IL-7 activation was not significantly different between CD4+ T cells and CD8+ T cells: 72 ng/ml for CD4+ T cells and 61 ng/mL for CD8+ T cells. For IL-15 activation the IC_{50} was 37 ng/ml in CD4+ T cells and 76 ng/mL in CD8+ T cells and again CD4+ T cells were more sensitive to STAT5 inhibition ($p < 0.01$, table 1). These data also showed a differential inhibitory effect of tofacitinib on cytokine-induced P-STAT5 between the three cytokines. P-STAT5 was more profoundly inhibited by tofacitinib in IL-2-activated cells than in IL-7 or IL-15-activated cells. The IC_{50} in IL-2-activated CD4+ T cells was 63% lower than in IL-7-activated CD4+ T cells ($p<0.01$) and 29% lower than in IL-15-activated CD4+ T cells ($p < 0.01$, table 1). The same differences in the inhibitory effect of tofacitinib on IL-2, IL-7 and IL-15-activated P-STAT5 were also significant in CD8+ T cells ($p<0.01$).

Table 1: Tofacitinib IC_{50} of cytokine-induced P-STAT5

IC_{50} mean (95% CI), ng/mL	**CD4+ T cells**	**CD8+ T cells**	***P***
IL-2	26 (23 - 30)	35 (31 - 39)	**<0.05**
IL-7	72 (62 - 84)	61 (52 - 70)	**N.S.**
IL-15	37 (33 - 41)	76 (67 - 82)	**<0.01**

Each IC50 value represents the mean of 8 healthy subjects. The P value is for the difference in IC50 of CD4+ compared with CD8+ T cells. CI, confidence interval; IL, interleukin; NS, not significant.

STAT5 Phosphorylation in Whole Blood T Cell Subsets of Kidney Transplant Recipients

The cellular drug sensitivity and efficacy of tofacitinib, basiliximab, and CNI was studied by analysis of cytokine-activated P-STAT5 in whole blood samples of kidney transplant patients. Figure 2 illustrates the flow cytometric analysis of the IgG-control, unstimulated samples and cytokine-stimulated samples, for one patient per treatment arm before and 7 days after transplantation. The unstimulated and cytokine-stimulated samples were stained with PE-conjugated P-STAT5 monoclonal antibody. In tofacitinib/basiliximab-treated patients, the IL-2, IL-7 and IL-15-induced STAT5 phosphorylation, MFI of cytokine-stimulated sample minus unstimulated sample, was significantly inhibited in both CD4+ and CD8+ T cells at day 7 compared with before transplantation (Fig. 3). An example of P-STAT5 kinetics in a tofacitinib/basiliximab-treated patient is shown in figure 4. Due to anaemia, this patient had to discontinue tofacitinib treatment on day 62 and as a result the induced STAT-5 phosphorylation recovered to pre-transplantation levels.

Analysis of the transplant patients per group showed that in the CNI (tacrolimus)-treated control patients, IL-2 [15], IL-7 and IL-15-activated P-STAT5 was not inhibited (Fig. 3). In CNI (CsA)/basiliximab-treated patients, IL-2-activated P-STAT5 in CD4+ T cells was inhibited on day 7 by 77 % vs. pre-transplantation and by 52% in CD8+ T cells ($p<0.05$), and gradually recovered to pretreatment levels 2 months after transplantation. No effect of this immuno-suppressive regimen was found on IL-7-activated and IL-15-activated P-STAT5. In tofacitinib/basiliximab-treated patients, the IL-2, IL-7 and IL-15-induced STAT5 activation was strongly inhibited and persisted for the 2-month study period (Fig. 3). On day 7 the IL-2-activated P-STAT5 in CD4+ T cells was decreased by 92 % ($p<0.01$ vs pre-transplantation), by 60 % ($p<0.01$) for IL-7-activation, and by 75 % ($p<0.01$) for IL-15-activation. The same profile was found for CD8+ T cells: 83%, 61% and 68% for IL-2, IL-7 and IL-15, respectively (all $p\leq0.05$). In the third month after transplantation three out of five tofacitinib/basiliximab-treated patients discontinued study medication due to complications (see clinical results). With just two patients left in this treatment arm the P-STAT5 analysis was stopped.

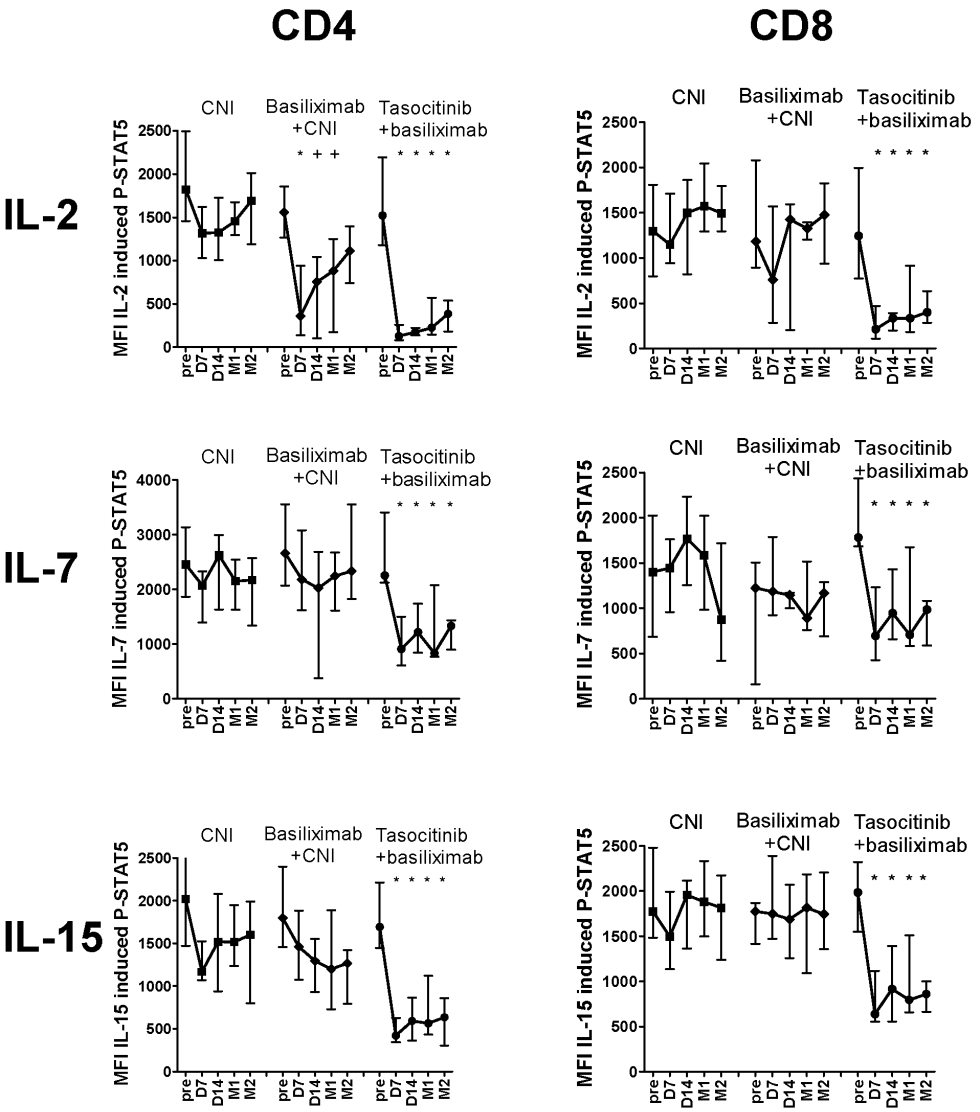


Figure 3: STAT-5 monitoring of kidney transplant patients in a tofacitinib phase II study: first 2 month.
Control patients treated with calcineurin inhibitor (CNI; tacrolimus), CNI (cyclosporine A)/basiliximab treated patients and tofacitinib/basiliximab treated patients are shown. Basiliximab induction therapy was given at day 0 and 4 after transplantation. Each point represents the cytokine-activated phosphorylated STAT (P-STAT) 5: Median fluorescence intensity (MFI) of cytokine stimulated P-STAT5 minus MFI of the unstimulated sample (median + interquartile range) (* $p<0.05$ and + $p<0.06$).

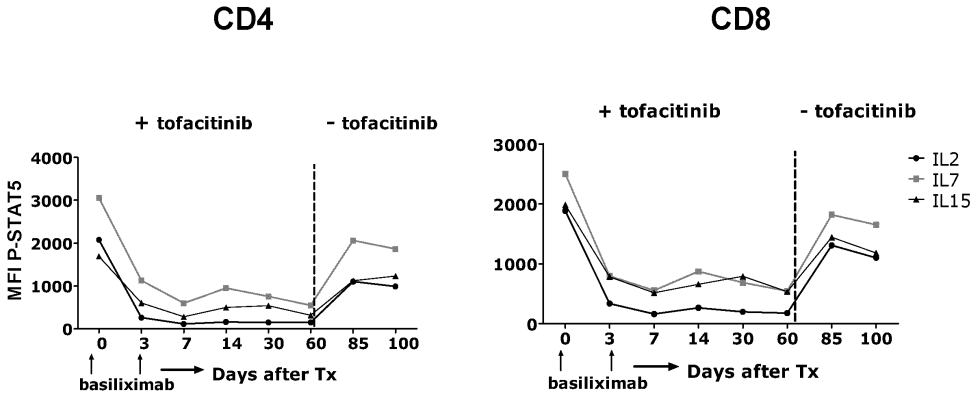


Figure 4: Tofacitinib withdrawal and cytokine-activated STAT5 phosphorylation. STAT5 phosphorylation after whole blood activation with IL-2, L-7 and IL-15 was inhibited in this patient on tofacitinib/basiliximab therapy and immediately recovered when the tofacitinib treatment was stopped due to an adverse event (anemia) on day 64. Cytokine-activated STAT5 phosphorylation is plotted on the y-axis as the median fluorescence intensity (MFI) of cytokine stimulated phosphorylated STAT (P-STAT) 5 minus the MFI of the unstimulated sample.

For the first two month after transplantation the tofacitinib blood concentrations were compared to the cytokine-induced P-STAT5 levels (Fig. 5). In CD4+ T cells tofacitinib levels did not significantly correlate with induced P-STAT5 ($p>0.05$), although IL-7-induced P-STAT5 was almost significantly inversely correlated to tofacitinib levels ($p=0.06$). In the CD8+ T cells there was a correlation between tofacitinib levels and both IL-7-activated and IL-15-activated P-STAT5 ($p<0.05$).

Discussion

Because of inter-individual variability in the pharmacokinetics of and response to immunosuppressive drugs, dose optimization for individual patients is often difficult to achieve. Pharmacodynamic monitoring by whole blood phosphospecific flow cytometry quantitatively measures the cellular response to an immunosuppressive agent and can give a better reflection of the patient-specific drug sensitivity [15]. In the current study, this novel technique was used to monitor the direct effect of tofacitinib and basiliximab on the γ_c cytokines-activated JAK-STAT signaling pathway in vitro and in kidney transplant patients.

In patients, tofacitinib inhibited STAT5 phosphorylation induced by γ_c cytokines (i.e., IL-2, IL-7, and IL-15), whereas basiliximab specifically suppressed IL-2-induced STAT5 activation. Both in vitro and in vivo data show the potency and selectivity of tofacitinib and basiliximab. Within CD4+ or CD8+ T cell subsets, the potency of tofacitinib for inhibiting IL-2 or IL-7/IL-15-activated STAT5 was significantly different. This difference might be the result of variations in cytokine receptor expression and binding affinity, which together with differences in JAK/STAT utilization may influence tofacitinib potency and selectivity. A distinctive feature of IL-7 for example compared to IL-2 and IL-15 relates to receptor expression. The IL-2R and IL-15R are upregulated after T cell receptor activation, whereas the IL-7R is expressed by resting T cells and downregulated after T cell receptor activation [16]. Further examination of the mechanisms behind differential tofacitinib sensitivity of IL-2 vs IL-7/IL-15 dependent STAT5 phosphorylation in CD4+ and CD8+ T cells is needed.

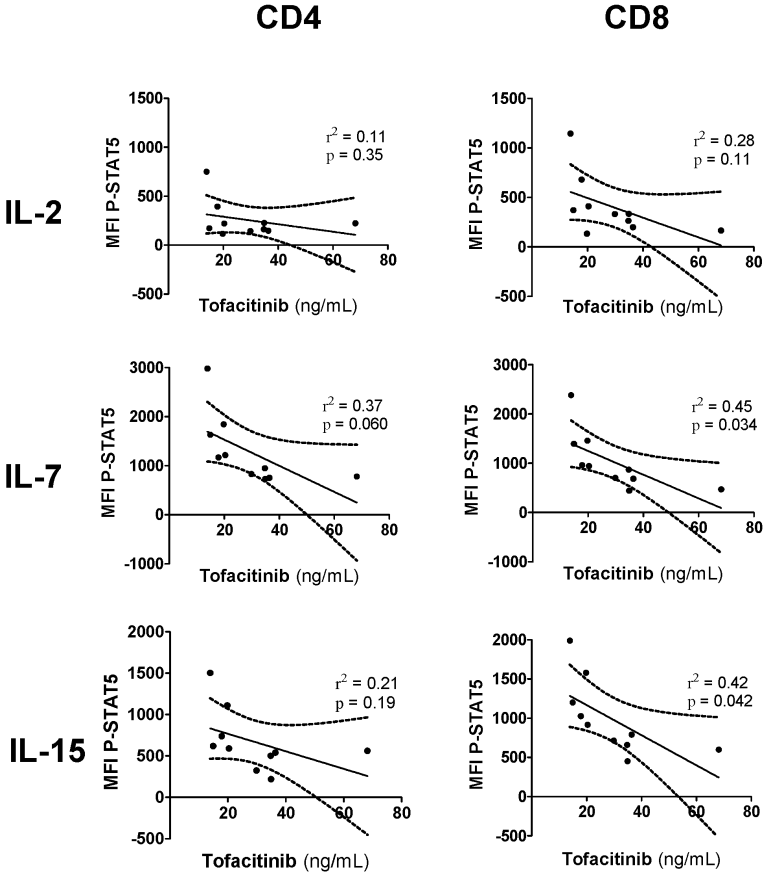


Figure 5: Correlation between tofacitinib levels and STAT-5 phosphorylation in kidney transplant patients.
In the first two month after transplantation, for the five tofacitinib-treated kidney transplant patients the P-STAT5 MFI for CD4+ and CD8+ T cells are plotted against the tofacitinib blood concentrations. P-STAT5 MFI and drug concentrations were both measured pre-dosing of tofacitinib.

In whole blood samples from our tofacitinib/basiliximab-treated kidney transplant patients, a strong inhibition of IL-2 and, to a lesser extent of IL-7-induced and IL-15-induced STAT5-activation in CD4+ and CD8+ T cells was found. The IL-7-activated and IL-15-activated P-STAT5 in CD8+ T cells and the IL-7-activated P-STAT5 in CD4+ T cells correlated with tofacitinib trough levels (Fig. 5).

In CNI/basiliximab-treated patients, IL-2-activated P-STAT5 was inhibited specifically with no effect on IL-7 and IL-15-activated P-STAT5. The broad inhibitory effect of tofacitinib on signal transduction, mediated by multiple γ_c cytokines, together with the persistence of such inhibition over the entire duration of tofacitinib treatment may explain the higher incidence of viral infections and malignancies compared to the CNI (CsA)/basiliximab arm of the phase II study [12-13] (Fig. 4). Immunosuppressive drugs are known to inhibit their therapeutic target less at trough levels than at peak levels [17-19]. However in tofacitinib-treated patients, inhibition of IL-2-activated STAT5 was almost complete even at trough concentrations. This also explains why no correlation was found between tofacitinib levels and IL-2-activated P-STAT5 in CD4+ and CD8+ T cells and IL-15-activated P-STAT5 in CD4+ T cells (Fig. 5), which in our in vitro experiments were the most sensitive to inhibition by tofacitinib (table 1). These observations suggest that the tofacitinib dosage regimen needs modification.

The observed adverse effects in some of the tofacitinib/basiliximab-treated patients might be the result of a combination of impaired T cell function and reduced NK cell numbers, which are consequences of blocked IL-2, IL-7, and IL-15 signaling pathways. Previously and in this study, we found a reduction of more than 70% on NK cell numbers in tofacitinib/basiliximab-treated patients [2]. IL-15 is an important growth and survival factor for NK-cells and memory T cells [16]. In IL-15 knock out (KO) and in IL-15R α-chain-deficient mice, diminished numbers of NK cells and memory phenotype CD8+ T-cells were seen. In addition, immune regulation by FOXP3 T cells seems less susceptible to tofacitinib and may suppress the effector T cell function in these patients [20]. Lower doses of tofacitinib might overcome the problems of overdosing without decreasing efficacy. For that purpose, PFC may provide a helpful tool to

determine patient-specific drug efficacy at the single cell level. Inhibition of STAT5-activation in the tofacitinib/basiliximab patient group was reversible after discontinuation (Fig. 3), which suggests whole blood PFC may also be useful for monitoring patient compliance. The results of the current study are preliminary due to the small number of patients and more elaborate studies are needed to assess the usefulness of this assay for predicting clinical parameters. Nonetheless, we think PFC is a suitable and robust assay for routine pharmacodynamic monitoring as it requires small amounts of whole blood, results are available within hours, the assay is sensitive, standardized [21] and has low variability (Fig. 1, 5) [22].

In conclusion, tofacitinib therapy strongly inhibits γ_c cytokine-induced JAK/STAT5 activation, while basiliximab suppresses IL-2-stimulated activation only. Pharmacodynamic monitoring by phosphospecific flow cytometry offers a unique tool to evaluate the biological effects of immunosuppressive drugs.

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Chapter 7

Summary and General Discussion

Summary

The aim of this thesis is to better understand the biological effects of immunosuppressive drugs within the quest to optimize individual treatment for the transplant recipient. The interest in the pharmacodynamic effects of drugs is increasing given the immense ongoing efforts made at transplant centers worldwide to personalize treatment, in order to increase efficacy and to minimize drug-related side effects such as cardiovascular disease, diabetes, infection and cancer [1-3].

Since the immunosuppressive drugs have a narrow therapeutic range and large inter- and intra-individual pharmacokinetic variability [4], treatment should be individualized to minimize drug exposure [4-6]. Currently routine therapeutic drug monitoring is based on measuring blood concentrations [7-8]. Nevertheless patients still suffer from rejection and toxicity [9-10]. These events occur at irregular drug concentrations as blood levels are an indirect predictor of the biological effects of immunosuppressants and do not consider that patients are genetically heterogenic and not equally sensitive to the effects of medication. The impact of genetic variation on the pharmacodynamics of immunosuppressive agents in transplant recipients is a highly interesting topic discussed further on in this section.

The technique phosphospecific flow cytometry was used in the study described in **chapter 2** to quantify MAPK immune activation and the inhibitory effect of tacrolimus, a first choice immunosuppressant after solid organ transplantation. Phosphorylation of p38 MAPK, a key intracellular signaling molecule [11-14] and upstream activator of NFAT (Fig. 1) [15-16], was measured. *In vitro* tacrolimus inhibited the p38 MAPK activation capacity in a dose dependent manner in CD4+ helper T cells and CD8+ cytotoxic T cells which mediate acute allograft rejection [17-18]. In healthy volunteers and in stable renal transplant recipients a reverse dose response was found between tacrolimus blood concentrations and p38 MAPK activation in the T cell subsets. P38 MAPK activation is inhibited by the calcineurin inhibitor tacrolimus in a dose dependent manner and therefore may be a potential marker for immune-monitoring in transplant recipients.

In **chapter 3** the effect of tacrolimus on NF-κB activation in peripheral human T cells was studied. The possibility that tacrolimus inhibits the NF-κB pathway in T cells was suggested by I. the study presented in Chapter 2 where tacrolimus affected a signaling pathway involved in NF-κB activity (Fig. 1) [19] and II. by studies which linked NF-κB activity to the calcineurin pathway in T cell lines [20]. We found a dose-dependent inhibitory effect of tacrolimus on

CD3/CD28 induced NF-κB phosphorylation in helper, cytotoxic and regulatory T cells. Downstream of NF-κB phosphorylation, the binding to target genes was also inhibited as well as the NF-κB dependent cytokine production. We here showed for the first time the suppressive effect of tacrolimus on this signaling pathway at clinically relevant concentrations in peripheral human T cell. This was proven by analysis at three specific positions in the NF-κB activation cascade which is pivotal for the development and activation of immune cells [21].

In **chapter 4** the influence of the *ABCB1* 3435C>T single-nucleotide polymorphism (SNP) on the pharmacodynamic effect of tacrolimus was studied. The ABCB1 efflux-pump is expressed by CD4+ T cells and CD8+ T cells. Our *in vitro* study showed that tacrolimus-mediated inhibition of cytokine production in T cells was enhanced by the ABCB1 blocker verapamil. In tacrolimus-treated renal transplant recipients with the 3435CC genotype, the more active ABCB1 variant, verapamil decreased IL-2 production, i.e. enhanced the biological effect of tacrolimus. In contrast, verapamil did not influence cytokine production in patients with the TT genotype. Furthermore, the ratio of tacrolimus trough levels and percentage of IL-2 producing T cells, a measure for the requirement of tacrolimus in renal transplant recipients was compared. This ratio was significantly higher in CD8+ T cells of patients who are carriers of the 3435CC genotype than in TT genotype carriers, showing that tacrolimus had a smaller effect in the renal transplant patients with the CC genotype. Hence the ABCB1 3435C>T SNP enhances ABCB1 activity of T cells and the pharmacodynamic effect of tacrolimus in kidney transplant patients.

While the previous results consider the effects of tacrolimus on signal 1 and 2 in T cells, the effects of immunosuppressive drugs on the consecutively activated signal 3 is discussed in **chapter 5 and 6**. Signal 3 is activated by cytokines and our studies focused on the IL-2 family or common-γ-chain (γ_c) cytokines which activate the JAK-STAT-pathway in lymphocytes. In chapter 5 we discussed analysis of the JAK-STAT signaling pathway by phosphospecific flow cytometry as a novel tool I. to determine the signaling responses of immune cells in response to antigen challenge, II. to unravel the signal transduction networks that drive functional cellular changes in diseased individuals, III. to perform drug screening; and IV. to monitor the specificity and efficacy of immunosuppressive agents in transplant recipients. In chapter 6, the effect of immunosuppressive drugs targeting this pathway, i.e. the JAK inhibitor tofacitinib (CP-690,550) and the anti-IL-2R-antibody basiliximab, was studied as part of a phase 2B study in renal allograft recipients. Both *in vitro* and *ex vivo* data showed the potency and selectivity of tofacitinib and basiliximab. By whole blood phosphospecific flow cytometry, we found that tofacitinib therapy

administered to renal transplant recipients strongly inhibited STAT5 phosphorylation induced by the γc receptor cytokines, IL-2, IL-7, and IL-15, while basiliximab specifically suppressed IL-2-induced STAT5 activation with no effect on IL-7-activated or IL-15-activated P-STAT5. In the CNI-treated control patients STAT5 phosphorylation was not inhibited. Hence we showed that whole blood pharmacodynamic monitoring by phosphospecific flow cytometry offers a unique tool to evaluate the biological effects of tofacitinib and basiliximab therapy.

General Discussion

Many drugs have taken up the unprofitable challenge against the currently used calcineurin inhibitors. Co-stimulation blockers, m-TOR inhibitors, B and T cell depleting monoclonal and polyclonal antibodies as well as several cellular therapies were or are being investigated [22-23]. Yet CNI are still used in the vast majority of immunosuppressive treatments after organ transplantation. This success can be attributed to the inhibition of a cluster of signaling pathways in immune competent cells, found in our studies, showing that tacrolimus inhibits the major signaling routes downstream of the T cell receptor (Fig. 1). In this context, the efficacy-toxicity profile of tacrolimus is favored by an incomplete inhibition of these activation pathways. However, the narrow therapeutic window of immunosuppressive agents puts patients at risk of rejection in case of under dosing or toxicity when overdosed. Consequently a biomarker to improve the efficacy-toxicity profile of CNI is eagerly anticipated.

Classically, pharmacodynamic monitoring of the direct effects of calcineurin inhibitors (CNI), tacrolimus and cyclosporine A, has focused on measuring calcineurin phosphatase activity [24] and NFAT regulated genes [25]. Other studied biomarkers are lymphocyte cytokine expression, lymphocyte proliferation and expression of cell surface activation antigens [9, 26-28] which measure the overall immunosuppressive load but are not drug-specific. Calcineurin activity was extensively studied in transplant recipients in the last decade [24, 29-30]. To our knowledge this biomarker is not used for pharmacodynamic monitoring due to insufficient evidence for its clinical usefulness. The partially drug-specific measurements of NFAT regulated gene expression have been studied by Sommerer et al. and could predict recurrent infections but not acute rejections [25]. Nevertheless, measurements of NFAT regulated genes has not been tested or confirmed as clinically useful by other independent study groups.

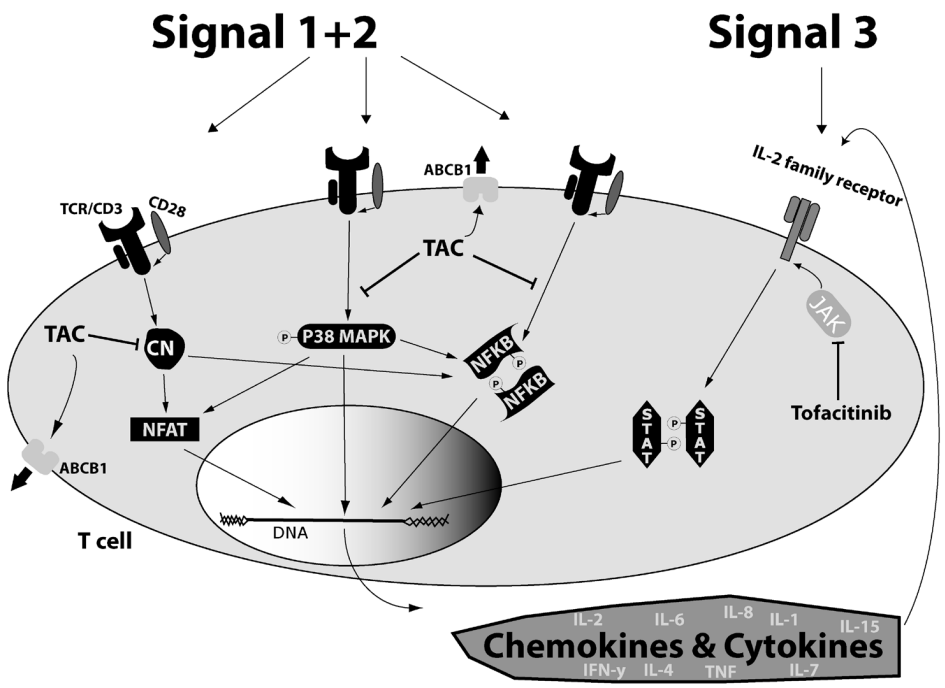

Figure 1: T cell Signaling and targets of immunosuppressive drugs studied in this thesis.
T cell receptor (TCR) activation (signal 1) in concert with CD28 activation (signal 2) provoces stimulation of the calcineurin (CN)-nuclear factor of activated T cells (NFAT) pathway, the mitogen activated protein kinase (MAPK) pathway and the nuclear factor of κB (NFκB) pathway. These pathways are highly interactive as , for example, MAPK signaling influences the CN-NFAT and NF-κB activation pathways. As described in chapter 2-4 of this thesis tacrolimus (TAC) supresses the signaling pathway downstream of the TCR and it is actively pumped out of the cell by the ABCB1 transport protein. After signal 1 and 2, a consecutive third signal is activated in T cells by cytokines and the intracellular JAK-STAT pathway. Activation of signal 3 by the IL-2 family cytokines is abrogated by the JAK-inhibitor tofacitinib as shown in chapter 6.

Therefore there is a need for new drug specific and practical methods to study the biological effects of immunosuppressive medication. We tested the potential of phosphospecific flow cytometry to measure activated signaling molecules as a tool for routine pharmacodynamic analysis. This quantitative tool is fast and provides unique information on drug effects as it measures the phosphorylation of activation-molecules at an early and drug-specific point in

the activation cascade of T lymphocytes. The Rotterdam Transplant Laboratory has been the first to publish on the use of this technique for measuring the inhibitory effects of tacrolimus on the activation of MAPK and NF-κB signaling in immune competent cells. Tacrolimus-induced NF-κB-inhibition might be exerted directly and indirectly via the calcineurin and MAPK activation pathway [19-20]. These signaling molecules are potential biomarkers for tacrolimus efficacy as they are major activation pathways in immune cells. NF-κB promotes the transcription of over 150 target genes and is pivotal for the development and activation of immune cells [21]. Furthermore, inhibition of NF-κB-activity in epidermal cells might explain the tumerigenic adverse effects of tacrolimus. Occurrence of squamous cell carcinoma (SCC) and other neoplasms are major complications of CNI treatment [1, 31-33], leading to a long-term incidence (20 years) of skin cancer of more than 50% [34]. Despite the role of NF-κB in the inhibition of apoptosis and tumorigenesis [21, 35-36], inhibition of the pathway increases sensitivity to developing SCC [37] and skin tumor formation [38-40]. Furthermore decreased IKKα expression and consequently NF-κB activity in the epidermis is associated with tumor progression in human skin and lung SCC [39, 41-42]. Other research groups have also explored the value of phosphospecific flow cytometry, for pharmacodynamic monitoring of the immunosuppressive mammalian target of rapamycin (mTOR)-inhibitors. These drugs are administered to patients who do not react satisfactory to CNI-treatment. They suppress the downstream phosphorylation of S6 ribosomal protein, which was used as the biomarker [43-44].

The only oral alternative that can challenge the tacrolimus predomination in transplantation the coming years is the JAK inhibitor tofacitinib. The clinical results of the phase 2B study showed non-inferiority in 6 month incidence of clinical acute rejection, better renal function, a lower incidence of chronic allograft nephropathy and new-onset diabetes in the tofacitinib-treated patients compared to CNI treatment, at the expense of an increased incidence of infections. The researchers of the clinical trial concluded that adjustments to the tofacitinib-dosing may permit the benefits of improved kidney function with less drug-related adverse effects [45]. Our results using phosphospecific flow cytometry suggest that this can be achieved without loss of efficacy. We found that tofacitinib trough-levels correlated with inhibition of IL-7-activated and IL-15-activated P-STAT5 levels in CD8+ T cells and IL-7-activated P-STAT5 in CD4+ T cells. In contrast, the STAT5 activities most sensitive to inhibition by tofacitinib, i.e. IL-2-induced P-STAT5 in CD4+ and CD8+ T cells and IL-15-induced P-STAT5 in CD4+ T cells, did not correlate to tofacitinib-levels, as they were abrogated even at trough concentrations. This complete

blockade of the STAT5 activation pathways by tofacitinib suggests over-exposure and explains the higher incidence of infections compared to the CNI arm of the phase 2B study [45]. The studies presented in this thesis illustrated a low intra-assay variation and robustness of the phosphospecific flow cytometry technique.

Our study on the ABCB1 efflux pump demonstrated that the 3435 C>T SNP affects the biological effect of tacrolimus in renal transplant recipients. Previous studies on this SNP have mainly focused on the association with tacrolimus pharmacokinetics, i.e. blood concentrations [46-47]. The intra-lymphocytic drug concentrations are however not well mirrored by whole blood concentrations since in blood, tacrolimus and cyclosporine are extensively distributed in erythrocytes. In plasma, more than 90% of the remaining drug is bound to proteins like 1-acid glycoprotein, albumin, globulins and lipoproteins, leaving around 1% associated with leukocytes [8, 48]. Interestingly, tacrolimus trough-concentrations do not correlate with acute rejection after liver transplantation, as found in a meta-analysis of 957 recipients [49]. In contrast, Zahir et al. showed that low intra-lymphocytic tacrolimus concentrations did correlate with acute rejection in liver transplant recipients while whole blood concentrations did not [48]. Similarly, in renal transplant recipients experiencing rejection the intracellular cyclosporine AUC 0-12 measured in T cells during the stable phase was lower compared to rejection-free patients and intra-T-cellular concentrations were lower starting three days before the rejection episodes were clinically recognized [50]. Together these data implicate the clinical relevance of our study in which T cells of renal transplant patients with the *ABCB1* 3435 TT phenotype were more susceptible to the drug, due to a lower pump activity that affects the intracellular drug concentrations [51].

Future Perspectives

Phosphospecific flow cytometry is a novel tool for routine pharmacodynamic monitoring offering a sensitive method for the detection of phosphorylated and active signaling molecules in immune cells by the complex multicolor analysis of whole blood samples [52]. Thus the critical cell subsets involved in allo-immune responses can be analyzed in the presence of therapeutic drug-concentrations. Such a biomarker added to the currently used pharmacokinetic monitoring methods needs to be clinically validated by independent research and fulfill the following requirements: reproducible, easy to perform, standardized, cost effective with results easy to interpret. The biomarkers introduced in this thesis meet most of these requirements while further standardization is needed by

studies which should investigate the variables that can influence the assay, such as sample collection and storage conditions.

To confirm the clinical usefulness, individual *ex vivo* susceptibility of p38 MAPK activation to tacrolimus should be evaluated prior to transplantation in a prospective study and correlated to the incidence of rejection and drug-related adverse effects. Measurements should be performed as in our study in healthy volunteers, where the biomarker was analyzed in T cells prior to and two hours after oral intake of a single tacrolimus dose. For the STAT5-biomarker that we used to measure the immunosuppressive effects of tofacitinib future studies should validate whether the level of inhibited STAT5 phospohrylation correlates with drug toxicity in the first months after transplantation.

The results of the tacrolimus studies show that this agent has broad specificity. Apart from calcineurin also the activation of MAPK and NF-κB are, at least in part, inhibited by this immunosuppressive agent (Fig. 1). These findings may explain both the efficacy and the toxicity profile of tacrolimus. This knowledge is also essential for paving the way towards more targeted therapy. Furthermore, availability of a monoclonal antibody for the detection of NF-κB phosphorylation by whole blood phosphospecific flow cytometry will permit the simultaneous pharmacodynamic analysis of this signaling molecule and p38 MAPK in patient lymphocytes, without drug-diluting PBMC isolation steps.

Whether the clinical impact of the *ABCB1* 3435 C>T SNP which influences tacrolimus pharmacodynamics is large enough to adjust CNI-dosing is an interesting question [53]. In a recently published study, patients treated with tacrolimus for inflammatory bowel disease, had a strikingly higher success rate if they were homozygous for one of the three *ABCB1* alleles 1236T, 2677T, and 3435T, while the highest odds ratio was found for the 3435C>T SNP [54]. Regarding CNI-related side effects, in a study involving 147 CNI-treated renal transplant recipients, carriers of the T allelic variant had significantly higher incidences of new-onset diabetes and cytomegalovirus reactivation compared with carriers of the wild-type genotype [53]. Summarizing our and these data, restrictive tacrolimus dosing in the first months after renal transplantation in homozygous TT patients should be investigated in a prospective study as a mean to decrease the incidence of adverse effects and to improve renal function.

In conclusion, the *ABCB1* 3435 C>T SNP enhances the biological effect of tacrolimus in renal transplant recipients. Phosphospecific flow cytometry provides a rapid and sensitive tool for monitoring the biological effects of immunosuppressive medication used after organ transplantation, such as tofacitinib and tacrolimus. Furthermore it offers a unique tool to explore the

mechanism of action, efficacy and specificity of immunosuppressive agents in immune cell subsets key in allo-reactivity.

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Chapter 8

Nederlandse Samenvatting

Samenvatting

Een persoon met een falend orgaan door ziekte of een aangeboren afwijking komt in aanmerking voor orgaantransplantatie. Hierbij wordt het orgaan van een donor overgeplaatst naar een ontvanger, de patiënt. Tenzij het afkomstig is van een eeneiïge tweeling zal het orgaan door het afweersysteem van de ontvanger als lichaamsvreemd worden aangevallen en verstoten. Derhalve is een goede onderdrukking van de afweerreactie tegen het ontvangen, lichaamsvreemde orgaan van groot belang. Om afstoting te voorkomen wordt de patiënt behandeld met meerdere medicijnen die het afweer- of immuunsysteem onderdrukken.

Deze medicijnen dienen het immuunsysteem zo te beïnvloeden dat een afstoting wordt onderdrukt en tegelijkertijd een normale afweer tegen schimmels, bacteriën en virussen intact blijft. De balans tussen werkzaamheid en schadelijkheid luistert dus heel nauw, mede doordat deze medicijnen bij een groot deel van de patiënten ernstige bijwerkingen veroorzaken zoals hart- en vaatziekten, suikerziekte, maligniteiten en nierfalen. In dit proefschrift zijn de biologische effecten van afweeronderdrukkende medicijnen op het immuunsysteem onderzocht met als doel een veiligere medicamenteuze behandeling voor orgaanontvangers mogelijk te maken.

Om de blootstelling van patiënten aan afweerremmende medicijnen te minimaliseren wordt gebruik gemaakt van een cocktail van medicijnen die ieder op een specifieke manier werkzaam zijn. Hierdoor kan de dosering relatief laag worden gehouden. De meeste patiënten krijgen direct vóór en in de dagen na een niertransplantatie inductietherapie met Basiliximab voor een snelle sterke afweeronderdrukking. Basiliximab is een medicijn die de signalen naar afweercellen, de zogenaamde witte bloedcellen, remt. Dit betreft het signaal dat door een ontstekingseiwit, de cytokine interleukine-2 (IL-2), wordt afgegeven. Als onderhoudstherapie na de transplantatie worden voor zolang nodig een corticosteroïd, mycofenolzuur en een calcineurineremmer tegelijk gegeven. Het corticosteroïd wordt na een aantal maanden gestopt en de andere twee medicijnen worden levenslang gebruikt. Mycofenolzuur remt de deling van afweercellen. Corticosteroïden zoals prednisolon en calcineurineremmers hebben een remmende werking op het functioneren van afweercellen. Er worden twee calcineurineremmers als medicijn toegepast bij orgaanontvangers: tacrolimus en cyclosporine. Hierbij wordt bij een overgrote meerderheid van de transplantatiecentra voor tacrolimus gekozen. Vooral deze na transplantatie onmisbare calcineurineremmers hebben een erg nauwe marge tussen werkzaamheid en schadelijkheid. Om bijwerkingen te voorkomen zouden we het liefst een zo laag mogelijke dosis van deze medicijnen aan patiënten geven.

Het is echter niet mogelijk om de dosering zomaar bij alle patiënten te verlagen omdat patiënten een hoog risico op afstoting hebben. Bij de nieren bijvoorbeeld is de vijf-jaars-overlevingskans van het overgeplaatste orgaan rond de 70% en na 10 jaar is dit 50%. Deze percentages zijn bereikt door de dosering van calcineurineremmers op individueel niveau aan te passen en daarmee de kans op bijwerkingen, door een te hoge dosis, en afstotingsreacties, door een te lage dosis, te verkleinen. Als patiënt A en B dezelfde hoeveelheid tabletten calcineurineremmer krijgen, is het niet ongebruikelijk als patiënt A wordt blootgesteld aan tien maal hogere concentraties van het medicijn in zijn lichaam. Dit wordt veroorzaakt doordat tacrolimus en cyclosporine grote individuele verschillen in opname en afscheiding vertonen. Daarom wordt de medicijnconcentratie in het bloed van transplantatiepatiënten regelmatig gemeten. Deze bloedwaardes zijn een maat voor de blootstelling van de patiënt aan de afweerremmende medicijnen en worden binnen bepaalde waardes gehouden door de arts. Dit geschiedt door de hoeveelheid in te nemen tabletten bij iedere patiënt aan te passen aan de hand van de bloedwaardes.

Ondanks deze maatregelen treedt bij een groep patiënten nog steeds afstotingsreacties op en krijgt een groot deel van de patiënten te maken met ernstige bijwerkingen. Dit komt doordat er geen rekening wordt gehouden met de grote individuele verschillen in gevoeligheid voor het geneesmiddel. Met andere woorden, niet iedere persoon reageert hetzelfde op dezelfde hoeveelheid medicijnen. Voor een verdere verbetering van de therapie is het derhalve wenselijk om de gevoeligheid van patiënten voor de afweer-onderdrukkende medicijnen te kunnen bepalen. Dit kan door middel van farmacogenetica en door het meten van de biologische effecten van medicijnen op cellulair niveau. Farmacogenetica maakt aan de hand van het genetisch profiel van een persoon een inschatting van de opname en uitscheiding van en de gevoeligheid voor de medicijnen. De biologische effecten of werkzaamheid van medicijnen kunnen gemeten worden door het meten van enzymactiviteit, ontstekingseiwitten die de geactiveerde afweercellen uitscheiden of tot expressie brengen en door te kijken naar celdeling. Men heeft in het verleden studies uitgevoerd waarbij de activiteit van het enzym calcineurine in afweercellen is gemeten als specifieke maat voor de gevoeligheid voor calcineurineremmers. Helaas is in patiënten echter geen duidelijk verband tussen deze enzymactiviteit en werkzaamheid of toxiciteit aangetoond.

In **hoofdstuk 2** wordt een geheel nieuwe methode gebruikt om het effect van tacrolimus op een belangrijk intracellulair signaalmolecuul in T cellen te meten. Deze cellen zijn een subpopulatie afweercellen die belangrijk zijn bij afstoting van het getransplanteerde orgaan doordat ze de lichaamsvreemde

antigenen (human leukocyte antigen) van het nieuwe orgaan (transplantaat) herkennen. Het signaalmolecuul dat we hebben gemeten in de T cellen was p38 mitogen activated protein kinase (MAPK). Dit molecuul is in ongeactiveerde toestand aanwezig en wordt na activatie gefosforyleerd: het krijgt een extra fosformolecuul waardoor het actief wordt. Na fosforylatie zet p38 MAPK de T cel aan tot celdeling en het maken van cytokines en oppervlakte-eiwitten waarmee ze in stelling komen om het lichaamsvreemde materiaal aan te vallen. Met de methode fosfospecifieke flowcytometrie op volbloed, kan de hoeveelheid gefosforyleerd p38 MAPK in T cellen gekwantificeerd worden. Dit is een praktische en snelle methode die in de toekomst gemakkelijk voor patiënten toepasbaar is. Tacrolimus remde in reageerbuisexperimenten (*in vitro*) de p38 MAPK activiteit in zowel de CD4+ helper T cel- als in de CD8+ killer T celpopulatie, en wel dosisafhankelijk. Dat laatste wil zeggen, hoe meer tacrolimus hoe sterker het signaalmolecuul was geremd. Na inname van een tablet met 10 mg tacrolimus door gezonde vrijwilligers bleek de p38 MAPK activiteit in de genoemde T celpopulaties omgekeerd evenredig met de bloedconcentraties medicijn. Ook in nierontvangers werd een omgekeerde relatie tussen de bloedconcentraties en p38 MAPK activiteit aangetoond. Hieruit volgt dat tacrolimus behalve calcineurine ook de p38 MAPK activatieroute in belangrijke afweercelpopulaties remt. Bovendien is dit signaalmolecuul dankzij de snelle en gevoelige fosfospecifieke flowcytometrie technologie een veelbelovende marker voor het routinematig meten van de gevoeligheid van patiënten voor tacrolimus. Vervolgonderzoek bij een groter aantal patiënten is nodig om te bewijzen dat de p38 MAPK activiteit een voorspellende waarde heeft voor de preventie van afstotingsreacties en tacrolimus gerelateerde bijwerkingen.

De MAPK activatieroute is gelieerd aan een ander belangrijk signaalmolecuul in afweercellen, namelijk NF-kB. Dit signaalmolecuul kan op een soortgelijke manier geactiveerd worden in T cellen als p38 MAPK. Het zet na activatie meer dan 150 genen aan tot het produceren van eiwitten die het afweersysteem in de aanvalsmodus zetten. Dat tacrolimus mogelijk de NF-kB route remt hebben enkele studies gesuggereerd in T cellijnen. Dit zijn cellen die jarenlang zijn doorgekweekt in laboratoria omdat ze gemakkelijk hanteerbaar zijn voor experimenten. In **hoofdstuk 3** hebben we onderzocht of tacrolimus ook NF-kB kan remmen in gewone T cellen afkomstig uit het bloed van vrijwilligers. Om de klinische situatie zoveel mogelijk na te bootsen is het onderzoek uitgevoerd met de lage medicijnconcentraties die ook in transplantatiepatiënten worden gemeten. We vonden dat tacrolimus de NF-kB activiteit in CD4+ T cellen, CD8+ T cellen en in regulatoïre T cellen op een

dosisafhankelijke wijze remde. Dit werd gemeten door de fosforylatiegraad van het NF-kB molecuul te bestuderen in geactiveerde T cellen. De mate waarin NF-kB genen aanzet was eveneens door tacrolimus geremd, voor ongeveer zestig procent. Na het aanzetten van genen door NF-kB worden ontstekingseiwitten geproduceerd, zoals TNFα, welke sterk geremd werd door tacrolimus. Dit onderzoek liet voor het eerst in humane bloed T cellen het effect van klinisch relevante tacrolimusconcentraties op NF-kB-activatie zien. De resultaten van hoofdstuk 2 en 3 tonen het brede werkingsmechanisme van tacrolimus op de NF-kB en MAPK activatieroutes in immuuncellen, terwijl in het algemeen wordt aangenomen dat dit afweerremmende geneesmiddel alleen de calcineurine-enzymactiviteit remt. Deze bevindingen helpen ons de werkzaamheid en toxiciteit van tacrolimus beter te verklaren en kunnen ons verder leiden naar nieuwe medicijnen met een gunstiger werkings-bijwerkingen profiel.

In **hoofdstuk 4** hebben wij in nierontvangers de invloed van een genpolymorfisme, genvariatie, op het biologisch effect van tacrolimus onderzocht. Het betreffende *ABCB1* 3435 gen is verantwoordelijk voor de expressie van het eiwit ABCB1 dat actief lichaamsvreemde stoffen en zo ook medicijnen uit cellen pompt. Eerdere studies hebben aangetoond dat dragers van de *ABCB1* 3435 CC genvariant een sterkere pompfunctie hebben dan dragers van het 3435 TT genotype. Deze pomp is actief in CD4+ T cellen en CD8+ T cellen. Dit leidde tot de onderzoeksvraag of deze genvariatie het effect van tacrolimus op het afweersysteem beïnvloedt. Onze i*n vitro* experimenten lieten zien dat na het remmen van deze pomp, tacrolimus de T cellen sterker onderdrukte in hun cytokineproductie. In de T cellen van nierontvangers die drager zijn van het *ABCB1* 3435CC genotype kon de pompfunctie worden geremd en daarmee het biologisch effect van tacrolimus op T cellen verhoogd. Dit was echter niet het geval bij de dragers van het 3435 TT genotype. Bovendien hadden de transplantaatontvangers met het 3435CC genotype gemiddeld een hogere tacrolimus bloedconcentratie nodig om een zelfde remming van hun afweercellen te krijgen, vergeleken met dragers van het 3435TT genotype. Dit onderzoek liet zien dat het *ABCB1* 3435C>T genpolymorfisme de gevoeligheid van afweercellen van patiënten voor tacrolimus beïnvloedt.

In hoofdstuk 5 en 6 zijn de biologische effecten bestudeerd van een veelbelovend nieuw afweerremmend geneesmiddel, tofacitinib. Dit middel bevindt zich momenteel in de laatste klinische onderzoeksfase en is reeds bij grote groepen patiënten getest op veiligheid en werkzaamheid. Tofacitinib is een janus kinase (JAK) remmer. De JAK-STAT signaal-transductieroute wordt in T cellen aangezet nádat de MAPK en NF-kB moleculen zijn geactiveerd. Deze twee

laatste moleculen zetten de cel aan tot het produceren van onder andere de IL-2 familie-cytokines welke de JAK-STAT route activeren. In **hoofdstuk 5** wordt dit mechanisme beschreven en de mogelijkheden om de JAK-STAT route in afweercellen te analyseren met behulp van fosfospecifieke flowcytometrie. Met deze methode kan de activiteit van STAT moleculen in meerdere subpopulaties cellen worden gemeten en het biedt de mogelijkheid om het ontwikkelproces dat afweercellen doormaken tijdens en na activatie van het afweersysteem te doorgronden. Dit geeft ons tevens inzicht in het ontstaansproces van cellulaire veranderingen die tot ziektes leiden. Fosfospecifieke flowcytometrie kan ook worden aangewend om het werkingsmechanisme, de specificiteit en de effectiviteit van medicijnen te bestuderen, zoals we in **hoofdstuk 6** lieten zien. Hier werd het effect van tofacitinib op de JAK-STAT activatieroute gemeten bij nierontvangers van de recente klinische fase 2B onderzoek. Door het toepassen van fosfospecifieke flowcytometrie op volbloed werden nauwkeurig de effecten van tofacitinib in kaart gebracht. Hiervoor werd het bloed van de patiënten gestimuleerd met een drietal afzonderlijke IL-2 famillie-cytokines, IL-2, IL-7 en IL-15, waarna de STAT5 fosforylatie werd gemeten. In T cellen van patiënten die met tofacitinib en basiliximab werden behandeld was de STAT5-fosforylatie geremd na stimulatie met alledrie de cytokines. In patiënten die behandeld werden met de IL-2 receptor blokker basiliximab zonder tofacitinib, was enkel de STAT5-fosforylatie t.g.v. stimulatie met IL-2 geremd. In patiënten die noch met basiliximab noch met tofacitinib maar met een calcineurineremmer werden behandeld was de STAT5 fosforylatie in alle gevallen intact. Bij de tofacitinib-behandelde patiënten was de STAT5-activatie zelfs net vóór het tweemaaldaagse innamemoment van de medicijn, wanneer de bloed-concentraties laag zijn, sterk geremd. Dit was een aanwijzing voor een te sterke remming van het afweersysteem en werd bevestigd door de klinische resultaten van het fase 2B onderzoek waaruit bleek dat deze patiënten een verhoogde kans hadden op infecties vergeleken met patiënten die calcineurineremmers kregen. Hetzelfde onderzoek liet een lagere kans op suikerziekte en nierschade zien bij patiënten die tofacitinib kregen, waardoor een vervolgstudie met lagere doses van dit medicijn geoorloofd is. Ons onderzoek liet zien dat volbloed fosfospecifieke flowcytometrie een unieke, nieuwe, sensitieve en gevoelige methode is waarmee de specificiteit en de biologische effecten van afweeronderdrukkende medicijnen te meten zijn door actieve intracellulaire signaalmoleculen te meten.

De studies beschreven in dit proefschrift laten zien dat orgaanontvangers met het *ABCB1* 3435 CC genotype minder gevoelig zijn voor de biologische effecten van tacrolimus. Daarnaast is het met de snelle en

gevoelige fosfospecifieke flowcytometrie techniek mogelijk om de biologische effecten van tacrolimus en tofacitinib in orgaanontvangers te kwantificeren. Het voorspellen van de individuele gevoeligheid van patiënten voor de werkzaamheid en toxiciteit van afweerremmende medicijnen met behulp van deze techniek zou de behandeling aanmerkelijk verbeteren.

Appendix

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PhD Portfolio

Abbreviations

Dankwoord

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Martin Hoogduijn en Nicolle Litjens, jullie hadden altijd tijd voor mij. We hebben vaak een goed gesprek gehad, over privé-zaken en over mijn data. Meermaals hebben jullie mij van goed advies voorzien over de te volgen strategie. Ik hoop dat jullie nog lang aan het laboratorium verbonden mogen blijven en vele subsidies binnenhalen.

De collega's verbonden aan het transplantatielaboratorium, **Anja, Andre, Anne, Annemiek, Elly, Fabian, Fane, Frieda, Iris, Jan, Joke, Karin, Marjolein, Marcia, Marieke, Mariska, Martijn, Nelly, Nicole, Monique, Perikles, Ronella, Ruben, Ruud, Saida, Sander, Stefan, Thea, Varsha en Wenda,** zijn stuk voor stuk mooie mensen met wie ik met veel plezier heb samengewerkt. Mocht ik iemand vergeten zijn te vermelden, dan is dat per abuis gebeurd. Ik heb de afgelopen jaren met plezier samengewerkt met iedereen in het Erasmus MC. Daar horen ook de Collega's van de D-vleugel bij.

Professor van Gelder, Rachida en Dennis, bedankt voor de fijne samenwerking in het ABCB1-project en de nuttige wetenschappelijke discussies.

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Adriaan Houtsmuller, jou wil ik bedanken omdat je veel tijd en moeite hebt geïnvesteerd in het mij leren omgaan met de confocaal-microscoop. Het is jammer dat we niet de tijd en mogelijkheid hebben gevonden om deze techniek toe te passen in mijn promotieonderzoek**.**

Professor Randall Morris, it is a great pleasure and honor to have you in my promotion committee.

Mijn para-nimfen zijn **Martijn Demmers** en **Marcel.la Franquesa**. Martijn, je bent altijd in voor een praatje, wat belangrijk was voor mij als afleiding van de vele uren schrijven en om nieuwe ideeën op te doen voor experimenten. Marcel.la, you have left your home for a new challenge in the Netherlands and I think you have made it a huge success. At the same time the Rotterdam transplantation laboratory has gained a very nice person. Ik ben blij dat jullie mij bij willen staan tijdens de promotieplechtigheid.

Luuk, jij hebt mij precies op tijd gewezen op het stellen van mijn prioriteiten. Ongeveer halverwege het promotietraject zei je dat het schrijven van de artikelen het belangrijkste is voor het afronden van de promotie. Dankzij onze gesprekken, heb ik op tijd nee kunnen zeggen tegen de drang om steeds weer nieuwe experimenten te doen en mij geconcentreerd op het schrijven en afronden van dit proefschrift.

Hormuz, toen ik naar Rotterdam verhuisde in 2008 had ik nog geen eigen huis. Jij bood mij niet alleen onderdak aan, maar ook een dierbare vriendschap. Je sliep zelfs op de bank zodat ik op jouw bed kon slapen. Gelukkig vond ik snel een eigen onderkomen. Bedankt voor alles.

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Mitra, jou heb ik tijdens mijn promotie-periode leren kennen. Gods wegen zijn ondoorgrondelijk en voor mij is het een wonder dat zo een sterke intelligente vrouw mijn leven is binnengestapt. Meermaals heb je mij moeten overhalen door te gaan met de promotie en het af te maken. **Shirwan**, jou wil ik bedanken omdat je papa iedere dag blij maakt met je brede glimlach.

Curriculum Vitae

Ramin Vafadari werd op 26 augustus 1978 geboren in de havenplaats Bandar Abbas te Iran. Op achtjarige leeftijd verhuisde hij naar Nederland waar hij in 1996 zijn VWO diploma behaalde aan het Corderius College in Amersfoort. Na zijn VWO begon hij met de studie farmacie aan de Universiteit Utrecht. In 2001 behaalde hij het doctoraal examen waarvoor een onderzoeksproject is voltooid aan *The University of Florida* op het onderwerp inhalatie-corticosteroïden. Na een stage in het Universitair Medisch Centrum te Utrecht legde hij zijn apothekersexamen af in 2003. Gedurende zijn studieperiode was hij werkzaam als apotheekmedewerker in de Nachtapotheek Overkapel te Utrecht. Na het behalen van het apothekersdiploma heeft hij gewerkt als beherend apotheker voor de Etos Apotheken te Den Bosch en als registratiemanager voor Boehringer Ingelheim in Alkmaar. In 2008 begon hij aan zijn promotie-onderzoek in het transplantatie-laboratorium van de afdeling Interne Geneeskunde in het Erasmus Universitair Medisch Centrum te Rotterdam. Onder begeleiding van Dr. Carla Baan en Prof. dr. Willem Weimar onderzocht hij de biologische effecten van immunosuppressieve medicijnen voor transplantatiepatiënten. Momenteel woont hij in Plochingen, Duitsland, met zijn vrouw Mitra en zoon Shirwan.

PhD Portfolio

Name PhD student:	Ramin Vafadari
Erasmus MC Department:	Internal Medicine - Transplantation
PhD period:	September 2008 - December 2012
Promotor:	Prof.dr. W. Weimar
Co-promotor:	Dr. C.C. Baan

1. PhD training

General academic skills

2008 Basic radiation protection 5B (Erasmus MC)
2010 Biomedical English Writing and Communication (Molmed)
2010 Brain training & mind mapping (MT company BV)
2011 Photoshop and Illustrator CS5 Workshop (Molmed)

Research skills

2009 Statistics: Classical Methods for Data Analysis (Nihes)
2010 Human Genetics (Molmed)
2011 Advanced Course on Molecular Immunology (Molmed)

(Inter)national conferences and presentations

2009	Bootcongres NTV[1], Zeewolde, the Netherlands	
2009	European Society for Organ Transplantation Basic Science Meeting, Brussels, Belgium	
2010	Wetenschapsdagen Internal Medicine Erasmus MC Antwerpen, BE	poster
2010	American Transplant Congres, San Diego, US	poster
2010	Bootcongres NTV[1], Rotterdam, NL	oral
2010	International Congress of the Transplantation Society Vancouver, CA	oral
2010	International Congress of the Transplantation Society, Vancouver, CA	poster
2011	Bootcongres NTV[1], Amsterdam, NL	oral
2011	Bootcongres NTV[1], Amsterdam, NL	poster

[1] NTV Nederlandse Transplantatie Vereniging

2011	American Transplant Congress, Philadelphia, US	poster
2011	European Society for Organ Transplantation Congress Glasgow, UK	oral
2011	European Society for Organ Transplantation Congress Glasgow, UK	poster
2011	International Congress of Therapeutic Drug Monitoring, Stuttgart, DE	oral
2012	Bootcongres NTV[I], Maastricht, NL	oral
2012	International Congress of the Transplantation Society Berlin, DE	2x poster
2012	European Society for Organ Transplantation & American Society of Transplantation Joint Meeting, Nice, FR	2x poster

Seminars and workshops

2009 Clinical Review Symposium NTV, Utrecht, NL
2009 MolMed Day, Rotterdam, NL
2010 Clinical Review Symposium NTV, Utrecht, NL
2010 MolMed Day, Rotterdam, NL
2010 Post Graduate Weekend of the Transplantation Society, Vancouver, CA

2010 Browsing Genes and Genomes with Ensembl, Rotterdam, NL
2011 Clinical Review Symposium NTV, Utrecht, NL

Scientific Awards

2010 American Transplant Congress – Poster of Distinction
2010 Novartis Pharma Transplantation Advisory Board – Travel Grant
2011 Genzyme Award – Best Poster Bootcongres
2011 American Transplant Congress – Poster of Distinction
2011 Novartis Pharma Transplantation Advisory Board – Travel Grant

2. Teaching activities

2009 Keuze beroeps onderwijs – supervision of first year medical students Erasmus MC

2011 Transplantation education – supervision of second year medical students Erasmus MC

2011 International Congress of Therapeutic Drug Monitoring, Stuttgart, Germany:
Workshop on Biomarkers in Transplantation Medicine (Invited Lecture)

Abbreviations

APC	Antigen presenting cells
CNI	Calcineurin inhibitor
CsA	Cyclosporin A
ELISA	Enzyme-Linked Immunosorbent Assay
IκB	Inhibitor of κB
IFNγ	Interferon γ
IL	Interleukin
IL-2R	Interleukin-2 receptor
JAK	Janus tyrosine kinase
Iono	Ionomycin
mAb	Monoclonal antibody
MAPK	Mitogen activated protein kinase
MFI	Median fluorescence intensity
MMF	Mycophenolate mofetil
MPA	Mycophenolic acid
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NFAT	Nuclear factor of activated T cells
NK cell	Natural killer cell
PBMC	Peripheral blood mononuclear cells
PMA	Phorbol-12-myristate-13-acetate
PFC	Phosphospecific flow cytometry
SCID	Severe combined immunodeficiency
SOCS	Suppressors of cytokine signaling
STAT	Signal transducer and activator of transcription
Treg	Regulatory T cell
TYK	Tyrosine kinase
γ_c	Y-chain
TAC	Tacrolimus
TCR	T cell receptor
TDM	Therapeutic drug monitoring
TNFα	Tumor necrosis factor α