Monitoring Signal Transduction after Kidney Transplantation

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“Caput esse sapientiae, ut temetipsum noris.”

“Het begin van wijsheid is dat je jezelf kent.”

Desiderius Erasmus

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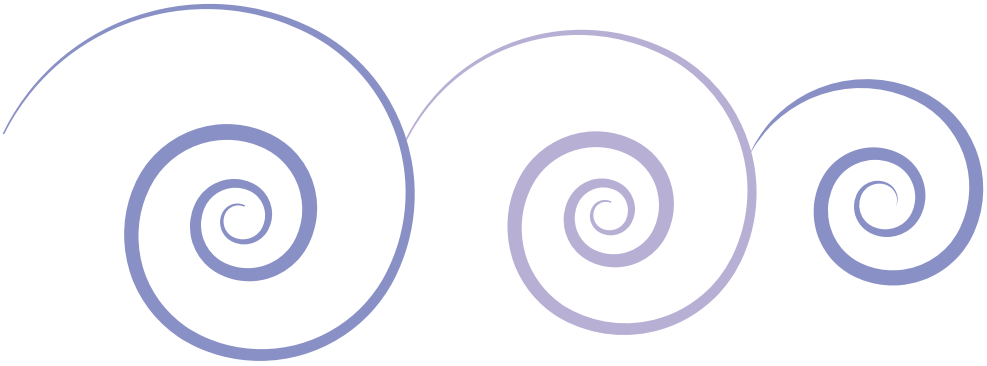

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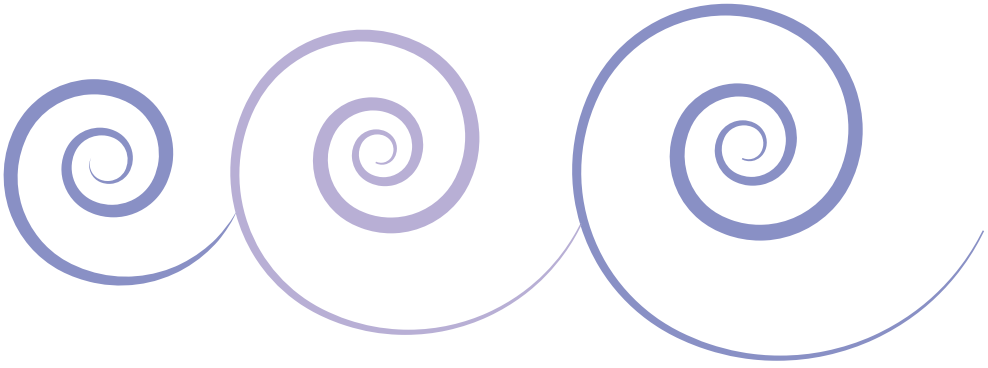
General introduction and outline of the thesis



Background

Kidney transplantation greatly improves the quality of life of patients with end-stage renal disease (ESRD) and reduces their mortality risk, even of elderly transplant recipients [1,2]. In addition, a successful kidney transplantation increases the psychosocial well-being of children. Finally, kidney transplantation is also the preferred treatment for ESRD from an economical perspective [3,4]. The short-term results of kidney transplantation have improved considerably over the last decades and 1-year graft survival (censored for death) is now 95% in most transplant centers [5]. One of the main factors responsible for the huge success of kidney transplantation has been the development of potent immunosuppressive (combination) drug therapy to prevent acute rejection.

There are several mechanisms by which kidney allografts can be rejected. In the classic model of rejection, activated cells from the innate immune system, such as dendritic cells (DCs) or macrophages, trigger the cells from the adaptive immune response, such as T and B cells. They can present donor-derived Human Leucocyte Antigen (HLA) molecules to naïve T cells of the transplant recipient. When T cells specifically recognize allo-antigens by their T cell receptor (TCR), this leads to clonal expansion and migration of these alloreactive cells from secondary lymphoid organs to the graft where they cause the classic acute T cell-mediated rejection response [6]. Three general pathways of allograft recognition exist: direct, indirect and cross-dressing [7]. In the direct pathway, donor-derived antigen presenting cells (APCs) present donor HLA molecules to T cells from the recipient. In the indirect pathway, donor-derived antigens are processed by the recipient's own APCs and presented to the cells of the adaptive immune system of the recipient. The third form of allograft recognition, cross-dressing, includes the fusion of a recipient APC with a donor-derived HLA molecule followed by activation of the T cells of the recipient.

Acute rejection can occur in the first few days after transplantation. Ischemia-reperfusion injury (IRI) is a process which augments acute rejection and is inevitable as a result of the necessary surgical procedure. Ischemic damage occurs when the blood flow in the transplanted kidney is interrupted during explantation and storage of the organ. Reperfusion of the already damaged ischemic tissue causes microvascular injury which is associated with hypoxia and generation of reactive oxygen species, due to the oxidative damage and resupply of oxygen [8]. Eventually, these processes will lead to an inflammatory response. IRI is an unavoidable consequence of transplantation and is a risk factor for acute rejection and also affects long-term graft survival [9].

The first responders to IRI-induced tissue injury are cells of the innate immune system, such as DCs, neutrophils, natural killer (NK) cells, monocytes and macrophages. These cells recognize so called damage-associated molecular patterns (DAMPS) (**Figure 1**) [10-12]. DAMPS are biomolecules released by injured cells and function as danger signals. Recognition of DAMPS leads to the activation of toll-like receptors (TLRs) on the surface of cells of the innate immune system and a full-blown inflammatory response, which is characterized by an inflammatory cell infiltrate, the production of cytokines and the activation of the

complement system. In the end, the injured cells will be monitored and removed by the innate immune system.

In addition, antibody-mediated rejection (ABMR) is now recognized as an important mechanism of allograft rejection. B cells, with help from T cells, turn into alloantibody-producing plasmablasts and plasma cells. These allo-antibodies are mostly directed against the HLA antigens of the donor (so-called donor-specific anti-HLA antibodies or DSA) but can also be directed against non-HLA antigens. Three types of ABMR are recognized clinically, ***i.e.*** hyperacute ABMR, acute ABMR and chronic ABMR. Acute ABMR is a relatively rare phenomenon but chronic ABMR is now considered to be the most important cause of late allograft loss [13]. Chronic ABMR often occurs years after transplantation and no established treatment is available for this type of rejection [6,14,15].

Without suppression of the above-described anti-donor responses, transplanted organs will reject and will ultimately fail. Fortunately, a number of immunosuppressive drugs that inhibit these responses are available. The drugs are mostly used as combination therapy in solid organ transplant (SOT) recipients to ensure maximum efficacy and limit toxicity of the individual agents. Currently, the most widely used immunosuppressive drug combination therapy after kidney transplantation consist of tacrolimus in combination with mycophe-

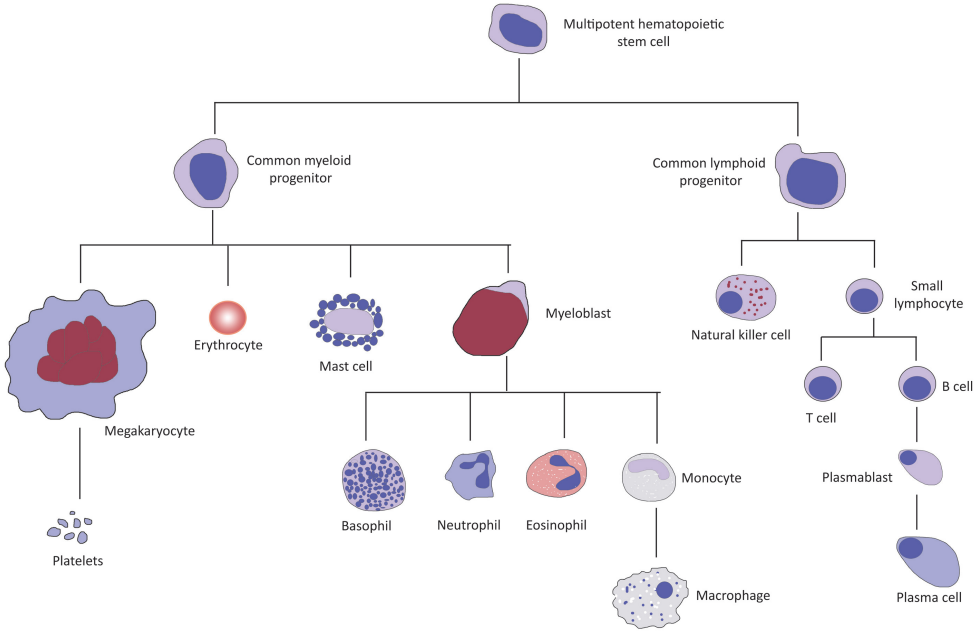


Figure 1. Myeloid cell differentiation. Multipotent hematopoietic stem cells develop into various types of cells, such as T cells, B cells (belonging to the adaptive immune system), natural killer cells and monocytes (belonging to the innate immune system). Upon activation, monocytes can differentiate into macrophages, which are phagocytes that can also present antigens to T cells.

nolic acid (MPA) and glucocorticoids [16]. Better diagnostic approaches and the development of potent and more specific immunosuppressive drug therapy have improved the clinical outcome after SOT [17,18]. However, tacrolimus, which is nowadays the cornerstone immunosuppressant, has several side effects, such as nephrotoxicity, neurotoxicity and diabetes mellitus. Furthermore, immunosuppression in general increases the chance of developing malignancy and infection [19]. In addition, the therapeutic window of tacrolimus is narrow, meaning that the dosage range for safe and effective treatment is small. The use of tacrolimus is also complicated by its high inter- and intra-patient variability [20]. An important problem is nonadherence of transplantation recipients to their immunosuppressive agents. Nonadherence is a cause of fluctuating drug concentrations and increases the chance of developing rejection. Nonadherence has been shown to increase when a drug is dosed more frequently and when therapy is chronic [21]. For this reason, treatment with tacrolimus, like many other immunosuppressants, is monitored by means of measuring (whole blood) drug concentrations, a practice known as therapeutic drug monitoring (TDM). By performing TDM, the time a patient is exposed to supra- or sub-therapeutic drug concentrations is limited. However, there is another problem with the TDM of tacrolimus, next to the high intra-patient variability. The tacrolimus pre-dose concentration has an imperfect correlation ($r_s \approx 0.7$) with the total exposure to tacrolimus during a dosing interval as measured by the area-under the concentration ***versus*** time-curve (AUC), and, as a consequence, the occurrence of acute rejection or side effects could not accurately predicted [22-24]. One way for solving these problems associated with TDM of tacrolimus is to focus on the biological effects of immunosuppressive drugs on T cells and other immune cells after transplantation [25,26].

T cell activation

T cells are arguably the most important players in acute rejection of SOT transplants. For a T cell response three signals are required: 1) antigenic stimulation of the T cell receptor (TCR) by the HLA – alloantigen complex on an APC, 2) a co-stimulatory signal and, 3) amplification of the T cell activation through the production and binding of cytokines to their corresponding receptors on T cells (**Figure 2**) [27,28]. The most extensively studied co-stimulatory signal is the interaction between CD28 molecules on the T cell surface and the CD80/86 molecules on APCs, such as macrophages and dendritic cells. Once activated, intracellular signaling pathways are triggered, such as the calcineurin, Mitogen-Activated Protein Kinase (MAPK) and PI3K pathways that control transcription factor activity (***e.g.*** NFκB and CREB) and gene transcription (**Figure 3**). As a consequence, cytokines are produced (***e.g.*** interleukin (IL)-2, interferon (IFN)-γ and tumor necrosis factor (TNF)-α) that cause the proliferation and differentiation of T cells.

The third signal, needed for T cell differentiation, consists of a positive feedback loop driven by cytokines that are produced by activated T cells after receiving signal 1 and 2. Examples of cytokines needed for the third signal are IL-2, IL-4, IL-7, IL-9, IL-15 and IL-21

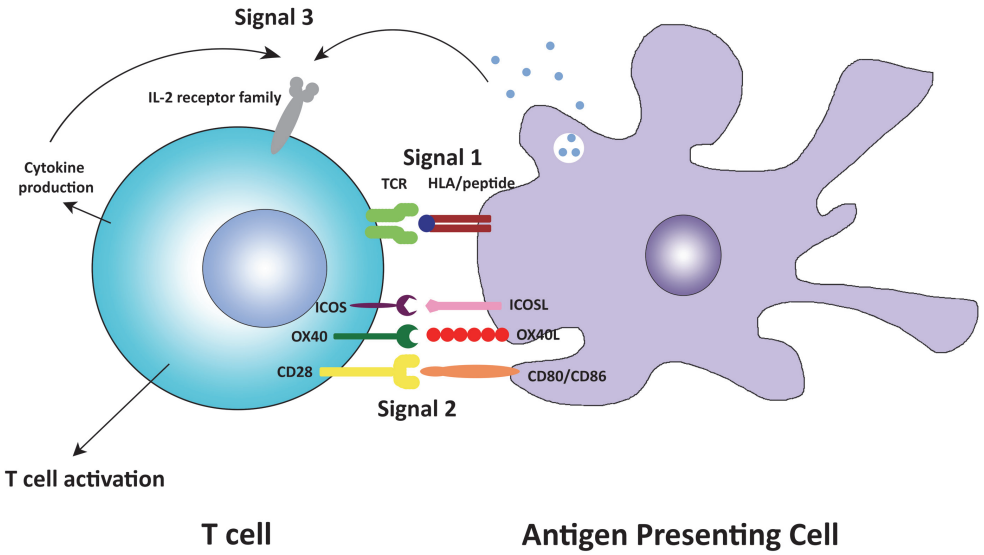


Figure 2. Overview of signals needed for T cell activation. T cells become activated upon receiving three separate signals: 1) antigenic stimulation of the T cell receptor (TCR) by the HLA–alloantigen complex on an APC; 2) co-stimulation, of which the interaction between CD28 molecules on T cells and CD80/86 molecules on the APC is one of the best studied pathways, and 3) binding of cytokines, such as IL-2 that can enhance the T cell response.

that bind to the IL-2 receptor family on the cell membrane of T cells. This interaction will activate the JAK/STAT (Janus activated kinase/Signal transducer and activator of transcription) signaling pathways intracellular of a T cell which then induces T cell differentiation [29].

Different T cell subsets exist, the main distinction being that between $CD4^+$ and $CD8^+$ T cells. $CD4^+$ T cells can be subdivided into naïve and memory T cells. Memory T cells are antigen-experienced cells and control a rapid and lifelong immune protection after responding to an antigen that they have previously encountered. Differentiated memory T cells can be further divided into T-helper (Th)1, Th2, Th9, Th17, Th22 and follicular Th cells [30]. In general, these cells provide help to $CD8^+$ T cells, B cells and cells of the innate immune system. $CD8^+$ T cells can cause the apoptosis (cell death) of a target cell in different ways. The first way is by the production of the cytokines perforin and granzyme that initiate the forming of pores in the membrane and induce the caspase cascade (consisting of cleaving enzymes) inside the target cell. Other ways to destroy their target cell is via the production of the cytokines TNF-α and IFN-γ, and via the interaction between the ligand FasL on the T cell and Fas receptor on the target cell. This will also activate the caspase cascade needed for apoptosis. In addition to alloreactive T cells, there also exist regulatory T cell (Treg) subsets that suppress the alloimmune response and may even be responsible for clinical tolerance [31,32].

The expression of the costimulatory molecule CD28 distinguishes several functionally different T cell subsets. The CD28 molecule provides, among others, co-stimulatory signals

required for T cell activation and survival by acting as a receptor for the B7-molecules CD80 and CD86 which are present on APCs. Upon activation, CD28 triggers T cell intracellular signaling pathways that are needed for T cell activation and proliferation, including the NFAT, MAPK, NFκB and PI3K pathways (**Figure 3**). The same ligands that control CD28 activity can also bind with a greater affinity to the CTLA-4 molecule, also known as CD152, on T cells. This interaction will cause the inhibition of a T cell and induces the regulatory function of a T cell.

$CD28^+$ T cells are naïve T cells which only become activated after encountering signal 1 and a costimulatory signal via CD28. In contrast, $CD28^-$ T cells are terminally-differentiated memory T cells, which do not require signaling via CD28 in order to become activated. Upon antigenic re-stimulation, $CD28^-$ T cells produce high levels of effector cytokines [33,34]. In addition, $CD28^-$ T cells are highly antigen-experienced and can react faster and stronger to antigen presentation than their positive counterpart [35]. An important and clinically relevant problem is that $CD28^-$ T cells are not susceptible to the immunosuppressive effects of the drug belatacept, which blocks the interaction between CD28 and CD80/CD86 and acts as CTLA-4 immunoglobulin (Ig) [36-38].

Monocyte activation

The role of monocytes in IRI and acute or chronic rejection is increasingly recognized [39-42]. For example, the occurrence of ABMR is characterized by the accumulation of monocytes and macrophages and these cells are also present in renal biopsies taken during acute cellular rejection [43,44]. In general, upon activation, monocytes differentiate into macrophages or DCs, after which they process and present alloantigen to the immune system of the recipient. They also play a role in tissue repair processes, providing co-stimulation signals and producing pro-inflammatory cytokines, such as TNF-α, IL-1 and IL-6 [45]. In IRI, monocytes are attracted to the site of injury by the binding of monocyte chemotactic protein 1 (MCP-1) to their CCR2 receptor, after which they differentiate into DCs or macrophages [46]. In ABMR, monocytes contribute to cell injury via the activation of their Fcγ-receptor (FcγRI or CD64) by allo-antibodies [47]. The signal received by the Fcγ-receptor will block apoptosis and cause the accumulation of monocytes at the site of rejection where they produce pro-inflammatory cytokines. Monocytes can be divided into three phenotypically and functionally distinct subsets, based on their expression of CD14 and CD16: $CD14^{++}CD16^-$ (classical), $CD14^{++}CD16^+$ (intermediate), and $CD14^+CD16^{++}$ (non-classical) monocytes [48]. Infiltrating monocytes can differentiate into classically and alternatively activated macrophages, generally called M1 and M2 macrophages, that are now recognized as two ends in a wide functional spectrum [49].

Although the knowledge about the role of monocytes in the alloimmune response in transplant patients is increasing, the impact of immunosuppressive drugs on monocyte/macrophage functions has hardly been studied [50-52]. It is, therefore, necessary to learn

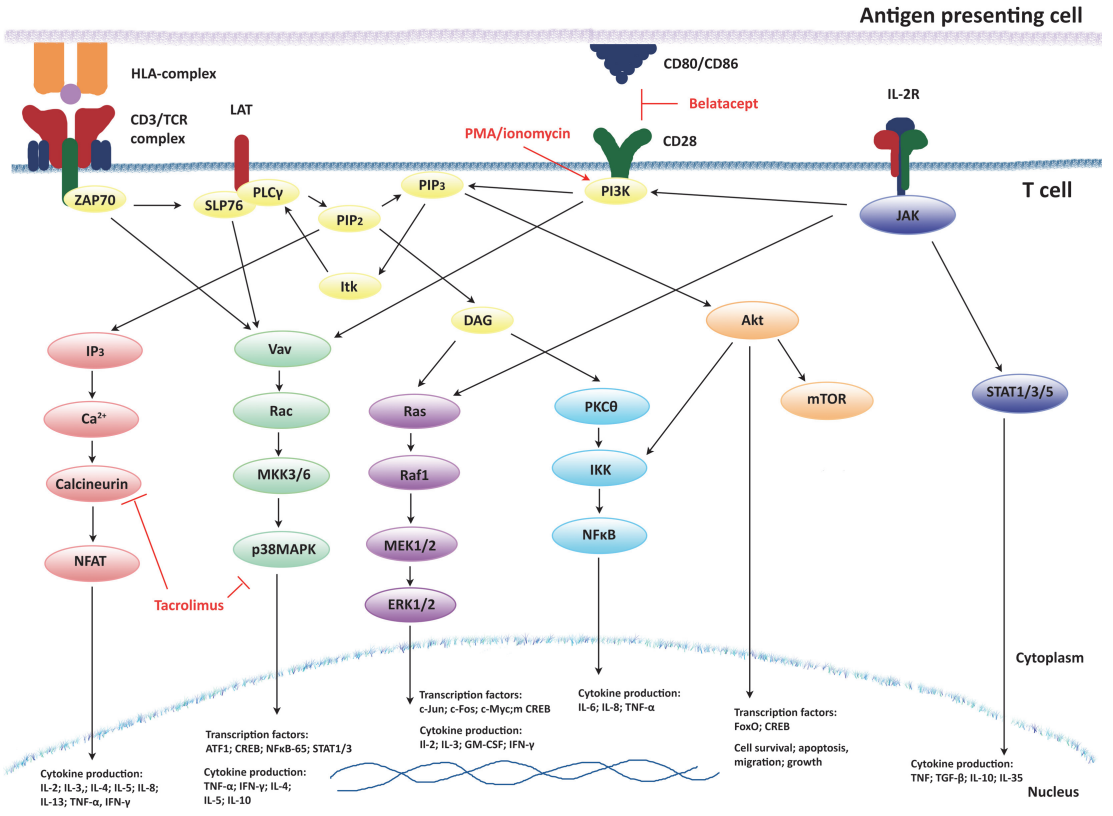


Figure 3. Schematic overview of intracellular signaling pathways in T cells. Antigenic stimulation can be mimicked by using phorbol myristate acetate (PMA)/ionomycin. T cell activation causes the phosphorylation of downstream signaling molecules including NFAT, p38MAPK, ERK, Akt and JAK/STAT. Signaling pathway induction will initiate transcription of, for example, the TNF-α and IFN-γ genes and controls T cell functions, such as cytokine production, cell survival, cell differentiation and cell apoptosis. Tacrolimus is a calcineurin inhibitor known to affect p38MAPK signaling. In contrast to tacrolimus, belatacept does not directly inhibit intracellular signaling pathways, but blocks the interaction between CD28 on the surface of T cells and CD80/86 on APCs.

more about the effects of currently prescribed immunosuppressive drugs on monocyte activation and function, in order to improve patient outcome after transplantation and to develop better strategies for patient treatment.

Immunosuppressive drug therapy

To prevent and overcome rejection responses, SOT recipients are treated with immunosuppressive drugs. The most frequently prescribed immunosuppressive drug therapy consists of the combination of a calcineurin inhibitor (CNI; either cyclosporine or tacrolimus), MPA (either mycophenolate mofetil (MMF) or mycophenolate sodium) and glucocorticoids with or without induction therapy, consisting of T cell-depleting antibody therapy or the IL-2 receptor blocker basiliximab [16,28]. After intake of the pro-drug MMF, this agent is converted to the active metabolite MPA, which inhibits the function of inosine monophosphate dehydrogenase (IMPDH). As a consequence, the production of guanosine nucleotides, required for DNA synthesis, is blocked and T and B cell proliferation is inhibited [53]. Glucocorticoids bind to the glucocorticoid receptor, intracellular of T cells, but can also bind to this receptor in many other cells that regulate the immune response. After translocation to the nucleus, glucocorticoids interact with the glucocorticoid response elements that interfere with the promotors of different genes, such as tyrosine aminotransferase and NFκB. Glucocorticoids, such as prednisolone, have a wide biological effect and inhibit the gene expression of numerous cytokines and chemokines, such as IL-2, IL-6, INF–γ and TNF–α [18].

CNIs inhibit intracellular T cell activation by blocking the calcineurin pathway **(Figure 2)**. This results in a reduction of cytokine production, including IL-2 [54]. The therapeutic window of tacrolimus is small, meaning that the range between an effective dose and the dose causing (nephro)toxicity or other drug-related side effects is narrow [55]. The most recent immunosuppressive drug to be approved for the prevention of acute rejection after kidney transplantation by the US Food and Drug Administration and the European Medicines Agency is belatacept. Belatacept is non-nephrotoxic and inhibits T cell responses more selectively than tacrolimus. Belatacept is a fusion protein consisting of the extracellular domain of the human cytotoxic T-lymphocyte antigen (CTLA)-4 linked to a Fc-fragment of immunoglobulin G1 [56]. It binds to the CD80/CD86 molecule on APCs, thereby blocking the second signal needed for T cell activation. Belatacept is more selective in inhibiting the T cell response after transplantation than other immunosuppressive drugs, due to its high affinity for the CD80/CD86 molecules. The co-stimulatory interaction between CD80/CD86 and CD28 is largely limited to APCs and T cells, which explains the low rate of side effects after belatacept treatment.

Altogether, the above-described immunosuppressive drugs mainly target the activation, proliferation and differentiation of T cells, while the knowledge of their effects on monocytes is limited. For example, ***in vitro*** studies have shown that tacrolimus and MPA affect cytokine production by isolated monocytes but their effect on monocytes in whole-blood samples of transplant recipients is unknown [57,58]. Therefore, research is needed to establish

the effect of immunosuppressive drugs on monocyte functions *in vivo* after transplantation.

Therapeutic drug monitoring: Pharmacokinetics *versus* pharmacodynamics

Problems arising from the small therapeutic window of tacrolimus can be limited by TDM, whereby tacrolimus blood concentrations are routinely monitored to adjust dosages. Currently, TDM of tacrolimus is based on pharmacokinetic (PK) approaches, of which the whole-blood pre-dose concentration (C_0) is the parameter of choice since it correlates well with total exposure to tacrolimus during a dosing interval [59]. However, the tacrolimus C_0 has a limited predictive power with regard to the occurrence of acute rejection episodes or the long-term outcome after transplantation [55,60]. In contrast, tacrolimus C_0 have a better correlation with the total tacrolimus exposure, measured as the AUC ($r_s \approx 0.7$), which is however more labor-intensive to measure [22].

A better or complementary way for TDM of immunosuppressive drugs may be to measure their biological effects directly. This is called pharmacodynamic (PD) monitoring [61-63]. Essential for PD monitoring is the knowledge about the pharmacological mechanism of action of a drug in order to develop a specific assay. Tacrolimus is known to inhibit the signaling molecule calcineurin within T cells, which controls the activation and translocation of nuclear factor of activated T-cells (NFAT) to the nucleus (**Figure 3**) [64]. Once in the nucleus, NFAT activation leads to transcription of several genes playing a role in T cell activation, differentiation and the production of cytokines. Other signaling pathways important for T cell activation are the Mitogen-Activated Protein Kinase (MAPK) and PI3K pathways [65]. Activation of these pathways is characterized by the phosphorylation of specific signaling molecules, such as p38MAPK, Extracellular signal-Regulated Kinases 1 and 2 (ERK1/2) and AKT8 virus oncogene cellular homolog (Akt).

Improving transplantation diagnostics: Novel concepts for TDM

Most studies focusing on the PD effects of immunosuppressive drugs performed to date failed to find a strong correlation with clinical outcomes or with PK parameters [66,67]. These studies include the measurement of calcineurin phosphatase activity, cytokine production by T cells, and the expression of NFAT-regulated genes [68,69]. So far, none of these techniques has found its way into routine clinical practice. Apart from their imperfect correlation with clinical outcomes, problems with these assays are the fact that they are time-consuming, costly, and importantly, measure the effect of a single immunosuppressive agent rather than the combined effect of several drugs [70]. Other reasons for the failure of these assays for routine clinical practice are the long turnaround time and the difficulties that arise in reproducing the results [71].

Phospho-specific flow cytometry is a relatively novel and potential clinically useful approach to directly measure and monitor PD drug effects in whole blood samples of transplant recipients [62,72-74]. This technique allows measurement and quantification of the

phosphorylation of intracellular signaling molecules in a rapid, sensitive way at the single-cell level. Previous studies using this technique have shown a dose-dependent response of tacrolimus on the inhibition of p38MAPK phosphorylation in T cells and have also demonstrated the strength of TDM by means of phospho-specific flow cytometry in the field of rheumatoid arthritis and oncology [75-77]. These studies indicate that phospho-specific flow cytometry could be a powerful technique tool to measure cell activation markers for PD TDM.

Aim and outline of the thesis

Despite intensive PK monitoring of immunosuppressive drugs after transplantation, a large number of patients suffer from a lack of efficacy or toxicity, due to the small therapeutic window of tacrolimus and the high intra-patient variability. A better way to control drug exposure might be PD monitoring. The aim of this thesis was to assess techniques for PD TDM of immunosuppressive drug effects after kidney transplantation. The work described in this thesis focused on two cell types involved in the immune response after transplantation. The first part of the thesis focusses on the effects of immunosuppressive drugs on monocyte activation; the second part aims to describe the effects of tacrolimus, belatacept and MPA on signaling transduction pathways in T cells and several T cell subsets. In more detail, this thesis will assess the following:

- To assess the role of monocytes in transplantation and the effects of currently prescribed immunosuppressive drugs on these cells (**Chapter 2**)
- To investigate the individual PD effects of tacrolimus and MPA on monocytes of healthy volunteers (**Chapter 3**)
- To determine the combined effects of immunosuppressive drug therapy on monocytes after kidney transplantation (**Chapter 4**)
- To assess the PD monitoring of tacrolimus and other immunosuppressive drugs by measuring the inducible isoform of NFAT in T cells (**Chapter 5**)
- To determine whether measuring p38MAPK phosphorylation can be a promising tool for monitoring the effects of conversion from the twice-daily tacrolimus formulation to the once-daily, prolonged-release tacrolimus formulation (**Chapter 6**)
- To study differences in the PD drug effects of tacrolimus-based therapy compared to belatacept-based therapy (**Chapter 7**)
- To review the use of the JAK inhibitor tofacitinib in kidney transplantation and to assess why the clinical trials of this drug were relatively unsuccessful (**Chapter 8**)

Chapter 9 and 10 summarize the findings of the studies described above and place them into a larger perspective.

References

1. Heldal K, Hartmann A, Grootendorst DC, et al. Benefit of kidney transplantation beyond 70 years of age. Nephrol Dial Transplant 2010;25:1680-7.
2. Laupacis A, Keown P, Pus N, et al. A study of the quality of life and cost-utility of renal transplantation. Kidney Int 1996;50:235-42.
3. van Heurn E, de Vries EE. Kidney transplantation and donation in children. Pediatr Surg Int 2009;25:385-93.
4. Howard K, Salkeld G, White S, et al. The cost-effectiveness of increasing kidney transplantation and home-based dialysis. Nephrology (Carlton) 2009;14:123-32.
5. Matas AJ, Smith JM, Skeans MA, et al. OPTN/SRTR 2013 Annual Data Report: kidney. Am J Transplant 2015;15 Suppl 2:1-34.
6. Nankivell BJ, Alexander SI. Rejection of the kidney allograft. N Engl J Med 2010;363:1451-62.
7. Lin CM, Gill RG. Direct and indirect allograft recognition: pathways dictating graft rejection mechanisms. Curr Opin Organ Transplant 2016;21:40-4.
8. Menke J, Sollinger D, Schamberger B, Heemann U, Lutz J. The effect of ischemia/reperfusion on the kidney graft. Curr Opin Organ Transplant 2014;19:395-400.
9. Salvadori M, Rosso G, Bertoni E. Update on ischemia-reperfusion injury in kidney transplantation: Pathogenesis and treatment. World J Transplant 2015;5:52-67.
10. Farrar CA, Kupiec-Weglinski JW, Sacks SH. The innate immune system and transplantation. Cold Spring Harb Perspect Med 2013;3:a015479.
11. LaRosa DF, Rahman AH, Turka LA. The innate immune system in allograft rejection and tolerance. J Immunol 2007;178:7503-9.
12. Wood KJ, Goto R. Mechanisms of rejection: current perspectives. Transplantation 2012;93:1-10.
13. Haas M, Sis B, Racusen LC, et al. Banff 2013 meeting report: inclusion of c4d-negative antibody-mediated rejection and antibody-associated arterial lesions. Am J Transplant 2014;14:272-83.
14. Sellares J, de Freitas DG, Mengel M, et al. Understanding the causes of kidney transplant failure: the dominant role of antibody-mediated rejection and nonadherence. Am J Transplant 2012;12:388-99.
15. Kim MG, Kim YJ, Kwon HY, et al. Outcomes of combination therapy for chronic antibody-mediated rejection in renal transplantation. Nephrology (Carlton) 2013;18:820-6.
16. Matas AJ, Smith JM, Skeans MA, et al. OPTN/SRTR 2011 Annual Data Report: kidney. Am J Transplant 2013;13 Suppl 1:11-46.
17. Grinyo JM. Why is organ transplantation clinically important? Cold Spring Harb Perspect Med 2013;3.
18. Taylor AL, Watson CJ, Bradley JA. Immunosuppressive agents in solid organ transplantation: Mechanisms of action and therapeutic efficacy. Crit Rev Oncol Hematol 2005;56:23-46.

19. Malvezzi P, Rostaing L. The safety of calcineurin inhibitors for kidney-transplant patients. Expert Opin Drug Saf 2015;14:1531-46.
20. Shuker N, van Gelder T, Hesselink DA. Intra-patient variability in tacrolimus exposure: causes, consequences for clinical management. Transplant Rev (Orlando) 2015;29:78-84.
21. Prendergast MB, Gaston RS. Optimizing medication adherence: an ongoing opportunity to improve outcomes after kidney transplantation. Clin J Am Soc Nephrol 2010;5:1305-11.
22. Hon YY, Chamberlain CE, Kleiner DE, et al. Evaluation of tacrolimus abbreviated area-under-the-curve monitoring in renal transplant patients who are potentially at risk for adverse events. Clin Transplant 2010;24:557-63.
23. Matas AJ, Smith JM, Skeans MA, et al. OPTN/SRTR 2012 Annual Data Report: kidney. Am J Transplant 2014;14 Suppl 1:11-44.
24. Saint-Marcoux F, Woillard JB, Jurado C, Marquet P. Lessons from routine dose adjustment of tacrolimus in renal transplant patients based on global exposure. Ther Drug Monit 2013;35:322-7.
25. Meier-Kriesche HU, Schold JD, Srinivas TR, Kaplan B. Lack of improvement in renal allograft survival despite a marked decrease in acute rejection rates over the most recent era. Am J Transplant 2004;4:378-83.
26. Tang IY, Meier-Kriesche HU, Kaplan B. Immunosuppressive strategies to improve outcomes of kidney transplantation. Semin Nephrol 2007;27:377-92.
27. Esposito P, Grosjean F, Rampino T, et al. Costimulatory pathways in kidney transplantation: pathogenetic role, clinical significance and new therapeutic opportunities. Int Rev Immunol 2014;33:212-33.
28. Halloran PF. Immunosuppressive drugs for kidney transplantation. N Engl J Med 2004;351:2715-29.
29. Rochman Y, Spolski R, Leonard WJ. New insights into the regulation of T cells by gamma(c) family cytokines. Nat Rev Immunol 2009;9:480-90.
30. Nakayamada S, Takahashi H, Kanno Y, O'Shea JJ. Helper T cell diversity and plasticity. Curr Opin Immunol 2012;24:297-302.
31. Braza F, Dugast E, Panov I, et al. Central Role of CD45RA- Foxp3hi Memory Regulatory T Cells in Clinical Kidney Transplantation Tolerance. J Am Soc Nephrol 2015;26:1795-805.
32. Hu M, Wang YM, Wang Y, et al. Regulatory T cells in kidney disease and transplantation. Kidney Int 2016;90:502-14.
33. Vallejo AN. CD28 extinction in human T cells: altered functions and the program of T-cell senescence. Immunol Rev 2005;205:158-69.
34. Arosa FA. CD8+CD28- T cells: certainties and uncertainties of a prevalent human T-cell subset. Immunol Cell Biol 2002;80:1-13.
35. Mou D, Espinosa J, Lo DJ, Kirk AD. CD28 negative T cells: is their loss our gain? Am J Transplant 2014;14:2460-6.

36. de Graav GN, Hesselink DA, Dieterich M, Kraaijeveld R, Weimar W, Baan CC. Down-Regulation of Surface CD28 under Belatacept Treatment: An Escape Mechanism for Antigen-Reactive T-Cells. PLoS One 2016;11:e0148604.
37. Betjes MG. Clinical consequences of circulating CD28-negative T cells for solid organ transplantation. Transpl Int 2016;29:274-84.
38. Ashokkumar C, Ganguly B, Townsend R, et al. Alloreactive CD154-expressing T-cell subsets with differential sensitivity to the immunosuppressant, belatacept: potential targets of novel belatacept-based regimens. Sci Rep 2015;5:15218.
39. Ysebaert DK, De Greef KE, Vercauteren SR, et al. Identification and kinetics of leukocytes after severe ischaemia/reperfusion renal injury. Nephrol Dial Transplant 2000;15:1562-74.
40. Hancock WW, Thomson NM, Atkins RC. Composition of interstitial cellular infiltrate identified by monoclonal antibodies in renal biopsies of rejecting human renal allografts. Transplantation 1983;35:458-63.
41. Rowshani AT, Vereyken EJ. The role of macrophage lineage cells in kidney graft rejection and survival. Transplantation 2012;94:309-18.
42. Girlanda R, Kleiner DE, Duan Z, et al. Monocyte infiltration and kidney allograft dysfunction during acute rejection. Am J Transplant 2008;8:600-7.
43. Bergler T, Jung B, Bourier F, et al. Infiltration of Macrophages Correlates with Severity of Allograft Rejection and Outcome in Human Kidney Transplantation. PLoS One 2016;11:e0156900.
44. Xu L, Collins J, Drachenberg C, Kukuruga D, Burke A. Increased macrophage density of cardiac allograft biopsies is associated with antibody-mediated rejection and alloantibodies to HLA antigens. Clin Transplant 2014;28:554-60.
45. Jiang X, Tian W, Sung YK, Qian J, Nicolls MR. Macrophages in solid organ transplantation. Vasc Cell 2014;6:5.
46. Zuidema MY, Zhang C. Ischemia/reperfusion injury: The role of immune cells. World J Cardiol 2010;2:325-32.
47. Wasowska BA. Mechanisms involved in antibody- and complement-mediated allograft rejection. Immunol Res 2010;47:25-44.
48. Wong KL, Yeap WH, Tai JJ, Ong SM, Dang TM, Wong SC. The three human monocyte subsets: implications for health and disease. Immunol Res 2012;53:41-57.
49. Mantovani A, Biswas SK, Galdiero MR, Sica A, Locati M. Macrophage plasticity and polarization in tissue repair and remodelling. J Pathol 2013;229:176-85.
50. Rogacev KS, Zawada AM, Hundsdorfer J, et al. Immunosuppression and monocyte subsets. Nephrol Dial Transplant 2015;30:143-53.
51. Vereyken EJ, Kraaij MD, Baan CC, et al. A shift towards pro-inflammatory CD16+ monocyte subsets with preserved cytokine production potential after kidney transplantation. PLoS One 2013;8:e70152.
52. Sekerkova A, Krepsova E, Brabcova E, et al. CD14+CD16+ and CD14+CD163+ monocyte subpopulations in kidney allograft transplantation. BMC Immunol 2014;15:4.

53. Allison AC, Eugui EM. Mycophenolate mofetil and its mechanisms of action. Immunopharmacology 2000;47:85-118.
54. Thomson AW, Bonham CA, Zeevi A. Mode of action of tacrolimus (FK506): molecular and cellular mechanisms. Ther Drug Monit 1995;17:584-91.
55. Bouamar R, Shuker N, Hesselink DA, et al. Tacrolimus predose concentrations do not predict the risk of acute rejection after renal transplantation: a pooled analysis from three randomized-controlled clinical trials(dagger). Am J Transplant 2013;13:1253-61.
56. Larsen CP, Pearson TC, Adams AB, et al. Rational development of LEA29Y (belatacept), a high-affinity variant of CTLA4-Ig with potent immunosuppressive properties. Am J Transplant 2005;5:443-53.
57. Weimer R, Mytilineos J, Feustel A, et al. Mycophenolate mofetil-based immunosuppression and cytokine genotypes: effects on monokine secretion and antigen presentation in long-term renal transplant recipients. Transplantation 2003;75:2090-9.
58. Chang KT, Lin HY, Kuo CH, Hung CH. Tacrolimus suppresses atopic dermatitis-associated cytokines and chemokines in monocytes. J Microbiol Immunol Infect 2016;49:409-16.
59. Schiff J, Cole E, Cantarovich M. Therapeutic monitoring of calcineurin inhibitors for the nephrologist. Clin J Am Soc Nephrol 2007;2:374-84.
60. Shuker N, Shuker L, van Rosmalen J, et al. A high intrapatient variability in tacrolimus exposure is associated with poor long-term outcome of kidney transplantation. Transpl Int 2016;29:1158-67.
61. Dieterlen MT, Eberhardt K, Tarnok A, Bittner HB, Barten MJ. Flow cytometry-based pharmacodynamic monitoring after organ transplantation. Methods Cell Biol 2011;103:267-84.
62. Baan C, Bouvy A, Vafadari R, Weimar W. Phospho-specific flow cytometry for pharmacodynamic monitoring of immunosuppressive therapy in transplantation. Transplant Res 2012;1:20.
63. Noceti OM, Woillard JB, Boumediene A, et al. Tacrolimus pharmacodynamics and pharmacogenetics along the calcineurin pathway in human lymphocytes. Clin Chem 2014;60:1336-45.
64. Macian F. NFAT proteins: key regulators of T-cell development and function. Nat Rev Immunol 2005;5:472-84.
65. Nakayama T, Yamashita M. The TCR-mediated signaling pathways that control the direction of helper T cell differentiation. Semin Immunol 2010;22:303-9.
66. Bergan S, Bremer S, Vethe NT. Drug target molecules to guide immunosuppression. Clin Biochem 2016;49:411-8.
67. Sommerer C, Giese T, Meuer S, Zeier M. Pharmacodynamic monitoring of calcineurin inhibitor therapy: is there a clinical benefit? Nephrol Dial Transplant 2009;24:21-7.
68. van Rossum HH, de Fijter JW, van Pelt J. Pharmacodynamic monitoring of calcineurin inhibition therapy: principles, performance, and perspectives. Ther Drug Monit 2010;32:3-10.
69. Abdel-Kahaar E, Giese T, Sommerer C, Rieger H, Shipkova M, Wieland E. Analytical Validation and Cross-Validation of an NFAT-Regulated Gene Expression Assay for Pharmacodynamic Monitoring of Therapy With Calcineurin Inhibitors. Ther Drug Monit 2016;38:711-6.

70. Klupp J, Holt DW, van Gelder T. How pharmacokinetic and pharmacodynamic drug monitoring can improve outcome in solid organ transplant recipients. Transpl Immunol 2002;9:211-4.
71. Dambrin C, Klupp J, Morris RE. Pharmacodynamics of immunosuppressive drugs. Curr Opin Immunol 2000;12:557-62.
72. Maguire O, Tario JD, Jr., Shanahan TC, Wallace PK, Minderman H. Flow cytometry and solid organ transplantation: a perfect match. Immunol Invest 2014;43:756-74.
73. Landskron J, Tasken K. Phosphoprotein Detection by High-Throughput Flow Cytometry. Methods Mol Biol 2016;1355:275-90.
74. Krutzik PO, Nolan GP. Intracellular phospho-protein staining techniques for flow cytometry: monitoring single cell signaling events. Cytometry A 2003;55:61-70.
75. Vafadari R, Hesselink DA, Cadogan MM, Weimar W, Baan CC. Inhibitory effect of tacrolimus on p38 mitogen-activated protein kinase signaling in kidney transplant recipients measured by whole-blood phosphospecific flow cytometry. Transplantation 2012;93:1245-51.
76. Irish JM, Hovland R, Krutzik PO, et al. Single cell profiling of potentiated phospho-protein networks in cancer cells. Cell 2004;118:217-28.
77. Galligan CL, Siebert JC, Siminovitch KA, et al. Multiparameter phospho-flow analysis of lymphocytes in early rheumatoid arthritis: implications for diagnosis and monitoring drug therapy. PLoS One 2009;4:e6703.

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Targeting the Monocyte–Macrophage Lineage in Solid Organ Transplantation

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Abstract

There is an unmet clinical need for immunotherapeutic strategies which specifically target the active immune cells participating in the process of rejection after solid organ transplantation. The monocyte-macrophage cell lineage is increasingly recognized as a major player in acute and chronic allograft immunopathology. The dominant presence of cells of this lineage in rejecting allograft tissue is associated with worse graft function and survival. Monocytes and macrophages contribute to alloimmunity via diverse pathways: antigen processing and presentation, co-stimulation, pro-inflammatory cytokine production and tissue repair. Cross talk with other recipient immune competent cells and donor endothelial cells leads to amplification of inflammation and a cytolytic response in the graft.

Surprisingly little is known about therapeutic manipulation of the function of cells of the monocyte-macrophage lineage in transplantation by immunosuppressive agents. Although not primarily designed to target monocyte-macrophage lineage cells, multiple categories of currently prescribed immunosuppressive drugs, such as mycophenolate mofetil, mTOR inhibitors and calcineurin inhibitors, do have limited inhibitory effects. These effects include diminishing the degree of cytokine production, blocking co-stimulation and inhibiting the migration of monocytes to the site of rejection. Outside the field of transplantation, some clinical studies have shown that the monoclonal antibodies canakinumab, tocilizumab and infliximab are effective in inhibiting monocyte functions. Indirect effects have also been shown for simvastatin, a lipid lowering drug, and BET (Bromodomain and Extra-Terminal motif) inhibitors that reduce the cytokine production by monocytes-macrophages in patients with diabetes mellitus and rheumatoid arthritis.

To date, detailed knowledge concerning the origin, the developmental requirements and functions of diverse specialized monocyte-macrophage subsets justifies research for therapeutic manipulation. Here, we will discuss the effects of currently prescribed immunosuppressive drugs on monocytes/macrophages features and the future challenges.

Introduction

Solid organ transplantation (SOT) is the preferred method to treat organ failure. Over the past decades, transplantation has become the preferred approach to treat solid organ failure. Striking improvement in short-term allograft survival, in particular of kidney allograft, has been achieved while long-term survival has lagged behind [1]. Intriguingly, this improvement is seen mainly in recipients who have never experienced a rejection episode, emphasizing the recipient's alloimmunity; in particular chronic antibody mediated rejection (cABMR) as a major determinant of overall transplant outcome [2,3]. At present, there is an unmet clinical need to apply immunotherapeutic strategies to specifically target the active immune cells crucially participating in the process of rejection after SOT.

However, treatment with immunosuppressive drugs has exchanged the morbidity and mortality of organ failure for the risks of infection, cancer and increased mortality from cardiovascular disease. Although acute and chronic rejection, regardless of the type and the time of occurrence, are still major contributors leading to graft failure [1,4,5], cABMR is the main concern for the long term graft survival. Chronic antibody mediated rejection arises, at least in part, because immunosuppressive strategies do not completely inhibit rejection-related alloimmune responses specifically, resulting in slow progressive deterioration of graft function.

The monocyte-macrophage cell lineage is increasingly recognized as a major player in acute and chronic allograft immunopathology [6,7]. The clinically used immunosuppressive drugs are not specifically directed against monocyte-macrophage lineage cells but still have some inhibitory effects. These cells contribute to alloimmunity via diverse pathways; antigen processing and antigen presentation, co-stimulation, pro-inflammatory cytokine production and tissue repair. Cross talk with other recipient immune competent cells and donor endothelial cells underlies amplification of inflammation at the graft site [8-10]. Interestingly, acute and chronic antibody mediated rejection are characterized amongst others by accumulation of monocyte-macrophage cells. Kidney graft infiltrating macrophages have been described to be a predictor of death-censored graft failure [11-21]. Macrophages are present in both acute antibody mediated rejection (ABMR) and acute cellular rejection (ACR) of solid organ transplants [19,22]. In rejecting cardiac tissue, interstitial and intraluminal macrophage density correlates with effector alloantibodies and clinical antibody mediated rejection [22]. Even more, histopathological staining's for macrophages have been found to be positive prior to the onset of graft dysfunction indicating that macrophages can serve as potential diagnostic markers for transplant rejection [18]. Intravascular macrophages in the capillaries of endomyocardial tissue are shown to be a distinguishing feature of ABMR and are considered as one of the important histopathological diagnostic criteria in cardiac transplantation [22,23].

A recent study showed that the severity of macrophage infiltration during ACR with arteritis is associated with impaired kidney function as measured by creatinine values up to 36 months post transplantation [19]. Importantly, Oberbarnscheidt et al. showed that monocyte

recognition of allogeneic non-self persists over time, long after acute surgical inflammation has been subsided, indicating the important role of monocytes in the principle of long-term graft failure [24]. Recently, the presence of smooth muscle like-precursor cells within the non-classical monocyte subset has been described in kidney transplant patients. Characterization of non-classical monocytes in peripheral blood of kidney transplant patients undergoing chronic transplant dysfunction showed lower numbers compared to patients without chronic transplant dysfunction. Within the total living cell percentages of CD14+ monocytes there was no change observed, suggesting a shift within different subsets. Non-classical monocytes being reduced in transplant recipients with chronic transplant dysfunction may indicate a vital role in interstitial and vascular remodelling [25].

In stable kidney transplant recipients, a skewed balance towards pro-inflammatory CD16+ monocytes was shown at the time of kidney transplantation and during the first 6 months post-transplant. These monocytes were able to produce IFNγ, which acts as an important bridge between innate and adaptive immunity [26,27].

In summary, the currently available knowledge concerning the immunobiology of specialized monocyte–macrophage subsets, their pathogenic role in rejection, and the still unmet clinical need to specifically prevent alloimmunity justify research on strategies for monocyte-macrophage directed therapeutics. In this review, we aim to discuss the relevant knowledge on monocyte-macrophage immunobiology. Briefly, to elaborate on the effects of currently available immunosuppressive drugs in relation to monocyte/macrophage lineage cells mainly focussed within, but also outside of the SOT field (**Table I and Figure 1**), and eventually touch upon the future challenges and developments.

Monocyte immunobiology

Monocytes and macrophages are mononuclear phagocytes with crucial and distinct roles in transplant immunity. Monocytes display a remarkable plasticity in response to signals from the microenvironment, enabling them to differentiate into various cell types. Several pro-inflammatory, metabolic and immune stimuli all increase the attraction of monocytes towards tissue [7]. Based on the expression of CD14 (LPS co-receptor) and CD16 (Fcγ receptor III), three phenotypically and functionally distinct human monocyte subsets: CD14++CD16- (classical), CD14++CD16+ (intermediate), and CD14+CD16++ (non-classical) monocytes can be defined [28-31]. Monocytes arise from myeloid precursor cells in primary and secondary lymphoid organs, such as liver and bone marrow. In humans, monocytes represent respectively 10% of the nucleated cells in peripheral blood, with 2 major reservoirs: the spleen and lungs that can mobilize monocytes on demand [32,33]. Classical monocytes are able to start proliferating in the bone marrow in response to infection or tissue damage, and subsequently be released into the circulation in a CCR2 dependent manner (**Figure 2**) [34]. Intermediate and non-classical monocytes are thought to be descendants of classical monocytes that have been under control of transcription factor Nur77 (NR4A1) returned to the bone marrow [35]. Non-classical monocytes show a patrolling, distinct motility and

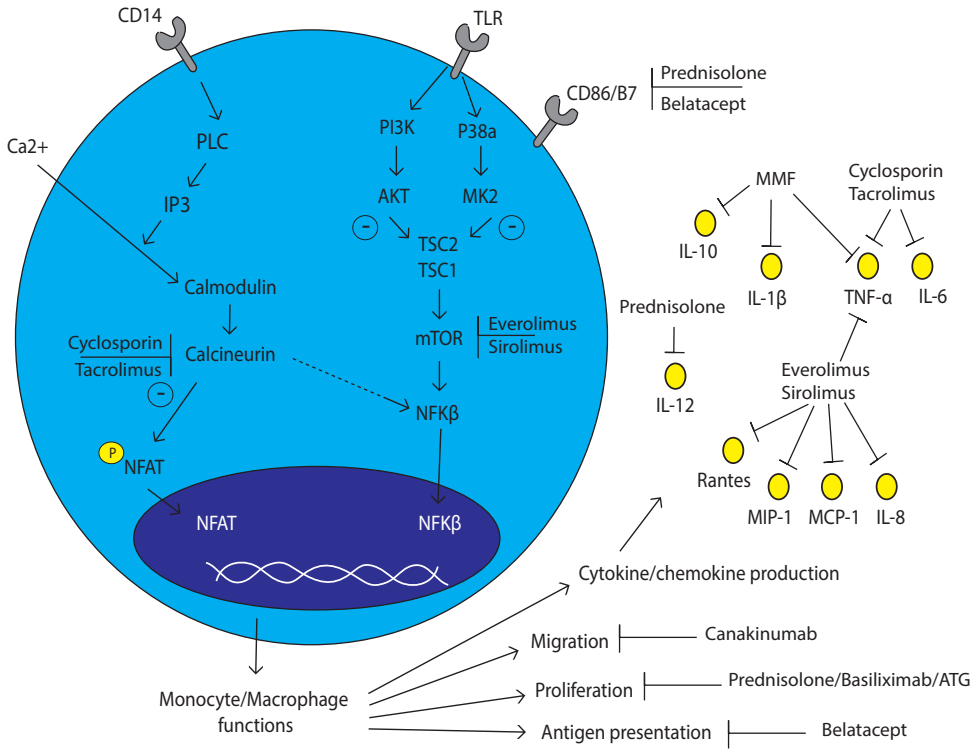

Figure 1. **Monocyte and macrophage lineage cells and the effect of immunosuppressive drugs.** The effect of currently prescribed immunosuppressive drugs with several inhibition spots on and in monocyte/macrophage lineage cells.

crawling pattern [36]. Interestingly, intermediate monocytes show higher expression of major histocompatibility (MHC) class II molecules and thereby more related to non-classical monocytes [37,38]. CD14+ monocytes can be recruited to the site of inflammation or areas of tissue injury where they can differentiate into macrophages and dendritic cells [39]. In steady state, circulating monocytes have minimal contribution to the maintenance of tissue resident macrophages [40,41]. Depending on the microenvironment, activation stimuli and cross talk with other immunological effector cells, activation of macrophages alters their cytokine profile and co-stimulatory molecule expression. Monocyte differentiation to tissue macrophages is Colony Stimulating Factor 1 Receptor (CSF1R) dependent. Most tissue macrophages are seeded before birth in embryonic state, with varying contributions of primitive-derived and definitive-derived cells. Monocytic input to tissue macrophage compartments seems to be restricted to inflammatory settings, such as infection and acute graft rejection [39]. Monocyte chemotactic peptide-1 (MCP-1) is an important regulator of macrophage recruitment and was shown to be highly expressed in the kidney allograft, supporting the concept of recruitment of monocytes from the circulation [42].

Table I. Immunosuppressive drugs and the monocyte/macrophage lineage

Drug type	Effects on monocytes/macrophages	Key references
Basiliximab & ATG	• Basliximab targets the CD25 molecule (the IL-2 receptor) on activated T cells • ATG binds to multiple T-cell specific antigens and causes cell death via complement mediated cytotoxicity • Reduced number of monocytes *in vivo* • Upregulation of the anti-inflammatory M2 macrophage subset CD14+ CD163+ *in vivo*	Sekerkova et al, 2014
Alemtuzumab	• Targets CD52 on B cells, T cells, NK cells, dendritic cells and monocytes • Less effective in depleting monocytes than depleting T cells • Leads to a relative high expression of co-stimulatory molecules, IL-6 and NFκB	Hale et al, 1990 Kirk et al, 2003 Fabian et al, 1993 Rao et al, 2012
Calcineurin inhibitors (tacrolimus & cyclosporin)	• No inhibitory effect on p38MAPK phosphorylation, but reduce cytokine production via ERK phosphorylation • Downregulate production of IL-6 and TNF-α after TLR stimulation *in vitro* • Impaired phagocytosis function and promotion of infection (CsA)	Escolano et al, 2014 Howell et al, 2013 Tourneur et al, 2013
Mycophenolate mofetil	• Diminished the production of IL-1β, IL-10 and TNF-α and decreased expression of TNF-receptor 1 on monocytes • Reduced monocyte migration through lower expression of adhesion molecules	Alisson et al, 2000 Weimer et al, 2003
Glucocorticoids	• Lower CD14+CD16++monocyte counts • Lower expression of B7 molecules leading to disturbed co-stimulation • Induction of anti-inflammatory response via increased IL-10 production • Impaired phagocytosis function	Rogacev et al, 2015 Girndt et al, 1998 Hodge et al, 2005 Blotta et al, 1997 Rinehart et al, 1974
mTor inhibitors	• Decreased chemokine and cytokine production • Combination therapy with steroids increased pro-inflammatory cytokine production	Lin et al, 2014 Oliveira et al, 2002 Weichhart et al, 2011
Belatacept/ abatacept	• Block CD80/86 molecules on antigen-presenting cells and inhibit co-stimulatory function • Lower migration and adhesion capacity • Decreased expression of the pro-inflammatory cytokines IL-12 and TNF-α	Latek et al, 2009 Bonelli et al, 2013 Wenink et al, 2011
Experimental drugs	• Canakinumab inhibits IL-1β production by monocytes • Sinomenine is associated with less monocyte migration, differentiation and maturation • 15-deoxyspergualin decreases monocyte proliferation, TNF-α production, phagocytosis and antigen presentation • Simvastatin and salsalate are associated with less monocyte activation and inhibition of IL-6 and IL-8 production in diabetes patients • Tocilizumab inhibits IL-6 production by monocytes • BET inhibitors are involved in epigenetic control of monocytes thereby preventing inflammation • Fish oils are associated with lower numbers of macrophages in obesitas patients and a reduced secretion of TNF-α *in vitro*	Hoffman et al, 1993 Ou Y et al, 2009 Wang et al, 2011 Perenyei et al, 2014 Donath et al, 2011 McCarty et al, 2010 Tono et al, 2015 Chan et al, 2015 Spencer et al, 2013 Zhao et al, 2013 Jialal et al. 2007

ATG anti-thymocyte globulin; **IL** interleukin; **NFκB** nuclear factor kappa-light-chain-enhancer of activated B cells; **MAPK**; mitogen-activated protein kinases; **ERK** extracellular signal–regulated kinase; **CsA** Cyclosporin A; **TNF** tumor necrosis factor; **BET** bromodomain and extra-terminal motif

Macrophages can be subdivided in 'classically activated' or 'alternatively activated'. Classically activated macrophages are described as M1 macrophages, which are developed upon response to IFNγ, LPS or TNF-α. M1 macrophages express surface markers: MHCII, CD40, CD80, CD86 and CD11b. They can produce inflammatory cytokines such as: TNF-α, IL-1, IL-6, IL-8, IL-12, CCL2, CXCL9 and CXCL10. M1 macrophages are linked to the Th1 response and are mainly considered as pro-inflammatory macrophages whereas M2 are considered as mainly anti-inflammatory. M2 macrophages can be subdivided in M2a, M2b and M2c. M2a macrophages are generated on response to IL-4 and IL-13. Immune complexes and TLR/IL-1R ligands activate M2b macrophages whereas M2c macrophages are activated by IL-10, TGF-β and glucocorticoids. M2 macrophages express surface markers: CD163, CD206 and CD209. M2 macrophages produce IL-10 and TGF-β mainly leading to tissue repair and scar formation. M2 macrophages are linked to Th2 response and show immune-modulatory functions [7,39,43]. Human regulatory macrophages (Mregs) are in a specific state of differentiation with a robust phenotype and potent T-cell suppressor function. These Mregs arise from CD14+ peripheral blood monocytes during 7-day culture exposed to M-CSF and activation by IFNγ [44]. Mregs express several molecules such as MHCII, FCγR, IFNγR, TLR-4 and PD-L1 as shown in **Figure 2** [45]. Shifting the balance between regulatory macrophages and/or monocytes on the one hand, and the effector macrophages and proinflammatory monocytes on the other hand could theoretically result in dampening the immune response against the graft and the immunological tolerance, or to aggravation of graft rejection. To date, two clinical trials investigated the feasibility of regulatory macrophages in promoting allograft acceptance with promising results [46,47]. Moreover, recently, a new homogeneous monocyte subpopulation of human G-CSF induced CD34+ monocytes with powerful immunosuppressive properties upon human allogeneic T-cell activation was described. Such tolerogenic monocytes could be used for novel immune-regulatory or cellular therapy development [48].

Recently, an adaptive feature of innate immunity has been described as "trained immunity". Trained immunity is defined as a nonspecific immunological memory resulting from rewiring the epigenetic program and the functional state of the innate immunity [49]. Twenty naïve patients were vaccinated for bacille Calmette-Guérin (BCG) to investigate mechanisms of the enhanced immune function. Interestingly, these authors identified trained monocytes in the circulation of ***BCG***-vaccinated individuals for at least 3 months suggesting that reprogramming takes place at the level of progenitor cells in the bone marrow [50]. Recent evidence emerged to indicate that innate immune memory could be transferred via hematopoietic stem and progenitor cells. In vitro studies showed effects lasting for days [51,52], whereas other reports showed memory effects for weeks [53]. These interesting observations might be explained by alterations in epigenetic (de)methylation profiles after antigenic stimulation. Altering the epigenetic program by pharmacological means leading to behavioral changes of monocytes could be a promising method to restore or modify the healthy gene/protein expression in the pro-inflammatory microenvironment.

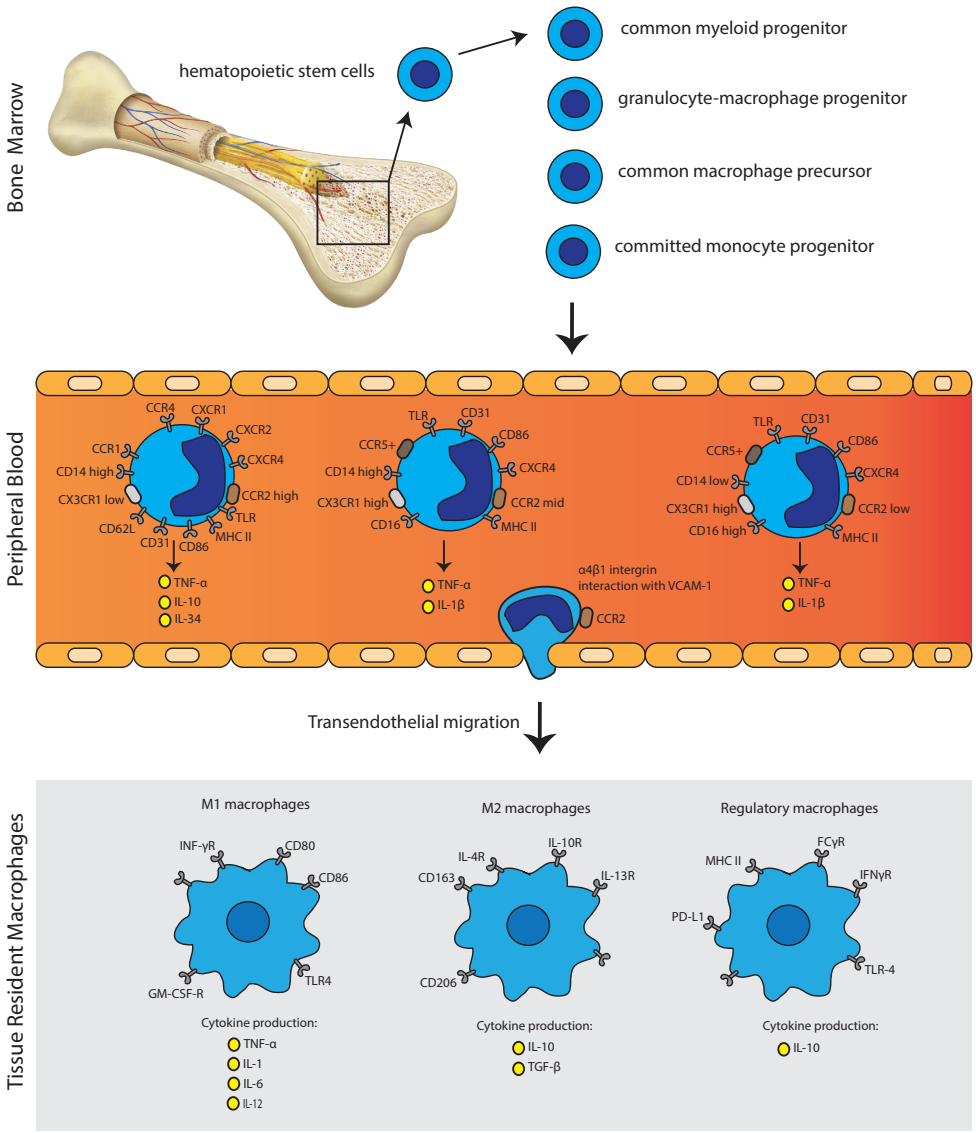

Figure 2. Monocyte immunobiology. Monocytes arise from myeloid precursor cells in primary lymphoid organs, including liver and bone marrow. In the peripheral blood, monocytes can be subdivided in three distinct subsets according to their CD14 and CD16 expression profile. Monocytes can undergo transendothelial migration through α4β1 integrin interaction with VCAM-1. Activation of monocytes is followed by the polarization of macrophages to acquire proinflammatory phenotype (M1), anti-inflammatory phenotype (M2) or the regulatory phenotype (Mreg). The secretion of distinct pro- or anti-inflammatory cytokines, next to expression patterns of surface molecules characterizes each phenotype.

The phenomenon of trained immunity in alloreactivity and transplantation may be a very interesting area of future research: i.e. innate memory towards donor antigens resulting from cross-reactivity with other microbial and/or viral agents.

rATG and basiliximab and monocyte/macrophage cell lineage

Rabbit Anti-thymocyte globulin (rATG) is a polyclonal antibody with mainly T cell depleting capacities. rATG can also induce B cell apoptosis, and stimulates Treg and NKT cell generation [54]. After rATG treatment, cytokine dependent homeostatic proliferation of T cells is initiated [55]. Basiliximab (anti CD25 monoclonal antibody) blocks the CD25 receptor on the surface of activated T cells. Studies on the effects of basiliximab or rATG on monocytes/macrophages are scarce. However, one report showed a reduction in the percentage of CD14+CD16+ monocytes when PBMC were cultured ***in vitro*** in the presence of rATG [56]. In contrast, this cell type was not affected by basiliximab, although low expression levels of CD25 on stimulated monocytes and macrophages are described [57,58]. These authors also reported a reduction of circulating CD14+CD16+ monocytes in kidney transplant patients treated with rATG during the first week after transplantation, while this was not seen for basiliximab induction therapy. Another part of the same study showed an upregulation of the percentage of CD14+CD163+ monocytes in either basiliximab or rATG -treated kidney transplant recipients, which could be detected for a longer time period in the circulation than in patients without induction therapy. CD14+CD163+ monocytes are precursors for M2 macrophages and these cells are well known for their anti-inflammatory effect, suggesting that the upregulation of CD14+CD163+ cells may contribute to a better outcome after transplantation. However, this study only described the changes in the CD14+CD16+ monocyte subset after rATG or basiliximab therapy, while the effect on other subsets such as the classical CD14++CD16- monocytes remains unknown. Therefore, it is unclear whether the pro-inflammatory immune response by monocytes is changed in the presence of rATG or basiliximab.

Alemtuzumab and monocyte/macrophage cell lineage

The humanized monoclonal antibody alemtuzumab targets the CD52 molecule which is expressed at different levels on B cells, T cells, NK cells, dendritic cells and monocytes. The CD52 molecule, also known as CAMPATH-1 antigen, is a glycoprotein of which the precise function is unclear, although it might be involved in T-cell migration and co-stimulation [59]. However, monocytes are known to be less sensitive for the depleting effects of alemtuzumab than lymphocytes, despite their high CD52 expression [60-63]. For example, in acute cellular rejection dominated by monocytes, alemtuzumab treatment did not show depletion of monocytes in tissue, confirming the low sensitivity of monocytes to alemtuzumab treatment [64]. An explanation for this low susceptibility could be the high expression levels of complement inhibitory proteins, which protect monocytes from complement mediated lysis [63]. Another study showed repopulation of monocytes within 3 months after alemtuzumab therapy, while the recovery of T and B cells takes usually more than 1 year. Consequently, the low susceptibility of monocytes for alemtuzumab is thought to be one of the reasons for renal graft dysfunction after induction therapy with alemtuzumab, such as reperfusion and rejection [65]. So far, this low susceptibility of monocytes to alemtuzumab

therapy could be partially explained by the high expression of complementary inhibitory proteins that protect monocytes from getting lysed after alemtuzumab treatment [63]. After alemtuzumab treatment, tissue monocytes in the rejecting graft showed an increased expression of the co-stimulatory molecules CD80 and CD86, a higher intracellular expression of NFκB and stronger production of IL-6 compared to patients without alemtuzumab therapy [61]. Moreover, this pro-inflammatory cytokine production could facilitate kidney allograft rejection after alemtuzumab therapy, although other cell types, such as NK cells, could also contribute to rejection processes after alemtuzumab therapy [66].

Calcineurin inhibitors and monocyte/macrophage cell lineage

Tacrolimus and cyclosporine A inhibit the calcineurin pathway in T cells, which is also present in other cell types. As a consequence, the activation of the Nuclear Factor of Activated T cells (NFAT) is blocked, leading to a reduced production of IL-2 and IFN-γ by T cells [67,68]. Calcineurin inhibitors (CNI) also have an effect on the MAPK signalling pathway via the inhibition of p38MAPK phosphorylation and consequently, reduced production of cytokines, such as IL-2, IL-10, TNF-α and IFN-γ [69]. The calcineurin and MAPK pathway are also present in macrophages, although the inhibitory effects of CNIs on T cells and macrophages are different [70]. In more detail, tacrolimus was found to have no inhibitory effect on p38MAPK phosphorylation at low (5 ng/ml) and high (50 ng/ml) concentrations in LPS-activated monocytic THP-1 leukaemia cells [71]. However, another member of the MAPK pathway, ERK, did show less phosphorylation in the presence of a high concentration (50 ng/ml) of tacrolimus in monocytes as measured by western blotting, leading to a lower production of TNF-α. Kang et al. reported that monocyte signalling pathways were activated instead of inhibited by CNI via the inhibition of the calcineurin pathway and, as a consequence, the activation of the NFκB signalling pathway [70]. However, the concentrations of CNIs used in this study were supratherapeutic. Therefore, the observed induction in cytokine production, shown in this study, could also be explained by toxic lysis of the monocytes [72]. Overall, these studies suggest that CNIs cannot supress the activation of monocytes to the same degree as in T-cells.

Recognition of damage-associated molecular patterns (DAMP's) by toll like receptors (TLR) on the surface of monocytes leads to the activation of these cells and plays an important pathogenic role during transplant rejection [73-75]. Both tacrolimus and cyclosporine can inhibit TLR signaling of PBMC in liver transplant patients, as shown by decreased production of IL-6 and TNF-α after TLR stimulation [72]. CNIs act differently in suppressing the cytokine production upon TLR activation. For example, cyclosporine inhibits the production of TNF-α mediated by TLR7/8 and the production of IL-6 mediated by TLR2 and TLR7/8 signalling significantly more than tacrolimus [72]. Moreover, monocytes from renal transplant recipients treated with tacrolimus showed an increased production of IL-1β, TNF-α, IL-6, IL-10 after stimulation with LPS, in comparison to cyclosporine treated patients [76]. Thus, the effect of CNIs on monocytes differs between tacrolimus and cyclosporine.

The different outcomes of tacrolimus and cyclosporine on cytokine production concerns only one of the monocyte/macrophage functions. Bacterial infections can have a significant impact on the graft after transplantation. Cyclosporine inhibits the phagocytosis of bacteria by macrophages via the alteration of NOD-1 expression. The NOD-1 expression depends on the activation of the transcription factor NFAT, which is the main target of CNI [77]. Thus, cyclosporine can promote bacterial infections after transplantation by altering phagocytic capacity of macrophages more rigorously.

Mycophenolate mofetil and monocyte/macrophage cell lineage

Mycophenolate mofetil (MMF) has led to significantly reduced rejection rate as compared to its counterpart azathioprine [78-80]. The active metabolite mycophenolic acid (MPA) reduces the synthesis of guanosine nucleotides via the inhibition of inosine monophosphate dehydrogenase, which is a more specific metabolic pathway for T and B cells than for other cell types [81,82] .

Circulating monocytes of kidney transplant recipients suffering from chronic rejection who were treated with MMF showed a decreased capacity to produce IL-1β, IL-10 and TNF-α as compared to circulating monocytes of chronic rejection patients who were not treated with MMF. Cytokine production capacity was measured by flow cytometry and confirmed by PCR on gene expression level [83]. Moreover, the expression of the TNF-receptor 1 was decreased in the MMF treated group, suggesting a favourable effect in patients with chronic rejection [83]. Furthermore, MMF reduced the expression of the adhesion molecules; intercellular adhesion molecule (ICAM)-1 and MHC II on isolated human monocytes [84].

Glucocorticoids and monocyte/macrophage cell lineage

The immunosuppressive and anti-inflammatory effects of glucocorticoids are redundant and cover different stages of alloreactivity triggered by activation of donor-specific T cells after transplantation. Steroids can bind via passive diffusion to the intracellular glucocorticoid receptor. After translocation to the nucleus, steroids bind to the glucocorticoid response elements (GRE's) that have a connection with promotors of different genes. The anti-inflammatory effect of glucocorticoids is based on the transrepression of inflammatory gene transcription, such as the inhibition of the transcription factors AP-1 and NFκB, and the transactivation of anti-inflammatory genes, including tyrosine aminotransferase (TAT) and the induction of IκB [85-88]. In this way, glucocorticoids control antigen presentation, cytokine production and proliferation of lymphocytes.

In monocytes, glucocorticoids specially affect the heterogeneity of monocyte subsets [89-91]. Flow cytometric analysis revealed that steroid treatment of stable kidney transplant patients for more than 12 months is associated with an increased absolute number of CD14++CD16- and CD14++CD16+ monocyte subsets compared to patients without steroid intake. As a consequence, the counts for the non-classical CD14+CD16++ monocyte subset, were significantly lower [89]. Furthermore, glucocorticoids inhibit the upregulation of

B7 molecules on the surface of human monocytes, which can negatively affect the antigen presenting function of the cell [92,93]. The B7 family consists of many peripheral membrane proteins, including CD80 and CD86, which are all involved in the co-stimulatory signal needed for T cell activation. This suggests that glucocorticoid therapy in combination with belatacept therapy (blocking CD80/CD86) could theoretically block the immune response by T cells induced via antigen presenting monocytes after transplantation.

The production of the anti-inflammatory cytokine IL-10 by monocytes is increased under treatment with methylprednisolone while the production of the pro-inflammatory cytokines IL-12, IL-1 and TNF-α are down-regulated in the presence of glucocorticoids [94,95]. Addition of 16 μg/ml of glucocorticoids ***in vitro*** leads to a decreased uptake of bacteria by monocytes, indicating that the phagocytosis of bacteria by monocytes is downregulated [96]. Glucocorticoids are also known to drive the polarization of macrophages to a M2 phenotype [43,97]. This indicates that glucocorticoids drive the cytokine production by monocyte to a more anti-inflammatory phenotype and inhibits the phagocytic function of monocytes. Glucocorticoids enhance the uptake of apoptotic cells by macrophages trough the induction of Mer-Tk (MER proto-oncogene tyrosine kinase), thereby inducing macrophage reprogramming toward a regulatory phenotype, also called Meff, for macrophages performing efferocytosis [98-100]. This approach has been evaluated in the treatment of collagen-induced arthritis (Bonnefoy F et al., Arthritis Res Ther. 2016 Aug 11;18(1):184), as well as acute graft rejection (Wang Z et al., Am J Transplant. 2006 Jun;6(6):1297-311.) justifying further exploration in the field of transplantation.

Inhibitors of the mammalian target of rapamycin (mTOR) and monocyte/macrophage cell lineage

The mammalian target of rapamycin (mTOR) signalling pathway is involved in the activation, proliferation, differentiation and translocation of T cells. Inhibitors of mTOR, such as everolimus and sirolimus, are therefore very useful after transplantation [101]. The same mTOR inhibitors do also have an inhibitory effect on human monocytes by suppressing the production of the chemokines MCP-1, RANTES, IL-8, MIP-1α and MIP-1β [102]. Furthermore, the downstream effects of rapamycin therapy are characterized by a decreased production of the monocyte-derived cytokine IL-6 and an increase of TGF-beta production in comparison to MMF, as it was shown by fine-needle biopsy cultures from kidney transplant patients treated with either a cyclosporine-rapamycin-prednisone or a cyclosporine-MMF-prednisone therapy one week after transplantation [103]. This resulted in a more tolerogenic effect of the monocytes and less graft rejection during the first 6 months after transplantation in comparison to a MMF based drug therapy. Moreover, combined therapy of mTOR inhibitors and glucocorticoid therapy increased the production of the pro-inflammatory cytokines IL-12, TNF-α and IL-1β [104]. Altogether, mTOR inhibitors can inhibit cytokine production by monocytes shortly after transplantation, although a combination therapy with prednisone should be regarded with caution.

Belatacept and monocyte/macrophage cell lineage

Belatacept, a fusion-protein consisting of the extracellular domain of the human cytotoxic T-lymphocyte antigen (CTLA)-4 antigen linked to a Fc-fragment of immunoglobulin G1 (IgG1), inhibits the co-stimulatory signal between the CD80/CD86 molecules on antigen presenting cells and the CD28 molecule on T cells, thereby preventing T cell activation [105]. Monocytes express CD80/CD86 molecules and, as a consequence, the antigen presenting function of monocytes is blocked by belatacept [106-108]. This suggests that belatacept inhibits the antigen presenting function of monocytes/macrophages. In one case of acute rejection within 3 months after transplantation, the blockade of CD80/CD86 was incomplete under belatacept treatment, suggesting the importance of higher belatacept tissue concentrations needed to completely block monocyte antigen presentation function [106]. Thus belatacept, in controlled dosages, blocks the expression of CD80/CD86 on monocytes, thereby inhibiting their antigen presenting function and activation of T cells.

The older variant of belatacept, abatacept (CTLA-4Ig), is frequently used in the treatment of patients with rheumatoid arthritis (RA) [109]. After treatment with abatacept, the number of circulating monocytes was increased, and the phenotype of these cells was significantly changed, due to down regulation of actin fibers. For example, the capability of monocyte migration was negatively changed even as the number of adhesion molecules *in vitro*. Data were verified with monocytes from healthy controls. The reduced number of adhesion molecules and migration capacity could be a reason for the increased number of monocytes in the peripheral blood that cannot pass endothelial barriers, whereby it is no longer possible for the monocyte to contribute in inflammation.

Binding of abatacept to the CD80/CD86 receptor on macrophages from healthy blood donors is associated with decreased production of the pro-inflammatory cytokines IL-12 and TNF-α, suggesting again a role for abatacept/belatacept in changing the pro-inflammatory environment via macrophages after transplantation [110].

Other experimental drugs and monocyte/macrophage cell lineage

Although no monocyte specific drugs as such exist now, multiple experimental and less known drugs do influence monocyte functions. Looking outside the box of currently used immunosuppressive drugs in solid organ transplantation, there are a few compounds with immune-inhibitory effects, which theoretically could be interesting in combating alloimmunity. For example, the human monoclonal antibody canakinumab, originally designed as an interleukin-beta (IL-1β) inhibitor for the repression of inflammation in autoimmune diseases, can also inhibit the IL-1β production by monocytes [111]. A high expression of IL-1β is noticed in the most severe liver transplant rejection episodes and at the time of kidney transplantation, suggesting the importance of blocking its production by monocytes [112,113]. However, treatment of kidney transplant recipients with canakinumab can inhibit IL-1β secretion in many other cell types, leading to undesirable side effects [114].

Infliximab, originally used in the treatment of autoimmune diseases, is another monoclonal antibody targeting monocyte TNF-α production. Monocytes and macrophages are main producers of TNF-α, suggesting the importance of infliximab for targeting monocytes [115]. Beside the effect on TNF-α production, monocytes from Crohn's disease patients treated with therapeutic concentrations of infliximab showed also increased apoptosis via the activation of caspase-3, 8 and 9 [116].

Furthermore, the herbal medicine sinomenine was found to reduce migration of activated human monocyte cells and inhibits human monocytes-derived DC differentiation and maturation [71,117]. In addition, peripheral blood monocytes from healthy donors cultured for 60 hours in the presence of different concentrations of sinomenine showed an enhanced production of IL-6 and a decreased expression of IL-8, which is important for cell migration [118]. This would suggest a positive effect of sinomenine on monocyte infiltration and migration, although there is still an increased production of pro-inflammatory cytokines. However, this research was performed using monocytic THP-1 cell-line, and isolated peripheral blood monocytes from healthy donors, so that possible effects with regard to transplantation are still unknown.

15-deoxyspergualin or gusperimus is a relatively long known immunosuppressive drug with an inhibitory effect on monocyte proliferation, TNF-α production and phagocytotic functions of monocytes. More recently, it was been suggested that gusperimus can also be effective in suppressing the antigen presentation function of monocytes in transplantation [119]. Another member of the spergualin family is LF15-0195. This drug is known for its inhibitory effect on monocyte accumulation in the tubulo-interstitial compartment of rat kidneys and was shown to have beneficial effects in the treatment of glomerulonephritis [120].

In diabetes mellitus, macrophage accumulation and activation play a central role in disease progression. Research on simvastatin, a drug to lower elevated lipid levels, has been shown to effectively lower IL-6, IL-8, TNF cytokine and superoxide anion production by monocytes isolated from human blood samples of patients with diabetes mellitus type 1 [121]. In addition, simvastatin reduces the NFκB activity in monocytes with approximately 60%, which causes the inhibition of IL-6 and IL-8 production. Treatment of IgA nephropathy with the drug atorvastatin showed a reduction of monocyte proliferation [122]. In diabetes mellitus type 2 patients this drug lowers the TNF-alpha production by monocytes [123]. Other studies in diabetes mellitus patients have shown potential effects of salsalate on macrophages activation. Salsalate, a prodrug of salicylic acid, is also known for the inhibition of the NFκB pathway in macrophages [124,125]. This suggests a working mechanism for salsalate that is similar to simvastatin. Both drugs can be promising compounds to inhibit monocyte and macrophage activation.

In RA, research on therapeutic drugs to target monocytes and macrophages is more common because of the important role of monocytes in developing this disease. In addition, TNF-α is a key player known to cause inflammation in RA and is mainly produced by monocytes [126]. Some of the drugs used to suppress inflammation in RA could also

have a potential in transplantation. For example, a decreased number of CD14+ CD16+ monocytes was found after treatment of RA patients with tocilizumab, an IL-6 receptor blocker [127]. In addition, production of IL-6 by monocytes from healthy donors was reduced when tocilizumab was added ***in vitro***. The drug also induces the apoptosis of SEB (staphylococcal enterotoxin B)-activated monocytes [128]. These results suggest that tocilizumab could theoretically impair the monocyte responses after transplantation. Furthermore, bromodomain and extra terminal (BET) inhibitors are developed to control the intracellular chromatin regulation responsible for the activation of monocytes, thereby inhibiting inflammation processes induced by monocytes. In more detail, CD14+ monocytes were isolated from blood samples of healthy volunteers and cultured in the presence of BET inhibitors and IFN-β, IFN-γ, IL-4 and IL-10 stimuli, where after the intracellular activation cytokine response were suppressed [129]. In RA patients this epigenetic control by BET inhibitors could suppress the production of pro-inflammatory cytokines and chemokines such as CXCL10. This would indicate that BET inhibitors could also inhibit monocyte activation after transplantation, although this is very speculative and require more research.

Fish oil based drugs, such as lovaza, are used to lower triglyceride levels in obesity. These fish oil compounds demonstrated a reduction in the number of macrophages and reduced MCP-1 blood levels [130]. Eicosapentaenoic acid, one of the major fatty acids in fish oil, reduces the secretion of TNF-α by human monocytic THP-1 cells, via the inhibition of the intracellular NFκB activation [131]. This suggests also a suppressing role for fatty acids in monocyte activation that could have a potential effect in transplantation as well.

Future challenges and developments

Therapies targeting monocytes and macrophages in (SOT) could intervene at different points with monocyte actions and their subsequent functions (**Figure 3**). First, the activation and function of the cells can be inhibited at multiple stages: Signaling pathway activation, antigen presentation and cytokine production. Blockade of the intracellular signaling pathways inhibits the activation of monocytes and macrophages. For example, the use of specific MAPK inhibitors, such as SB203580, blocks the activation of monocytes [132]. However, these drugs will also block the activation of many other cell types. Targeting antigen presentation is even more difficult than targeting signaling pathways. It is known that the Fcγ-receptor on monocytes is involved in the recognition and processing of donor antigen specific antibodies [133,134]. Blocking this receptor with specific antibodies could inhibit the antigen presentation function of monocytes. Furthermore, already existing drugs that reduce the cytokine production by monocytes and macrophages, for example canakinumab, infliximab and tocilizumab, mainly target the inhibition of one single cytokine. To be more effective, monocyte specific drugs should be developed to inhibit the production or the effects of multiple cytokines at once, thereby reducing side effects.

Second, delivering any potential new drug to the target cell, in this case, monocytes and macrophages, is a major point of intervention, which could lower the side effects. One can

envision a delivery system using the phagocytosis function of the monocyte/macrophage, whereby macrophages can ingest immunosuppressive drug loaded-inactivated bacteria or liposomes carrying the potential new drug [135]. However, the monocyte is not the only cell type with a phagocytic system. Therefore the surface of these bacteria or liposomes should be modified to facilitate the specific recognition by the monocyte/macrophage in order to overcome side effects. Another approach to target monocytes and macrophages via their phagocytotic function is to use apoptotic cells through a process that is known as efferocytosis [99,100]. Phagocytosis of these apoptotic cells by monocytes and macrophages will induce an anti-inflammatory response at the tissue level and may induce immunological tolerance. Furthermore, ***ex vivo*** experiments showed a decrease in CD11b expression on macrophages [136], suggesting that treatment with apoptotic cells induces the generation of Mregs. As mentioned above (paragraph "Glucocorticoids and monocyte/macrophage cell lineage"), the uptake of apoptotic cells can be enhanced by treatment with glucocorticoids [100].

The third point of therapeutic efficacy would be the manipulation of the nature of these cells. The future of ***in vivo*** manipulation of macrophages is intriguing; phenotypes could be changed by transfection with adenovirus, modulation of nuclear transcription factor NR4A1 (Nur77) or by modulation of local microenvironment with cytokines to polarize

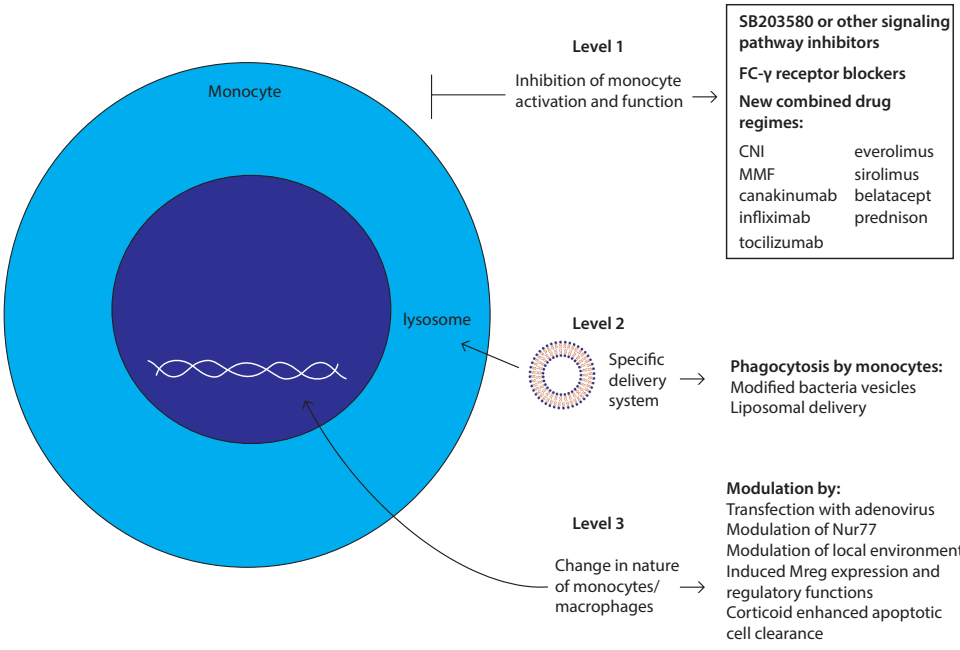

Figure 3. Future challenges and developments: Strategies to target monocytes/macrophages. New therapies targeting monocytes and macrophages could intervene at three levels with monocyte actions and their subsequent functions as depicted, and described in manuscript body.

macrophages to reparative phenotype [35]. Targeting all monocytes and macrophages indiscriminately could also be a disadvantage as regulatory and effector macrophages also have beneficial effects including the control of infections and the induction of regulatory cells [137].

Moreover, inhibition of all macrophages will also affect the number of Mregs, which are important for inducing tolerance after transplantation [138]. Too much inhibition of effector macrophages or Mregs could lead to graft rejection or complications, such as atherosclerosis and cardiovascular diseases. Furthermore, currently pre-scribed immunosuppressive drugs might miss the power to upregulate Mregs efficiently. In experimental mouse models, Mregs have demonstrated anti-inflammatory and T-cell suppressing effects (Other beneficial effects of Mregs are described in the paragraph "Monocyte immunobiology") [139,140]. A more specific upregulation of these cells could be an approach to beneficially shift the balance towards macrophages controlling immune responses including those in organ transplant patients. Ideally, after SOT, the balance of macrophage subsets should be in favour of macrophages that control the anti-donor response, while the accumulation of macrophages with pro-inflammatory and antigen presentation characteristics should be decreased [141,142]. For example reduced function of the detrimental functions of macrophages involved in alloreactivity might be a useful therapy, although more research is needed to find a specific approach. Another way to differentiate between effector and controlling functions of macrophages could be by polarizing cells into M1 and M2 subsets. Targeting specific signaling pathways involved in this polarization process like the Notch signaling pathway could change the nature of these cells to an more anti-inflammatory phenotype [143]. NFκB signaling, controlled by the Notch pathway, is associated with pro-inflammatory macrophage responses, while a more anti-inflammatory phenotype is induced via the ERK pathway [143,144]. Targeting these pathways with specific stimuli may change the phenotype of macrophages. Stimuli that induce macrophage polarization towards a M1 phenotype are GM-CSF, IFN-γ and LPS, while IL-4, IL-13 and IL-10 enhance a M2 macrophage phenotype [145]. Future insight and research are necessary to investigate the effect of these manipulated macrophages on healthy and diseased tissue.

Ideally, a potential new drug inhibiting monocytes-macrophages at these three levels would change the spectrum of not only rejection treatment or prevention after (SOT) but also the course of many autoimmune mediated diseases. Either alone or in combination with other existing immunosuppressive drugs, this field constitutes a challenging area of future therapeutic research.

References

1. Koo EH, Jang HR, Lee JE, et al. The impact of early and late acute rejection on graft survival in renal transplantation. Kidney Res Clin Pract 2015;34:160-4.
2. Sellares J, de Freitas DG, Mengel M, et al. Understanding the causes of kidney transplant failure: the dominant role of antibody-mediated rejection and nonadherence. Am J Transplant 2012;12:388-99.
3. Einecke G, Sis B, Reeve J, et al. Antibody-mediated microcirculation injury is the major cause of late kidney transplant failure. Am J Transplant 2009;9:2520-31.
4. Meier-Kriesche HU, Schold JD, Srinivas TR, Kaplan B. Lack of improvement in renal allograft survival despite a marked decrease in acute rejection rates over the most recent era. Am J Transplant 2004;4:378-83.
5. Nankivell BJ, Alexander SI. Rejection of the kidney allograft. N Engl J Med 2010;363:1451-62.
6. Mannon RB. Macrophages: Contributors to Allograft Dysfunction, Repair or Innocent Bystanders? Curr Opin Organ Transplant 2012;17:20-5.
7. Rowshani AT, Vereyken EJF. The Role of Macrophage Lineage Cells in Kidney Graft Rejection and Survival. Transplantation 2012;94:309-18.
8. Girlanda R, Kleiner DE, Duan Z, et al. Monocyte Infiltration and Kidney Allograft Dysfunction During Acute Rejection. Am J Transplant 2008;8:600.
9. van Kooten C, Daha MR. Cytokine cross-talk between tubular epithelial cells and interstitial immunocompetent cells. Curr Opin Nephrol Hypertens 2001;10:55-9.
10. Moreau A, Varey E, Anegon I, Cuturi MC. Effector Mechanisms of Rejection. Cold Spring Harb Perspect Med 2013;3.
11. Toki D, Zhang W, Hor KLM, et al. The Role of Macrophages in the Development of Human Renal Allograft Fibrosis in the First Year After Transplantation. Am J Transplant 2014;14:2126-36.
12. Sentís A, Kers J, Yapici U, et al. The prognostic significance of glomerular infiltrating leukocytes during acute renal allograft rejection. Transpl Immunol 2015;33:168-75.
13. Kwan T, Wu H, Chadban SJ. Macrophages in renal transplantation: Roles and therapeutic implications. Cell Immunol 2014;291:58-64.
14. Kozakowski N, Böhmig GA, Exner M, et al. Monocytes/macrophages in kidney allograft intimal arteritis: no association with markers of humoral rejection or with inferior outcome. Nephrol Dial Transplant 2009;24:1979-86.
15. Tinckam KJ, Djurdjev O, Magil AB. Glomerular monocytes predict worse outcomes after acute renal allograft rejection independent of C4d status. Kidney Int 2005;68:1866-74.
16. Fahim T, Böhmig GA, Exner M, et al. The Cellular Lesion of Humoral Rejection: Predominant Recruitment of Monocytes to Peritubular and Glomerular Capillaries. Am J Transplant 2007;7:385-93.
17. Özdemir BH, Demirhan B, Güngen Y. The Presence and Prognostic Importance of Glomerular Macrophage Infiltration in Renal Allografts. Nephron 2002;90:442-6.

18. Grimm PC, McKenna R, Nickerson P, et al. Clinical Rejection Is Distinguished from Subclinical Rejection by Increased Infiltration by a Population of Activated Macrophages. Clin J Am Soc Nephrol 1999;10:1582-9.
19. Bergler T, Jung B, Bourier F, et al. Infiltration of Macrophages Correlates with Severity of Allograft Rejection and Outcome in Human Kidney Transplantation. PLoS One 2016;11:e0156900.
20. Om A BA, Raja R, Kim P, Bannett AD. The prognostic significance of the presence of monocytes in glomeruli of renal transplant allografts. Transplant Proc 1987;19:1618-22.
21. Copin MC, Noel C, Hazzan M, et al. Diagnostic and predictive value of an immunohistochemical profile in asymptomatic acute rejection of renal allografts. Transpl Immunol 1995;3:229-39.
22. Xu L, Collins J, Drachenberg C, KuKuruga D, Burke A. Increased macrophage density of cardiac allograft biopsies is associated with antibody-mediated rejection and alloantibodies to HLA antigens. Clin Transplant 2014;28:554-60.
23. Fishbein GA, Fishbein MC. Morphologic and immunohistochemical findings in antibody-mediated rejection of the cardiac allograft. Hum Immunol 2012;73:1213-7.
24. Oberbarnscheidt MH, Zeng Q, Li Q, et al. Non-self recognition by monocytes initiates allograft rejection. J Clin Invest 2014;124:3579-89.
25. Boersema M, van den Born JC, van Ark J, et al. CD16+ monocytes with smooth muscle cell characteristics are reduced in human renal chronic transplant dysfunction. Immunobiology 2015.
26. Vereyken EJF, Kraaij MD, Baan CC, et al. A Shift towards Pro-Inflammatory CD16+ Monocyte Subsets with Preserved Cytokine Production Potential after Kidney Transplantation. PLoS One 2013;8:e70152.
27. Kraaij MD, Vereyken EJF, Leenen PJM, et al. Human monocytes produce interferon-gamma upon stimulation with LPS. Cytokine 2014;67:7-12.
28. Ziegler-Heitbrock L. Blood monocytes and their subsets: established features and open questions. Front Immunol 2015;6.
29. Ziegler-Heitbrock L. Monocyte subsets in man and other species. Cell Immunol 2014;289:135-9.
30. Ziegler-Heitbrock L, Hofer TP. Toward a refined definition of monocyte subsets. Front Immunol 2013;4:23.
31. Wong KL, Yeap WH, Tai JJ, Ong SM, Dang TM, Wong SC. The three human monocyte subsets: implications for health and disease. Immunol Res 2012;53:41-57.
32. van Furth R, Sluiter W. Distribution of blood monocytes between a marginating and a circulating pool. J Exp Med 1986;163:474-9.
33. Swirski FK, Nahrendorf M, Etzrodt M, et al. Identification of Splenic Reservoir Monocytes and Their Deployment to Inflammatory Sites. Science 2009;325:612-6.
34. Terry RL, Miller SD. Molecular control of monocyte development. Cell Immunol 2014;291:16-21.

35. Hanna RN, Carlin LM, Hubbeling HG, et al. The transcription factor NR4A1 (Nur77) controls bone marrow differentiation and the survival of Ly6C- monocytes. Nat Immunol 2011;12:778-85.

36. Ghattas A, Griffiths HR, Devitt A, Lip GYH, Shantsila E. Monocytes in Coronary Artery Disease and Atherosclerosis: Where Are We Now? J Am Coll Cardiol 2013;62:1541-51.

37. Frankenberger M, Hofer TPJ, Marei A, et al. Transcript profiling of CD16-positive monocytes reveals a unique molecular fingerprint. Eur J Immunol 2012;42:957-74.

38. Brooks CF, Moore M. Differential MHC class II expression on human peripheral blood monocytes and dendritic cells. Immunology 1988;63:303-11.

39. Ginhoux F, Jung S. Monocytes and macrophages: developmental pathways and tissue homeostasis. Nat Rev Immunol 2014;14:392-404.

40. Yona S, Kim K-W, Wolf Y, et al. Fate Mapping Reveals Origins and Dynamics of Monocytes and Tissue Macrophages under Homeostasis. Immunity 2013;38:79-91.

41. Hashimoto D, Chow A, Noizat C, et al. Tissue-Resident Macrophages Self-Maintain Locally throughout Adult Life with Minimal Contribution from Circulating Monocytes. Immunity 2013;38:792-804.

42. W. Prodjosudjadi MRD, J. S. J. Gerritsma, K. W. Florijn, J. N. M. Barendregt , J. A.Bruijn, F.J. van der Woude and L.A. van Es. Increased urinary excretion of monocyte ehemoattractant protein-1 during acute renal allograft rejection. Nephrol Dial Transplant 1996;11:7.

43. Martinez FO, Gordon S. The M1 and M2 paradigm of macrophage activation: time for reassessment. F1000Prime Rep 2014;6:13.

44. Hutchinson JA, Riquelme P, Sawitzki B, et al. Cutting Edge: Immunological Consequences and Trafficking of Human Regulatory Macrophages Administered to Renal Transplant Recipients. J Immunol 2011;187:2072-8.

45. Fleming BD, Mosser DM. Regulatory macrophages: Setting the Threshold for Therapy. Eur J Immunol 2011;41:2498-502.

46. Hutchinson JA, Riquelme P, Brem-Exner BG, et al. Transplant acceptance-inducing cells as an immune-conditioning therapy in renal transplantation. Transpl Int 2008;21:728-41.

47. Hutchinson JA, Brem-Exner BG, Riquelme P, et al. A cell-based approach to the minimization of immunosuppression in renal transplantation. Transpl Int 2008;21:742-54.

48. D'Aveni M, Rossignol J, Coman T, et al. G-CSF mobilizes CD34+ regulatory monocytes that inhibit graft-versus-host disease. Sci Transl Med 2015;7:281ra42-ra42.

49. Netea MG, Joosten LAB, Latz E, et al. Trained immunity: A program of innate immune memory in health and disease. Science 2016;352.

50. Kleinnijenhuis J, Quintin J, Preijers F, et al. Bacille Calmette-Guérin induces NOD2-dependent nonspecific protection from reinfection via epigenetic reprogramming of monocytes. Proc Natl Acad Sci U S A 2012;109:17537-42.

51. Ostuni R, Piccolo V, Barozzi I, et al. Latent Enhancers Activated by Stimulation in Differentiated Cells. Cell 2013;152:157-71.

52. Quintin J, Saeed S, Martens Joost HA, et al. Candida albicans Infection Affords Protection against Reinfection via Functional Reprogramming of Monocytes. Cell Host & Microbe 2012;12:223-32.
53. Yoshida K, Ishii S. Innate immune memory via ATF7-dependent epigenetic changes. Cell Cycle 2016;15:3-4.
54. Mohty M. Mechanisms of action of antithymocyte globulin: T-cell depletion and beyond. Leukemia 2007;21:1387-94.
55. Bouvy AP, Kho MM, Klepper M, et al. Kinetics of homeostatic proliferation and thymopoiesis after rATG induction therapy in kidney transplant patients. Transplantation 2013;96:904-13.
56. Sekerkova A, Krepsova E, Brabcova E, et al. CD14+CD16+ and CD14+CD163+ monocyte subpopulations in kidney allograft transplantation. BMC Immunol 2014;15:4-.
57. Bosco MC, Espinoza-Delgado I, Schwabe M, et al. Regulation by interleukin-2 (IL-2) and interferon gamma of IL-2 receptor gamma chain gene expression in human monocytes. Blood 1994;83:2995.
58. Valitutti S, Carbone A, Castellino F, et al. The expression of functional IL-2 receptor on activated macrophages depends on the stimulus applied. Immunology 1989;67:44-50.
59. Hu Y, Turner MJ, Shields J, et al. Investigation of the mechanism of action of alemtuzumab in a human CD52 transgenic mouse model. Immunology 2009;128:260-70.
60. Hale G, Xia M-Q, Tighe HP, Dyer MJS, Waldmann H. The CAMPATH-1 antigen (CDw52). Tissue Antigens 1990;35:118-27.
61. Kirk AD, Hale DA, Mannon RB, et al. Results from a human renal allograft tolerance trial evaluating the humanized CD52-specific monoclonal antibody alemtuzumab (CAMPATH-1H). Transplantation 2003;76:120-9.
62. Fabian I, Flidel O, Gadish M, Kletter Y, Slavin S, Nagler A. Effects of CAMPATH-1 antibodies on the functional activity of monocytes and polymorphonuclear neutrophils. Exp Hematol 1993;21:1522-7.
63. Rao SP, Sancho J, Campos-Rivera J, et al. Human Peripheral Blood Mononuclear Cells Exhibit Heterogeneous CD52 Expression Levels and Show Differential Sensitivity to Alemtuzumab Mediated Cytolysis. PLoS One 2012;7:e39416.
64. Zhang PL, Malek SK, Prichard JW, et al. Acute cellular rejection predominated by monocytes is a severe form of rejection in human renal recipients with or without Campath-1H (alemtuzumab) induction therapy. Am J Transplant 2005;5:604-7.
65. Bloom D, Chang Z, Pauly K, et al. BAFF Is Increased in Renal Transplant Patients Following Treatment with Alemtuzumab. Am J Transplant 2009;9:1835-45.
66. Lenihan CR, Tan JC, Kambham N. Acute transplant glomerulopathy with monocyte rich infiltrate. Transpl Immunol 2013;29:114-7.
67. Liu J, Farmer JD, Jr., Lane WS, Friedman J, Weissman I, Schreiber SL. Calcineurin is a common target of cyclophilin-cyclosporin A and FKBP-FK506 complexes. Cell 1991;66:807-15.
68. Fruman DA, Klee CB, Bierer BE, Burakoff SJ. Calcineurin phosphatase activity in T lymphocytes is inhibited by FK 506 and cyclosporin A. Proc Natl Acad Sci U S A 1992;89:3686-90.

69. Vafadari R, Hesselink DA, Cadogan MM, Weimar W, Baan CC. Inhibitory effect of tacrolimus on p38 mitogen-activated protein kinase signaling in kidney transplant recipients measured by whole-blood phosphospecific flow cytometry. Transplantation 2012;93:1245-51.

70. Kang YJ, Kusler B, Otsuka M, et al. Calcineurin Negatively Regulates TLR-Mediated Activation Pathways. J Immunol 2007;179:4598-607.

71. Ou Y-q, Chen L-h, Li X-j, Lin Z-b, Li W-d. Sinomenine influences capacity for invasion and migration in activated human monocytic THP-1 cells by inhibiting the expression of MMP-2, MMP-9, and CD147. Acta Pharmacol Sin 2009;30:435-41.

72. Howell J, Sawhney R, Testro A, et al. Cyclosporine and tacrolimus have inhibitory effects on toll-like receptor signaling after liver transplantation. Liver Transpl 2013;19:1099-107.

73. Zhuang Q, Lakkis FG. Dendritic cells and innate immunity in kidney transplantation. Kidney Int 2015;87:712-8.

74. Rao DA, Pober JS. Endothelial injury, alarmins, and allograft rejection. Crit Rev Immunol 2008;28:229-48.

75. Kono H, Rock KL. How dying cells alert the immune system to danger. Nat Rev Immunol 2008;8:279-89.

76. Weimer R, Melk A, Daniel V, Friemann S, Padberg W, Opelz G. Switch from cyclosporine A to tacrolimus in renal transplant recipients: impact on Th1, Th2, and monokine responses. Hum Immunol 2000;61:884-97.

77. Tourneur E, Ben Mkaddem S, Chassin C, et al. Cyclosporine A Impairs Nucleotide Binding Oligomerization Domain (Nod1)-Mediated Innate Antibacterial Renal Defenses in Mice and Human Transplant Recipients. PLoS Pathog 2013;9:e1003152.

78. Zuckermann A, Klepetko W, Birsan T, et al. Comparison between mycophenolate mofetil- and azathioprine-based immunosuppressions in clinical lung transplantation. J Heart Lung Transplant 1999;18:432-40.

79. Rigotti P, Cadrobbi R, Baldan N, et al. Mycophenolate mofetil (MMF) versus azathioprine (AZA) in pancreas transplantation: a single-center experience. Clin Nephrol 2000;53:suppl 52-4.

80. van Gelder T, Hesselink DA. Mycophenolate revisited. Transpl Int 2015;28:508-15.

81. Allison AC, Eugui EM. Mycophenolate mofetil and its mechanisms of action. Immunopharmacology 2000;47:85-118.

82. Allison AC, Eugui EM. Immunosuppressive and other effects of mycophenolic acid and an ester prodrug, mycophenolate mofetil. Immunol Rev 1993;136:5-28.

83. Weimer RM, Joannis; Feustel, Andreas; Preiss, Astrid; Daniel, Volker; Grimm, Helmut; Wiesel, Manfred; Opelz, Gerhard. Mycophenolate mofetil-based immunosuppression and cytokine genotypes: effects on monokine secretion and antigen presentation in long-term renal transplant recipients. Transplantation 2003;75:2090-9.

84. Glomsda BA, Blaheta RA, Hailer NP. Inhibition of monocyte//endothelial cell interactions and monocyte adhesion molecule expression by the immunosuppressant mycophenolate mofetil. Spinal Cord 2003;41:610-9.

85. Newton R, Holden NS. Separating transrepression and transactivation: a distressing divorce for the glucocorticoid receptor? Mol Pharmacol 2007;72:799-809.

86. Jantzen HM, Strahle U, Gloss B, et al. Cooperativity of glucocorticoid response elements located far upstream of the tyrosine aminotransferase gene. Cell 1987;49:29-38.

87. Rigaud G, Roux J, Pictet R, Grange T. In vivo footprinting of rat TAT gene: dynamic interplay between the glucocorticoid receptor and a liver-specific factor. Cell 1991;67:977-86.

88. Cato AC, Wade E. Molecular mechanisms of anti-inflammatory action of glucocorticoids. Bioessays 1996;18:371-8.

89. Rogacev KS, Zawada AM, Hundsdorfer J, et al. Immunosuppression and monocyte subsets. Nephrol Dial Transplant 2015;30:143-53.

90. Orii M, Imanishi T, Teraguchi I, et al. Circulating CD14++CD16+ Monocyte Subsets as a Surrogate Marker of the Therapeutic Effect of Corticosteroid Therapy in Patients With Cardiac Sarcoidosis. Circ J 2015;79:1585-92.

91. Sumegi A, Antal-Szalmas P, Aleksza M, et al. Glucocorticosteroid therapy decreases CD14- expression and CD14-mediated LPS-binding and activation of monocytes in patients suffering from systemic lupus erythematosus. Clin Immunol 2005;117:271-9.

92. Girndt M, Sester U, Kaul H, Hünger F, Köhler H. Glucocorticoids inhibit activation-dependent expression of costimulatory molecule B7-1 in human monocytes. Transplantation 1998;66:370-5.

93. Jirapongsananuruk O, Leung DY. The modulation of B7.2 and B7.1 on B cells by immunosuppressive agents. Clin Exp Immunol 1999;118:1-8.

94. Hodge G, Hodge S, Reynolds PN, Holmes M. Up-regulation of Interleukin-8, Interleukin-10, Monocyte Chemotactic Protein-1, and Monocyte Chemotactic Protein-3 in Peripheral Blood Monocytes in Stable Lung Transplant Recipients: Are Immunosuppression Regimens Working? Transplantation 2005;79:387-91.

95. Blotta MH, DeKruyff RH, Umetsu DT. Corticosteroids inhibit IL-12 production in human monocytes and enhance their capacity to induce IL-4 synthesis in CD4+ lymphocytes. J Immunol 1997;158:5589-95.

96. Rinehart JJ, Balcerzak SP, Sagone AL, LoBuglio AF. Effects of corticosteroids on human monocyte function. J Clin Invest 1974;54:1337-43.

97. Italiani P, Boraschi D. From Monocytes to M1/M2 Macrophages: Phenotypical vs. Functional Differentiation. Front Immunol 2014;5:514.

98. Schif-Zuck S, Gross N, Assi S, Rostoker R, Serhan CN, Ariel A. Saturated-efferocytosis generates pro-resolving CD11b low macrophages: modulation by resolvins and glucocorticoids. Eur J Immunol 2011;41:366-79.

99. Saas P, Daguindau E, Perruche S. Concise Review: Apoptotic Cell-Based Therapies-Rationale, Preclinical Results and Future Clinical Developments. Stem Cells 2016;34:1464-73.

100. Poon IK, Lucas CD, Rossi AG, Ravichandran KS. Apoptotic cell clearance: basic biology and therapeutic potential. Nat Rev Immunol 2014;14:166-80.

101. Shipkova M, Hesselink DA, Holt DW, et al. Therapeutic Drug Monitoring of Everolimus: A Consensus Report. Ther Drug Monit 2016;38:143-69.
102. Lin HY-H, Chang K-T, Hung C-C, et al. Effects of the mTOR inhibitor Rapamycin on Monocyte-Secreted Chemokines. BMC Immunol 2014;15:1-9.
103. Oliveira JGG, Xavier P, Sampaio SM, et al. Compared to mycophenolate mofetil, rapamycin induces significant changes on growth factors and growth factor receptors in the early days postkidney transplantation1. Transplantation 2002;73:915-20.
104. Weichhart T, Haidinger M, Katholnig K, et al. Inhibition of mTOR blocks the anti-inflammatory effects of glucocorticoids in myeloid immune cells. Blood 2011;117:4273-83.
105. Graav GNd, Bergan S, Baan CC, Weimar W, van Gelder T, Hesselink DA. Therapeutic Drug Monitoring of Belatacept in Kidney Transplantation. Ther Drug Monit 2015;37:560-7.
106. Graav GNd, Hesselink DA, Dieterich M, et al. An Acute Cellular Rejection With Detrimental Outcome Occurring Under Belatacept-Based Immunosuppressive Therapy: An Immunological Analysis. Transplantation 2015;Online First.
107. Latek R, Fleener C, Lamian V, et al. Assessment of Belatacept-Mediated Costimulation Blockade Through Evaluation of CD80/86-Receptor Saturation. Transplantation 2009;87:926-33.
108. Ford ML, Adams AB, Pearson TC. Targeting co-stimulatory pathways: transplantation and autoimmunity. Nat Rev Nephrol 2014;10:14-24.
109. Bonelli M, Ferner E, Goschl L, et al. Abatacept (CTLA-4IG) treatment reduces the migratory capacity of monocytes in patients with rheumatoid arthritis. Arthritis Rheum 2013;65:599-607.
110. Wenink MH, Santegoets KCM, Platt AM, et al. Abatacept modulates proinflammatory macrophage responses upon cytokine-activated T cell and Toll-like receptor ligand stimulation. Ann Rheum Dis 2011.
111. Dhimolea E. Canakinumab. mAbs 2010;2:3-13.
112. Hoffmann MW, Wonigeit K, Steinhoff G, Herzbeck H, Flad HD, Pichlmayr R. Production of cytokines (TNF-alpha, IL-1-beta) and endothelial cell activation in human liver allograft rejection. Transplantation 1993;55:329-35.
113. Vereyken EJ, Kraaij MD, Baan CC, et al. A shift towards pro-inflammatory CD16+ monocyte subsets with preserved cytokine production potential after kidney transplantation. PLoS One 2013;8:e70152.
114. Wanderer AA. Rationale and timeliness for IL-1β-targeted therapy to reduce allogeneic organ injury at procurement and to diminish risk of rejection after transplantation. Clin Transplant 2010;24:307-11.
115. Ebert EC. Infliximab and the TNF-α system. Am J Physiol Gastrointest Liver Physiol 2009;296:G612-G20.
116. Lugering A, Schmidt M, Lugering N, Pauels HG, Domschke W, Kucharzik T. Infliximab induces apoptosis in monocytes from patients with chronic active Crohn's disease by using a caspase-dependent pathway. Gastroenterology 2001;121:1145-57.

117. Wang Q, Li X-K. Immunosuppressive and anti-inflammatory activities of sinomenine. Int Immunopharmacol 2011;11:373-6.

118. Shenghao T, Yonghong H, Fu'er L. Effect of sinomenine on IL-8, IL-6, IL-2 produced by peripheral blood mononuclear cells. J Tongji Med Univ 1999;19:257-9.

119. Perenyei M, Jayne DR, Flossmann O. Gusperimus: immunological mechanism and clinical applications. Rheumatology 2014;53:1732-41.

120. Tesch GH, Hill PA, Wei M, Nikolic-Paterson DJ, Dutartre P, Atkins RC. LF15-0195 prevents the induction and inhibits the progression of rat anti-GBM disease. Kidney Int 2001;60:1354-65.

121. Jialal I, Miguelino E, Griffen SC, Devaraj S. Concomitant reduction of low-density lipoprotein-cholesterol and biomarkers of inflammation with low-dose simvastatin therapy in patients with type 1 diabetes. J Clin Endocrinol Metab 2007;92:3136-40.

122. Lundberg S, Lundahl J, Gunnarsson I, Jacobson SH. Atorvastatin-induced modulation of monocyte respiratory burst in vivo in patients with IgA nephropathy: a chronic inflammatory kidney disease. Clin Nephrol 2010;73:221-8.

123. Mandosi E, Fallarino M, Gatti A, et al. Atorvastatin downregulates monocyte CD36 expression, nuclear NFkappaB and TNFalpha levels in type 2 diabetes. J Atheroscler Thromb 2010;17:539-45.

124. Donath MY, Shoelson SE. Type 2 diabetes as an inflammatory disease. Nat Rev Immunol 2011;11:98-107.

125. McCarty MF. Salsalate may have broad utility in the prevention and treatment of vascular disorders and the metabolic syndrome. Med Hypotheses 2010;75:276-81.

126. Davignon J-L, Hayder M, Baron M, et al. Targeting monocytes/macrophages in the treatment of rheumatoid arthritis. Rheumatology 2012.

127. Kikuchi J, Hashizume M, Kaneko Y, Yoshimoto K, Nishina N, Takeuchi T. Peripheral blood CD4(+)CD25(+)CD127(low) regulatory T cells are significantly increased by tocilizumab treatment in patients with rheumatoid arthritis: increase in regulatory T cells correlates with clinical response. Arthritis Res Ther 2015;17:10.

128. Tono T, Aihara S, Hoshiyama T, Arinuma Y, Nagai T, Hirohata S. Effects of anti-IL-6 receptor antibody on human monocytes. Mod Rheumatol 2015;25:79-84.

129. Chan CH, Fang C, Qiao Y, Yarilina A, Prinjha RK, Ivashkiv LB. BET bromodomain inhibition suppresses transcriptional responses to cytokine-Jak-STAT signaling in a gene-specific manner in human monocytes. Eur J Immunol 2015;45:287-97.

130. Spencer M, Finlin BS, Unal R, et al. Omega-3 fatty acids reduce adipose tissue macrophages in human subjects with insulin resistance. Diabetes 2013;62:1709-17.

131. Zhao Y, Joshi-Barve S, Barve S, Chen LH. Eicosapentaenoic acid prevents LPS-induced TNF-alpha expression by preventing NF-kappaB activation. J Am Coll Nutr 2004;23:71-8.

132. Kaminska B. MAPK signalling pathways as molecular targets for anti-inflammatory therapy--from molecular mechanisms to therapeutic benefits. Biochim Biophys Acta 2005;1754:253-62.

133. Gosselin J, Flamand L, D'Addario M, et al. Modulatory effects of Epstein-Barr, herpes simplex, and human herpes-6 viral infections and coinfections on cytokine synthesis. A comparative study. J Immunol 1992;149:181-7.

134. Castro-Dopico T, Clatworthy MR. Fcγ Receptors in Solid Organ Transplantation. Curr Transplant Rep 2016:1-10.

135. Kelly C, Jefferies C, Cryan S-A. Targeted Liposomal Drug Delivery to Monocytes and Macrophages. J Drug Deliv 2011;2011:11.

136. Pashover-Schallinger E, Aswad M, Schif-Zuck S, Shapiro H, Singer P, Ariel A. The atypical chemokine receptor D6 controls macrophage efferocytosis and cytokine secretion during the resolution of inflammation. Faseb J 2012;26:3891-900.

137. Murray PJ, Wynn TA. Protective and pathogenic functions of macrophage subsets. Nat Rev Immunol 2011;11:723-37.

138. Zhang C, Wang S, Yang C, Rong R. The Crosstalk between Myeloid Derived Suppressor Cells and Immune Cells: To Establish Immune Tolerance in Transplantation. J Immunol Res 2016;2016:4986797.

139. Brem-Exner BG, Sattler C, Hutchinson JA, et al. Macrophages driven to a novel state of activation have anti-inflammatory properties in mice. J Immunol 2008;180:335-49.

140. Kraaij MD, van der Kooij SW, Reinders ME, et al. Dexamethasone increases ROS production and T cell suppressive capacity by anti-inflammatory macrophages. Mol Immunol 2011;49:549-57.

141. Conde P, Rodriguez M, van der Touw W, et al. DC-SIGN(+) Macrophages Control the Induction of Transplantation Tolerance. Immunity 2015;42:1143-58.

142. Scalea JR, Tomita Y, Lindholm CR, Burlingham W. Transplantation Tolerance Induction: Cell Therapies and Their Mechanisms. Front Immunol 2016;7:87.

143. Quillard T, Charreau B. Impact of notch signaling on inflammatory responses in cardiovascular disorders. Int J Mol Sci 2013;14:6863-88.

144. Zhang Q, Wang C, Liu Z, et al. Notch signal suppresses Toll-like receptor-triggered inflammatory responses in macrophages by inhibiting extracellular signal-regulated kinase 1/2-mediated nuclear factor kappaB activation. J Biol Chem 2012;287:6208-17.

145. Wang N, Liang H, Zen K. Molecular mechanisms that influence the macrophage m1-m2 polarization balance. Front Immunol 2014;5:614.

3

The Effect of Tacrolimus and Mycophenolic Acid on CD14+ Monocyte Activation and Function

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Abstract

Monocytes and macrophages play key roles in many disease states, including cellular and humoral rejection after solid organ transplantation (SOT). To suppress alloimmunity after SOT, immunosuppressive drug therapy is necessary. However, little is known about the effects of the immunosuppressive drugs tacrolimus and mycophenolic acid (MPA) on monocyte activation and function. Here, the effect of these immunosuppressants on monocytes was investigated by measuring phosphorylation of three intracellular signaling proteins which all have a major role in monocyte function: p38MAPK, ERK and Akt. In addition, biological functions downstream of these signaling pathways were studied, including cytokine production, phagocytosis and differentiation into macrophages. To this end, blood samples from healthy volunteers were spiked with diverse concentrations of tacrolimus and MPA.

Tacrolimus (200 ng/ml) inhibited phosphorylation of p38MAPK by 30% (mean) in CD14+ monocytes which was significantly less than in activated CD3+ T cells (max 60%; $p < 0.05$). This immunosuppressive agent also partly inhibited p-Akt (14%). MPA, at a therapeutic concentration showed the strongest effect on p-Akt (27% inhibition). p-ERK was inhibited with a maximum of 15% after spiking with either tacrolimus or MPA. The production of IL-1β and phagocytosis by monocytes were not affected by tacrolimus concentrations, whereas MPA did inhibit IL-1β production by 50%. Monocyte/macrophage polarization was shifted to an M2-like phenotype in the presence of tacrolimus, while MPA increased the expression of M2 surface markers, including CD163 and CD200R, on M1 macrophages.

These results show that tacrolimus and MPA do not strongly affect monocyte function, apart from a change in macrophage polarization, to a clinically relevant degree.

Introduction

Monocytes have numerous biologic functions that make them key players in solid organ transplantation (SOT)-related conditions, including ischemia-reperfusion injury and its repair, as well as regulation of allograft rejection [1-5]. After SOT, cells of the monocyte/macrophage lineage, process and present alloantigen to the recipients' immune system, induce inflammation and contribute to allograft rejection, through the secretion of pro-inflammatory cytokines and by providing help to alloreactive T- and B-cells. For example, after ischemia and reperfusion injury, monocytes infiltrate the allograft where, after differentiation into macrophages, they produce inflammatory cytokines, can present donor antigen and also contribute to tissue injury and repair processes [6]. Furthermore, immunohistochemistry of acute rejection kidney specimens demonstrated massive infiltration of the transplanted organ by CD68+ monocyte/macrophages [3,7]. In antibody-mediated rejection, monocytes control and induce cell injury via the activation of their Fcγ-receptor by allo-antibodies [8,9].

In tissue, monocytes differentiate into different macrophages subsets depending on the environmental cues the cells encounter. In general, classically and alternatively activated macrophages, termed M1 and M2 macrophages, respectively, represent the ends of a spectrum and can be distinguished by a unique set of cell surface markers [10-12]. Typically, M1 macrophages have a pro-inflammatory function and secrete large amounts of IL-12 and low levels of IL-10, while M2 macrophages can be divided into functionally different subsets. M2a macrophages are involved in T-helper type 2 (Th2) immune responses and have pro-fibrotic properties. M2b macrophages are considered immunoregulatory because they secrete large amounts of IL-10 in combination with TNF-α, IL-1 and IL-6 [13]. Just like the M2a cells, M2b macrophages are also involved in Th2 immune responses [14]. M2c macrophages are anti-inflammatory and characterized by their capacity to produce large amounts of TGF-ß and IL-10 [14,15].

Despite their clinical importance, surprisingly little is known about the effects of immunosuppressive drugs on monocyte/macrophage differentiation and function. Currently, most kidney transplant recipients, as well as the majority of recipients of other solid organ transplants receive combination immunosuppressive therapy consisting of tacrolimus and mycophenolic acid (MPA; either in the form of mycophenolate mofetil or mycophenolate-sodium) [16-24]. In addition, tacrolimus has also shown to be effective in the treatment of patients with ulcerative colitis and atopic dermatitis [25-28] while MPA is used for the treatment of auto-immune disease such as systemic lupus erythematosus [29]. The limited number of studies on the effect of tacrolimus on monocyte functions have been mostly performed in animal models, immortalized cell lines and cord blood cells. These studies report that tacrolimus can suppress the production of IL-1β, IL-10 and TNF-α by polyclonally activated monocytes [26,30,31].

A limited number of studies report on the effects of MPA on macrophage functions. The study by Weimer et al. showed that MPA can suppress the production of IL-1β and IL-6 by staphylococcal superantigens activated monocytes, while the effects of MPA on phagocytosis and monocyte differentiation are unknown [32].

Monocyte/macrophage responses to environmental triggers are controlled by the activation of multiple intracellular signaling pathways, in which p38 mitogen-activated protein kinases (p38MAPK), extracellular signal-regulated kinases (ERK) and Akt play important roles (**Figure 1**) [33-36]. Activation of these pathways initiates a complex cascade leading to binding of transcription factors to DNA followed by cytokine gene expression and production, phagocytosis and other functions. Previous studies in T-lymphocytes have demonstrated that tacrolimus does not only inhibit the calcineurin pathway, but also affects the MAPK pathway, while the effects of MPA are unknown [37]. It is unknown if immunosuppressive drugs also inhibit these same pathways in monocytes, and affect related biological functions.

Given the important role of monocytes in immune responses after SOT, the effects on monocyte function of the two most commonly prescribed immunosuppressive agents, tacrolimus and MPA, have been studied. Monocytes have been studied in peripheral blood samples of healthy volunteers that were incubated with tacrolimus or MPA and subsequently their activation, signal transduction, phenotypic differentiation, phagocytic capacity and cytokine production upon PMA/ionomycin stimulation were investigated.

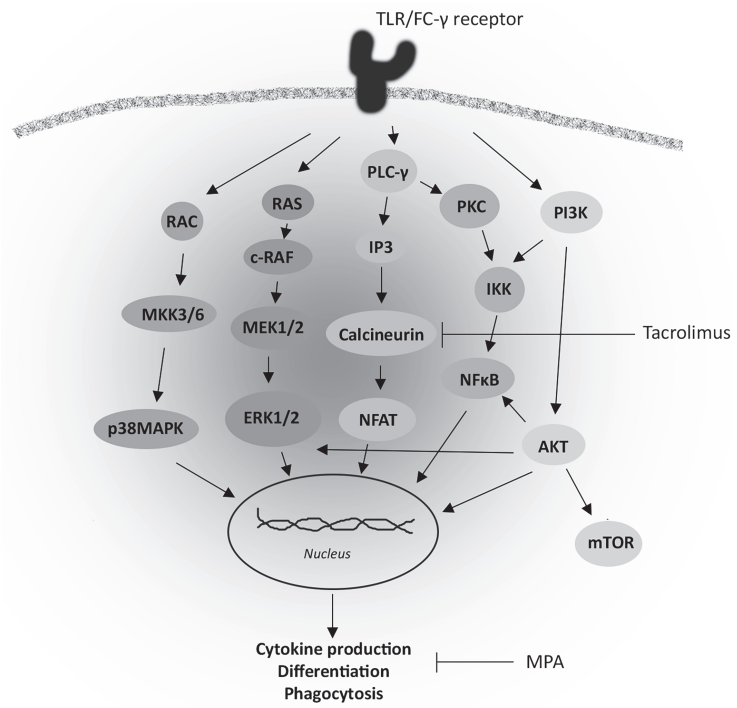


Figure 1. Simplified overview of intracellular signaling pathways involved in monocyte activation. After toll like receptor (TLR) or FC-γ receptor activation the MAPK, Akt and NFAT pathways are activated upstream. The phosphorylation of the intracellular signaling proteins leads to the activation of transcription factors, such as CREB and NF-KB p65, which after activation leads to gene transcription. This process determines the function of monocytes including phenotypic differentiation, cytokine production and phagocytosis.

Materials and Methods

In vitro phosphorylation study and whole–blood phospho–specific flowcytometry

To measure the effect of tacrolimus and MPA on signaling molecules, heparinized blood samples were drawn from healthy volunteers (n = 5). The study protocol was approved by the local ethics committee of the Erasmus medical center and written informed consent was obtained from each individual after receiving detailed information about the aims of the study. Samples were incubated for one hour at 37 °C with either vehicle, tacrolimus (10, 50 or 200 ng/ml; Prograf®, Astellas Pharma Inc., Tokyo, Japan), MPA (10 µg/ml; Sigma-Aldrich, Steinheim, Germany) or the p38MAPK inhibitor SB203580 (20 µM; Invivogen, San Diego, CA). The vehicle used was IMDM medium (Gibco BRL, Carlsbad, CA) supplemented with 10% heat-inactivated fetal bovine serum (BioWhittaker, Verviers, Belgium). Phosphorylation of p38MAPK, ERK and Akt was measured according to the manufacturer's instructions for phosphoprotein analysis (BD Biosciences, San Jose, CA). In brief, 200 µl heparinized blood was stained with Brilliant Violet (BV)510-labelled mouse anti-human CD3 (Biolegend, San Diego, CA) and Fluorescein Isothiocyanate (FITC)-labelled mouse anti-human CD14 (Serotec, Oxford, UK) for 30 minutes at 37˚C. After 15 minutes, blood samples were activated with PMA/ionomycin (25 µg per ml/500 µg per ml for p38MAPK and Akt; 5 µg per ml/100 µg per ml for ERK, Sigma-Aldrich, Steinheim, Germany) for 15 minutes. To compare phosphorylation of p38MAPK in CD3+ T-cells and CD14+ monocytes, samples were also stimulated for 30 minutes with PMA/ionomycin. Then cells were fixed for 10 minutes with Lyse/Fix buffer (BD Biosciences) and permeabilized with 90% methanol at -20˚C for 30 minutes. Samples were stained intracellular with fluorchrome conjugated mAb phycoerythrin (PE)-labelled mouse anti-p38MAPK (clone pT180/pY182), PE-labelled mouse anti-pAkt (clone pS473) or AlexaFluor647 (AF647)-labelled mouse anti-pERK1/2 (pT202/pY204) (all from BD Biosciences) for 30 minutes at room temperature and analyzed on a FACS Canto II flowcytometer (BD Biosciences). Unstimulated samples were used as negative controls. Isotype controls; mouse IgG1-PE (p38, Akt Biolegend) and mouse IgG1-AF647 (ERK; Biolegend); were included in separated tubes. Interday-variability of the flowcytometer was corrected by using Cytocalbeads (Thermo Scientific, Fremont, CA) according to the manufacturer's instructions.

Cytokine production

Heparinized blood samples from healthy volunteers were incubated for 1 hour at 37°C with either vehicle, tacrolimus (10 ng/ml and 50 ng/ml), MPA (10 µg/ml) and activated with PMA/ionomycin (25 µg per ml/400 µg per ml) for four hours at 37°C. Golgiplug (BD Biosciences) was added during the incubation phase to accumulate cytokines intracellularly. Subsequently, EDTA was added for 15 minutes to stop activation. Cells were then stained with BV510-labelled mouse anti-human CD3 (Biolegend) and FITC-labelled mouse anti-human CD14 (Serotec) for 30 minutes at 37 °C, fixed for 10 minutes with FACS lysing

solution (BD Biosciences) and treated with permeabilization buffer II (BD Biosciences) for 10 min. AF647-labelled anti-IL-1β (detecting the bioactive form of IL-1β, 17.3 kD, clone JK1B-1, BD Biosciences) was used for intracellular cytokine staining for 30 minutes at 4°C.

Phagocytosis

To assess phagocytosis by monocytes, whole-blood samples of healthy volunteers were incubated for 1 hour at 37°C with either vehicle, tacrolimus (10, 50 and 200 ng/ml) or MPA (10 µg/ml). Then 100 µl of spiked blood per sample was tested for phagocytosis according to the manufacturer's instructions of the Phagotest (Glycotope Biotechnology, Heidelberg, Germany), which used FITC- labelled E. coli-bacteria.

Macrophage phenotypic differentiation

Peripheral blood mononuclear cells (PBMC) were isolated from heparinized blood samples by density-gradient centrifugation using Ficoll-paque (GE Healthcare, Uppsala, Sweden). Subsequently, monocytes were isolated by MACS magnetic cell separation (Miltenyi Biotec, Bergisch Gladbach, Germany) using anti-human CD14 magnetic microbeads (Miltenyi). Then, monocytes were cultured in a 12 wells plate at a concentration of 75 $x10^4$ cells/ml in RPMI 1640 culture medium with glutaMAX and 25 mM HEPES (Gibco, Life technologies, Paisley, UK), supplemented with 10% heat inactivated fetal bovine serum (FBS, BioWhittaker, Verviers, Belgium) and M-CSF (macrophage colony-stimulating factor; 5ng/ml, Bioconnect, Huissen, the Netherlands). As positive controls, cells were polarized with recombinant human IFN-γ, IL-10 and IL-4 (all 50 ng/ml, Bioconnect) according to Ambarus et al. [38]. Effects of immunosuppressive drugs on monocyte differentiation were examined by culturing monocytes in the presence of either vehicle, tacrolimus (10 ng/ml and 50 ng/ml) or MPA (10 µg/ml). At baseline (day 0) and after 4 days of differentiation, monocytes were tested for their polarization profile after incubation on ice for 1 h by using the following markers [38]: BV421-labelled mouse anti-human CD80 (Biolegend), FITC-labelled mouse anti-human CD163 (Serotec), allophycocyanin (APC)-H7-labelled mouse anti-human CD14 (BD Biosciences), BV510-labelled mouse anti-human CD64 (BD Biosciences), PE-Cyanine7 (PE-Cy7)-labelled mouse anti-human CD16 (BD Biosciences), peridinin-chlorophyll-protein (PERCP)-labelled mouse anti-human CD200R (eBioscience, Vienna, Austria) and APC-labelled rat anti-mouse/human CD11b (Biolegend). Unstained samples were used as a negative control. Background fluorescence levels of isotype controls were used as negative reference.

Data analysis and statistics

The phosphorylation of p38MAPK, Akt and ERK was measured as the Median Fluorescence Intensity (MFI) and normalized using Cytocalbeads (Thermo Scientific). MFI values of the unstimulated samples were subtracted from the stimulated MFI values. Data and statistical analysis was performed with Diva-version 6.0 software (BD Biosciences) and Graph Pad Prism 5.0 (Graph Pad Software Inc., La Jolla, CA) by using paired t-tests (for the *in vitro*

phosphorylation study after performing log transformation and after finding a p-value >0.05 with an F-test). A two-sided p-value < 0.05 was considered statistically significant.

Results

Inhibitory effect of tacrolimus and MPA in monocyte signaling pathway activation

A typical example of monocytes (CD14+) and T-cells (CD3+) gating strategies is depicted in **Figure 2A**. In line with previous observations, activation of whole blood samples by PMA/ionomycin significantly increased the expression of phosphorylated p38MAPK in CD3+ T-cells compared to the *ex vivo* (unstimulated) whole blood level and isotype controls [37] (**Figures 2B and 2C**). In CD14+ monocytes, an increase in phosphorylation levels after stimulation was observed for p38MAPK, ERK and Akt compared to the isotype controls and *ex vivo* samples (**Figures 2D and 2E**). Again, this increase is in line with previous studies [39-41].

To study the effect of tacrolimus on CD3+ T-cells and CD14+ monocytes, cells were incubated with tacrolimus in a dose dependent manner. In this study, T-cell p38MAPK phosphorylation levels served as controls. At a high concentration (200 ng/mL), tacrolimus inhibited p-p38MAPK in T-cells with a mean of 60%, which was significantly higher than the percentage of inhibition in monocytes (30%, $p < 0.05$) (**Figure 3**). For the monocytes this was not different over time.

Next, the effect of tacrolimus and MPA on the phosphorylation of each signaling protein in CD14+ monocytes was measured and compared to the phosphorylation in the samples without drugs. The p38MAPK inhibitor SB203580, used as a control, showed the maximal inhibitory effect on p38MAPK phosphorylation (inhibition 48%; $p < 0.001$, **Figures 4A and 4B**). Apart from the effect on p38MAPK, SB203580 also suppressed the phosphorylation of ERK (mean inhibition 13%; $p < 0.05$), and Akt (mean inhibition 59%; $p < 0.001$).

In the presence of high tacrolimus concentrations (50 ng/ml, 200 ng/ml), phospho-p38MAPK was significantly lower expressed ($p < 0.05$ and $p < 0.01$, respectively) than in the samples without tacrolimus **Figure 4A**). This decrease was not seen in the presence of a therapeutic tacrolimus concentration (10 ng/ml). The mean maximal p38MAPK phosphorylation inhibition in monocytes was 30% and 33% at 50 and 200 ng/ml tacrolimus, respectively (**Figure 4B**). Furthermore, in the presence of 200 ng/ml tacrolimus the PMA/iono activated phosphorylation of both ERK and Akt were suppressed, although with small percentages (mean 7% and 14%, respectively, $p < 0.05$, **Figure 4**) On the other hand, MPA (10 µg/ml) downregulated p-ERK and p-Akt (mean inhibition 14% and 27%, respectively, $p < 0.01$), but not phospho-p38MAPK ($p = 0.51$, **Figure 4**).

Taken together, these experiments demonstrate that tacrolimus at high concentrations inhibits phosphorylation of p38MAPK more than Akt and ERK, while at therapeutic concentrations, tacrolimus did not affect the activation of these molecules. In addition, MPA suppresses the phosphorylation of Akt more than the phosphorylation of ERK or p38MAPK.

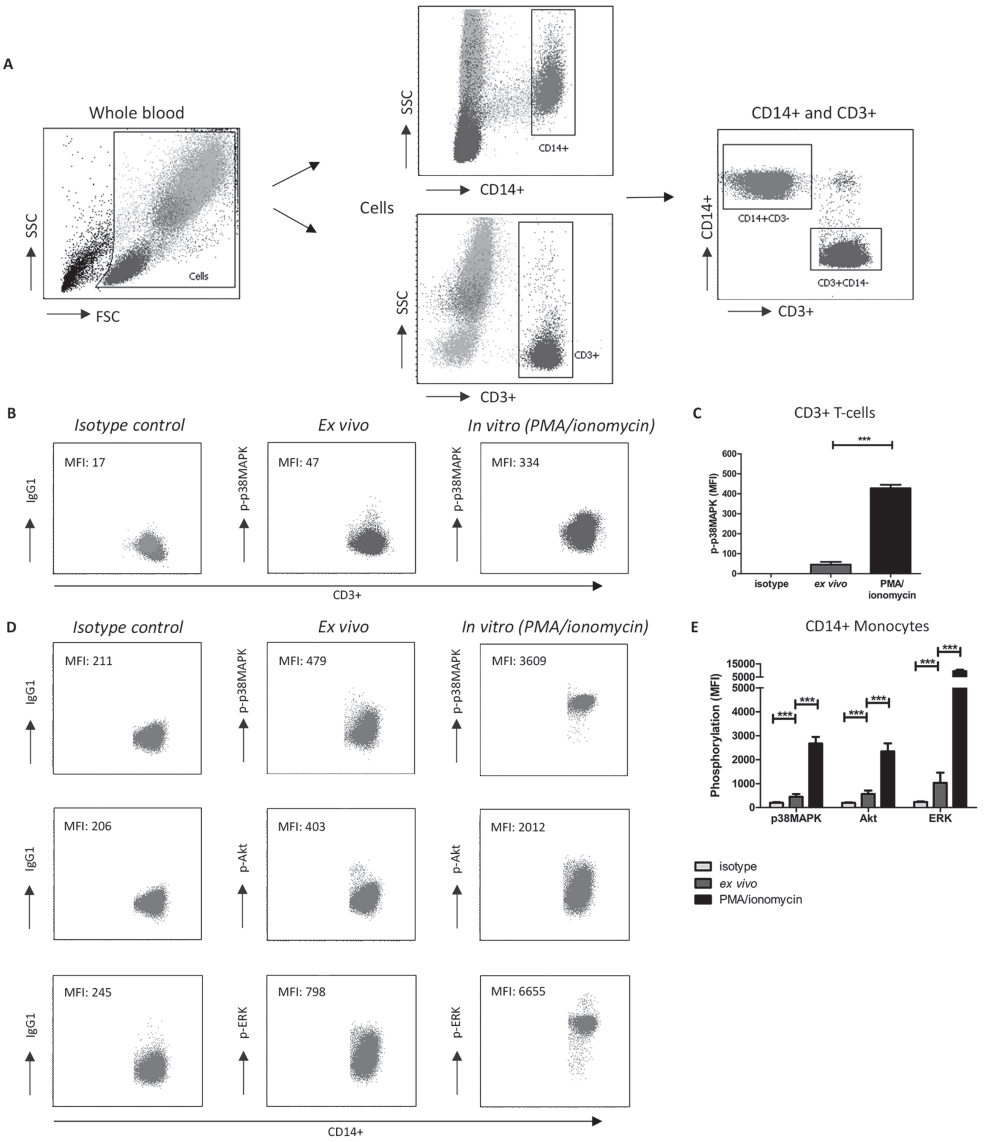


Figure 2. Gating strategy for the selection of monocytes and T-cells, and the measurement of p38MAPK, ERK and Akt phosphorylation. (A) Scatter dot plots to define the monocyte population in blood samples from healthy controls. Cells were selected in whole blood samples for each healthy control and then gated for their expression of either CD3 or CD14. Then, CD3+ and CD14+ cells were combined in one dotplot, to make sure that there were no double positive cells in the analysis. (B) An example of p38MAPK phosphorylation in CD3+ T cells, measured as the median fluorescence intensity (MFI) prior to (*ex vivo*) or after stimulation with PMA/ionomycin (*in vitro*) compared to the isotype control. (C) p38MAPK phosphorylation in CD3+ T-cells was increased after stimulation with PMA/ionomycin compared to isotype controls and *ex vivo* (unstimulated) samples. (D) Examples of the phosphorylation of p38MAPK, Akt and ERK in CD14+ monocytes of isotype controls, *ex vivo* (unstimulated) and PMA/Ionomycin stimulated (*in vitro*) samples. (E) Phosphorylation (MFI) of p38MAPK, Akt and ERK in CD14+ monocytes is increased after PMA/ionomycin stimulation compared to isotype controls and *ex vivo* samples and showed the maximum phosphorylation capacity for each protein. FSC, forward scatter; SSC, side scatter; MFI, median fluorescence intensity.(Data are plotted as the mean ±SEM; n=5)

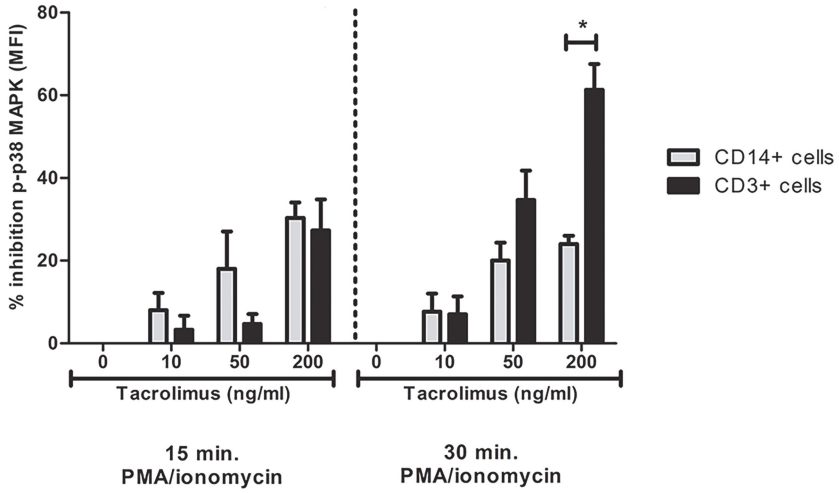


Figure 3. p38MAPK phosphorylation is inhibited more in T-cells than in monocytes. Blood samples from healthy volunteers were spiked with either vehicle, 10 ng/ml tacrolimus, 50 ng/ml tacrolimus or 200 ng/ml tacrolimus. Thereafter, the phosphorylation of p38MAPK was determined in T-cells and monocytes after 15 or 30 min. of stimulation with PMA/ionomycin. After 30 min. stimulation, T-cells were significantly more inhibited than monocytes. (Data are plotted as the mean ±SEM; n=3) *) $p < 0.05$; **) $p < 0.01$; ***) $p < 0.001$

IL–1β production decreased in the presence of MPA but not after tacrolimus spiking

The percentage of IL-1β-producing cells was studied to determine whether the significant alterations in phosphorylation of the MAPK pathway members p38 and ERK by tacrolimus has an effect on cytokine production by monocytes. Again, whole blood samples were stimulated with PMA/ionomycin after which IL-1β protein expression was measured (**Figure 5A**). After stimulation, more than 10% of the monocytes expressed the 17.3 kD form of IL-1β, which is the active IL-1β protein (in contrast to the 31kD precursor protein which is not biologically active). No significant change in IL-1β protein expression was found when blood samples were spiked with tacrolimus at either a therapeutic concentration (10 ng/ml, $p = 0.28$) or a concentration of 50 ng/ml or 200 ng/ml ($p = 0.36$ and $p = 6758$, respectively) (**Figure 5B**). A significantly lower percentage of IL-1β-producing cells was found in the presence of 10 µg/ml MPA (about 50% inhibition; $p < 0.05$).

Effects of tacrolimus and MPA on phagocytosis by monocytes

Subsequently, phagocytosis, one of the primary biological functions of monocytes, was studied in the presence and absence of immunosuppressive drugs. The percentage of monocytes that phagocytized labeled bacteria after incubation at 37°C, was more than 90% (**Figure 6**). This percentage was not influenced by tacrolimus at therapeutic (10 ng/ml, $p = 0.44$) or high (50 ng/ml, $p = 0.29$ and 200 ng/ml, $p = 0.33$) concentrations nor by a high concentration of MPA (10 µg/ml, $p = 0.45$).

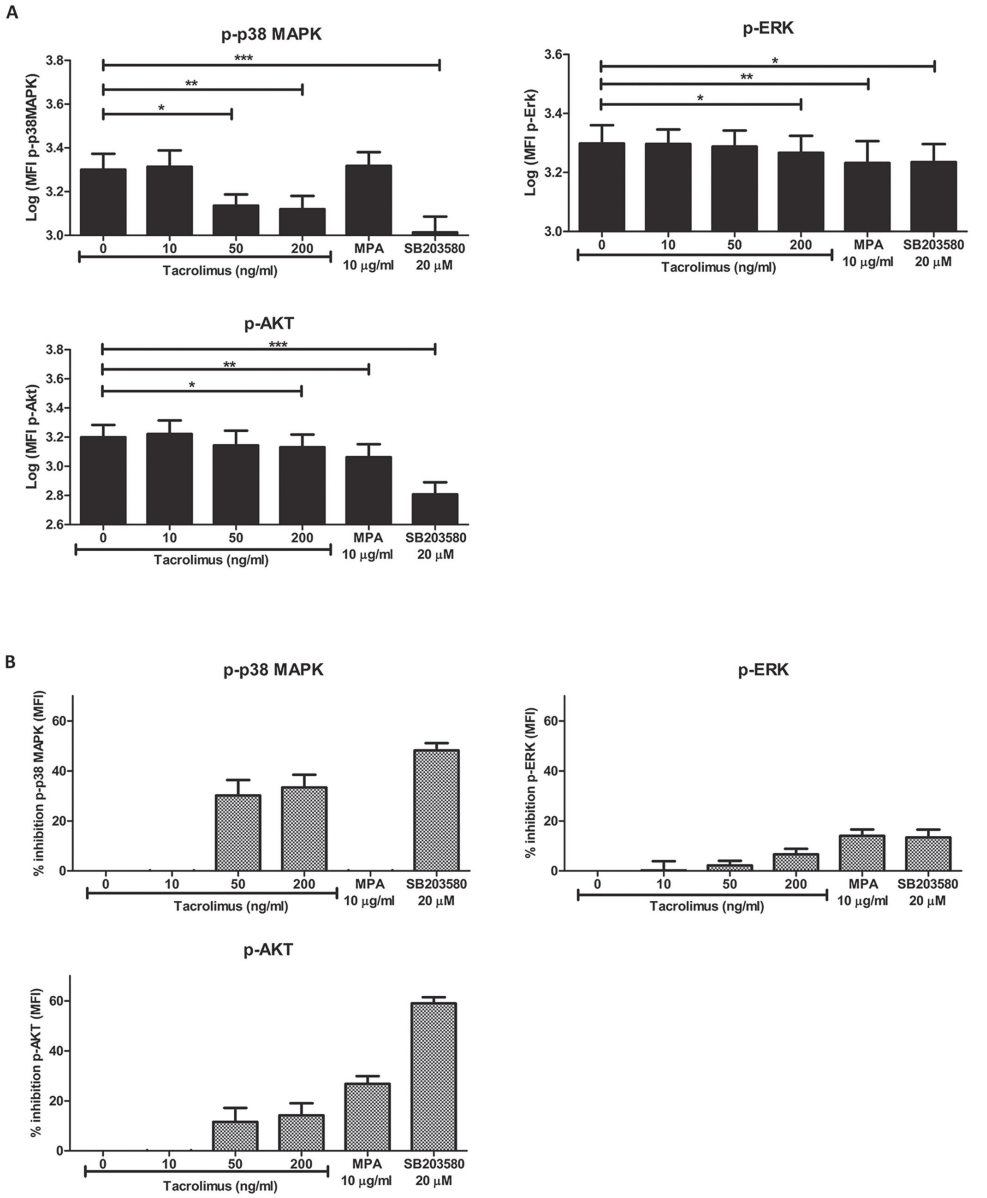


Figure 4. Tacrolimus and MPA can inhibit signaling pathway activation in whole-blood samples. (A) Phospho-p38MAPK (upper left panel), p-ERK (upper right panel) and p-Akt (lower panel) phosphorylation in monocytes was measured as MFI level. Blood samples from healthy volunteers were spiked with vehicle, 10 ng/ml tacrolimus, 50 ng/ml tacrolimus, 200 ng/ml tacrolimus, 10 μg/ml MPA or 20 μM of the MAPK inhibitor SB203580. The effect of tacrolimus and MPA was based on the stimulated samples without the addition of drugs. The MAPK inhibitor was used as a positive control. Gating was performed according to figure 2. Tacrolimus was found to have an effect on p38MAPK, ERK and Akt phosphorylation. Akt and ERK phosphorylation was decreased in the presence of MPA. (B) Percentages of inhibition for the phosphorylation of p38MAPK (upper left panel), ERK (upper right panel) and Akt (lower panel). Data are normalized for the MFI values of the stimulated samples without the addition of immunosuppressive drugs (Data are plotted as the mean ±SEM; n = 5) *) $p < 0.05$; **) $p < 0.01$; ***) $p < 0.001$

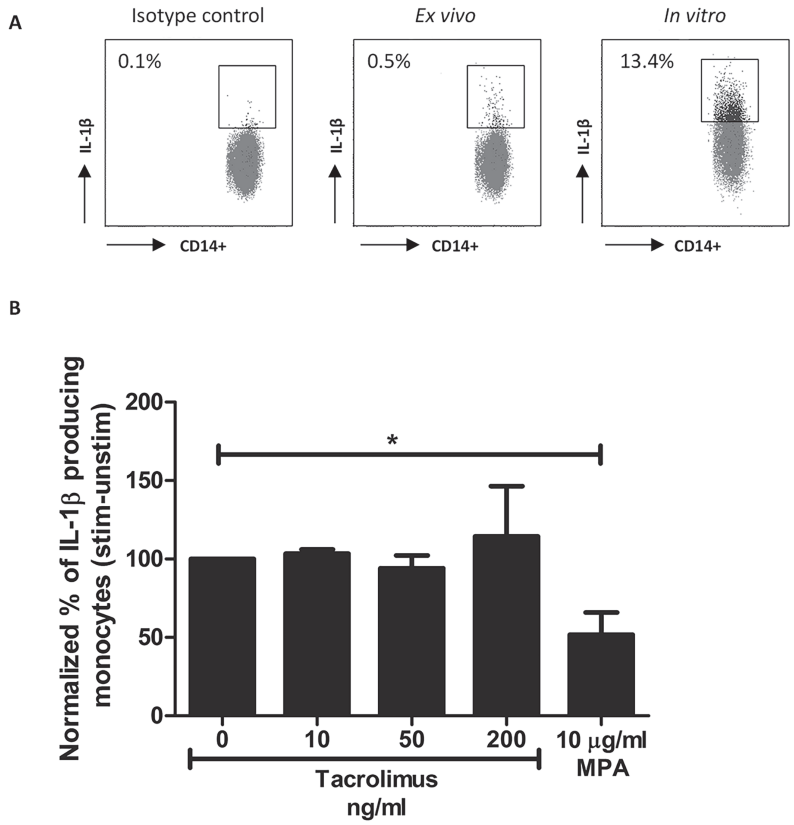


Figure 5. **IL-1β production by monocytes of healthy controls is suppressed in the presence of MPA but not in the presence of tacrolimus.** (A) Dot plots showing IL-β production with or without stimulation in monocytes. Cells were gated of whole blood samples according to figure 2a. Isotype controls were used as negative controls and were used to set the gate for the positive IL-1β expression. Results are shown as the percentage of IL-1β producing monocytes compared to the isotype control. Samples were stimulated with PMA/ionomycin for maximum production of IL-1β. (B) Mean percentages of IL-1β producing monocytes after spiking with vehicle, 10 ng/ml tacrolimus, 50 ng/ml tacrolimus or 10 μg/ml MPA. Samples were corrected for the unstimulated results and then normalized to the samples without drug exposure. IL-1β production in monocytes was significantly suppressed by a concentration of 10 μg/ml MPA. (Data are plotted as the mean ±SEM; n = 5) *) $p < 0.05$

Phenotypic macrophage differentiation is influenced by tacrolimus and MPA

After stimulation with M-CSF, freshly isolated monocytes differentiate into M1 or M2 subsets when additionally treated with the appropriate triggers (a schematic flow diagram of these experiments is given in **Supplementary Figure 1A**). In line with previously published data, M1 macrophages had a higher expression of CD80 and CD64, M2a macrophages had a lower expression of CD14 and a higher expression of CD200R, and M2c macrophages had a higher expression of CD163 and CD16 on their surface compared to macrophages cultured without the addition of cytokines (**Supplementary Figure 1B**) [38].

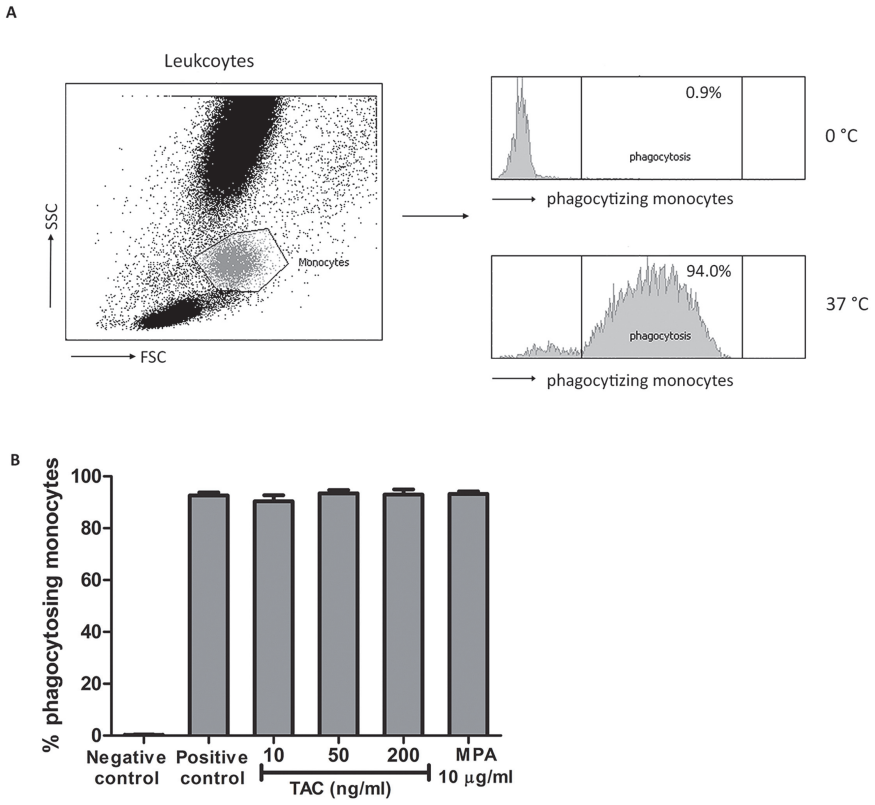


Figure 6. **The percentage of phagocytosis by monocytes from healthy controls was not changed in the presence of tacrolimus or MPA.** (A) Monocytes were selected from the leukocyte population by a forward and side scatter. Analysis was based on the phagocytosis of the FITC-labeled bacteria. Incubation with FITC-labeled bacteria on 37°C showed a high percentage of phagocytosing monocytes (positive control) compared to monocytes on 0°C (negative control). (B) Mean percentage of phagocytosing monocytes after spiking with either vehicle, 10 ng/ml tacrolimus, 50 ng/ml tacrolimus, 200 ng/ml tacrolimus or 10 µg/ml MPA. Incubation at 37°C increased the percentage of phagocytosing monocytes by more than 90%. Effects of tacrolimus and MPA on phagocytosis were determined as the percentage of phagocytosing monocytes compared to the positive control without immunosuppressive drugs. (Data are plotted as the mean ±SEM; n=4)

Differentiation of monocytes/macrophages can be divided into two processes: maturation of the monocyte into a macrophage and the subsequent polarization of a macrophage into an M1 or M2 type. First, the effect of tacrolimus and MPA on monocyte maturation was studied. After maturation of monocytes under M-CSF culture conditions only, the expression of all six tested markers increased significantly (**Figure 7**). During maturation, tacrolimus (10 ng/ml), but not MPA, slightly increased the expression of CD200R and CD16 (both markers for M2 macrophages, $p < 0.05$ **Figure 7**), compared to the maturation without the presence of immunosuppressive drugs. A high concentration of tacrolimus (50 ng/ml) also increased the expression of CD16 (MFI increase: 5692 to 6607 $p < 0.05$). Second, the polarization of these mature macrophages was determined. The addition of IFN-γ, IL-4 and IL-10 was used as a positive control for the differentiation assays to compare the polarization

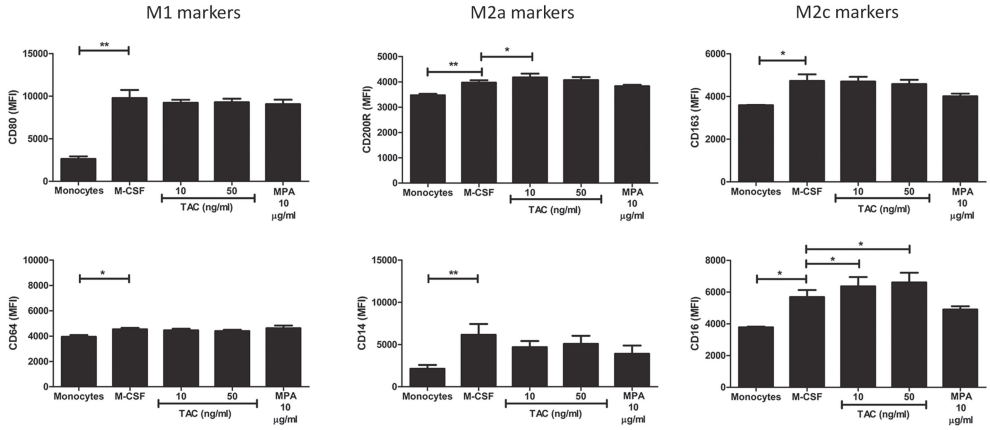


Figure 7. **Monocyte differentiation in the presence of tacrolimus and MPA causes a small shift in macrophages subsets.** CD14+ monocytes were freshly isolated from whole blood samples of healthy volunteers (n = 5) and then cultured for 4 days with either vehicle, 10 ng/ml tacrolimus, 50 ng/ml tacrolimus, 200 ng/ml tacrolimus or 10 µg/ml MPA. The addition of cytokines was used a positive control. Differentiated macrophages were gated based on their location on the forward sideward scatter. After 4 days of culturing, the expression of all tested surface markers was increased compared to freshly isolated monocytes. The addition of tacrolimus, but not MPA, resulted in an increase of the expression of M2 markers (CD16 and CD200R). (Data are plotted as the mean ±SEM; n = 5 *) $p < 0.05$; **) $p < 0.01$; ***) $p < 0.001$

of the macrophages after their maturation from monocytes. An increase of CD200R and CD16 expression on monocytes was seen after the addition of IL-4 and IL-10 to the culture medium, which are both stimuli to induce M2 macrophages (**Supplementary Figure 1B**). Thus, tacrolimus increased the expression of markers for M2 macrophages, while this was not seen for MPA.

Next, the influence of tacrolimus and MPA on the polarization of macrophage subsets was determined, by measuring the expression of M1- and M2-related surface markers. Monocytes were cultured for 4 days in the presence of M-CSF as a maturation stimulus supplemented with M1 (IFN-γ), M2a (IL-4), and M2c (IL-10) stimulants and different concentrations of tacrolimus or MPA.

In the presence of IFN-γ, a stimulus for M1, the addition of tacrolimus (50 ng/ml and 200 ng/ml) led to the increased expression of the M2 marker CD16 ($p < 0.01$ and $p < 0.05$, respectively) and a lower expression of CD14 ($p < 0.05$ and $p < 0.01$, respectively, **Figure 8**, left column). In addition, tacrolimus at a therapeutic concentration (10 ng/ml) also lowered CD14 expression ($p < 0.05$). MPA caused a significant increase of the other two M2 markers, CD200R and CD163 ($p< 0.05$) and, although not significant, seemed to lower the expression of the M1 markers CD80 and CD64.

In the presence of the M2a stimulus IL-4, high concentrations of tacrolimus (50 and 200 ng/ml) increased the expression of CD163, although this difference was small (MFI: from 3906 to 3958 and 3957, respectively, $p < 0.05$, **Figure 8**, middle column). MPA significantly decreased the expression of CD80 (M1 marker, $p < 0.05$).

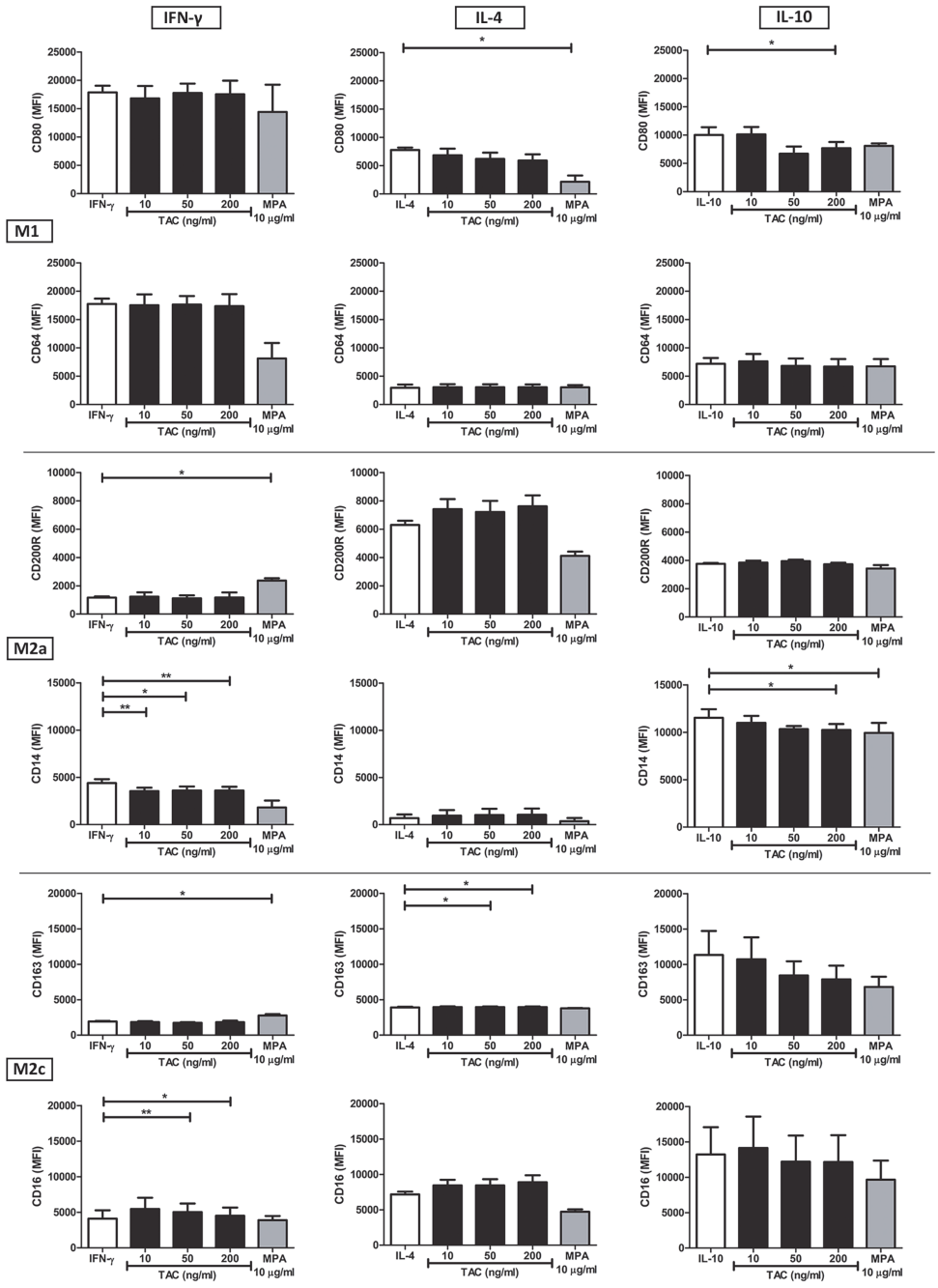


Figure 8. MPA and tacrolimus act differentially on the polarization of M1 and M2 macrophages. CD14+ isolated monocytes were cultured for 4 days in the presence of either M-CSF and IFN-γ (left graphs), M-CSF and IL-4 (middle graphs) or M-CSF and IL-10 (right graphs). In addition, vehicle, 10 ng/ml tacrolimus, 50 ng/ml tacrolimus, 200 ng/ml tacrolimus or 10 μg/ml MPA were added to each culture condition. Tacrolimus changed the expression of M2 markers under a M1-driven condition and decreases the expression of M1 markers under M2 conditions. MPA reduced the expression of CD80 under a M2 inducing condition and increased M2 expression under IFN-γ stimulation. (Data are plotted as the mean ±SEM n = 3 *) $p < 0.05$; **) $p < 0.01$; ***) $p < 0.001$.

Finally, tacrolimus, but only at a concentration of 200 ng/ml, decreased CD14 and CD80 expression in the presence of IL-10 (M2c stimulus) ($p < 0.05$), while MPA decreased CD14 expression ($p < 0.05$) and did not affect the other markers (**Figure 8,** right column). Thus, both tacrolimus and MPA increased the expression of M2 markers and decreased the expression of M1 markers in the presence of different M1 and M2 stimuli.

Discussion

Cells from both the adaptive and innate immune system play an important role in the immune response after SOT but the effects of the immunosuppressants tacrolimus and MPA on human monocyte differentiation and functions have not been studied in-depth in previous studies. This study demonstrates that 1) these drugs partially inhibit phosphorylation of signaling molecules involved in CD14+ monocyte activation, i.e., p38MAPK, ERK and Akt kinases, but 2) have only limited effects on cytokine production, phagocytosis, phenotypic maturation, and polarization (an overview of de results is given in **Supplementary Table I**).

The inhibitory effect of tacrolimus on the calcineurin pathway and the effect of MPA on the reduction of guanosine nucleotide synthesis in T-cells are well-known [19,42]. Tacrolimus also proved to inhibit p38MAPK phosphorylation in T-cells by more than 60%, which contrasts with the inhibition in monocytes [37]. As demonstrated here, in monocytes the suppression of phosphorylation of intracellular signaling molecules was not more than 35%. Tacrolimus partly inhibited p38 phosphorylation, while MPA mainly inhibited Akt phosphorylation, although this inhibitory effect was also limited. Furthermore, phagocytosis and differentiation were minimally influenced by tacrolimus and MPA, showing that monocytes could be functional in the presence of tacrolimus and MPA. This may imply that cells of the innate immune system are less susceptible for the immunosuppressive effects of tacrolimus or MPA in comparison to cells of the adaptive immune system. The difference in inhibition between the two immune responses could be explained by the working mechanism of the immunosuppressive drugs. For example, the main target of tacrolimus is the calcineurin pathway and it may be that this pathway plays a more important role in T-cell activation than in monocyte activation [43].

The limited effect of tacrolimus on p-ERK, a member of the MAPK pathway, may explain the incomplete inhibition of monocyte activity and function as demonstrated in this study [44]. P-ERK is involved in processes leading to IL-10 production (M2 macrophages), while phosphorylated p38MAPK is essential for IL-12 production, which is in agreement with the observed shift in macrophage polarization [45]. These data are in contradiction with the findings reported by Chang ***et al***. who described an inhibitory effect of tacrolimus on p-ERK but not on p38MAPK phosphorylation [26]. However, in contrast to the present study in which primary monocytes were investigated, the experiments by Chang ***et al.*** were performed with LPS-stimulated monocytic leukemia cells (THP-1) and phosphorylation was measured by means of Western blot. In the present study, phospho-specific flowcytometry was used to

quantify the biological effects of tacrolimus and MPA at the single-monocyte level, which is a more sensitive tool for pharmacodynamic monitoring of drug effects [46,47].

In contrast to tacrolimus, MPA (at a therapeutic concentration of 10 μg/ml) did not suppress the phosphorylation of p38MAPK, affected p-ERK to some degree, and had the largest effect on p-Akt. MPA inhibits inosine monophosphate dehydrogenase, an enzyme that is responsible for the ***de novo*** synthesis of guanosine nucleotides. As a consequence, the proliferation of B and T-cells is inhibited. The only described effect of MPA on monocyte function is the reduced production of IL-6 and IL-10, which are both downstream products of the Akt/mTOR pathway [42,48]. It has also been reported that IL-1β is a downstream molecule of the Akt pathway [49,50]. Here we found that MPA indeed partly inhibited IL-1β production, suggesting that, in combination with the preferred inhibition of p-Akt, MPA affects cytokine production via p-Akt.

The inhibition of signaling molecule phosphorylation by tacrolimus and MPA was smaller than by the positive control SB203580, showing the limited effects of both immunosuppressive drugs. However, the inhibition of phosphorylated p38MAPK by the positive control was also not more than 50%. SB203580 was designed as an inhibitor of the phosphorylation induced by p38MAPK on other molecules and on its own molecule, but cannot inhibit phosphorylation of p38MAPK by other kinases [51]. Here, a significantly inhibitory effect of the MAPK inhibitor on phosphorylated p38MAPK was found, which can probably be ascribed to auto-phosphorylation in monocytes [52-54]. This alternative, non-canonical pathway for p38MAPK phosphorylation could be another partial explanation for the incomplete inhibition of monocytes by tacrolimus and MPA, besides the unaffected ERK phosphorylation.

The residual phosphorylation of the signaling molecules after tacrolimus or MPA treatment may imply that monocyte functions, such as phagocytosis, remain intact. After SOT, phagocytosis by monocytes/macrophages is one of the mechanisms to overcome infections. Here, phagocytosis of E.coli-bacteria by monocytes was not affected by either tacrolimus or MPA, suggesting that this monocyte function is still active during immunosuppressive drug treatment.

The present study reports for the first time on the change of human macrophage maturation and polarization in the presence of tacrolimus or MPA. Only high concentrations of tacrolimus affect the maturation and change the polarization of macrophages to some extent. Addition of tacrolimus or MPA did not affect the expression of CD80/CD64 (M1). However, tacrolimus did stimulate the expression of CD16 and CD200R (M2). A similar change in polarization by tacrolimus was previously found in mouse studies [55]. In addition, the effect of tacrolimus and MPA on macrophage polarization in the presence of specific stimuli was investigated. A shift to an M2 phenotype was noticed when monocytes were cultured with tacrolimus or MPA in combination with IFN-γ, a cytokine that induces the differentiation of monocytes into M1 macrophages. CD200R and CD163 (M2) expression was increased, although the expression in M1 markers did not change. However, in the presence of M2-driving cytokines (IL-4 or IL-10) the CD80 marker for M1 macrophages was

diminished by tacrolimus, again pointing to induction of M2 differentiation. Altogether, these findings suggest that tacrolimus and MPA have a limited effect on the function of monocytes by driving their differentiation towards an M2 phenotype.

In order to reveal the clinical relevance of the limited effect of tacrolimus and MPA on monocytes, the drug effects should be studied in a clinical setting. The role of monocytes in alloreactivity after SOT includes the recognition of non-self antigens during the cellular immune response and danger signals (PAMP's) induced by ischemia-reperfusion injury [56,57]. The humoral immune response includes the activation of Fcγ-receptors on monocytes by allo-antibodies [8]. This immune response plays an important role in chronic antibody-mediated rejection which is the main reason for chronic graft loss [58]. The residual monocyte activity, a consequence of the limited effect of tacrolimus and MPA treatment, may partly explain why chronic antibody-mediated rejection occurs after SOT. Furthermore, our previous study on monocytes from kidney transplant patients showed a similarly limited functional effect of immunosuppressive drugs on monocytes, suggesting that these cells can still play a role in early post-transplant cellular immunity [59]. As readout in a new study, signaling protein phosphorylation could be measured, thereby relating the results to the outcomes of the present paper. Here, we focused on the activation and function of CD14+ monocytes, without dividing them into CD16-positive and -negative subsets, which show differential responses in an inflammatory setting [60]. Future studies can focus on these subsets to reveal the effects of immunosuppressive medication on these clinically relevant monocyte subsets. In addition, studies in patients can unveil the effect of combination therapy on monocyte activation and function, since the present study focused on the individual immunosuppressive drug effects. It must also be considered that the role of macrophages may be different in different types of organ transplantation. For example, cardiac macrophages are involved in tissue remodeling and repair after myocardial infarction, while in the lungs their primary role is immune surveillance. In the liver, Kupffer cells are involved in the breakdown of erythrocytes and the response to infections, toxins, ischemia and other stress conditions [61,62]. These cells are involved in allograft rejection and may also play an important role in the development of immune tolerance after transplantation, suggesting that (partly) inhibition of these cells with tacrolimus and MPA may will cause negative effects on graft survival [62]. In addition, macrophages are known for their heterogeneity and it is possible that the composition of macrophage subsets is different for different organs [63,64]. For example, the polarization of tissue-resident macrophages is dependent on their local environment, suggesting high heterogeneity in macrophage subsets between different organs [65]. Research is needed to show the functional effects of tacrolimus and MPA on these cells. However, each macrophage does have targets for tacrolimus and MPA (e.g. calcineurin pathway and inosine monophosphate dehydrogenase) suggesting that the difference in sensibility for these drugs between different macrophage subsets at the single cell level may be limited.

In conclusion, tacrolimus and MPA hardly suppress monocyte signaling pathway activation. The residual phosphorylation of signaling proteins explains the limited effect of both immunosuppressive drugs on cytokine production and phagocytosis, apart from monocyte differentiation. This suggests that innate immune responses induced by monocytes after SOT may still occur despite immunosuppressive therapy.

References

1. Rowshani AT, Vereyken EJ. The role of macrophage lineage cells in kidney graft rejection and survival. Transplantation 2012;94:309-18.
2. Nagata Y, Fujimoto M, Nakamura K, et al. Anti-TNF-α Agent Infliximab and Splenectomy Are Protective Against Renal Ischemia-Reperfusion Injury. Transplantation 2016;100:1675-82.
3. Girlanda R, Kleiner DE, Duan Z, et al. Monocyte infiltration and kidney allograft dysfunction during acute rejection. Am J Transplant 2008;8:600-7.
4. Kraaij MD, Vereyken EJ, Leenen PJ, et al. Human monocytes produce interferon-gamma upon stimulation with LPS. Cytokine 2014;67:7-12.
5. Grigoryev YA, Kurian SM, Avnur Z, et al. Deconvoluting post-transplant immunity: cell subset-specific mapping reveals pathways for activation and expansion of memory T, monocytes and B cells. PLoS One 2010;5:e13358.
6. Day YJ, Huang L, Ye H, Linden J, Okusa MD. Renal ischemia-reperfusion injury and adenosine 2A receptor-mediated tissue protection: role of macrophages. Am J Physiol Renal Physiol 2005;288:F722-31.
7. Mannon RB. Macrophages: contributors to allograft dysfunction, repair, or innocent bystanders? Curr Opin Organ Transplant 2012;17:20-5.
8. Wasowska BA. Mechanisms involved in antibody- and complement-mediated allograft rejection. Immunol Res 2010;47:25-44.
9. Schmidt RE, Gessner JE. Fc receptors and their interaction with complement in autoimmunity. Immunol Lett 2005;100:56-67.
10. Mantovani A, Biswas SK, Galdiero MR, Sica A, Locati M. Macrophage plasticity and polarization in tissue repair and remodelling. J Pathol 2013;229:176-85.
11. Udalova IA, Mantovani A, Feldmann M. Macrophage heterogeneity in the context of rheumatoid arthritis. Nat Rev Rheumatol 2016;12:472-85.
12. Clavel C, Ceccato L, Anquetil F, Serre G, Sebbag M. Among human macrophages polarised to different phenotypes, the M-CSF-oriented cells present the highest pro-inflammatory response to the rheumatoid arthritis-specific immune complexes containing ACPA. Ann Rheum Dis 2016;75:2184-91.
13. Mosser DM. The many faces of macrophage activation. J Leukoc Biol 2003;73:209-12.
14. Mantovani A, Sica A, Sozzani S, Allavena P, Vecchi A, Locati M. The chemokine system in diverse forms of macrophage activation and polarization. Trends Immunol 2004;25:677-86.
15. Murray PJ, Allen JE, Biswas SK, et al. Macrophage activation and polarization: nomenclature and experimental guidelines. Immunity 2014;41:14-20.
16. van Gelder T, Hesselink DA. Mycophenolate revisited. Transpl Int 2015;28:508-15.
17. Meier-Kriesche HU. Mycophenolate mofetil-based immunosuppressive minimization and withdrawal strategies in renal transplantation: possible risks and benefits. Curr Opin Nephrol Hypertens 2006;15 Suppl 1:S1-5.

18. Kidney-Disease:-Improving-Global-Outcomes-(KDIGO)-Transplant-Work-Group. KDIGO Clinical Practice Guidelines for the Care of Kidney Transplant Recipients. Am J Transplant 2009;9:S1-S157.
19. Halloran PF. Immunosuppressive drugs for kidney transplantation. N Engl J Med 2004;351:2715-29.
20. Matas AJ, Smith JM, Skeans MA, et al. OPTN/SRTR 2011 Annual Data Report: kidney. Am J Transplant 2013;13 Suppl 1:11-46.
21. Jasiak NM, Park JM. Immunosuppression in Solid-Organ Transplantation: Essentials and Practical Tips. Crit Care Nurs Q 2016;39:227-40.
22. Kaufman DB, Shapiro R, Lucey MR, Cherikh WS, R TB, Dyke DB. Immunosuppression: practice and trends. Am J Transplant 2004;4 Suppl 9:38-53.
23. Christie JD, Edwards LB, Kucheryavaya AY, et al. The Registry of the International Society for Heart and Lung Transplantation: 29th adult lung and heart-lung transplant report-2012. J Heart Lung Transplant 2012;31:1073-86.
24. Meier-Kriesche HU, Li S, Gruessner RW, et al. Immunosuppression: evolution in practice and trends, 1994-2004. Am J Transplant 2006;6:1111-31.
25. Cury Martins J, Martins C, Aoki V, Gois AF, Ishii HA, da Silva EM. Topical tacrolimus for atopic dermatitis. Cochrane Database Syst Rev 2015:CD009864.
26. Chang KT, Lin HY, Kuo CH, Hung CH. Tacrolimus suppresses atopic dermatitis-associated cytokines and chemokines in monocytes. J Microbiol Immunol Infect 2016;49:409-16.
27. Komaki Y, Komaki F, Ido A, Sakuraba A. Efficacy and Safety of Tacrolimus Therapy for Active Ulcerative Colitis; A Systematic Review and Meta-analysis. J Crohns Colitis 2016;10:484-94.
28. Okada Y, Maeda N, Takakura S, Miyata K, Koshiba M. Preventive and therapeutic effects of tacrolimus in an interleukin-10-deficient mouse model of colitis. Inflamm Res 2011;60:1049-59.
29. Dall'Era M. Mycophenolate mofetil in the treatment of systemic lupus erythematosus. Curr Opin Rheumatol 2011;23:454-8.
30. Howell J, Sawhney R, Testro A, et al. Cyclosporine and tacrolimus have inhibitory effects on toll-like receptor signaling after liver transplantation. Liver Transpl 2013;19:1099-107.
31. Puzik A, Schultz C, Iblher P, Muller-Steinhardt M, Hartel C. Effects of ciclosporin A, tacrolimus and sirolimus on cytokine production in neonatal immune cells. Acta Paediatr 2007;96:1483-9.
32. Weimer R, Mytilineos J, Feustel A, et al. Mycophenolate mofetil-based immunosuppression and cytokine genotypes: effects on monokine secretion and antigen presentation in long-term renal transplant recipients. Transplantation 2003;75:2090-9.
33. O'Neill LA, Golenbock D, Bowie AG. The history of Toll-like receptors - redefining innate immunity. Nat Rev Immunol 2013;13:453-60.
34. Luyendyk JP, Schabbauer GA, Tencati M, Holscher T, Pawlinski R, Mackman N. Genetic analysis of the role of the PI3K-Akt pathway in lipopolysaccharide-induced cytokine and tissue factor gene expression in monocytes/macrophages. J Immunol 2008;180:4218-26.

35. Wang N, Liang H, Zen K. Molecular mechanisms that influence the macrophage m1-m2 polarization balance. Front Immunol 2014;5:614.

36. Li LB, Leung DY, Goleva E. Activated p38 MAPK in Peripheral Blood Monocytes of Steroid Resistant Asthmatics. PLoS One 2015;10:e0141909.

37. Vafadari R, Hesselink DA, Cadogan MM, Weimar W, Baan CC. Inhibitory Effect of Tacrolimus on p38 Mitogen-Activated Protein Kinase Signaling in Kidney Transplant Recipients Measured by Whole-Blood Phosphospecific Flow Cytometry. Transplantation 2012;93:1245-51.

38. Ambarus CA, Krausz S, van Eijk M, et al. Systematic validation of specific phenotypic markers for in vitro polarized human macrophages. J Immunol Methods 2012;375:196-206.

39. Badou A, Bennasser Y, Moreau M, Leclerc C, Benkirane M, Bahraoui E. Tat protein of human immunodeficiency virus type 1 induces interleukin-10 in human peripheral blood monocytes: implication of protein kinase C-dependent pathway. J Virol 2000;74:10551-62.

40. Foey AD, Brennan FM. Conventional protein kinase C and atypical protein kinase Czeta differentially regulate macrophage production of tumour necrosis factor-alpha and interleukin-10. Immunology 2004;112:44-53.

41. Visintin A, Mazzoni A, Spitzer JH, Wyllie DH, Dower SK, Segal DM. Regulation of Toll-like receptors in human monocytes and dendritic cells. J Immunol 2001;166:249-55.

42. Allison AC, Eugui EM. Mycophenolate mofetil and its mechanisms of action. Immunopharmacology 2000;47:85-118.

43. Shao K, Lu Y, Wang J, et al. Different Effects of Tacrolimus on Innate and Adaptive Immune Cells in the Allograft Transplantation. Scand J Immunol 2016;83:119-27.

44. Numazawa S, Watabe M, Nishimura S, Kurosawa M, Izuno M, Yoshida T. Regulation of ERK-mediated signal transduction by p38 MAP kinase in human monocytic THP-1 cells. J Biochem 2003;133:599-605.

45. Feng GJ, Goodridge HS, Harnett MM, et al. Extracellular signal-related kinase (ERK) and p38 mitogen-activated protein (MAP) kinases differentially regulate the lipopolysaccharide-mediated induction of inducible nitric oxide synthase and IL-12 in macrophages: Leishmania phosphoglycans subvert macrophage IL-12 production by targeting ERK MAP kinase. J Immunol 1999;163:6403-12.

46. Baan C, Bouvy A, Vafadari R, Weimar W. Phospho-specific flow cytometry for pharmacodynamic monitoring of immunosuppressive therapy in transplantation. Transplant Res 2012;1:20.

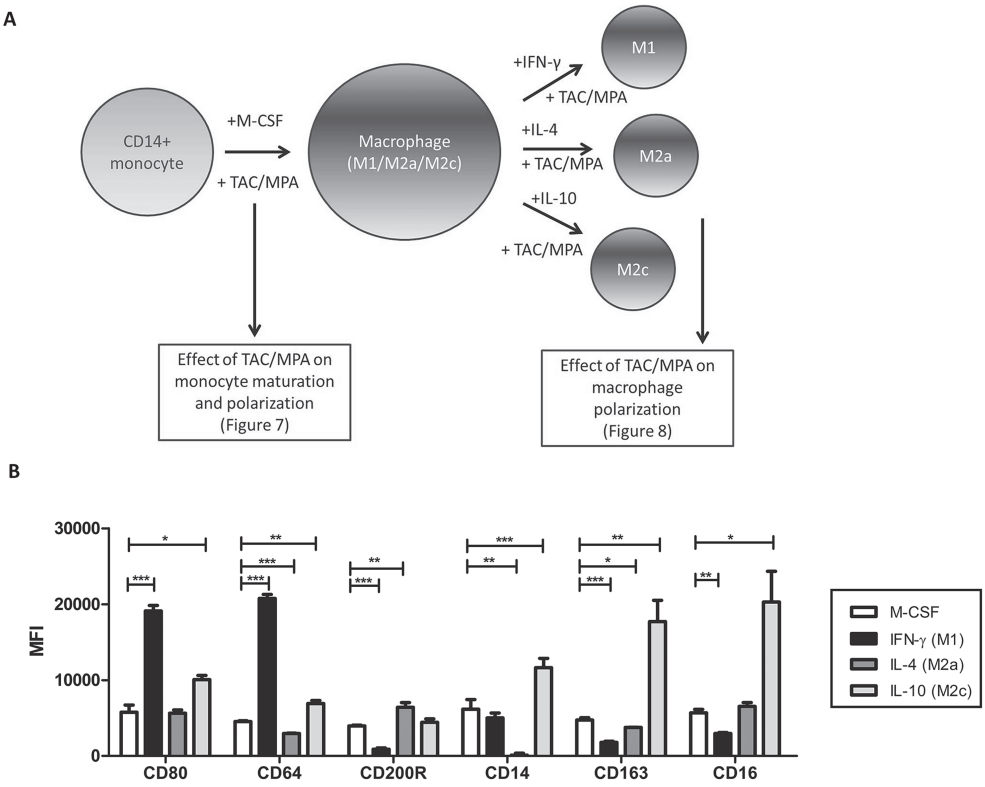
47. Vafadari R, Kraaijeveld R, Weimar W, Baan CC. Tacrolimus inhibits NF-kappaB activation in peripheral human T cells. PLoS One 2013;8:e60784.

48. Schaeffer V, Arbabi S, Garcia IA, et al. Role of the mTOR pathway in LPS-activated monocytes: influence of hypertonic saline. J Surg Res 2011;171:769-76.

49. Molnarfi N, Gruaz L, Dayer JM, Burger D. Opposite regulation of IL-1beta and secreted IL-1 receptor antagonist production by phosphatidylinositide-3 kinases in human monocytes activated by lipopolysaccharides or contact with T cells. J Immunol 2007;178:446-54.

50. Lodermann B, Wunderlich R, Frey S, et al. Low dose ionising radiation leads to a NF-kappaB dependent decreased secretion of active IL-1beta by activated macrophages with a discontinuous dose-dependency. Int J Radiat Biol 2012;88:727-34.
51. Kim L, Del Rio L, Butcher BA, et al. p38 MAPK autophosphorylation drives macrophage IL-12 production during intracellular infection. J Immunol 2005;174:4178-84.
52. Mittelstadt PR, Salvador JM, Fornace JAJ, Ashwell JD. Activating p38 MAPK: New Tricks for an Old Kinase. Cell Cycle 2005;4:1189-92.
53. Pimienta G, Pascual J. Canonical and Alternative MAPK Signaling. Cell Cycle 2007;6:2628-32.
54. Salvador JM, Mittelstadt PR, Guszczynski T, et al. Alternative p38 activation pathway mediated by T cell receptor-proximal tyrosine kinases. Nat Immunol 2005;6:390-5.
55. Bai L, Gabriels K, Wijnands E, et al. Low- but not high-dose FK506 treatment confers atheroprotection due to alternative macrophage activation and unaffected cholesterol levels. Thromb Haemost 2010;104:143-50.
56. Oberbarnscheidt MH, Zeng Q, Li Q, et al. Non-self recognition by monocytes initiates allograft rejection. J Clin Invest 2014;124:3579-89.
57. Zhuang Q, Lakkis FG. Dendritic Cells and Innate Immunity in Kidney Transplantation. Kidney international 2015;87:712-8.
58. Sellares J, Reeve J, Loupy A, et al. Molecular diagnosis of antibody-mediated rejection in human kidney transplants. Am J Transplant 2013;13:971-83.
59. Vereyken EJ, Kraaij MD, Baan CC, et al. A shift towards pro-inflammatory CD16+ monocyte subsets with preserved cytokine production potential after kidney transplantation. PLoS One 2013;8:e70152.
60. Ziegler-Heitbrock L. Blood Monocytes and Their Subsets: Established Features and Open Questions. Front Immunol 2015;6:423.
61. Ju C, Tacke F. Hepatic macrophages in homeostasis and liver diseases: from pathogenesis to novel therapeutic strategies. Cell Mol Immunol 2016;13:316-27.
62. Bilzer M, Roggel F, Gerbes AL. Role of Kupffer cells in host defense and liver disease. Liver Int 2006;26:1175-86.
63. Gordon S, Pluddemann A, Martinez Estrada F. Macrophage heterogeneity in tissues: phenotypic diversity and functions. Immunol Rev 2014;262:36-55.
64. Epelman S, Lavine KJ, Randolph GJ. Origin and functions of tissue macrophages. Immunity 2014;41:21-35.
65. Lavin Y, Winter D, Blecher-Gonen R, et al. Tissue-resident macrophage enhancer landscapes are shaped by the local microenvironment. Cell 2014;159:1312-26.

Supplementary Figures and Tables



Supplementary Figure 1. Monocyte differentiation experiments. (A) Schematic overview of the monocyte differentiation experiments. For the first part of the differentiation study, monocytes were cultured in the presence of M-SCF to induce maturation into macrophages. Next, the expression of the surface markers for M1, M2a or M2c macrophages was determined after addition of tacrolimus or MPA to the culture system. In the second part of the experiments, monocytes were induced to polarize into a specific macrophage subtype. Addition of IFN-γ drives the monocytes to polarize into M1 macrophages, IL-4 induces M2a and IL-10 increases M2c macrophages. Then, tacrolimus and MPA were added to the cultured cells to determine the capability of both drugs to change the expression of surface markers on M1, M2a and M2c differentiated macrophages. (B) Validation of the differentiation assay. After addition of IFN-γ to the culture medium, monocytes are stimulated to differentiate into M1 macrophages with a significantly higher expression of the CD80 ($p < 0.001$) and CD64 ($p < 0.001$) compared to monocytes cultured without the addition of extra cytokines**.** Culturing with IL-4 increased the expression of CD200R ($p < 0.01$), lowered CD14 expression ($p < 0.01$), and thus drove the differentiation into M2a macrophages. IL-10 stimulation induced the expression of CD163 ($p < 0.01$) and CD16 ($p < 0.05$) and resulted in M2c macrophage differentiation. (Data are plotted as the mean ±SEM; n = 5) *) $p < 0.05$; **) $p < 0.01$; ***) $p < 0.001$

Supplementary Table I. Overview of the effects of tacrolimus and MPA on monocyte activation and function

	Tacrolimus	MPA	Comments
Signaling pathways	+	+	Maximum of 30%
Cytokine production	-	+	50% inhibition by MPA
Phagocytosis	-	-	
Differentiation	+/-	+/-	Change in M2 expression markers, but not all subset markers

+) effect; +/-) small effect; -) no effect

4

Pharmacodynamic Monitoring of Tacrolimus–Based Immunosuppression in CD14$^+$ Monocytes after Kidney Transplantation

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Abstract

Background

Monocytes significantly contribute to ischemia reperfusion injury and allograft rejection after kidney transplantation. However, the knowledge about the effects of immunosuppressive drugs on monocyte activation is limited. Conventional pharmacokinetic methods for immunosuppressive drug monitoring are not cell type-specific. In this study, phosphorylation of three signaling proteins was measured to determine the pharmacodynamic effects of immunosuppression on monocyte activation in kidney transplant patients.

Methods

Blood samples from 20 kidney transplant recipients were monitored before and during the first year after transplantation. All patients received induction therapy with basiliximab, followed by tacrolimus (TAC), mycophenolate mofetil (MMF), and prednisolone maintenance therapy. TAC whole-blood pre-dose concentrations were determined using an antibody-conjugated magnetic immunoassay. Samples were stimulated with PMA/ionomycin and phosphorylation of p38MAPK, ERK, and Akt in $CD14^+$ monocytes was quantified by phospho-specific flow cytometry.

Results

Phosphorylation of p38MAPK and Akt in monocytes of immunosuppressed recipients was lower after 360 days compared with before transplantation in the unstimulated samples (mean median fluorescence intensity (MFI) reduction 36%; range -28% to 77% for p-p38MAPK and 20%; range -22% to 53% for p-Akt; $p < 0.05$). P-ERK was only decreased at day 4 after transplantation (mean inhibition 23%; range -52% to 73%; $p < 0.05$). At day 4, when the highest whole-blood pre-dose TAC concentrations were measured, p-p38MAPK and p-Akt, but not p-ERK, correlated inversely with TAC ($r_s = -0.65$; $p = 0.01$ and $r_s = -0.58$; $p = 0.03$, respectively).

Conclusions

Immunosuppressive drug combination therapy partially inhibits monocyte activation pathways after kidney transplantation. This inhibition can be determined by phospho-specific flow cytometry, which enables the assessment of the pharmacodynamic effects of immunosuppressive drugs in a cell-type-specific manner.

Introduction

Monocytes and macrophages contribute to the immune responses after kidney transplantation, which include tissue repair after ischemia-reperfusion injury, as well as acute cellular and antibody-mediated allograft rejection [1-5]. After ischemia-reperfusion injury, monocytes are activated, in particular via their Toll-like receptor (TLR)-4, and infiltrate the allograft [6,7]. Directly after transplantation and during acute cellular rejection, recipient monocytes migrate to the site of tissue injury at the graft and differentiate into $CD68^+$ macrophages, where the presence of these macrophages is associated with graft dysfunction [4,8]. Infiltrating monocytes can differentiate locally into macrophages, which may be polarized into pro- or anti-inflammatory phenotypes. These have previously been indicated as M1 and M2 macrophages, respectively, and these are now recognized as extremes in a wide functional spectrum [8-10]. Macrophages are key players in the initiation of anti-donor responses through their antigen-presenting function and production of cytokines. In addition to their role in acute cellular rejection, these cells are also involved in antibody-mediated rejection. After binding of monocyte Fc-γ receptors to donor allo-antibodies, the signal will block apoptosis and cause the accumulation of monocytes at the site of inflammation, where they produce pro-inflammatory cytokines [11-14].

Activation of monocytes and macrophages is controlled, among others, by the three intracellular signaling molecules p38 Mitogen-Activated Protein Kinase (p38MAPK), Extracellular signal-Regulated Kinases 1 and 2 (ERK1/2), and AKT8 virus oncogene cellular homolog (Akt) [15-20]. Phosphorylation of these molecules by upstream kinases in the signaling pathway causes them to act on transcription factors. Phosphorylation of the MAPK members p38MAPK and ERK will lead to the activation of transcription factors (e.g., NFκB, CREB, ATF-1) that regulate the transcription and translation of several genes involved in cytokine production (e.g., TNF-α, IL-1β and IL-6). In the end, activation of the MAPK pathway will affect many other monocyte functions, such as phagocytosis and differentiation into distinct macrophage activation stages [21-23]. Similarly, Akt plays a central role in several pathways (PI3K, NFκB, and mTOR) involved in cytokine production, macrophage differentiation, and phagocytosis [24-26].

After kidney transplantation, most patients are treated with combination immunosuppressive drug therapy consisting of tacrolimus (TAC), mycophenolic acid (MPA), and glucocorticoids to prevent allograft rejection [27]. The effects of these drugs on alloreactive T-cell function have been extensively characterized, but the knowledge of their effect on monocytes is limited [5]. The few ***in vitro*** studies that have been conducted in this respect have indicated that TAC and MPA affect cytokine production by monocytes [28,29]. Furthermore, TAC did not affect phagocytosis or production of IL-1β ***in vitro***, whereas MPA did reduce the production of IL-1β [30].

Given the important role of monocyte/macrophages in the immune responses following kidney transplantation, a deeper understanding of the effect of immunosuppressive drugs on their activation is important. Furthermore, there is an unmet need for laboratory

techniques that can reliably measure such effects to guide clinical immunosuppression. The conventional method of therapeutic drug monitoring (TDM) of immunosuppressive drugs is pharmacokinetic monitoring by determining the (pre-dose) concentration of these drugs in whole blood (in case of TAC) or plasma (in case of MPA). This, however, disregards putative differences in individual responsiveness to these agents. Possibly, cell-specific and pharmacodynamic monitoring of the effects of immunosuppressive drug therapy on monocyte signaling pathway activation may be a superior strategy for TDM [31-34].

To define a new method for monitoring the impact of immunosuppression on monocyte activation we monitored and quantified the phosphorylation of p38MAPK, ERK, and Akt by phospho-specific flow cytometry, in whole-blood samples of kidney transplant patients before and after transplantation during treatment with TAC, MPA, and glucocorticoids.

Materials and Methods

Kidney transplant patients

To determine the effect of immunosuppressive drugs on $CD14^+$ monocyte activation, we studied 20 renal transplant patients who were followed during the first 12 months after transplantation. The present study was part of a clinical study that was approved by the Medical Ethical Committee of the Erasmus MC, University Medical Center (MEC number 2012-421, EudraCT # 2012-003169-16) [35,36]. All participants gave written consent for collecting their blood samples. Patients were treated with 20 mg basiliximab intravenously (Simulect®, Novartis, Basel, Switzerland) on the day of transplantation and day 4 after transplantation. During the first three post-operative days, prednisolone was administered intravenously in a dosage of 100 mg/day. Subsequently, prednisolone was given orally in a dose of 20 mg and tapered to 5 mg/day by month 3. Mycophenolate mofetil (MMF; Cellcept®; Roche, Basel, Switzerland) was given in a starting dose of 2000 mg/day equally divided in two doses, and then adjusted to pre-dose concentrations (target concentration range: 1.5-3.0 µg/mL). Patients received TAC (Prograf®, Astellas Pharma Inc., Tokyo, Japan) from the day of transplantation twice a day with a starting dose of 0.2 mg/kg/day. Thereafter, TAC was adjusted to pre-dose concentrations: 10-15 ng/mL (week 1-2), 8-12 ng/mL (week 3-4), and 5-10 ng/mL (from week 5 onwards). Heparin blood samples were collected pre-transplantation and 4 days, 1 month, and 3, 6, and 12 months post-transplantation.

Absolute numbers of $CD14^+$ monocytes were measured with BD multi-test 6-colour in BD TruCount Tubes (BD Biosciences, San Jose, CA). TAC whole-blood and MPA plasma pre-dose concentrations were determined in EDTA blood using the antibody-conjugated magnetic immunoassay on a Dimension Xpand analyzer (Siemens HealthCare Diagnostics Inc., Newark, DE) according to the manufacturer's instructions. The lower and upper limits of quantification of TAC were 1.5 and 30 ng/mL and for MPA 0.5 µg/mL and 15 µg/mL, respectively. For TAC, the coefficients of variation (CV) were 15.0%, 8.9%, and 11.2% for the

low, middle, and high control samples, respectively. For MPA, the CV were 3.9% and 3.7%, for the low and high controls, respectively. Proficiency samples were obtained from the UK Quality Assessment Scheme (Analytical Services International Ltd, London, UK) and the laboratory successfully participates in this international proficiency testing scheme.

Whole–blood phospho–specific flow cytometry

Phosphorylation of p38MAPK, ERK, and Akt was measured in whole-blood samples according to the manufacturer's instructions for phosphoprotein analysis (BD Biosciences; CV: 5.6%) and as described previously [37,38]. In short, 200 µL heparinized blood was stained for 30 minutes at 37ºC with Fluorescein Isothiocyanate (FITC)-labeled mouse anti-human CD14 (Serotec, Oxford, UK) and Brilliant Violet (BV) 510-labeled mouse anti-human CD3 (Biolegend, San Diego, CA). After 15 minutes of staining, PMA/ionomycin (Sigma-Aldrich, Steinheim, Germany) was added for 15 minutes to activate the blood cells. Applied final concentrations of PMA/ionomycin were 500 ng per mL/5 µg per mL for samples stained for p38MAPK and Akt, and 100 ng per mL/1 µg per mL was used for ERK, based on prior titration for optimal detection of phosphorylated protein. Thereafter, cells were fixed for 10 minutes with Lyse/Fix buffer (BD Biosciences). After permeabilization with 90% methanol at -20ºC for 30 minutes, intracellular staining was performed with phycoerythrin (PE)-labeled mouse anti-p-p38MAPK (clone pT180/pY182), PE-labeled mouse anti-p-Akt (clone pS473), or AlexaFluor647 (AF647)-labeled mouse anti-p-ERK1/2 (pT202/pY204) mAB (all from BD Biosciences) for 30 minutes at room temperature. Samples were analyzed on a FACS Canto II flow cytometer (BD Biosciences). Isotype controls; mouse IgG1-PE (p38MAPK and Akt, Biolegend) and mouse IgG1-AF647 (ERK; Biolegend); were included in separate tubes and served as negative controls. Interday-variability of the flow cytometer was corrected by using Cytocalbeads (Thermo Scientific, Fremont, CA) according to the manufacturer's instructions.

Statistical analysis

The Median Fluorescence Intensity (MFI) was measured for the phosphorylation of p38MAPK, ERK, and Akt and data analysis was performed with Diva-version 6.0 software (BD Bioscience). MFI values were normalized using Cytocalbeads (Thermo Scientific). Statistical analysis was performed with Graph Pad Prism 5.0 (Graph Pad Software Inc., La Jolla, CA) by using paired and unpaired t-tests (after finding a p-value > 0.05 with the Kolmogorov-Smirnov test for normality for the study population). Correlations between drug concentrations and phosphorylation were calculated as the Spearman correlation coefficient. Associations between phosphorylation levels and covariates were tested by linear regression with IBM SPSS statistics software (version 21; IBM Analytics, Chicago, Illinois, USA). Bonferroni correction was used to correct for multiple testing. A two-sided p-value < 0.05 was considered statistically significant, and for the association calculations, a two-sided p value < 0.006 was considered statistically significant after Bonferroni correction.

Results

Patient characteristics

Baseline characteristics of the kidney transplant patients at the time of transplantation are shown in **Table I**. Two patients suffered from an acute T-cell mediated rejection corresponding to an overall one-year acute rejection incidence of 10%. The rejections were classified as Banff type 1B and 2A and occurred on post-operative days 152 and 10, respec-

Table I: Patient demographics and baseline characteristics

	Study population (n = 20)
Age in years	55 (21-76)
Male/female	16 (80%)/4 (20%)
Ethnicity	
• Caucasian	16 (80%)
• African	2 (10%)
• Asian	2 (10%)
Body weight (kg, mean and range)	88.5 (51.4-120.0)
HLA A mismatch	1.4 (± 0.5)
HLA B mismatch	1.5 (± 0.5)
HLA DR mismatch	1.3 (± 0.4)
Current PRA (%) (mean and range)	2.5 (0-17)
Peak PRA (%) (mean and range)	4.2 (0-21)
Donor age in years	51 (22-80)
Living-related/living-unrelated donor	5 (25%)/15 (75%)
Cause of end-stage renal disease	
• Diabetes mellitus	7 (35%)
• Hypertension	5 (25%)
• IgA nephropathy	3 (15%)
• Polycystic kidney disease	3 (15%)
• Obstructive nephropathy	1 (5%)
• Unknown	0 (0%)
• Other	1 (5%)
Renal replacement therapy prior to transplantation	
• None (pre-emptive)	12 (60%)
• Hemodialysis	6 (30%)
• Peritoneal dialysis	2 (10%)
Time on dialysis (days) (mean and range)	783 (465-1519)

Continuous variables are presented as means (± SD) or medians (range) and categorical variables as numbers (plus percentages), unless otherwise specified.
All patients received their first kidney transplant.
HLA, human leukocyte antigen; PRA, panel reactive antibodies (current = PRA at time of transplantation, peak = historically highest measured PRA); SD, standard deviation.

tively [39,40]. Samples from these patients were excluded for further analysis after the rejection time point. Absolute monocyte counts before and after transplantation were measured. An increase in the absolute monocyte count was measured at day 4 after transplantation (mean increase of 224 monocytes/µL whole blood; $p < 0.01$), which can be due to the surgical procedure (**Figure 1A and Supplementary Table I**). At months 1 and 3, the absolute counts were decreased in comparison to the baseline value ($p < 0.01$ and $p < 0.001$, respectively), while at months 6 and 12 the monocyte numbers recovered to the baseline level. As expected, the TAC pre-dose concentrations were higher at day 4 than at the later time points ($p < 0.001$) with a median concentration of 15.3 ng/mL (9.1 to 28.4) at day 4 ***vs.*** 6.8 ng/mL (4.4 to 13.3) at day 360 (**Figure 1B and Supplementary Table I**). In contrast to TAC, the MPA pre-dose concentrations did not significantly change over time, which reflects TDM and the intention to keep MPA exposure constant **(Supplementary Table I**).

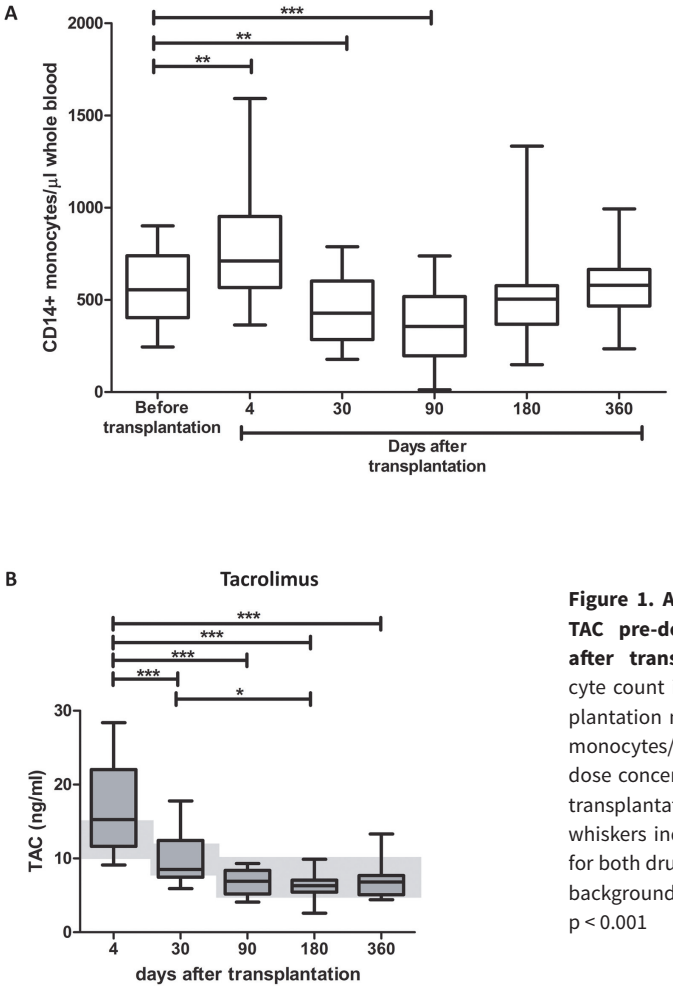

Figure 1. Absolute monocyte numbers and TAC pre-dose concentrations before and after transplantation. A) Absolute monocyte count in patients before and after transplantation measured as the number of $CD14^+$ monocytes/µL whole blood. B) TAC blood pre-dose concentrations within the first year after transplantation. Data are plotted as box and whiskers indicating total range. Target ranges for both drugs are indicated in light grey in the background. ($n = 20$) *) $p < 0.05$, **) $p < 0.01$, ***) $p < 0.001$

Phosphorylation of p38MAPK, ERK, and Akt in kidney transplant patients

To assess the effects of immunosuppression on the potential of monocytes to become activated, the phosphorylation levels of p38MAPK, ERK, and Akt were measured in whole-blood samples from kidney transplant patients either directly or after stimulation with PMA/ionomycin. (**Supplementary Figure 1A and B**)

In the unstimulated samples (directly analyzed in fresh blood), the baseline phosphorylation levels of p38MAPK, ERK, and Akt were higher before transplantation than for the isotype control ($p < 0.001$ for all tested proteins) (**Figure 2**). The phosphorylation level of p38MAPK in these samples was significantly lower compared to pre-transplant levels at all test days through day 360 ($p < 0.01$), except at day 90 (**Figure 2A**). In contrast, the

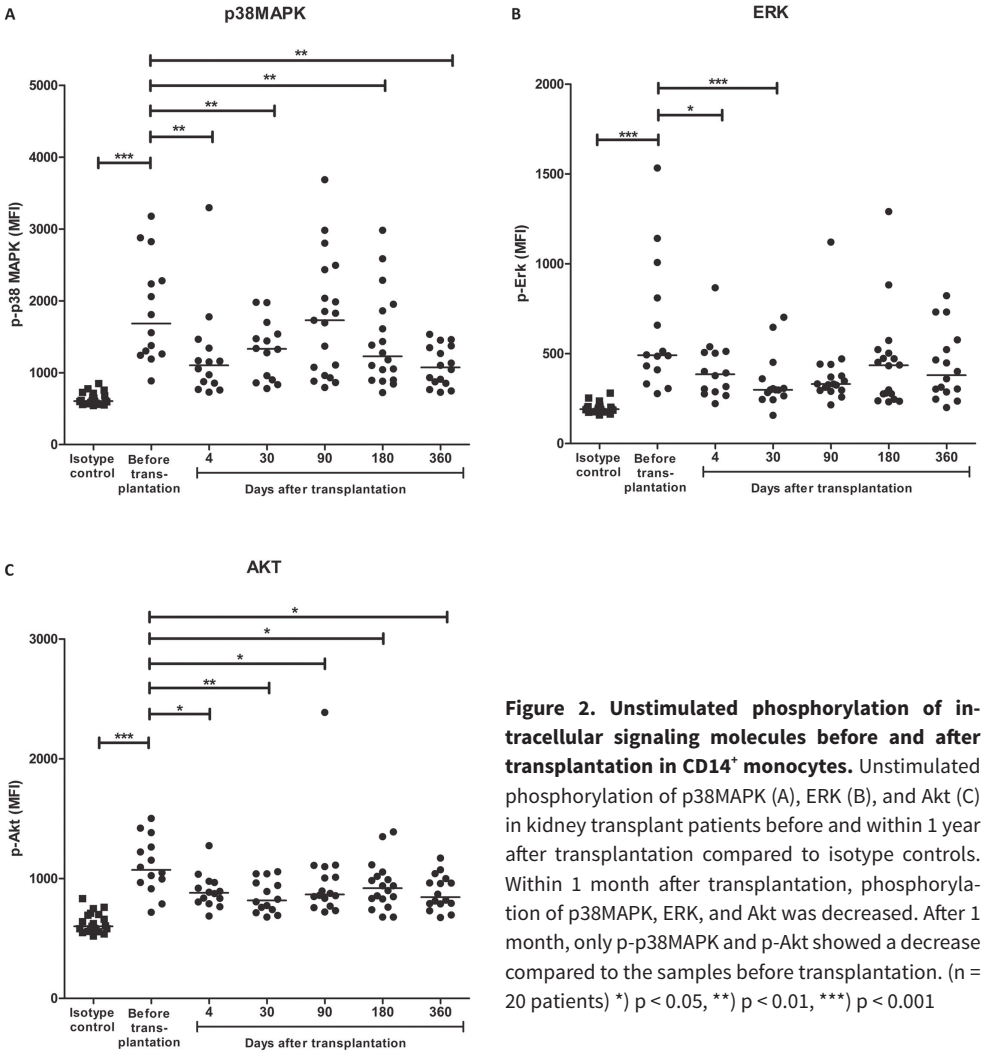

Figure 2. Unstimulated phosphorylation of intracellular signaling molecules before and after transplantation in CD14$^+$ monocytes. Unstimulated phosphorylation of p38MAPK (A), ERK (B), and Akt (C) in kidney transplant patients before and within 1 year after transplantation compared to isotype controls. Within 1 month after transplantation, phosphorylation of p38MAPK, ERK, and Akt was decreased. After 1 month, only p-p38MAPK and p-Akt showed a decrease compared to the samples before transplantation. (n = 20 patients) *) $p < 0.05$, **) $p < 0.01$, ***) $p < 0.001$

other MAPK member, ERK, showed only an inhibited phosphorylation at day 4 and day 30 ($p < 0.05$ and $p < 0.001$, respectively) and a constant phosphorylation pattern between day 90 and day 360 (**Figure 2B**). The MFI values were comparable with the levels before transplantation. The third signaling protein, Akt, showed a decrease in phosphorylation levels at all time points compared with baseline (pre-transplantation; $p < 0.05$) (**Figure 2C**). The strongest reduction was measured at day 30 ($p < 0.01$).

To determine the effects of immunosuppression on the maximum phosphorylation capacity of each tested signaling protein, whole-blood samples were stimulated with PMA/ionomycin for 15 minutes. In these stimulated whole-blood samples, the baseline phosphorylation levels of p38MAPK, ERK, and Akt were higher before transplantation than for the

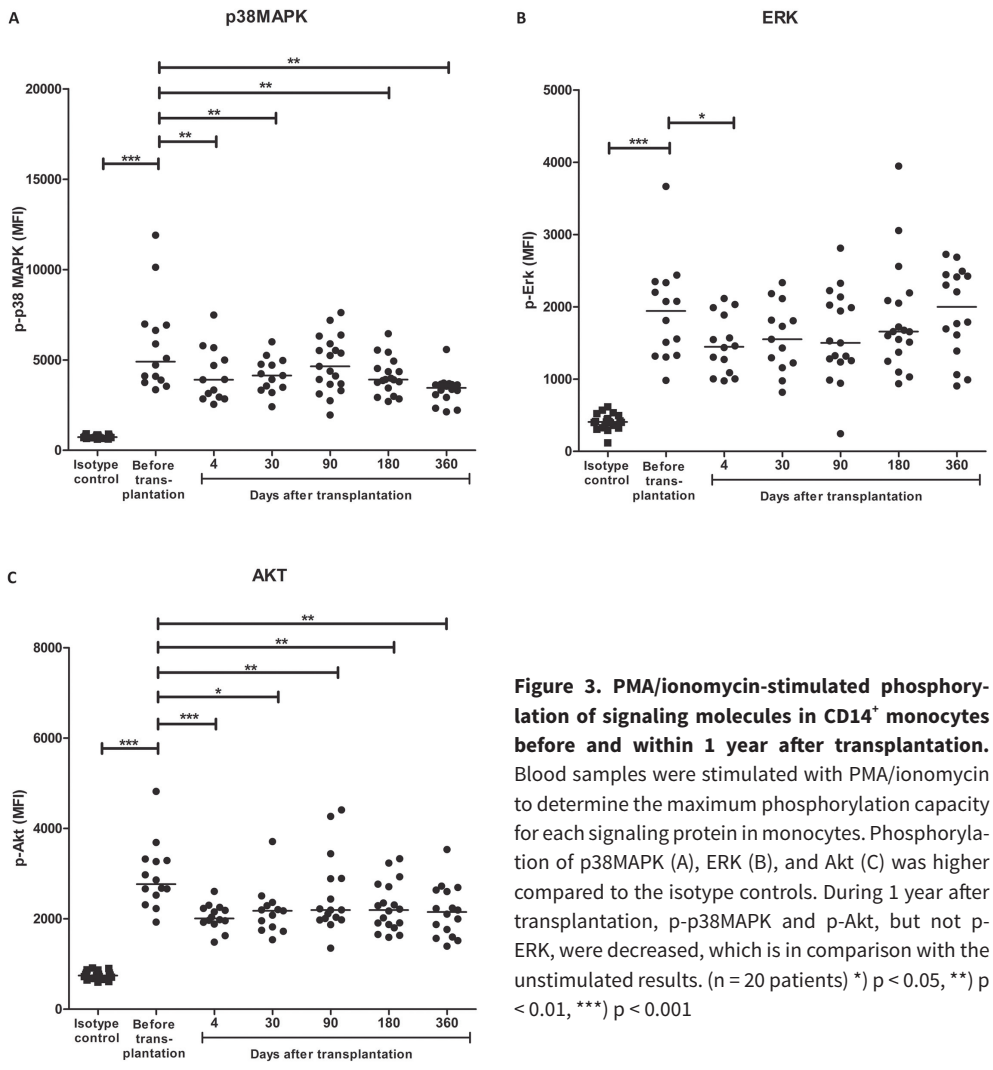

Figure 3. PMA/ionomycin-stimulated phosphorylation of signaling molecules in CD14$^+$ monocytes before and within 1 year after transplantation. Blood samples were stimulated with PMA/ionomycin to determine the maximum phosphorylation capacity for each signaling protein in monocytes. Phosphorylation of p38MAPK (A), ERK (B), and Akt (C) was higher compared to the isotype controls. During 1 year after transplantation, p-p38MAPK and p-Akt, but not p-ERK, were decreased, which is in comparison with the unstimulated results. (n = 20 patients) *) $p < 0.05$, **) $p < 0.01$, ***) $p < 0.001$

isotype control (**Figure 3**). Again, phosphorylation of p38MAPK and Akt was decreased after transplantation compared to pre-transplant phosphorylation (**Figures 3A and 3B**), which was comparable with the results obtained with the directly measured samples. However, p-ERK expression showed only a decrease at day 4 (**Figure 3C**), which was in contrast to the significant decrease observed at both day 4 and day 30 in the unstimulated samples.

One patient, who was diagnosed with acute rejection on day 152, also showed an increase in p-ERK expression over time after stimulation with PMA/ionomycin, while this was not seen for the expression of p-p38MAPK or p-Akt (**Supplementary Figure 2**).

We also calculated the percentage of phosphorylation reduction (**Table II**). In line with the absolute data, the decrease of p-p38MAPK was highest at day 360 (36% (SD ±31%) and 34% (SD ±28%) for the unstimulated and PMA/ionomycin stimulated samples, respectively). At the other time points tested, the decrease was 31% at most. Finally, p-Akt was reduced, with a maximum of 27%, and showed the smallest decrease at day 90 and 180.

Table II: Reduction of signaling molecule phosphorylation

% inhibition	p38MAPK		ERK		Akt	
(mean ± SD)	Unstim	PMA/iono	Unstim	PMA/iono	Unstim	PMA/iono
Day 4	30% (±26%) **	24% (±14%) **	23% (±34%) *	18% (±25%) *	16% (±20%) *	27% (±18%) ***
Day 30	24% (±24%) **	16% (±24%) **	39% (±22%) ***	12% (±37%)	20% (±17%) **	21% (±22%) *
Day 90	13% (±49%)	17% (±31%)	19% (±51%)	7% (±31%)	17% (±21%) *	21% (±18%) **
Day 180	31% (±16%) **	27% (±27%) **	16% (±49%)	-5% (±38%)	13% (±26%) *	25% (±27%) **
Day 360	36% (±31%) **	34% (±28%) **	16% (±61%)	-28% (±60%)	20% (±23%) *	27% (±25%) **

Unstim) unstimulated samples; **PMA/iono**) PMA/ionomycin stimulated samples
Significant difference with baseline phosphorylation: *) $p < 0.05$; **) $p < 0.01$; ***) $p < 0.001$

Correlations of monocyte signaling protein phosphorylation with patient treatment and demographics

To determine a putative association between phosphorylation for all tested signaling proteins and the given immunosuppressive therapy, correlations between immunosuppressive drug pre-dose concentrations and MFI levels at day 4 (n = 14) and 360 (n = 19) in the unstimulated samples were calculated (**Table III**). Both p-p38MAPK and p-AKT, but not p-ERK, showed an inverse correlation with TAC at day 4 ($r_s = -0.65$; $p < 0.05$ and $r_s = -0.58$; $p < 0.05$, respectively) (**Supplementary Figure 3**). At day 360, none of the tested signaling proteins was correlated with TAC pre-dose concentrations.

To define whether the demographic parameters were confounding variables in this study, linear regression analysis was performed (**Supplementary Table II**). After correction for multiple testing, no association between the demographic characteristics of patients and the level of phosphorylation of p38MAPK, ERK, and Akt was found before transplantation or 4 and 360 days after transplantation, indicating that these parameters did not confound the results (**Supplementary Table II**).

Table III: Correlation between signaling molecule phosphorylation (unstimulated) and immunosuppressive drug trough blood concentrations at day 4 and day 360 after transplantation

		p-p38MAPK		p-ERK		p-Akt	
Correlation		r_s	p value	r_s	p value	r_s	p value
day 4	Tacrolimus	**-0.65**	**0.012**	-0.15	0.615	**-0.58**	**0.030**
day 360	Tacrolimus	-0.21	0.512	0.20	0.563	-0.10	0.780

r_s = Spearman's Rank Correlation Coefficient

Discussion

Monocytes and macrophages are crucial cells in the innate immune response and are involved in the adaptive immune response via antigen presentation after kidney transplantation [3,4]. In this pilot study, phospho-specific flow cytometry was used to monitor the effects of immunosuppressive drugs on $CD14^+$ monocyte activation by measuring phosphorylation of three major signaling molecules: p38MAPK, ERK, and Akt.

Phospho-specific flow cytometry is a relatively novel technique useful for studying the pharmacodynamic effects of immunosuppressive drug combination therapy in whole-blood samples of kidney transplant patients at the single-cell level [41-43]. In most transplant centers, TDM is performed by measuring immunosuppressive drug blood concentrations. However, this method is not cell type-specific and does not completely reflect the pharmacodynamic effects of immunosuppressants on monocytes and other immune cells [44]. Furthermore, classic, pharmacokinetic TDM of TAC in general is based on pre-dose concentrations that have an imperfect correlation with the area under the concentration ***vs.*** time curve and do not accurately predict acute rejection [45,46].

In recent years, several methods for pharmacodynamic TDM have been investigated, including the measurement of calcineurin phosphatase activity, cytokine production, and the expression of NFAT-regulated genes [47-50]. However, until now these methods have not found their way into routine clinical practice, because of poor correlation with clinical outcomes, controversial data on the correlation with pharmacokinetic parameters, and high interindividual variability, respectively [44]. Furthermore, these pharmacodynamic assays were developed to study the effect on T-cells and it is unknown whether these methods can also be used for studying the effect of immunosuppressants on monocytes.

A correlation between reduction of p-p38MAPK levels in T-cells and TAC blood concentrations after kidney transplantation was previously found with phospho-specific flow cytometry [37,51]. In the present study, $CD14^+$ monocytes from kidney transplant patients showed a decrease in p-p38MAPK and p-Akt with a maximum of 36% and 20%, respectively, as compared to pre-transplant levels. These results are in line with previous ***in vitro*** findings: healthy control blood samples spiked with TAC showed a maximum p-p38MAPK and p-Akt inhibition in monocytes of 33% and 14%, respectively [30]. In the ***in vitro*** study, spiking with TAC did not affect the production of IL-1β or the phagocytosis by monocytes. Only a slight change in monocyte differentiation toward an M2-like phenotype was measured in the

presence of high TAC concentrations [30]. In contrast to the reduction of p-p38MAPK and p-Akt, phosphorylation of the other MAPK member, ERK, was only significantly reduced within the first month after transplantation. The maximum decrease at day 4 and day 30 was 39%, indicating a stronger reduction of p-ERK than for p-p38MAPK and p-Akt. Of note, the expression of p-ERK after stimulation increased with time after transplantation in the one patient suffering from an acute rejection, indicating a potential role for this signaling molecule in acute rejection.

The decreased phosphorylation found for the signaling molecules indicates that the innate immune response of monocytes is not completely inhibited after kidney transplantation. The incomplete inhibition causes a residual monocyte activity that may contribute to immune responses after kidney transplantation, such as chronic antibody-mediated rejection. The residual monocyte activity is reflected in the retained ability of monocytes to produce pro- and anti-inflammatory cytokines after transplantation [52]. Moreover, the PI3K/Akt pathway is the main regulator of cell survival in human monocytes and decreased activation of this pathway is associated with immunological quiescence after kidney transplantation [53,54]. It has also been suggested that p-Akt inhibition causes impairment in IL-10 production and upregulation of p-p38MAPK and p-ERK1/2 after transplantation [16,55]. Furthermore, MAPK pathways are involved in monocyte adhesion (ERK) and chemotaxis (p38MAPK) [56,57]. p-ERK controls the differentiation, survival, and homeostasis of monocytes when the cells are stimulated with a growth or survival factor, such as M-CSF (macrophage colony-stimulating factor), while inhibition of p-ERK causes cell apoptosis [58,59]. Altogether, this shows that monitoring of signaling pathway activation is important to control monocyte-mediated immune responses after transplantation.

Multiple factors can influence signaling protein phosphorylation, including immunosuppressive drugs. TAC showed a significant negative correlation with phosphorylation intensity of p38MAPK and Akt, suggesting that the inhibition of p-p38MAPK and p-Akt is TAC concentration dependent. However, these correlations were only observed at day 4, when TAC pre-dose concentrations were highest, and are in line with the findings of the previous *in vitro* study [30]. No other associations were observed between patient demographics and signaling protein phosphorylation, indicating that phospho-specific flow cytometry is a promising tool to detect TAC effects after transplantation.

These data also indicate that TAC has the most important role in the inhibition of intracellular signaling pathways in monocytes within 4 days after transplantation, while the inhibition at later time points may be due to the presence of prednisolone in the blood samples. In mouse peritoneal macrophages, the glucocorticoid receptor is involved in the inhibition of p-p38MAPK, while p-ERK and p-Akt are not affected by glucocorticoid signaling [60]. This suggests that the given prednisolone doses in the present study could only inhibit p-p38MAPK. However, the prednisolone blood concentrations were not measured in this study, and more research is needed to distinguish between the individual effects of glucocorticoids on monocyte intracellular activation pathways.

The present study provides preliminary data on the use of phospho-specific flow cytometry for clinical diagnostics. More research is needed to translate the present findings on phosphorylation status into meaningful clinical diagnostics. For example, all tested proteins in the present study showed the least percentage of phosphorylation inhibition between day 90 and 180 after transplantation, but it is unknown whether this will also increase the risk of monocyte-mediated rejection. The technique is ready to be used for clinical diagnostics of malignancies in the field of hematology and oncology [61,62]. However, for daily clinical TDM of immunosuppressive drugs, this technique needs more validation to become a standardized procedure. The labor intensity, reproducibility, and cost-effectiveness of the technique should be established. However, compared to western blotting, phospho-specific flow cytometry is cell specific and a relatively rapid method to measure cell signaling pathway activation. For example, the turnaround time of the test used in the present study is only 4 hours. The next step would be to study the correlation of phosphorylation profiles with pharmacokinetic parameters and to find a threshold of phosphorylation that indicates a risk for rejection. In a future prospective study, blood samples from kidney transplant patients who might develop rejection should be measured and the predictive value of the phosphorylation status of the different molecules in monocytes, p-p38MAPK, p-ERK, and p-Akt, should be assessed. It could also be informative to combine phospho-specific flow cytometry with the measurement of intra-lymphocytic or tissue TAC concentrations. The latter directly quantifies the TAC concentration in its target compartment and therefore possibly relates more closely to efficacy and toxicity. Studies in intracellular TAC concentrations have hitherto been performed in lymphocytes and peripheral blood mononuclear cells, as well as in kidney and liver tissue, but not in purified monocytes [63-67]. It may also be of interest to combine phospho-specific flow cytometry with novel biomarkers such as graft-derived cell-free DNA. Graft-derived cell-free DNA may serve as a “liquid biopsy” in transplantation, although this biomarker requires further validation and it remains to be determined whether it may aid in improving TDM of TAC and other immunosuppressive drugs [68,69].

Conclusion

Phospho-specific flow cytometry is a technique to measure the pharmacodynamic effects of immunosuppressive drug therapy on $CD14^+$ monocytes. The use of this technique demonstrated that monocyte activation pathways are only partially inhibited by TAC, MMF, and prednisolone combination therapy after kidney transplantation.

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References

1. Ysebaert DK, De Greef KE, Vercauteren SR, et al. Identification and kinetics of leukocytes after severe ischaemia/reperfusion renal injury. Nephrol Dial Transplant 2000;15:1562-74.
2. Hancock WW, Thomson NM, Atkins RC. Composition of interstitial cellular infiltrate identified by monoclonal antibodies in renal biopsies of rejecting human renal allografts. Transplantation 1983;35:458-63.
3. Rowshani AT, Vereyken EJ. The role of macrophage lineage cells in kidney graft rejection and survival. Transplantation 2012;94:309-18.
4. Girlanda R, Kleiner DE, Duan Z, et al. Monocyte infiltration and kidney allograft dysfunction during acute rejection. Am J Transplant 2008;8:600-7.
5. van den Bosch TP, Kannegieter NM, Hesselink DA, Baan CC, Rowshani AT. Targeting the Monocyte-Macrophage Lineage in Solid Organ Transplantation. Front Immunol 2017;8:153.
6. Li L, Okusa MD. Macrophages, dendritic cells, and kidney ischemia-reperfusion injury. Semin Nephrol 2010;30:268-77.
7. Pulskens WP, Teske GJ, Butter LM, et al. Toll-like receptor-4 coordinates the innate immune response of the kidney to renal ischemia/reperfusion injury. PLoS One 2008;3:e3596.
8. Jiang X, Tian W, Sung YK, Qian J, Nicolls MR. Macrophages in solid organ transplantation. Vasc Cell 2014;6:5.
9. Mantovani A, Biswas SK, Galdiero MR, Sica A, Locati M. Macrophage plasticity and polarization in tissue repair and remodelling. J Pathol 2013;229:176-85.
10. Schultze JL, Schmidt SV. Molecular features of macrophage activation. Semin Immunol 2015;27:416-23.
11. Valenzuela NM, Mulder A, Reed EF. HLA class I antibodies trigger increased adherence of monocytes to endothelial cells by eliciting an increase in endothelial P-selectin and, depending on subclass, by engaging FcgammaRs. J Immunol 2013;190:6635-50.
12. Wasowska BA. Mechanisms involved in antibody- and complement-mediated allograft rejection. Immunol Res 2010;47:25-44.
13. Schmidt RE, Gessner JE. Fc receptors and their interaction with complement in autoimmunity. Immunol Lett 2005;100:56-67.
14. Wang ZQ, Bapat AS, Rayanade RJ, Dagtas AS, Hoffmann MK. Interleukin-10 induces macrophage apoptosis and expression of CD16 (FcgammaRIII) whose engagement blocks the cell death programme and facilitates differentiation. Immunology 2001;102:331-7.
15. O'Neill LA, Golenbock D, Bowie AG. The history of Toll-like receptors - redefining innate immunity. Nat Rev Immunol 2013;13:453-60.
16. Martin M, Schifferle RE, Cuesta N, Vogel SN, Katz J, Michalek SM. Role of the phosphatidylinositol 3 kinase-Akt pathway in the regulation of IL-10 and IL-12 by Porphyromonas gingivalis lipopolysaccharide. J Immunol 2003;171:717-25.
17. Luyendyk JP, Schabbauer GA, Tencati M, Holscher T, Pawlinski R, Mackman N. Genetic analysis of the role of the PI3K-Akt pathway in lipopolysaccharide-induced cytokine and tissue factor gene expression in monocytes/macrophages. J Immunol 2008;180:4218-26.

18. Wang N, Liang H, Zen K. Molecular mechanisms that influence the macrophage m1-m2 polarization balance. Front Immunol 2014;5:614.
19. Wang Y, Zeigler MM, Lam GK, et al. The role of the NADPH oxidase complex, p38 MAPK, and Akt in regulating human monocyte/macrophage survival. Am J Respir Cell Mol Biol 2007;36:68-77.
20. Liu HS, Pan CE, Liu QG, Yang W, Liu XM. Effect of NF-kappaB and p38 MAPK in activated monocytes/macrophages on pro-inflammatory cytokines of rats with acute pancreatitis. World J Gastroenterol 2003;9:2513-8.
21. Wen AY, Sakamoto KM, Miller LS. The role of the transcription factor CREB in immune function. J Immunol 2010;185:6413-9.
22. Zou J, Shankar N. Roles of TLR/MyD88/MAPK/NF-kappaB Signaling Pathways in the Regulation of Phagocytosis and Proinflammatory Cytokine Expression in Response to E. faecalis Infection. PLoS One 2015;10:e0136947.
23. Zhou Y, Zhang T, Wang X, et al. Curcumin Modulates Macrophage Polarization Through the Inhibition of the Toll-Like Receptor 4 Expression and its Signaling Pathways. Cell Physiol Biochem 2015;36:631-41.
24. Zhang W, Xu W, Xiong S. Macrophage differentiation and polarization via phosphatidylinositol 3-kinase/Akt-ERK signaling pathway conferred by serum amyloid P component. J Immunol 2011;187:1764-77.
25. Verschoor CP, Johnstone J, Loeb M, Bramson JL, Bowdish DM. Anti-pneumococcal deficits of monocyte-derived macrophages from the advanced-age, frail elderly and related impairments in PI3K-AKT signaling. Hum Immunol 2014;75:1192-6.
26. Molnarfi N, Brandt KJ, Gruaz L, Dayer JM, Burger D. Differential regulation of cytokine production by PI3Kdelta in human monocytes upon acute and chronic inflammatory conditions. Mol Immunol 2008;45:3419-27.
27. Matas AJ, Smith JM, Skeans MA, et al. OPTN/SRTR 2011 Annual Data Report: kidney. Am J Transplant 2013;13 Suppl 1:11-46.
28. Weimer R, Mytilineos J, Feustel A, et al. Mycophenolate mofetil-based immunosuppression and cytokine genotypes: effects on monokine secretion and antigen presentation in long-term renal transplant recipients. Transplantation 2003;75:2090-9.
29. Chang KT, Lin HY, Kuo CH, Hung CH. Tacrolimus suppresses atopic dermatitis-associated cytokines and chemokines in monocytes. J Microbiol Immunol Infect 2016;49:409-16.
30. Kannegieter NM, Hesselink DA, Dieterich M, et al. The Effect of Tacrolimus and Mycophenolic Acid on CD14+ Monocyte Activation and Function. PLoS One 2017;12:e0170806.
31. Blanchet B, Duvoux C, Costentin CE, et al. Pharmacokinetic-pharmacodynamic assessment of tacrolimus in liver-transplant recipients during the early post-transplantation period. Ther Drug Monit 2008;30:412-8.
32. Picard N, Bergan S, Marquet P, et al. Pharmacogenetic Biomarkers Predictive of the Pharmacokinetics and Pharmacodynamics of Immunosuppressive Drugs. Ther Drug Monit 2016;38 Suppl 1:S57-69.

33. Wallemacq P, Armstrong VW, Brunet M, et al. Opportunities to optimize tacrolimus therapy in solid organ transplantation: report of the European consensus conference. Ther Drug Monit 2009;31:139-52.

34. Brunet M, Shipkova M, van Gelder T, et al. Barcelona Consensus on Biomarker-Based Immunosuppressive Drugs Management in Solid Organ Transplantation. Ther Drug Monit 2016;38 Suppl 1:S1-20.

35. EU Clinical Trials Register. European Medicines Agency, 1995-2017. (Accessed 22 February 2017, at https://www.clinicaltrialsregister.eu/ctr-search/trial/2012-003169-16/NL.)

36. Graav de G, Hesselink DA, Dieterich M, Kraaijeveld R, Weimar W, Baan CC. Belatacept Does Not Inhibit Plasmablast Formation Supported by Follicular T Helper Cells, but Favors the Development of Transitional Regulatory B Cells in Kidney Transplant Patients. Am J Transplant 2016;16:suppl 3.

37. Kannegieter NM, Shuker N, Vafadari R, Weimar W, Hesselink DA, Baan CC. Conversion to Once-Daily Tacrolimus Results in Increased p38MAPK Phosphorylation in T Lymphocytes of Kidney Transplant Recipients. Ther Drug Monit 2016;38:280-4.

38. Vafadari R, Weimar W, Baan CC. Phosphospecific flow cytometry for pharmacodynamic drug monitoring: analysis of the JAK-STAT signaling pathway. Clin Chim Acta 2012;413:1398-405.

39. Racusen LC, Solez K, Colvin RB, et al. The Banff 97 working classification of renal allograft pathology. Kidney Int 1999;55:713-23.

40. Loupy A, Haas M, Solez K, et al. The Banff 2015 Kidney Meeting Report: Current Challenges in Rejection Classification and Prospects for Adopting Molecular Pathology. Am J Transplant 2017;17:28-41.

41. Landskron J, Tasken K. Phosphoprotein Detection by High-Throughput Flow Cytometry. Methods Mol Biol 2016;1355:275-90.

42. Krutzik PO, Trejo A, Schulz KR, Nolan GP. Phospho flow cytometry methods for the analysis of kinase signaling in cell lines and primary human blood samples. Methods Mol Biol 2011;699:179-202.

43. Baan C, Bouvy A, Vafadari R, Weimar W. Phospho-specific flow cytometry for pharmacodynamic monitoring of immunosuppressive therapy in transplantation. Transplant Res 2012;1:20.

44. Sommerer C, Giese T, Meuer S, Zeier M. Pharmacodynamic monitoring of calcineurin inhibitor therapy: is there a clinical benefit? Nephrol Dial Transplant 2009;24:21-7.

45. Saint-Marcoux F, Woillard JB, Jurado C, Marquet P. Lessons from routine dose adjustment of tacrolimus in renal transplant patients based on global exposure. Ther Drug Monit 2013;35:322-7.

46. Bouamar R, Shuker N, Hesselink DA, et al. Tacrolimus predose concentrations do not predict the risk of acute rejection after renal transplantation: a pooled analysis from three randomized-controlled clinical trials. Am J Transplant 2013;13:1253-61.

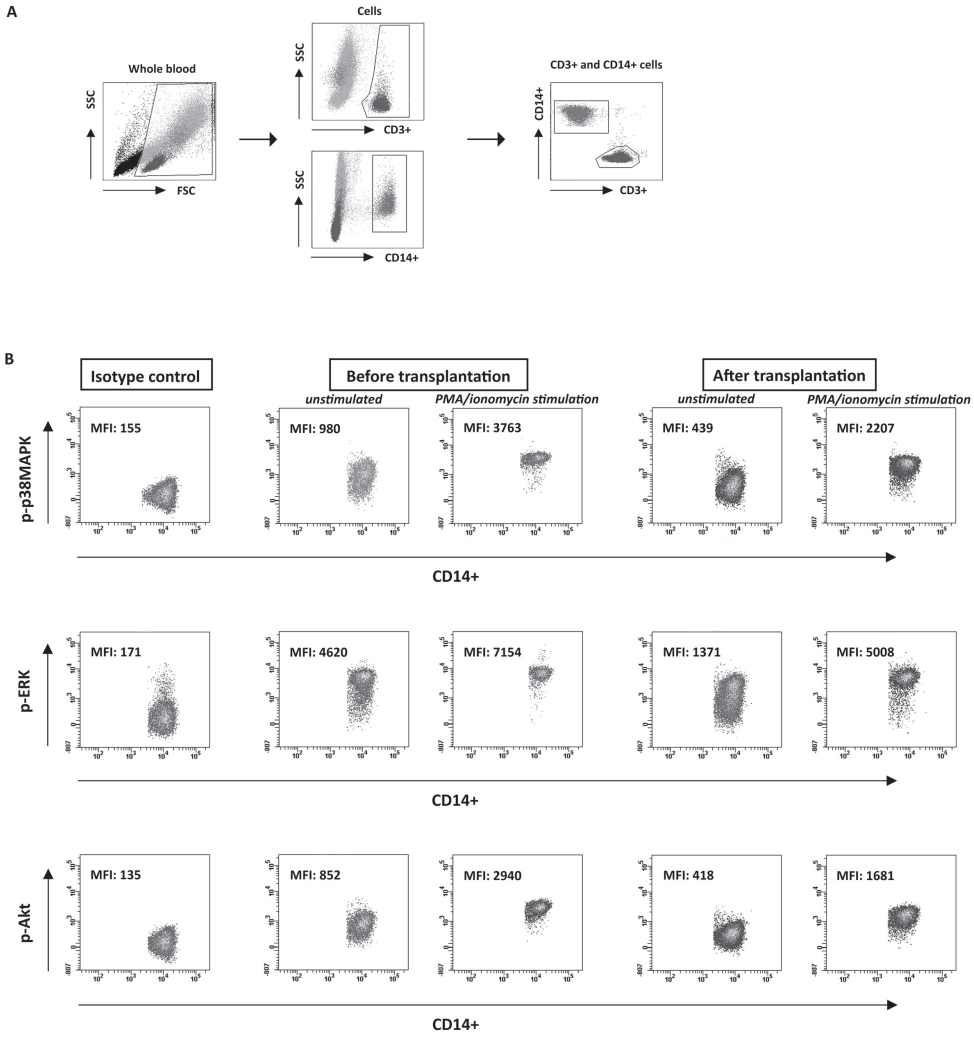
47. Steinebrunner N, Sandig C, Sommerer C, et al. Pharmacodynamic monitoring of nuclear factor of activated T cell-regulated gene expression in liver allograft recipients on immunosup-

pressive therapy with calcineurin inhibitors in the course of time and correlation with acute rejection episodes—a prospective study. Ann Transplant 2014;19:32-40.

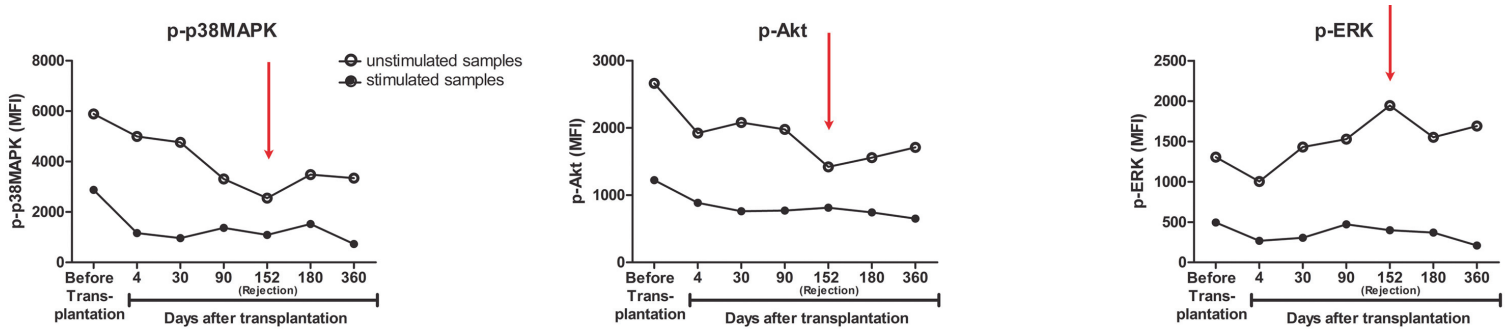
48. Keller F, Sommerer C, Giese T, Zeier M, Schroppel B. Correlation between pharmacokinetics of tacrolimus and pharmacodynamics on NFAT-regulated gene expression in stable kidney transplant recipients. Clin Nephrol 2017;87 (2017):93-9.
49. Albring A, Wendt L, Harz N, et al. Relationship between pharmacokinetics and pharmacodynamics of calcineurin inhibitors in renal transplant patients. Clin Transplant 2015;29:294-300.
50. Fukudo M, Yano I, Katsura T, et al. A transient increase of calcineurin phosphatase activity in living-donor kidney transplant recipients with acute rejection. Drug Metab Pharmacokinet 2010;25:411-7.
51. Vafadari R, Hesselink DA, Cadogan MM, Weimar W, Baan CC. Inhibitory effect of tacrolimus on p38 mitogen-activated protein kinase signaling in kidney transplant recipients measured by whole-blood phosphospecific flow cytometry. Transplantation 2012;93:1245-51.
52. Vereyken EJ, Kraaij MD, Baan CC, et al. A shift towards pro-inflammatory CD16+ monocyte subsets with preserved cytokine production potential after kidney transplantation. PLoS One 2013;8:e70152.
53. Hunter M, Wang Y, Eubank T, Baran C, Nana-Sinkam P, Marsh C. Survival of monocytes and macrophages and their role in health and disease. Front Biosci (Landmark Ed) 2009;14:4079-102.
54. Becker LE, de Oliveira Biazotto F, Conrad H, et al. Cellular infiltrates and NFkappaB subunit c-Rel signaling in kidney allografts of patients with clinical operational tolerance. Transplantation 2012;94:729-37.
55. Weichhart T, Saemann MD. The PI3K/Akt/mTOR pathway in innate immune cells: emerging therapeutic applications. Ann Rheum Dis 2008;67 Suppl 3:iii70-4.
56. Ashida N, Arai H, Yamasaki M, Kita T. Distinct signaling pathways for MCP-1-dependent integrin activation and chemotaxis. J Biol Chem 2001;276:16555-60.
57. Arefieva TI, Kukhtina NB, Antonova OA, Krasnikova TL. MCP-1-stimulated chemotaxis of monocytic and endothelial cells is dependent on activation of different signaling cascades. Cytokine 2005;31:439-46.
58. Richardson ET, Shukla S, Nagy N, et al. ERK Signaling Is Essential for Macrophage Development. PLoS One 2015;10:e0140064.
59. Bhatt NY, Kelley TW, Khramtsov VV, et al. Macrophage-colony-stimulating factor-induced activation of extracellular-regulated kinase involves phosphatidylinositol 3-kinase and reactive oxygen species in human monocytes. J Immunol 2002;169:6427-34.
60. Bhattacharyya S, Brown DE, Brewer JA, Vogt SK, Muglia LJ. Macrophage glucocorticoid receptors regulate Toll-like receptor 4-mediated inflammatory responses by selective inhibition of p38 MAP kinase. Blood 2007;109:4313-9.
61. Covey TM, Cesano A. Modulated multiparametric phosphoflow cytometry in hematological malignancies: technology and clinical applications. Best Pract Res Clin Haematol 2010;23:319-31.

62. Hasegawa D, Bugarin C, Giordan M, et al. Validation of flow cytometric phospho-STAT5 as a diagnostic tool for juvenile myelomonocytic leukemia. Blood Cancer J 2013;3:e160.
63. Capron A, Haufroid V, Wallemacq P. Intra-cellular immunosuppressive drugs monitoring: A step forward towards better therapeutic efficacy after organ transplantation? Pharmacol Res 2016;111:610-8.
64. Capron A, Musuamba F, Latinne D, et al. Validation of a liquid chromatography-mass spectrometric assay for tacrolimus in peripheral blood mononuclear cells. Ther Drug Monit 2009;31:178-86.
65. Noll BD, Coller JK, Somogyi AA, et al. Validation of an LC-MS/MS method to measure tacrolimus in rat kidney and liver tissue and its application to human kidney biopsies. Ther Drug Monit 2013;35:617-23.
66. Lemaitre F, Blanchet B, Latournerie M, et al. Pharmacokinetics and pharmacodynamics of tacrolimus in liver transplant recipients: inside the white blood cells. Clin Biochem 2015;48:406-11.
67. Han SS, Yang SH, Kim MC, et al. Monitoring the Intracellular Tacrolimus Concentration in Kidney Transplant Recipients with Stable Graft Function. PLoS One 2016;11:e0153491.
68. Gielis EM, Ledeganck KJ, De Winter BY, et al. Cell-Free DNA: An Upcoming Biomarker in Transplantation. Am J Transplant 2015;15:2541-51.
69. Oellerich M, Schutz E, Kanzow P, et al. Use of graft-derived cell-free DNA as an organ integrity biomarker to reexamine effective tacrolimus trough concentrations after liver transplantation. Ther Drug Monit 2014;36:136-40.

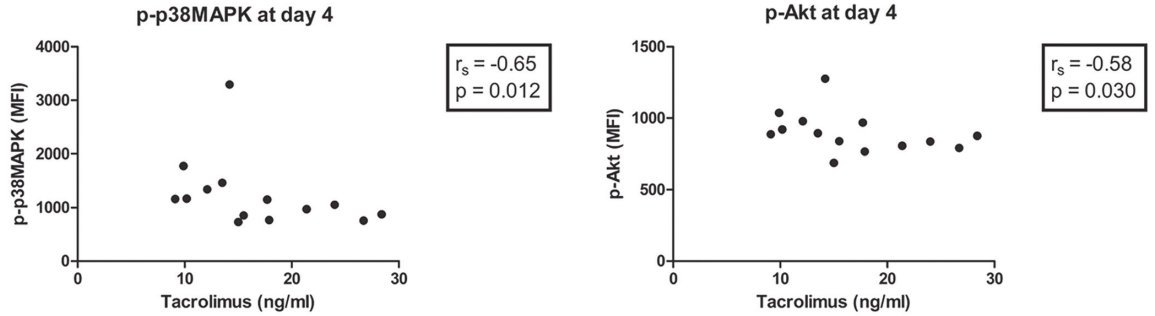
Supplementary Figures and Tables



Supplementary Figure 1. Gating strategy and phosphorylation example of CD14$^+$ monocytes. A) Gating strategy for CD14$^+$ monocytes. Nucleated cells were selected from whole blood samples via a FSC/SSC dotplot. These cells were then gated based on their CD3 and CD14 expression. Events double positive for CD14 and CD3 were gated out to show pure populations for CD14$^+$ monocytes. B) An example of measured median fluorescence intensities in CD14$^+$ monocytes for p-p38MAPK, p-ERK and p-Akt before and after transplantation(unstimulated and PMA/ionomycin stimulated) compared to their isotype control measurements. FSC) Forward scatter; SSC) sideward scatter.



Supplementary Figure 2. Phosphorylation measurements for a patient diagnosed with a BPAR on day 152 after transplantation. Phosphorylation of p38MAPK (*left*), Akt (*middle*), and ERK (*right*) was measured in $CD14^+$ monocytes before and after transplantation and in both unstimulated and stimulated samples. Red arrows indicate the rejection time point.



Supplementary Figure 3. Correlation between *ex vivo* p-p38MAPK (*left*) and p-Akt (*right*) and TAC pre-dose concentrations at day 4 after transplantation. Correlations were calculated as the Spearman correlation coefficient. (n=20 patients).

Supplementary Table I: Absolute monocyte counts and medication overview

	Monocytes count monocytes /µL whole blood		TAC dose mg	TAC C_0 ng/mL Mean (±SD)	TAC C_0 ng/mL Median (range)		MPA dose mg	MPA C_0 µg/mL Mean (±SD)	MPA C_0 µg/mL Median (range)
Before transplantation	566.4 (± 194.3)	** day 4, 30/ *** day 90							
Day 4	790.3 (± 323.4)	** before	16.7 (± 2.5)	16.8 (± 6.2)	15.3 (9.1-28.4)	*** day 30, 90, 180, 360	2000 (± 0)	3.35 (± 1.59)	2.85 (0.86-6.77)
Day 30	431.7 (± 182.5)	** before	8.9 (± 4.6)	9.8 (± 3.3)	8.5 (5.9- 17.8)	*** day 4/ * day 180	1750 (± 546)	2.69 (± 1.57)	2.32 (0.50-5.72)
Day 90	368.5 (± 204.5)	*** before	4.7 (± 1.4)	6.8 (± 1.7)	6.9 (4.1-9.3)	*** day 4	1342 (± 473)	2.83 (± 1.62)	2.48 (1.03-8.30)
Day 180	529.7 (± 264.9)		4.3 (± 1.1)	6.2 (± 1.5)	6.3 (2.6-9.9)	*** day 4/ * day 30	1171 (± 486)	2.14 (± 0.97)	1.73 (0.96-4.24)
Day 360	575.9 (± 211.2)		4.2 (± 1.2)	6.9 (± 2.2)	6.8 (4.4-13.3)	*** day 4	1097 (± 479)	1.96 (± 0.92)	1.83 (0.57-3.67)

*) $p < 0.05$; **) $p < 0.01$; ***) $p < 0.001$

Supplementary Table II: Univariate analysis of the association between patient demographic characteristics and signaling protein phosphorylation

		p-p38MAPK		p-ERK		p-AKT	
	Predictor	Univariate regression coefficient	p value	Univariate regression coefficient	p value	Univariate regression coefficient	p value
	Age (years)	-0.103	0.726	-0.357	0.210	-0.181	0.535
	Gender (male vs. female)	-0.323	0.260	0.330	0.249	-0.212	0.467
	Ethnicity (Caucasian vs. non-Caucasian)	-0.345	0.227	-0.261	0.367	-0.365	0.200
Before	Renal replacement therapy (no vs. yes)	-0.075	0.799	-0.428	0.127	-0.308	0.284
Transplantation	Cause of ESRD: diabetes (no vs. yes)	0.030	0.918	-0.428	0.127	-0.152	0.605
	Cause of ESRD: hypertension (no vs. yes)	0.344	0.229	-0.343	0.231	0.317	0.270
	Creatinine Se-GFR (mL/min)	0.044	0.880	0.131	0.655	0.272	0.348
	Creatinine (µmol/L)	-0.084	0.774	-0.386	0.173	-0.305	0.289
	Age (years)	-0.134	0.649	-0.212	0.466	-0.288	0.318
	Gender (male vs. female)	-0.287	0.320	-0.030	0.918	-0.400	0.157
	Ethnicity (Caucasian vs. non-Caucasian)	-0.280	0.332	-0.182	0.533	-0.343	0.230
	Renal replacement therapy (yes vs. no)	0.012	0.969	-0.476	0.085	-0.030	0.918
Day 4	Cause of ESRD: diabetes (no vs. yes)	0.100	0.734	-0.467	0.092	-0.054	0.854
	Cause of ESRD: hypertension (no vs. yes)	0.277	0.338	-0.488	0.108	0.138	0.638
	Creatinine Se-GFR (mL/min)	0.024	0.935	-0.024	0.934	-0.208	0.467
	Creatinine (µmol/L)	-0.126	0.667	-0.053	0.858	0.046	0.875
	Age (years)	0.205	0.401	-0.015	0.954	0.069	0.784
	Gender (male vs. female)	-0.081	0.741	-0.027	0.915	0.101	0.691
	Ethnicity (Caucasian vs. non-Caucasian)	-0.006	0.982	0.261	0.295	0.416	0.086
	Renal replacement therapy (yes vs. no)	-0.132	0.590	-0.170	0.500	-0.134	0.596
Day 360	Cause of ESRD: diabetes (no vs. yes)	-0.077	0.753	0.383	0.117	0.357	0.146
	Cause of ESRD: hypertension (no vs. yes)	0.337	0.158	0.087	0.732	0.011	0.965
	Creatinine Se-GFR (mL/min)	0.590	0.034	-0.100	0.758	0.129	0.689
	Creatinine (µmol/L)	-0.481	0.096	-0.017	0.959	-0.312	0.324

ESRD) End stage renal disease; GFR) Glomerular filtration rate

A two-sided p value < 0.006 was considered statistically significant after Bonferroni correction.

5

Analysis of NFATc1 Amplification in T Cells for Pharmacodynamic Monitoring of Tacrolimus in Kidney Transplant Recipients

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Submitted

Abstract

Therapeutic drug monitoring (TDM) of tacrolimus, based on blood concentrations, shows an imperfect correlation with the occurrence of rejection. Here, we tested whether measuring NFATc1 amplification, a member of the calcineurin pathway, is suitable for TDM of tacrolimus.

NFATc1 amplification was monitored in T cells of kidney transplant recipients who received either tacrolimus- (n = 11) or belatacept-based (n = 10) therapy. Individual drug effects on NFATc1 amplification were studied ***in vitro,*** after spiking blood samples of healthy volunteers with either tacrolimus, belatacept or mycophenolate mofetil.

At day 30 after transplantation, in tacrolimus-treated patients, NFATc1 amplification was inhibited in $CD4^+$ T cells expressing the co-stimulation receptor CD28 (mean inhibition 37%; p = 0.01) and in $CD8^+CD28^+$ T cells (29% inhibition; p = 0.02), while this was not observed in $CD8^+CD28^-$ T cells or belatacept-treated patients. Tacrolimus pre-dose concentrations of these patients correlated inversely with NFATc1 amplification in $CD28^+$ T cells (r_s = -0.46; p < 0.01). ***In vitro*** experiments revealed that 50 ng/ml tacrolimus affected NFATc1 amplification by 58% (mean; p = 0.02).

In conclusion, measuring NFATc1 amplification is a direct tool for monitoring biological effects of tacrolimus on T cells in whole blood samples of kidney transplant recipients. This technique has potential that requires further development before it can be applied in daily practice.

Introduction

Therapeutic drug monitoring (TDM) is routinely used to optimize tacrolimus (TAC) dosing after organ transplantation [1-3]. Traditionally, the TAC dose is adjusted based on whole blood pre-dose concentrations (C_0), that have an imperfect relationship with the occurrence of acute rejection and adverse events, such as nephrotoxicity and infection [4-10]. A promising strategy to overcome the limitations of traditional pharmacokinetic TDM may be to measure the biological effects of immunosuppressive drugs (pharmacodynamics).

The primary biological target of TAC in T cells is the calcineurin pathway, of which the nuclear factor of activated T cells (NFAT) is one of the most important signaling proteins [11]. The NFAT family consists of 5 members: NFATc1 (NFAT-2), NFATc2 (NFAT-1), NFATc3 (NFAT-4), NFATc4 (NFAT-3) and TonEBP (NFAT-5) [12]. NFAT molecules are key players in the immune response after transplantation and are involved in T cell development, activation, differentiation, as well as in the production of cytokines like interleukin (IL)-2 [11,13,14].

Activation of the NFAT family member NFATc1 is initiated when both the T cell receptor (TCR) and co-stimulatory molecules, such as CD28, become activated (**Figure 1**). Upon activation, the phosphatase calcineurin is triggered, which then dephosphorylates NFATc1. In turn, dephosphorylated NFATc1 is translocated to the nucleus where it interacts with other transcription factors, such as AP-1, and induces gene transcription.

In contrast to other members of the NFAT family that are mainly known for their role in cytokine production, NFATc1 is also known for its strongly inducible isoform NFATc1/A. NFATc1/A is the only NFAT member that can be enhanced upon antigenic stimulation and maintained by positive autoregulation in T cells (**Figure 1**) [11,15-19]. The calcineurin inhibitor (CNI) cyclosporine A is known to inhibit both the dephosphorylation of NFATc1 and the upregulation of NFATc1/A, but the effect of tacrolimus on NFATc1/A amplification is still unknown [20].

At present, clinically applicable pharmacodynamic assays to monitor the biological effect of TAC are in development, of which the NFAT regulate IL-2 gene expression is the most promising. It was recognized that measuring NFAT-regulated genes might be a good method to assess the risk of opportunistic infections, malignancy and acute rejection after transplantation [21-24]. However, NFAT-regulated genes, such as IL-2, IFN-γ and GM-CSF are activated downstream in the calcineurin pathway. Subsequently, the activation of these genes can be influenced by other immunosuppressive drugs, such as glucocorticoids, and influenced by other signaling pathways, such as the JAK-STAT signaling pathway [25,26]. Moreover, the measuring of NFAT-regulated gene expression is a non-validated tool to monitor the immunosuppressive effects in tacrolimus-treated patients. A better way for monitoring the direct biological effects of tacrolimus might be the measurement of the immunosuppressive effect on NFATc1 amplification. Flow cytometry offers the opportunity to quantify the amplification of NFATc1/A at the single cell level with a short turnaround time when blood samples were spiked with cyclosporine A [20]. This technique enables the measurement of the immunosuppressive drug effects on the calcineurin pathway directly

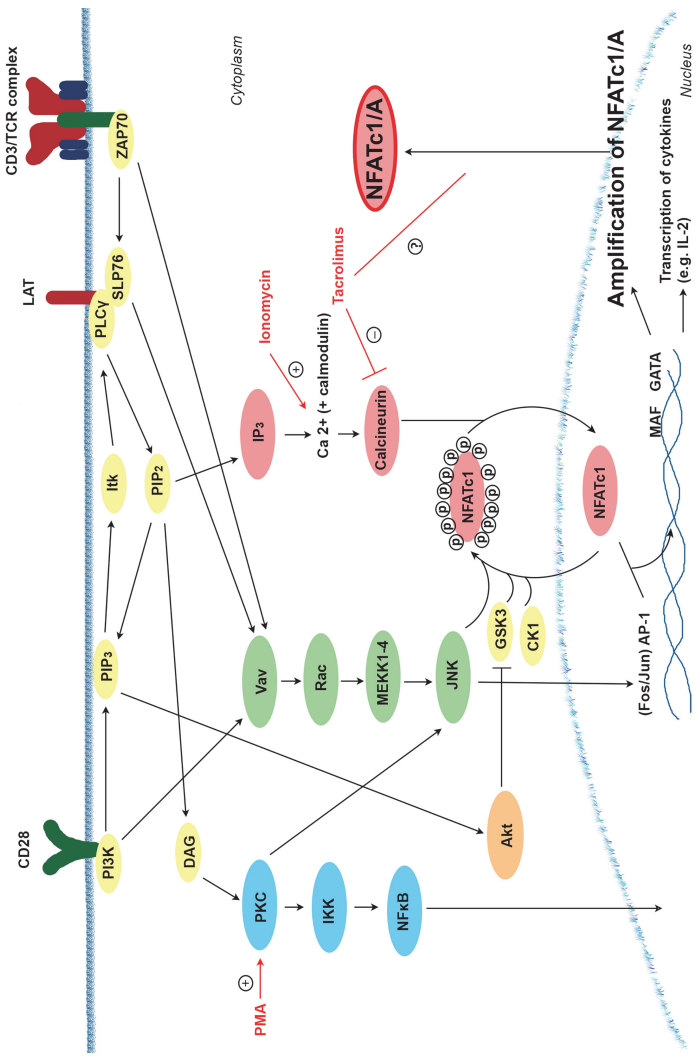

Figure 1. Schematic overview of the intracellular calcineurin pathway in T cells and amplification of the NFATc1/A isoform[11]. The calcineurin pathway is activated upon antigenic stimulation of the CD3/TCR complex in combination with co-stimulatory signals. This in turn activates the signal molecules phospholipase C-γ (PLC-γ) and inositol 1,4,5-trisphosphate (IP_3) causing an influx of calcium and the opening of calcium channels in the membrane to maintain intracellular calcium levels. Upon interaction between calcium and the small calcium-binding protein, calmodulin, the phosphatase calcineurin is activated, which dephosphorylates NFAT. There are 13 phosphorylation sites present on NFAT that are known to be dephosphorylated upon activation. Dephosphorylation causes the translocation of NFAT to the nucleus where it will initiate gene transcription trough the interaction with other transcription factors, such as AP-1. The signaling pathway is regulated by other signaling pathways, such as the MAPK pathway (JNK) and NFκB pathway. Once in the nucleus, NFAT will act as transcription factor and regulates the production of cytokines and the amplification of the isoform NFATc1/A as a positive autoregulatory feedback loop. The intracellular signaling pathways can also be activated by using PMA/ionomycin as a stimulus, while CNI's, such as tacrolimus, are known to inhibit the calcineurin pathway.

rather than measuring the end-products of this pathway, such as IL-2. Here, the applicability of the NFATc1 amplification assay is tested for the first time in whole-blood samples of TAC-treated kidney transplant recipients and we explored whether this method can be translated to daily clinical practice and can be an additional tool for monitoring the effects of TAC in different T cell subsets. Kidney transplant recipients, receiving a belatacept (BELA)-based maintenance therapy, served as a CNI-free control group, since BELA cannot directly inhibit the calcineurin/NFAT and other signaling pathways in T cells [27,28]. In addition, NFATc1 amplification was also measured in different T cells subsets, ***i.e.*** $CD4^+$ T cells, $CD8^+$ T cells, $CD28^+$ T cells, that need the expression of CD28 for co-stimulation and activation, and in the antigen experienced, potentially harmful, $CD28^-$ T cells [29,30]. Here, the difference between these cell subsets was measured to assess their susceptibility for immunosuppressive drug therapy.

Material and Methods

Kidney transplant recipients

Peripheral blood samples of 21 kidney transplant recipients were analyzed for the expression of NFATc1 in T cell subsets. The current study is a substudy of a prospective, randomized, clinical trial of which the results were published previously [31]. The study was approved by the Medical Ethical Committee of the Erasmus MC, University Medical Center (MEC number 2012-421, EudraCT # 2012-003269-16, registered October 17th 2013) and samples were collected according to the biobank protocol that was also approved by the local ethics committee (MEC-2010-022). The work was performed in accordance with the Declaration of Helsinki. All patients gave written informed consent before the start of the study.

For this study, 21 of the total of 40 kidney transplant recipients that were randomized in the trial were included, since these patients were also analyzed for their NFATc1 expression before transplantation [31]. Of these, 11 patients received TAC-based and 10 patients received belatacept (BELA)-based immunosuppressive treatment. Samples from patients treated with BELA, that blocks the co-stimulatory CD80/86-CD28 pathway, were used as a control, because of the indirect effect of BELA on intracellular signaling pathways in T cells. Patients received a TAC starting dose (Prograf®, Astellas Pharma Inc., Tokyo, Japan) based on bodyweight (0.2 mg/kg/day) in two equally divided doses starting on the day of transplantation. Thereafter, the TAC dose was adjusted according to whole-blood pre-dose concentrations: 10-15 ng/mL (week 1-2), 8-12 ng/mL (week 3-4), and 5-10 ng/mL (from week 5 onwards). BELA (Nulojix®, Bristol-Myers Squibb, New York, USA) was given according to the less intensive regimen [32]: a dose of 10 mg/kg administered intravenously on day 0, 4, 15, 30, 60 and 90 after transplantation and then a reduced dose of 5 mg/kg as monthly intravenous infusions. All patients received an additional treatment consisting of mycophenolate mofetil (MMF; Cellcept®; Roche, Basel, Switzerland), prednisolone and basiliximab

induction therapy (Simulect®, Novartis, Basel, Switzerland). MMF was administered at a starting dose of 1000 mg twice a day and then adjusted to pre-dose plasma concentrations between 1.5 and 3.0 mg/L. During the first three post-operative days, all patients received prednisolone intravenously in a dose of 100 mg/day. Thereafter, prednisolone was given orally in a dose of 20 mg/day and tapered to 5 mg/day by month 3 after transplantation. Basiliximab (20 mg) was given intravenously at day 0 and day 4 after transplantation.

Blood samples and tacrolimus pre–dose concentrations

To measure the expression of NFATc1, blood samples were collected in heparin tubes (BD Biosciences, San Jose, CA) by venipuncture at days 0 (pre-transplantation), 4, 30, 90, 180 and 360 after transplantation and before anti-rejection therapy was started (in the case of an (suspected) acute rejection). Samples were stored at room temperature on a tube-roller and prepared within 2 hours after venipuncture. TAC whole-blood pre-dose concentrations and mycophenolic acid (MPA) plasma pre-dose concentrations were measured in EDTA blood at the same time points by use of the antibody-conjugated magnetic immunoassay on a Dimension Xpand analyzer (Siemens HealthCare Diagnostics Inc., Newark, DE) according to the manufacturer's instructions. The lower and upper limit of TAC detection were 1.5 and 30 ng/mL, respectively, and the coefficient of variation was 15.0%, 8.9% and 11.2% for the low, middle and high control samples, respectively. For MPA, the lower and upper limits of detection were 0.5 µg/ml and 15 µg/ml, respectively, and the coefficient of variation was 3.9% and 3.7%, for the low and high controls, respectively. Proficiency samples were obtained from the UK Quality Assessment Scheme (Analytical Services International Ltd, London, UK). Our laboratory successfully participates in the international proficiency testing program.

Whole–blood intracellular staining for NFATc1

Heparinized blood samples were stimulated within 2 hours after blood collection with a final concentration of 0.5 µg/ml phorbol myristate acetate (PMA) and 10 µg/ml ionomycin for four hours at 37 °C and in the presence of Golgiplug (BD Biosciences) to maximize the expression of NFATc1 and to induce NFATc1 amplification intracellularly [20]. Thereafter, 100 µl 20 mM EDTA was added to remove adherent cells from the activation tube and incubated for 15 minutes at room temperature. Samples were then stained with fluorescein isothiocyanate (FITC)-labeled mouse anti-human CD14 (clone UCHM1, Serotec, Oxford, UK), brilliant violet (BV) 510-labeled mouse anti-human CD3 (clone OKT3, Biolegend, San Diego, CA), peridinin chlorophyll (PERCP)-labeled mouse anti-human CD4 (clone SK3, BD Biosciences), allophycocyanin (APC)-Cy7-labeled mouse anti-human CD8 (clone SK1, Biolegend) and BV421-labeled mouse anti-human CD28 (clone CD28.2, BD Biosciences) for 30 minutes at room temperature. Subsequently, samples were lysed and fixed twice for 10 minutes with FACS lysing solution (BD Biosciences) and treated with permeabilization buffer II (BD Biosciences) for 10 minutes at room temperature. Phycoerythrin (PE)-labeled

mouse anti-human NFATc1 (clone 7A6; Biolegend) was then added and incubated for 30 minutes on ice to determine the intracellular expression of NFATc1 in T cell subsets. Samples were analyzed on a FACS Canto II flow cytometer (BD Biosciences). Unstimulated samples were used to calculate NFATc1 amplification. An isotype control, mouse IgG1-PE, was included in a separate tube to see the background effect of antibodies binding on the NFAT molecule. Cytocalbeads (Thermo Scientific, Fremont, CA) were used to correct for interday-variability of the flow cytometer according to the manufacturer's instructions. The conditions and concentrations used in this assay were established after optimization of the intracellular staining protocol in our lab.

In vitro experiments

To measure the effect of the individual immunosuppressive drugs on NFATc1 amplification in T cell subsets, heparinized blood samples were drawn from healthy volunteers (n = 5). Samples were incubated for 16 hours overnight at 37 °C with either vehicle (ethanol dissolved 1:8000 in distilled water), TAC (10 or 50 ng/ml), MPA (10 µg/ml; Sigma-Aldrich, Steinheim, Germany), prednisolone (100 ng/ml) or BELA (5 µg/ml), to be sure that the samples are well mixed with the immunosuppressive drug concentrations. Thereafter, samples were treated in the same way as the blood-samples of kidney transplant patients and stimulated for 4 hours with PMA/ionomycin at 37 °C. After incubation, expression of NFATc1 was measured according to the protocol described in section 2.3.

Statistical analysis

The median fluorescence intensity (MFI) of NFATc1 was measured and data-analysis was performed with Diva-version 6.0 software (BD Bioscience). MFI values were normalized using Cytocalbeads (Thermo Scientific). To calculate the total amplification of the inducible NFATc1/A isoform**,** samples were further analyzed by correcting the stimulated total expression of NFATc1 (both phosphorylated and dephosphorylated) for the MFI value in unstimulated samples (also both phosphorylated and dephosphorylated). Statistical analysis was performed with Graph Pad Prism 5.0 (Graph Pad Software Inc., La Jolla, CA) by using paired and unpaired t-tests (after finding a p-value > 0.05 with the Kolmogorov-Smirnov test for normality of the study population). Correlations between drug concentrations and the expression of NFATc1 were calculated as the Spearman correlation coefficient. A two-sided p-value < 0.05 was considered statistically significant.

Results

Patient demographics and clinical outcomes

Supplementary Table I summarizes patient baseline characteristics, the incidence of rejection, and the medication of both TAC- and BELA-treated patients. In brief, the two

study populations did not differ in their baseline characteristics. The incidence of rejection was lower in the TAC-treated group than in the BELA-treated group (1 out of 11 TAC-treated patients *versus* 7 out of 10 BELA-treated patients, respectively) [31,33]. In the current study, patients were censored from the moment of rejection onwards, since the expression of NFATc1 might be influenced by the anti-rejection therapy. Prednisolone doses were not significantly different between the two study groups, but the MPA pre-dose plasma concentrations were significantly lower in the TAC-treated group than in the BELA-treated group ($p = 0.04$). Further details regarding the clinical outcomes were published previously [31,33].

NFATc1 amplification in T cell subsets

The effects of immunosuppressive drug therapy were determined in $CD4^+CD28^+$, $CD8^+CD28^+$ and $CD8^+CD28^-$ T cells. Upon stimulation, the expression of NFATc1 (expressed as MFI) increased compared to the unstimulated samples (**Figures 2B and 3**). After activation, no difference in the expression level of NFATc1 was found between healthy controls and patients before transplantation (**Figure 3**). **Figure 2A** shows the gating strategy for NFATc1 expression in $CD3^+$ T cells.

Before transplantation, the highest amplification of NFATc1 was found in $CD4^+CD28^+$ T cells, compared to $CD8^+CD28^+$ and $CD8^+CD28^-$ T cells (**Figure 4A**). After transplantation, in patients receiving TAC-based therapy, NFATc1 amplification was inhibited at day 30, 180 and day 360 in $CD4^+CD28^+$ T cells (mean inhibition of 37%, 24% and 28%; $p = 0.01$, $p = 0.03$ and $p = 0.03$, respectively; **Figure 4B**). In comparison to pre-transplantation, $CD8^+CD28^+$ T cells show a lower amplification of NFATc1 at day 30 and 360 after transplantation (mean inhibition of 29% and 15%; $p = 0.02$ and $p = 0.03$, respectively; **Figure 4C**). In contrast, no effect of TAC-based therapy was observed in $CD8^+CD28^-$ T cells (**Figure 4D**). T cell subsets of BELA-treated patients were also not affected by the immunosuppressive drug therapy (**Figures 4B-4D**). However, as depicted in **Figures 4B-4D,** a wide range of NFATc1 expression was observed due to the small number of patients on belatacept treatment at day 180 and onwards.

Correlation with TAC pre–dose concentrations

To determine whether TAC pre-dose concentrations correlated with the inhibition of NFATc1 amplification, correlations were calculated over time. For these calculations only samples from TAC-treated patients were included that were taken at each tested time point: day 0, 4, 30, 90, 180 and 360 ($n = 7$). Inverse correlations were found between TAC pre-dose concentrations and NFATc1 amplification in both $CD4^+CD28^+$ ($r_s = -0.463$; $p < 0.01$) and $CD8^+CD28^+$ ($r_s = -0.464$; $p < 0.01$) T cells, but not in $CD8^+CD28^-$ T cells (**Figure 5A**). No correlations were found between MPA pre-dose concentrations in TAC-treated patients and NFATc1 amplification for all three T cell subsets (**Figure 5B**).

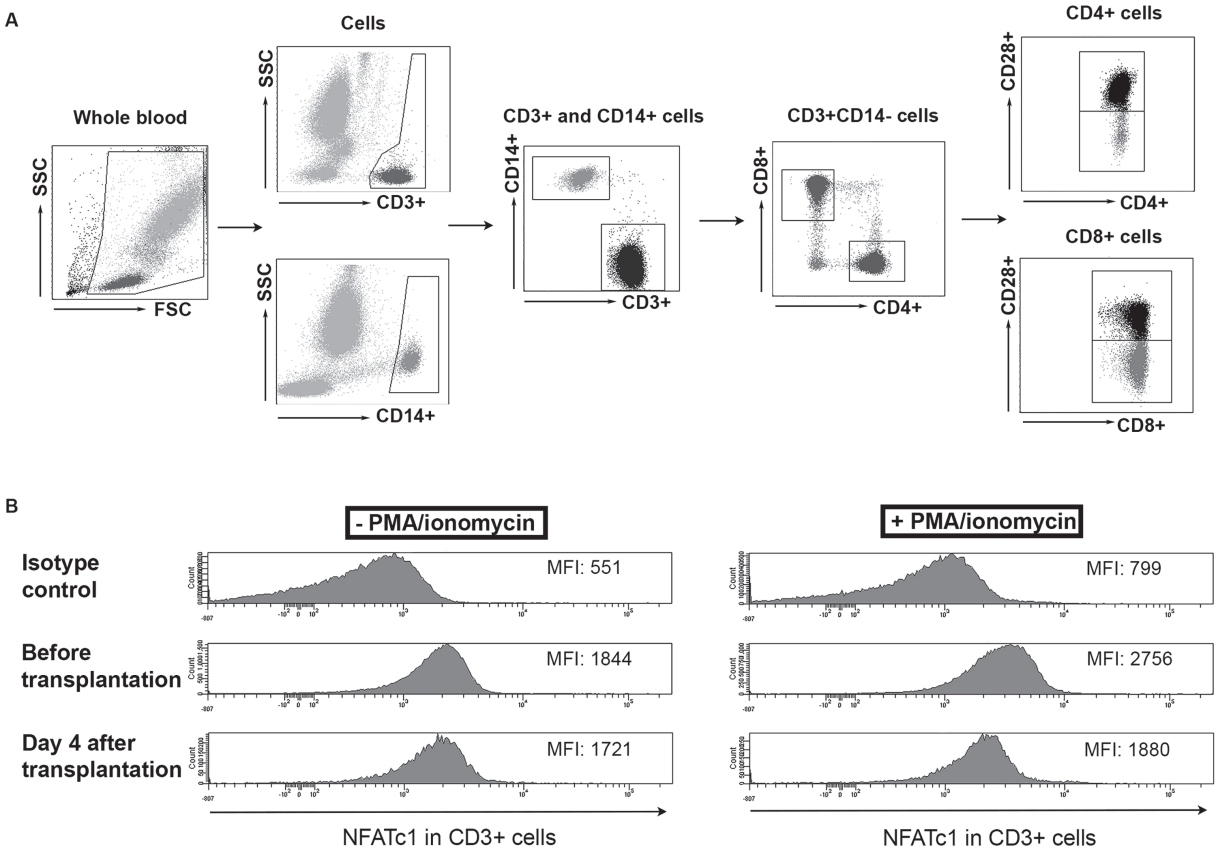

Figure 2. Gating strategy for the total NFATc1 expression in T cell subsets. (A) Gating of $CD3^+$ T cell subsets. Cells were gated for their expression of CD14 and CD3, where after $CD3^+CD14^-$ T cells were gated for their expression of CD4 or CD8. Within these populations, the expression of CD28 was determined to identify the $CD4^+CD28^+$, $CD8^+CD28^+$ and $CD8^+CD28^-$ T cell subsets. B) Example of the total NFATc1 expression in $CD3^+CD14^-$ cells, either unstimulated or stimulated with PMA/ionomycin. FSC) Forward scatter; SSC) sideward scatter

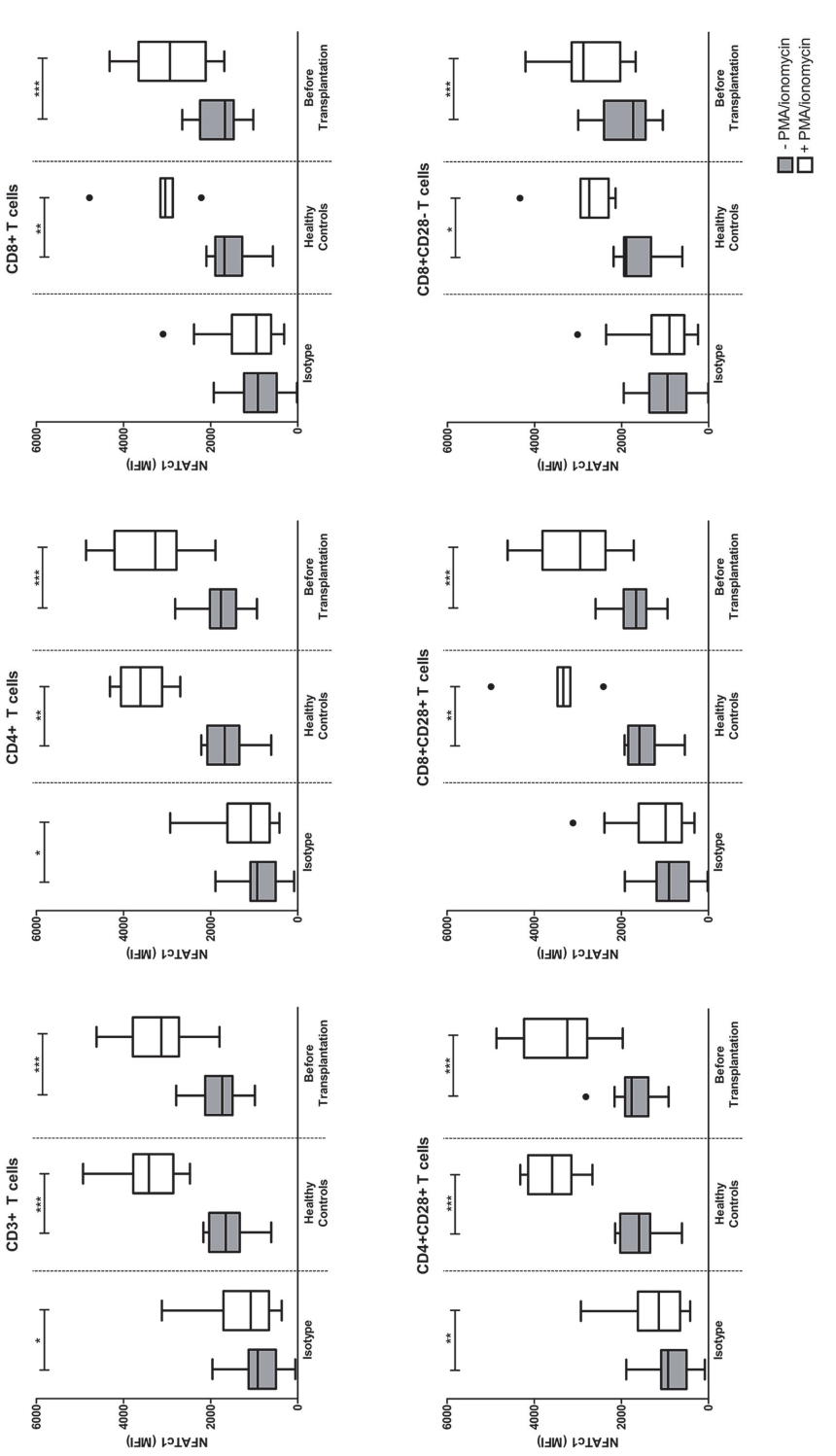

Figure 3. NFATc1 expression (MFI) in the total study population of TAC-treated patients. Unstimulated (*grey*) and PMA/ionomycin stimulated (*white*) blood samples were stained for the expression of NFATc1 in $CD3^+$ (*upper left graph*), $CD4^+$ (*upper middle graph*), $CD8^+$ (*upper right graph*), $CD4^+CD28^+$ (*lower left graph*), $CD8^+CD28^+$ (*lower middle graph*) and $CD8^+CD28^-$ (*lower right graph*) T cells. Data are plotted as box and whiskers (Tukey style); n = 23 isotype controls, n = 10 healthy controls, n = 11 TAC patients before transplantation; *) $p < 0.05$, **) $p < 0.01$, ***) $p < 0.001$

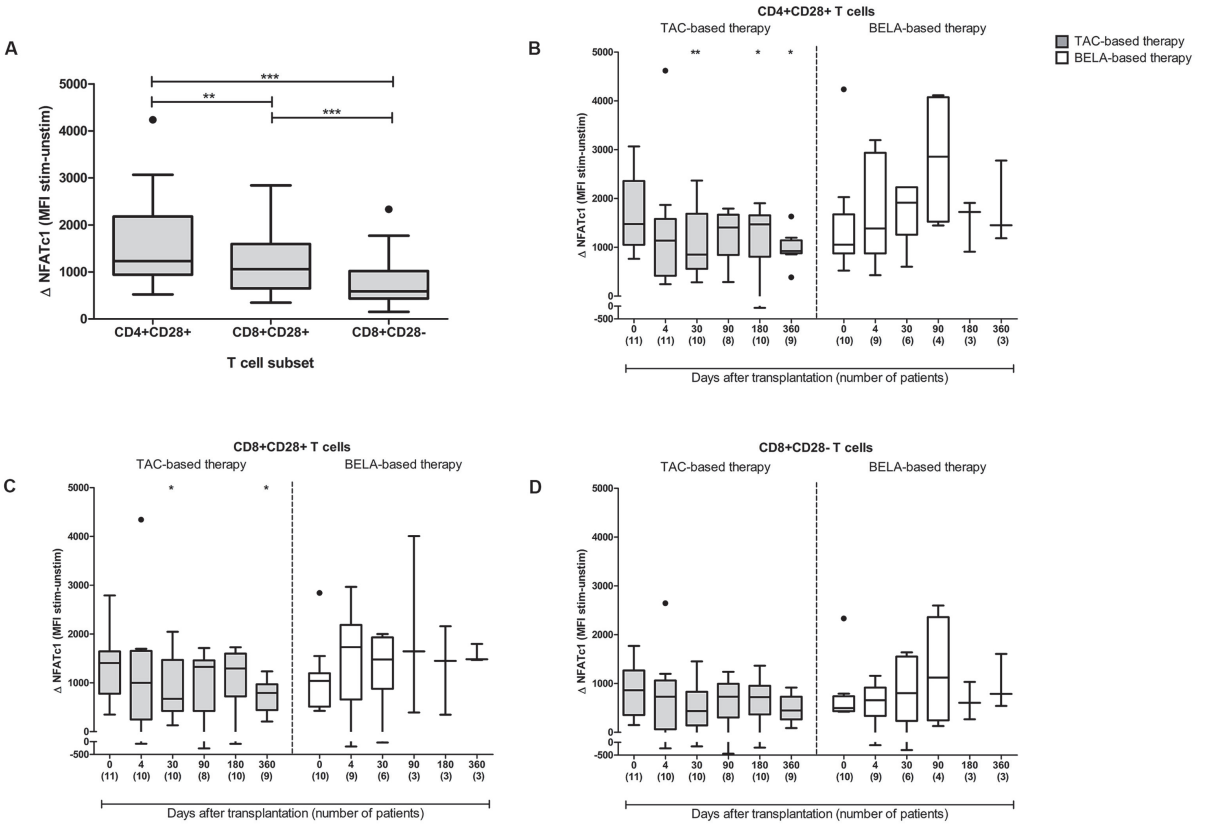

Figure 4. Immunosuppressive drug therapy effects on NFATc1 amplification in T cell subsets. (A) NFATc1 amplification in $CD4^+CD28^+$, $CD8^+CD28^+$ and $CD8^+CD28^-$ T cells, before transplantation. (n = 21 kidney transplant patients) (B, C, D) Inhibition of NFATc1 amplification (MFI) after a TAC-(*grey*) or BELA-(*white*) based therapy. Delta NFATc1 expression (amplification) was determined at different time points after transplantation and compared to the samples before transplantation in $CD4^+CD28^+$ (B), $CD8^+CD28^+$ (C) and $CD8^+CD28^-$ T cells (D). The number of patients that were measured at each time point is shown on the x-axis in parentheses. n = 11 TAC-treated patients and n = 10 BELA-treated patients at day 0. Data are plotted as box and whiskers (Tukey style); *) $p < 0.05$, **) $p < 0.01$ compared to day 0.

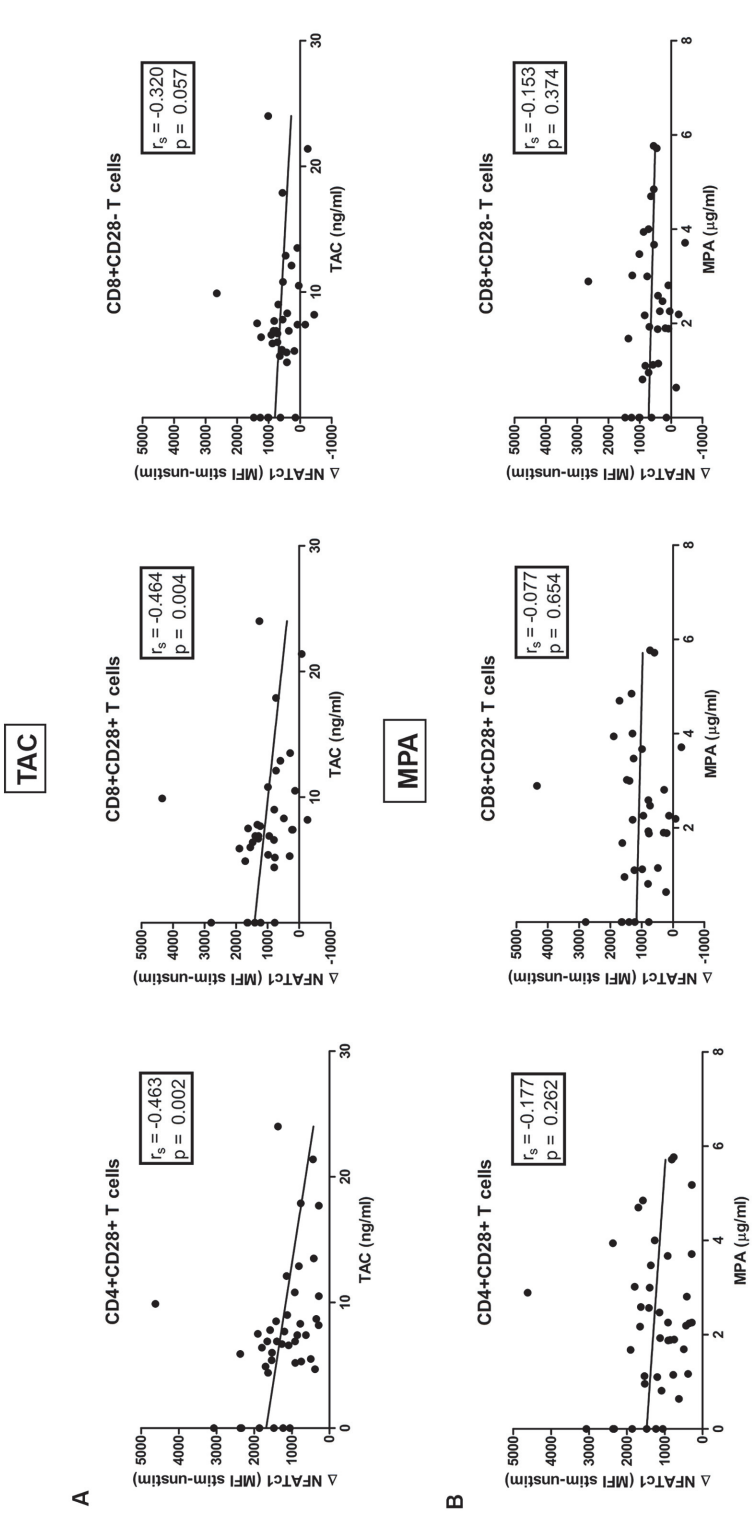

Figure 5. Spearman correlations of NFATc1 amplification with TAC or MPA pre-dose concentrations. (A) NFATc1 amplification inversely correlated with TAC pre-dose concentrations in time. The correlation was only seen in $CD4^+CD28^+$ (*left graph*) and $CD8^+CD28^+$ cells (*middle graph*) and not in $CD8^+CD28^-$ cells (*right graph*). (B) MPA pre-dose concentrations were not correlated to NFATc1 amplification levels in $CD4^+CD28^+$ (*left graph*) and $CD8^+CD28^+$ cells (*middle graph*) nor in $CD8^+CD28^-$ cells (*right graph*). TAC-treated patients were included for this analysis when blood samples were analyzed for NFATc1 amplification at all time points: before transplantation and day 4, 30, 90, 180 and 360 after transplantation. n = 7; r_s, spearman correlation coefficient.

Immunosuppressive drug effect on NFATc1 amplification: *In vitro* study

Next, the individual immunosuppressive drug effects on NFATc1 expression were determined in blood samples of healthy controls to define whether TAC was the responsible drug for the observed effects on NFATc1 amplification in the studied patient samples (who used a combination of immunosuppressive drugs). At a concentration of 10 ng/ml TAC, a small, non-significant decrease in NFATc1 expression was noted in $CD4^+CD28^+$, $CD8^+CD28^+$ and $CD8^+CD28^-$ T cells (**Figure 6**). However, at a concentration of 50 ng/ml, TAC decreased NFATc1 expression (58%, 58% and 60% for the three T cell subsets; $p = 0.02$, $p = 0.02$ and $p= 0.04$, respectively), which is in contrast with the patient experiments which showed only an effect of TAC-based therapy on $CD28^+$ T cells. MPA, prednisolone or the negative control BELA did not inhibit NFATc1 (**Figure 6**).

Discussion

TDM after transplantation is necessary to avoid problems with the small therapeutic window of TAC. To date, measuring whole-blood TAC concentrations is the method of choice for TDM in most clinics [2]. However, these (pharmacokinetic) measurements are not ideal, due to their poor correlation with clinical outcomes, such as acute rejection [6]. An additional pharmacodynamic tool for TDM is needed that measures the biological effects of TAC on its direct targets, such as the calcineurin pathway member NFAT. Up until now, no clinically applicable pharmacodynamic assay is available to monitor the direct effect of TAC on NFAT expression after kidney transplantation, although several studies have attempted to develop such an assay that measures NFAT-regulated genes [34]. For example, Maguire ***et al.*** studied the relation between TAC pre-dose concentrations and the translocation of NFAT to the nucleus [35]. Other studies have tried to correlate TAC pre-dose or spiked concentrations to the expression of NFAT-related genes [36-38].

This study shows that the amplification of total NFATc1 expression, measured in whole blood samples by means of intracellular staining, is related to the pre-dose concentration of TAC after transplantation. Upon stimulation of blood samples with PMA/ionomycin for 4 hours, only the amplification of the inducible NFAT isoform, NFATc1/A, was enhanced, which is consistent with previous studies [20,39]. Under TAC-based therapy, both $CD4^+CD28^+$ and $CD8^+CD28^+$ T cells, which express the CD28 molecule (needed for co-stimulation and proliferation), showed an inhibition of NFATc1 amplification. This was not the case when patients were treated with BELA-based therapy, indicating that TAC is responsible for the reduced NFATc1 amplification. Furthermore, the correlation between TAC pre-dose concentrations and the magnitude of NFATc1 amplification indicates that TAC is an important factor for the decreased NFATc1 amplification in kidney transplant recipients. These results are in line with the previous findings of the ***in vitro*** study by Brandt ***et al.***, which showed that the other CNI, cyclosporine A, is also responsible for the inhibition of NFATc1 amplification [20].

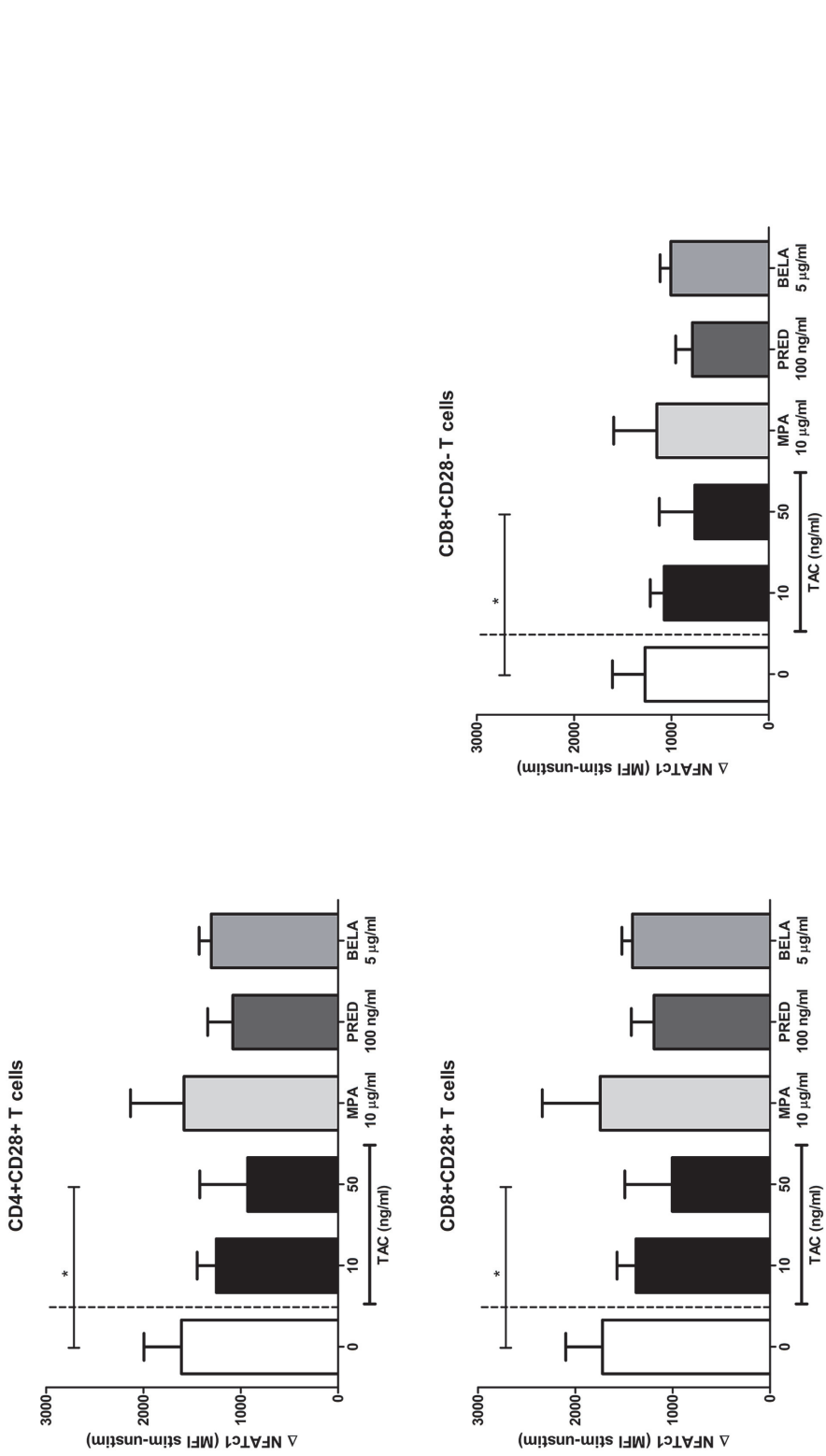

Figure 6. Individual drug effects on NFATc1 amplification in T cell subsets. TAC, at a high concentration of 50 ng/ml, significantly inhibited the expression of NFATc1 in both $CD4^+CD28^+$ (*upper graph*), $CD8^+CD28^+$ (*lower left graph*) and $CD8^+CD28^-$ cells (*lower right graph*) compared to the sample without drugs. Data are plotted as mean ±SEM; n = 5; *) $p < 0.05$

In contrast to $CD28^+$ T cells, no effect of TAC-based therapy on NFATc1 amplification was noted in $CD8^+CD28^-$ T cells. These cells are known to be more antigen-experienced than their CD28-positive counterpart and are insensitive to immunosuppressive drug therapy that targets co-stimulatory molecules, but up until now it has not been demonstrated that $CD28^-$ T cells are also less responsive to TAC-based therapy [29,30]. The overall amount of NFATc1 amplification was also lower in $CD28^-$ T cells than in $CD28^+$ T cells. An explanation could be that CD28 costimulatory signaling is needed for the inhibition of NFAT export from the nucleus back to the cytoplasm (deactivation) [40]. In the presence of CD28, the signaling molecule glycogen synthase kinase 3 (GSK3) is inhibited, resulting in decreased phosphorylation of NFATc1 and, as a consequence, in the inhibition of nuclear export (**Figure 1**). Without CD28 expression, more NFATc1 molecules become phosphorylated and will be retransferred to the cytoplasm, causing less amplification of NFATc1/A. However, in the current study, T cells were stimulated with PMA/ionomycin, which is a CD28-independent stimulation. This suggests that another and unknown mechanism may be responsible for the lower NFATc1 amplification in $CD28^-$ T cells. TAC-based therapy had no effect on NFATc1 amplification in $CD28^-$ T cells, confirming that these cells are indeed less sensitive to immunosuppressive drugs, probably due to the already low expression of NFATc1 in the nucleus. $CD8^+CD28^-$ T cells are also known for their immunoregulatory function, next to their more aggressive role in autoimmune diseases and malignancies, suggesting that the ineffective inhibition of these cells by immunosuppressive drug therapy could have a positive influence on graft survival after transplantation [41,42].

In line with the results found in patient samples, individual drug experiments also showed a decrease in NFATc1 amplification when a high concentration of TAC was used (50 ng/ml). However, in this setting, the high concentration of TAC also inhibited $CD28^-$ T cells, although the total NFATc1 amplification was again lower than in the $CD28^+$ T cells. MPA did not show any significant effect on NFATc1 amplification, whereas prednisolone seemed to have a small effect in some individual cases. This can be explained by the inhibitory effect of glucocorticoids on the transcription factor AP-1 via the glucocorticoid receptor [43,44]. NFATc1 and AP-1 cooperate to induce gene transcription, for example the amplification of NFATc1. Without the function of AP-1, NFATc1 is less effective in the transcription of genes, which will cause the reduced NFATc1/A production.

The present study has limitations. First, the amplification of NFATc1 is still not the most direct way to measure the effects of TAC, although NFATc1/A is the only NFAT member of which the total expression can be induced after T cell receptor activation. Other factors can also regulate the induction of NFATc1, including the expression of AP-1 [45]. The most ideal assay to monitor TAC exposure remains the measurement of NFAT dephosphorylation of all NFAT family members. However, since no such monoclonal antibody is available, the current assay can also provide sufficient information about TAC effects on NFAT functioning, since the induced NFATc1/A molecules contribute to the NFAT pathway as a positive feedback loop. Even more, the whole-blood assay presented here will give more sustained

information about TAC exposure than assays performed with isolated T cells, because the tacrolimus concentrations are present during the whole stimulation procedure and are not washed away. In blood samples of transplant patients, we here showed that it is possible to measure the NFATc1 amplification in the presence of tacrolimus. This has clear advantages over isolated T cell procedures where tacrolimus and other immunosuppressive drugs are washed away during the T cell isolation process. Another advantage of the whole-blood assay is that the impact of immunosuppression can be determined in different T cell subsets, such as $CD4^+CD28^+$, $CD8^+CD28^+$ and $CD8^+CD28^-$ T cells. The assay is also drug specific, as is shown by the individual drug experiments. However, it remains unknown whether the other NFAT members that are present in T cells (***e.g.*** NFATc2 and NFATc3) are also affected by TAC in the same way and to the same extent, since those NFAT members cannot be amplified [11]. Secondly, the small group size of this study might be a restriction for implementing the technique in daily clinical routine measurements. This is a single-center pilot study and it can only be recommended as a new method for biomarker when the individual responses to immunosuppressive drugs are validated [21]. For that a larger study population is needed to reveal the effect of baseline demographics on NFATc1 amplification. For the use of this assay in a clinical setting, future studies need to focus also on correlations between NFATc1 amplification and clinical outcomes, such as acute rejection [46]. The present study included only one patient suffering from a rejection under TAC-based therapy, which makes it impossible to draw conclusions on the association between NFATc1 amplification and rejection risk. We feel that in order to optimize TDM of TAC, the pharmacodynamic measurement of NFATc1 as described here should be combined with classic pharmacokinetic TDM or with the alternative pharmacokinetic measuring of intracellular tacrolimus concentrations [47].

In conclusion, measuring NFATc1 amplification is a direct method to determine the biological effects of TAC on divers T cell subsets in whole blood samples of kidney transplant recipients. This technique has potential but requires further development before it can be applied in clinical practice.

References

1. Meier-Kriesche HU, Li S, Gruessner RW, et al. Immunosuppression: evolution in practice and trends, 1994-2004. Am J Transplant 2006;6:1111-31.
2. Matas AJ, Smith JM, Skeans MA, et al. OPTN/SRTR 2013 Annual Data Report: kidney. Am J Transplant 2015;15 Suppl 2:1-34.
3. Wong SH. Therapeutic drug monitoring for immunosuppressants. Clin Chim Acta 2001;313:241-53.
4. Wallemacq P, Armstrong VW, Brunet M, et al. Opportunities to optimize tacrolimus therapy in solid organ transplantation: report of the European consensus conference. Ther Drug Monit 2009;31:139-52.
5. Shuker N, van Gelder T, Hesselink DA. Intra-patient variability in tacrolimus exposure: causes, consequences for clinical management. Transplant Rev (Orlando) 2015;29:78-84.
6. Bouamar R, Shuker N, Hesselink DA, et al. Tacrolimus predose concentrations do not predict the risk of acute rejection after renal transplantation: a pooled analysis from three randomized-controlled clinical trials(dagger). Am J Transplant 2013;13:1253-61.
7. Whalen HR, Glen JA, Harkins V, et al. High Intrapatient Tacrolimus Variability Is Associated With Worse Outcomes in Renal Transplantation Using a Low-Dose Tacrolimus Immunosuppressive Regime. Transplantation 2017;101:430-6.
8. Moscato D, Nonnato A, Adamo R, Vancheri M, Caropreso A. Therapeutic monitoring of tacrolimus: aberrant results by an immunoassay with automated pretreatment. Clin Chim Acta 2010;411:77-80.
9. Shi WL, Tang HL, Zhai SD. Effects of the CYP3A4*1B Genetic Polymorphism on the Pharmacokinetics of Tacrolimus in Adult Renal Transplant Recipients: A Meta-Analysis. PLoS One 2015;10:e0127995.
10. Andrews LM, Li Y, De Winter BCM, et al. Pharmacokinetic considerations related to therapeutic drug monitoring of tacrolimus in kidney transplant patients. Expert Opin Drug Metab Toxicol 2017;13:1225-36.
11. Macian F. NFAT proteins: key regulators of T-cell development and function. Nat Rev Immunol 2005;5:472-84.
12. Hogan PG, Chen L, Nardone J, Rao A. Transcriptional regulation by calcium, calcineurin, and NFAT. Genes Dev 2003;17:2205-32.
13. Rudensky AY, Gavin M, Zheng Y. FOXP3 and NFAT: partners in tolerance. Cell 2006;126:253-6.
14. Sundrud MS, Rao A. New twists of T cell fate: control of T cell activation and tolerance by TGF-beta and NFAT. Curr Opin Immunol 2007;19:287-93.
15. Serfling E, Chuvpilo S, Liu J, Hofer T, Palmetshofer A. NFATc1 autoregulation: a crucial step for cell-fate determination. Trends Immunol 2006;27:461-9.
16. Chuvpilo S, Jankevics E, Tyrsin D, et al. Autoregulation of NFATc1/A expression facilitates effector T cells to escape from rapid apoptosis. Immunity 2002;16:881-95.
17. Zhou B, Cron RQ, Wu B, et al. Regulation of the murine Nfatc1 gene by NFATc2. J Biol Chem 2002;277:10704-11.

18. Serfling E, Avots A, Klein-Hessling S, Rudolf R, Vaeth M, Berberich-Siebelt F. NFATc1/alphaA: The other Face of NFAT Factors in Lymphocytes. Cell Commun Signal 2012;10:16.
19. Muller MR, Rao A. NFAT, immunity and cancer: a transcription factor comes of age. Nat Rev Immunol 2010;10:645-56.
20. Brandt C, Liman P, Bendfeldt H, et al. Whole blood flow cytometric measurement of NFATc1 and IL-2 expression to analyze cyclosporine A-mediated effects in T cells. Cytometry A 2010;77:607-13.
21. Brunet M, Shipkova M, van Gelder T, et al. Barcelona Consensus on Biomarker-Based Immunosuppressive Drugs Management in Solid Organ Transplantation. Ther Drug Monit 2016;38 Suppl 1:S1-20.
22. Sommerer C, Brocke J, Bruckner T, et al. Improved pulse wave velocity and renal function in individualized calcineurin-inhibitor treatment by immunomonitoring: the randomized controlled Calcineurin Inhibitor-Sparing (CIS) Trial. Transplantation 2017.
23. Sommerer C, Zeier M, Meuer S, Giese T. Monitoring of calcineurin inhibitors by NFAT-regulated gene expression in de novo renal allograft recipients on cyclosporine A. Clin Nephrol 2015;84:165-72.
24. Giese T, Zeier M, Meuer S. Analysis of NFAT-regulated gene expression in vivo: a novel perspective for optimal individualized doses of calcineurin inhibitors. Nephrol Dial Transplant 2004;19 Suppl 4:iv55-60.
25. Northrop JP, Crabtree GR, Mattila PS. Negative regulation of interleukin 2 transcription by the glucocorticoid receptor. J Exp Med 1992;175:1235-45.
26. Baan CC, Kannegieter NM, Felipe CR, Tedesco Silva H, Jr. Targeting JAK/STAT Signaling to Prevent Rejection After Kidney Transplantation: A Reappraisal. Transplantation 2016;100:1833-9.
27. Vincenti F. Are calcineurin inhibitors-free regimens ready for prime time? Kidney Int 2012;82:1054-60.
28. Kannegieter NM, Hesselink DA, Dieterich M, de Graav GN, Kraaijeveld R, Baan CC. Differential T Cell Signaling Pathway Activation by Tacrolimus and Belatacept after Kidney Transplantation: Post Hoc Analysis of a Randomised-Controlled Trial. Sci Rep 2017;7:15135.
29. Betjes MG. Clinical consequences of circulating CD28-negative T cells for solid organ transplantation. Transpl Int 2016;29:274-84.
30. de Graav GN, Hesselink DA, Dieterich M, Kraaijeveld R, Weimar W, Baan CC. Down-Regulation of Surface CD28 under Belatacept Treatment: An Escape Mechanism for Antigen-Reactive T-Cells. PLoS One 2016;11:e0148604.
31. de Graav G, Baan CC, Clahsen-van Groningen MC, et al. A Randomized Controlled Clinical Trial Comparing Belatacept With Tacrolimus After De Novo Kidney Transplantation. Transplantation 2017.
32. Vincenti F, Charpentier B, Vanrenterghem Y, et al. A phase III study of belatacept-based immunosuppression regimens versus cyclosporine in renal transplant recipients (BENEFIT study). Am J Transplant 2010;10:535-46.

33. Kannegieter NM, Hesselink DA, Dieterich M, et al. Pharmacodynamic Monitoring of Tacrolimus-based Immunosuppression in CD14+ Monocytes after Kidney Transplantation. Ther Drug Monit 2017.

34. Bergan S, Bremer S, Vethe NT. Drug target molecules to guide immunosuppression. Clin Biochem 2016;49:411-8.

35. Maguire O, Tornatore KM, O'Loughlin KL, Venuto RC, Minderman H. Nuclear translocation of nuclear factor of activated T cells (NFAT) as a quantitative pharmacodynamic parameter for tacrolimus. Cytometry A 2013;83:1096-104.

36. Abdel-Kahaar E, Giese T, Sommerer C, Rieger H, Shipkova M, Wieland E. Analytical Validation and Cross-Validation of an NFAT-Regulated Gene Expression Assay for Pharmacodynamic Monitoring of Therapy With Calcineurin Inhibitors. Ther Drug Monit 2016;38:711-6.

37. Keller F, Sommerer C, Giese T, Zeier M, Schroppel B. Correlation between pharmacokinetics of tacrolimus and pharmacodynamics on NFAT-regulated gene expression in stable kidney transplant recipients. Clin Nephrol 2017;87 (2017):93-9.

38. Sommerer C, Meuer S, Zeier M, Giese T. Calcineurin inhibitors and NFAT-regulated gene expression. Clin Chim Acta 2012;413:1379-86.

39. Chuvpilo S, Zimmer M, Kerstan A, et al. Alternative polyadenylation events contribute to the induction of NF-ATc in effector T cells. Immunity 1999;10:261-9.

40. Pan M, Winslow MM, Chen L, Kuo A, Felsher D, Crabtree GR. Enhanced NFATc1 nuclear occupancy causes T cell activation independent of CD28 costimulation. J Immunol 2007;178:4315-21.

41. Mou D, Espinosa J, Lo DJ, Kirk AD. CD28 negative T cells: is their loss our gain? Am J Transplant 2014;14:2460-6.

42. Colovai AI, Mirza M, Vlad G, et al. Regulatory CD8+CD28- T cells in heart transplant recipients. Hum Immunol 2003;64:31-7.

43. Barnes PJ. Molecular mechanisms and cellular effects of glucocorticosteroids. Immunol Allergy Clin North Am 2005;25:451-68.

44. Herrlich P. Cross-talk between glucocorticoid receptor and AP-1. Oncogene 2001;20:2465-75.

45. Hock M, Vaeth M, Rudolf R, et al. NFATc1 induction in peripheral T and B lymphocytes. J Immunol 2013;190:2345-53.

46. Baan CC. Basic Sciences in Development: What Changes Will We See in Transplantation in the Next 5 Years? Transplantation 2016;100:2507-11.

47. Han SS, Yang SH, Kim MC, et al. Monitoring the Intracellular Tacrolimus Concentration in Kidney Transplant Recipients with Stable Graft Function. PLoS One 2016;11:e0153491.

Supplementary Figures and Tables

Supplementary Table I. Summary of patient baseline characteristics, incidence of rejection and medication

	Tacrolimus group (n = 11)	Belatacept group (n = 10)	p
Age (years)	55 (21-71)	46 (25-76)	*0.46*
Male / female	9 (82%) / 2 (18%)	6 (60%) / 4 (40%)	*0.30*
Ethnicity			*1.00*
• **Caucasian**	10 (91%)	9 (90%)	
• **African**	1 (9%)	1 (10%)	
Body weight (kg)	96.0 (63.3-103.0)	78.7 (56.6-111.4)	*0.10*
Donor age (years)	48 (22-80)	55.5 (39-70)	*0.46*
BPAR (median time to rejection in days)	1(152)	7(13)	
TAC C_0 (ng/ml ±SD)			
• **Day 4**	15.1 (± 4.8)	-	
• **Day 30**	9.9 (± 3.7)	-	
• **Day 90**	6.7 (± 1.7)	-	
• **Day 180**	6.7 (± 1.4)	-	
• **Day 360**	6.5 (± 2.0)	-	
BELA dose (mg ±SD)			
• **Day 4**	-	798 (± 180)	
• **Day 30**	-	740 (± 127)	
• **Day 90**	-	780 (± 180)	
• **Day 180**	-	358 (± 80)	
• **Day 360**	-	379 (± 69)	
MPA C_0 (ng/ml ±SD)			*0.04*
• **Day 4**	3.73 (± 1.52)	3.55 (± 1.10)	
• **Day 30**	2.89 (± 1.64)	3.67 (± 2.12)	
• **Day 90**	2.63 (± 0.94)	4.37 (± 1.76)	
• **Day 180**	2.16 (± 1.12)	2.78 (± 2.12)	
• **Day 360**	2.01 (±1.00)	1.99 (± 0.89)	
PRED dose (mg ±SD)			*0.16*
• **Day 4**	20.0 (± 0)	20.0 (± 0)	
• **Day 30**	12.7 (± 3.4)	11.6 (± 2.6)	
• **Day 90**	5.7 (± 1.6)	5.0 (± 0)	
• **Day 180**	5.0 (± 0)	5.0 (± 0)	
• **Day 360**	4.4 (± 1.1)	5.0 (± 0)	

Continuous variables are presented as medians (plus ranges) and categorical variables as numbers (plus percentages), unless otherwise specified

BPAR: The incidence of the first rejection episodes is given. The highest Banff score is depicted if sequential biopsies were performed.

BPAR, biopsy-proven acute rejection; C_0, predose concentration; MPA, mycophenolate mofetil; PRED, prednisolone; SD, standard deviation; TAC, tacrolimus

6

Conversion to Once-daily Tacrolimus Results in increased p38MAPK Phosphorylation in T-lymphocytes of Kidney Transplant Recipients

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Abstract

Background

The once-daily formulation of tacrolimus (TAC_{OD}) has been developed to overcome adherence problems. Conversion from the twice-daily TAC (TAC_{BID}) formulation to TAC_{OD} on a 1:1 basis, however, often leads to a decrease of TAC pre-dose concentrations which averages ~15%. Switching between the two TAC formulations may thus influence drug efficacy and necessitates therapeutic drug monitoring. As an additional tool in transplantation diagnostics, phospho-specific flow cytometry was used to study the biological effects of conversion on p38MAPK phosphorylation, a kinase involved in T-lymphocyte activation.

Methods

Stable renal transplant recipients (n=12), at least one year after their transplantation, were converted from TAC_{BID} to TAC_{OD} on 1:1 mg for mg base. Co-medication consisted of mycophenolate mofetil (n=10) and prednisolone (n=3). TAC whole-blood pre-dose concentrations were determined by immunoassay before and 3 months after conversion. P38MAPK phosphorylation was measured in T-lymphocytes by whole-blood phospho-specific flow cytometry.

Results

Three months after conversion, no significant decreases in TAC pre-dose concentrations (C_0) were found ($p = 0.54$), while p38MAPK phosphorylation increased with 11.4% ($p<0.05$) in CD4+ and with 15.6% ($p<0.05$) in CD8+ T-lymphocytes. The TAC C_0 during treatment with TAC_{BID} correlated inversely with p38MAPK phosphorylation in T-lymphocytes ($r_s= -0.638$, $p <0.05$).

Conclusions

These results suggest that measurement of p38MAPK phosphorylation status in T-lymphocytes is a sensitive method to determine the biological effects of TAC before and after conversion from TAC_{BID} to TAC_{OD}. This method could be a more sensitive tool for therapeutic drug monitoring of TAC.

Introduction

Therapy with the immunosuppressant tacrolimus (TAC) is routinely monitored by measuring whole-blood pre-dose concentrations (C_0). However, lack of efficacy (***i.e.*** the occurrence of acute rejection) or toxicity does occur in solid organ transplant recipients who have TAC concentrations that are considered therapeutic. A better way to perform therapeutic drug monitoring (TDM) of TAC may be to measure the drug's pharmacodynamic effects.

TAC inhibits the calcineurin pathway of activated T-lymphocytes resulting in decreased levels of de-phosphorylated Nuclear Factor of Activated T-lymphocytes (NFAT), less production of the cytokine IL-2, and ultimately, inhibition of T-lymphocyte proliferation. Earlier studies demonstrated that the protein expression of IL-2 in cell samples can be used as a pharmacodynamic tool for TDM of TAC [1]. However, this assay measures the effects of other immunosuppressive drugs, such as the effect of steroids, as well, and therefore is not specific for TAC [2]. In addition, this assay is time-consuming, costly and may not reflect TAC toxicity [3]. Thus there is an unmet need of better pharmacodynamic assays to monitor TAC treatment, leading to more customized immunosuppressive therapy [2].

Apart from its effects on the NFAT pathway, TAC also suppresses the phosphorylation of the mitogen activated protein kinase (MAPK) pathway [4]. The amount of phosphorylation of this signaling molecule was recently found to be inversely correlated with TAC whole-blood C_0 of kidney transplant patients. Furthermore, increased p38MAPK phosphorylation was associated with a higher T-lymphocyte activation status, which was inhibited by TAC in a dose dependent manner ***in vitro*** [4,5].

Here, the effect of conversion from the standard, twice-daily TAC formulation (TAC_{BID}) to the once-daily, prolonged-release TAC formulation (TAC_{OD}) on p38MAPK phosphorylation in kidney transplant recipients is reported. The novel TAC_{OD} formulation was developed to overcome adherence problems. However, whole-blood TAC C_0 may decrease by 9-15% following 1:1 conversion on a mg for mg basis [6,7]. This may lead to sub-therapeutic TAC exposure and may put certain patients at risk for rejection. We speculated that the present assay may be more sensitive than conventional C_0 monitoring and may reveal subtle changes in TAC effects.

Patients and Methods

Study design and determination of tacrolimus blood concentrations

All twelve patients reported here (for their characteristics see **Table I**) participated in a substudy of a larger clinical trial that was reported previously.[7] The aim of the clinical trial was to study the safety of conversion from TAC_{BID} (Prograf®, Astellas Pharma, Leiden, the Netherlands) to TAC_{OD} (Advagraf®, Astellas Pharma, Leiden, the Netherlands) on a 1:1 (mg:mg) basis. The aim of the substudy, which is presented here, was to investigate the effects of this conversion on p38MAPK phosphorylation status. For this pilot study n = 12

consecutive patients visiting the outpatient clinic and meeting the following inclusion criteria were asked to participate: 1) >1 year after kidney transplantation; 2) stable kidney function; 3) proteinuria <2 g/day; 4) age >18 years; 5) equal TAC dose before and 3 months after conversion. [7]

Table I. Patient characteristics (n=12)

Gender (male/female):	2 (16.7%) / 10 (83.6%)
Age (years):	54.7 (19.6-69.7)*
Time from transplantation to conversion (years):	4.92 (1-12)*
Number of subjects with concomitant MMF therapy:	10 (83.3%)
Number of subjects with concomitant steroid therapy:	3 (17.6%)
TAC dose (mg/day):	4.3 (1.5-10)*
TAC C_0 before conversion (ng/ml):	5.6 (1.9-9.8)*

*mean (range)

Heparin blood samples for p38MAPK phosphorylation status were collected 1 day before (visit 1) and 3 months after conversion to TAC_{OD} (visit 2). TAC whole-blood C_0 were determined in EDTA blood by using the antibody-conjugated magnetic immunoassay (ACMIA) on a Dimension Xpand analyzer (Siemens HealthCare Diagnostics Inc., Newark, DE) according to the manufacturer's instructions. The lower and upper limits of detection were 1.5 ng/mL and 30 ng/mL, respectively. Proficiency samples were obtained from the United Kingdom Quality Assessment Scheme (Dr. Holt, St George's Hospital Medical School, London, UK). The laboratory successfully participates in international proficiency testing schemes.

Whole–blood phospho–specific flowcytometry

p38MAPK phosphorylation was measured according to the manufacturer's instructions and as described previously.[4] In brief, 200 µl of heparinized blood was activated with PMA/Ionomycin (1.6 µmol/L/10 µg/mL, Sigma-Aldrich, Steinheim, Germany) and stained with APC-labelled mouse anti-human CD3, Pacific Blue mouse anti-human CD4 and PE-Cy7 mouse anti-human CD8 (BD Biosciences, San Jose, CA) for 30 minutes at 37˚C. Then cells were fixed for 10 minutes with Lyse/fix buffer and treated with permeabilization buffer III (both from BD biosciences) at -20˚C. Samples were stained with the fluorochrome-conjugated mAb PE mouse anti-p38MAPK (clone pT180/pY182, BD Biosciences) for 30 minutes at room temperature and analysed on a FACS Canto II flow cytometer (BD Biosciences). Isotype control IgG1-PE (clone X40, BD Biosciences) tubes were included. Interday-variability of the flowcytometer was corrected by using Quantibrite PE beads (BD Biosciences) according to the manufacturer's instructions.

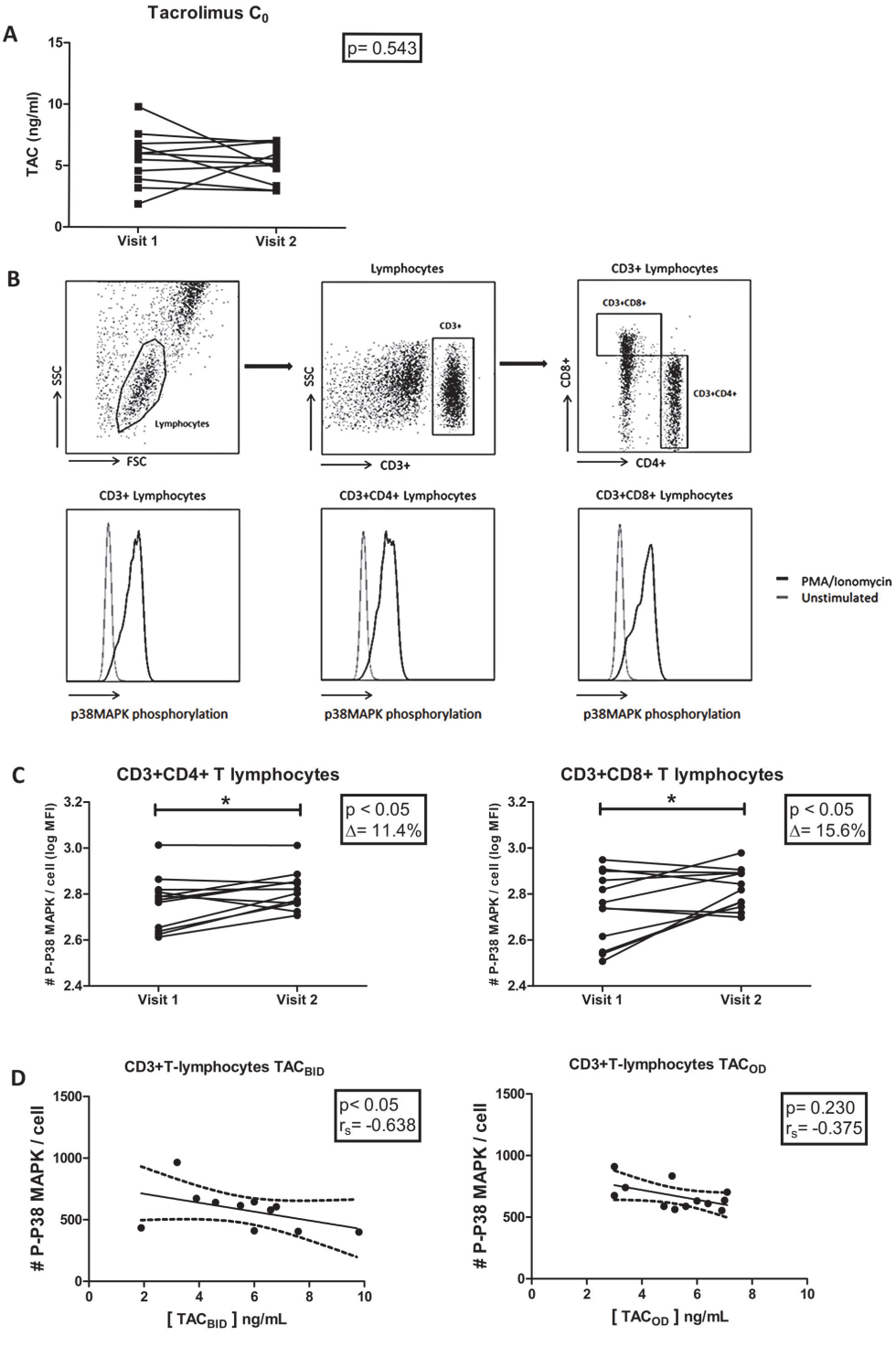
A
Tacrolimus C0
p= 0.543
TAC (ng/ml)
Visit 1
Visit 2
B
Lymphocytes
CD3+ Lymphocytes
SSC
FSC
CD3+
CD8+
CD4+
CD3+CD8+
CD3+CD4+
CD3+ Lymphocytes
CD3+CD4+ Lymphocytes
CD3+CD8+ Lymphocytes
p38MAPK phosphorylation
PMA/Ionomycin
Unstimulated
C
CD3+CD4+ T lymphocytes
CD3+CD8+ T lymphocytes
P-P38 MAPK / cell (log MFI)
p < 0.05
Δ= 11.4%
p < 0.05
Δ= 15.6%
D
CD3+T-lymphocytes TACBID
CD3+T-lymphocytes TACOD
P-P38 MAPK / cell
p< 0.05
rs= -0.638
p= 0.230
rs= -0.375
[TACBID] ng/mL
[TACOD] ng/mL

Figure 1. Tac blood C_0 after conversion from TAC_{BID} to TAC_{OD} and p38MAPK phosphorylation status. A) Tac C_0 in kidney transplant patients before and 3 months after conversion from TAC_{BID} to $TAC_{OD.}$ B) Dot plots demonstrating the selection of lymphocytes and the CD3+CD4+ and CD3+CD8+ T-lymphocytes subsets from whole blood samples. The histograms show the p38 phosphorylation for each subset after PMA/Ionomycin stimulation of whole blood for 30 min. C) P38 phosphorylation per cell before and 3 months after conversion from TAC_{BID} to TAC_{OD} in CD3+CD4+ (left panel) and CD3+CD8+ (right panel) T-lymphocytes. D) Correlation between p38 phosphorylation per cell and TAC_{BID} C_0 (left panel) or TAC_{OD} C_0 (right panel) in CD3+ T-lymphocytes. FSC, forward scatter; SSC, side scatter (n=12)

Data analysis and statistics

The p38MAPK phosphorylation was calculated as the Median Fluorescence Intensity (MFI) and normalized using Quantibrite-PE beads. Data and statistical analysis was performed with diva-version 6.0 software (BD Biosciences) and Graph Pad Prism 5.0 (Graph Pad Software Inc., La Jolla, CA) by using paired t-test (for p38MAPK phosphorylation after performing log transformation and after finding a P-value >0.05 with an F-test). Spearman's test was used to determine the correlation between TAC C_0 and p38MAPK phosphorylation. A two-sided p-value <0.05 was considered statistically significant.

Results

Conversion from TAC_{BID} to TAC_{OD} resulted in a decrease in C_0 of 6.0% (**Figure 1A**), which was not statistically significant (p= 0.54). Before conversion the median TAC C_0 was 6.0 ng/ml (range: 1.9 - 9.8 ng/ml); after conversion the median TAC C_0 was 5.4 ng/ml (range: 3 – 7.1 ng/ml).

A typical example of induced p38MAPK phosphorylation in CD3+, CD3+CD4+ and CD3+CD8+ T-lymphocytes, respectively, is shown in **Figure 1B**. The MFI levels of p38MAPK increased after stimulation with PMA/Ionomycin.

After conversion to TAC_{OD}, p38MAPK phosphorylation increased significantly, both in CD3+CD4+ (11.4% increase, p =0.034) and CD3+CD8+ T-lymphocytes (15.6% increase, p =0.038, **Figure 1C**).

Next, the phosphorylation was correlated to corresponding TAC C_0. A significant inverse correlation between TAC C_0 and p38MAPK phosphorylation in CD3+ T-lymphocytes was found for TAC_{BID} (r_s = -0.638, p <0.05, **Figure 1D**), but not for TAC_{OD} (r_s = -0.375, p=0.230, **Figure 1D**).

Discussion

p38MAPK phosphorylation potential increases significantly after 1:1 conversion from TAC_{BID} to TAC_{OD}, despite unchanged TAC whole-blood C_0. This observation suggests that measuring p38MAPK phosphorylation may be a more sensitive method to measure the effects of TAC therapy at the single-cell level as compared with conventional pharmacokinetic TDM.

The method may be able to provide more precise information on T-lymphocyte activation status and could thus guide TAC dosing in daily clinical practice. However, whether measuring p38MAPK phosphorylation status will better predict the occurrence of acute rejection under TAC therapy as compared to conventional TAC C_0 monitoring, remains to be established.

In this study, whole-blood single-cell phospho-specific flowcytometry was used to measure p38MAPK phosphorylation at the single cell level. This technique was earlier used by Nolan et al. who investigated the consequences of growth factor treatment on the profiles of cancer cell signaling networks of T-lymphocytes in tumor immunology [8]. Measuring the p38MAPK phosphorylation holds an advantage over the classic pharmacodynamic parameter IL-2 as the phosphorylation -in contrast to cytokine concentrations- corresponds with the upstream effects in the signaling cascade and consequently may be associated more strongly to clinical outcomes specific for TAC therapy. In addition, phosphorylation of molecules is a rapid process that can be measured with single-cell phospho-specific flow cytometry in whole-blood T-lymphocyte subsets within hours [4,9].

TAC_{OD} has the same safety and efficacy profile as TAC_{BID} [10-13]. Furthermore, the use of TAC_{OD} has been associated with improved adherence, a flatter pharmacokinetic profile and better glycemic control [14]. Nonetheless, C_0 may drop considerably after conversion from TAC_{BID} on a 1:1 basis in individual patients and close monitoring of TAC exposure after switching is recommended [14-16]. In the conversion trial, of which the present study was a substudy, TAC C_0 indeed decrease by an average of 12% after conversion.[7] In addition, one patient experienced a late acute cellular rejection that was associated with a marked drop in TAC exposure: TAC C_0 decreased from 6,9 ng/ml immediately before conversion to 3,6 ng/ml shortly after conversion [7].

Increased p38MAPK phosphorylation has been associated with more T-lymphocyte activation [17] and may theoretically result in a higher risk of acute cellular rejection for kidney transplant patients [18]. Despite the non-significant change in TAC exposure, that was observed to be significant in the whole study cohort but not in the n = 12 patients participating in this substudy, a significant 11.8% increase in p38MAPK phosphorylation was observed demonstrating the high sensitivity of this assay. This present finding suggests that conversion to TAC_{OD} alters exposure to the drug and that this may translate into a change in biologic effect at the single-cell level which is not detected by routine C_0 monitoring using immunoassays. Possibly, this subtle change in p38MAPK phosphorylation would have been reflected by the total TAC exposure during the dosing interval (measured by means of an area under the concentration ***versus*** time-curve) rather than a mere C_0. Alternatively, the lack of a significant change in C_0 after conversion from TAC_{BID} to TAC_{OD} and of a significant correlation between p38 phosphorylation status and C_0 during treatment with TAC_{OD} could also be related to the limited statistical power of this study.

In addition, a significant correlation between p38MFI and TAC C_0 during treatment with TAC_{BID} was observed, while remarkably, this correlation was not significant during treat-

ment with TAC_{OD}. The reason for this observation is unclear but again, it may be explained by small sample size.

Another limitation of this study is that TAC C_0 was measured with an immunoassay, which not only measures the parent compound but also cross-reacts with several TAC metabolites. Nonetheless, although measurement of TAC concentrations by LC-MS has increased, many transplant centers throughout the world still rely on immunoassays. Second, we tested only a limited number of patients. Therefore, the present findings should be interpreted with caution and be considered as hypothesis-generating.

In the future, it can be interesting to determine the risk of rejection during TAC therapy in kidney transplant patients with the help of phospho-specific flow cytometry. This pharmacodynamical approach could detect the risk of rejection in a more sensitive way than the standard pharmacokinetic method.

Furthermore, the assessment of the p38MAPK phosphorylation is a rapid method for therapeutic drug monitoring of TAC and can be used to consider dose adjustment during therapy.

Furthermore, it will be essential to start a longitudinal study to investigate the long-term effects of TAC conversion on p38MAPK phosphorylation to assess the phosphorylation change over time and to define if this change is related to a higher risk for rejection [12]. In addition, the results of the present study suggest that the increase of the p38MAPK phosphorylation status after conversion depends on the starting MFI of p38MAPK phosphorylation. It could be interesting to study intra-individual differences with the help of a larger study cohort.

Conclusion

Conversion from TAC_{BID} to TAC_{OD} is associated with a significant increase in p38MAPK phosphorylation which was not reflected by TAC whole-blood exposure as determined by C_0. Measurement of p38MAPK phosphorylation status seems feasible and could be a sensitive tool to assess the biological effects of TAC at the single T-lymphocyte level.

References

1. Sommerer C, Giese T, Meuer S, Zeier M. Pharmacodynamic monitoring of calcineurin inhibitor therapy: Is there a clinical benefit? Nephrol Dial Transplant 2009;24:21-7.
2. van Rossum HH, de Fijter JW, van Pelt J. Pharmacodynamic Monitoring of Calcineurin Inhibition Therapy: Principles, Performance, and Perspectives. Ther Drug Monit 2010;32:3-10.
3. Klupp J, Holt DW, van Gelder T. How pharmacokinetic and pharmacodynamic drug monitoring can improve outcome in solid organ transplant recipients. Transpl Immunol 2002;9:211-4.
4. Vafadari R, Hesselink DA, Cadogan MM, Weimar W, Baan CC. Inhibitory Effect of Tacrolimus on p38 Mitogen-Activated Protein Kinase Signaling in Kidney Transplant Recipients Measured by Whole-Blood Phosphospecific Flow Cytometry. Transplantation 2012;93:1245-51
5. Matsuda S, Koyasu S. Regulation of MAPK Signaling Pathways Through Immunophilin-ligand Complex. Curr Top Med Chem 2003;3:1358-67.
6. Barraclough KA, Isbel NM, Johnson DW, Campbell SB, Staatz CE. Once-Versus Twice-Daily Tacrolimus. Drugs 2011;71:1561-77.
7. Shuker N, Cadogan M, van Gelder T, et al. Conversion from Twice-Daily to Once-Daily Tacrolimus Does not Reduce Intra-Patient Variability in Tacrolimus Exposure. Ther Drug Monit 2015;37:262-9.
8. Irish JM, Hovland R, Krutzik PO, et al. Single Cell Profiling of Potentiated Phospho-Protein Networks in Cancer Cells. Cell 2004;118:217-28.
9. Baan C, Bouvy A, Vafadari R, Weimar W. Phospho-specific flow cytometry for pharmacodynamic monitoring of immunosuppressive therapy in transplantation. Transplant Res 2012;1:2047-1440.
10. Krämer BK, Charpentier B, Bäckman L, et al. Tacrolimus Once Daily (ADVAGRAF) Versus Twice Daily (PROGRAF) in De Novo Renal Transplantation: A Randomized Phase III Study. Am J Transplant 2010;10:2632-43.
11. Albano L, Banas B, Klempnauer JL, Glyda M, Viklicky O, Kamar N. OSAKA Trial: A Randomized, Controlled Trial Comparing Tacrolimus QD and BD in Kidney Transplantation. Transplantation 2013;96:897-903
12. van Hooff JP, Alloway RR, Trunečka P, Mourad M. Four-year experience with tacrolimus once-daily prolonged release in patients from phase II conversion and de novo kidney, liver, and heart studies. Clin Transplant 2011;25:E1-E12.
13. Tsuchiya T, Ishida H, Tanabe T, et al. Comparison of Pharmacokinetics and Pathology for Low-Dose Tacrolimus Once-Daily and Twice-Daily in Living Kidney Transplantation: Prospective Trial in Once-Daily Versus Twice-Daily Tacrolimus. Transplantation 2013;96:198-204
14. Hougardy J, Broeders N, Kianda M, et al. Conversion From Prograf to Advagraf Among Kidney Transplant Recipients Results in Sustained Decrease in Tacrolimus Exposure. Transplantation 2011;91:566-9
15. Wu M, Cheng C, Chen C, et al. Lower Variability of Tacrolimus Trough Concentration After Conversion From Prograf to Advagraf in Stable Kidney Transplant Recipients. Transplantation 2011;92:648-52

16. Lapeyraque A, Kassir N, Théorêt Y, et al. Conversion from twice- to once-daily tacrolimus in pediatric kidney recipients: a pharmacokinetic and bioequivalence study. Pediatr Nephrol 2014;29:1081-8.
17. Cook R, Wu CC, Kang YJ, Han J. The role of the p38 pathway in adaptive immunity. Cell Mol Immunol 2007;4:253-9.
18. Heeger PS. T-Cell Allorecognition and Transplant Rejection: A Summary and Update. Am J Transplant 2003;3:525-33.

7

Differential T Cell Signaling Pathway Activation by Tacrolimus and Belatacept after Kidney Transplantation: Post Hoc Analysis of a Randomised–Controlled Trial

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Abstract

Pharmacokinetic immunosuppressive drug monitoring poorly correlates with clinical outcomes after solid organ transplantation. A promising method for pharmacodynamic monitoring of tacrolimus (TAC) in T cell subsets of transplant recipients might be the measurement of (phosphorylated) p38MAPK, ERK1/2 and Akt (activated downstream of the T cell receptor) by phospho-specific flow cytometry.

Here, blood samples from n = 40 kidney transplant recipients (treated with either TAC-based or belatacept (BELA)-based immunosuppressive drug therapy) were monitored before and throughout the first year after transplantation.

After transplantation and in unstimulated samples, p-p38MAPK and p-Akt were inhibited in $CD8^+$ T cells and p-ERK in $CD4^+$ T cells but only in patients who received TAC-based therapy. After activation with PMA/ionomycin, p-p38MAPK and p-AKT were significantly inhibited in $CD4^+$ and $CD8^+$ T cells when TAC was given, compared to pre-transplantation. Eleven BELA-treated patients had a biopsy-proven acute rejection, which was associated with higher p-ERK levels in both $CD4^+$ and $CD8^+$ T cells compared to patients without rejection.

In conclusion, phospho-specific flow cytometry is a promising tool to pharmacodynamically monitor TAC-based therapy. In contrast to TAC-based therapy, BELA-based immunosuppression does not inhibit key T cell activation pathways which may contribute to the high rejection incidence among BELA-treated transplant recipients.

Introduction

Pharmacokinetic monitoring of the most frequently used immunosuppressive drug after solid organ transplantation, tacrolimus (TAC), is most often based on whole-blood, pre-dose concentrations. The TAC pre-dose concentration, however, has an imperfect correlation ($r_s \approx 0.7$) with the total exposure to TAC during a dosing interval as measured by the area-under the concentration ***versus*** time-curve [1-3]. As a consequence, and due to a high intra-patient variability in TAC exposure, the occurrence of acute rejection or side effects is not accurately predicted by TAC pre-dose concentrations [4-7].

T cells are the main target of most immunosuppressive drugs used in transplantation. T cells become activated upon three separate stimulation signals: 1) antigen recognition by the T cell receptor (TCR) with the help of antigen presenting cells (APC); 2) co-stimulation, of which the interaction between CD28 molecules on T cells and CD80/86 molecules on the APC is the best known pathway, and 3) binding of cytokines [8]. This will activate intracellular signaling pathways downstream of the TCR [including the calcineurin, Mitogen-Activated Protein Kinase (MAPK) and PI3K pathways] and initiate the activation of transcription factors that regulate the production of cytokines (***e.g.*** IL-2, IFN-γ and TNF-α) [9]. The activation of these pathways is characterized by (de-)phosphorylation of specific signaling molecules: Nuclear Factor of Activated T cells (NFAT), p38 MAPK, Extracellular signal-Regulated Kinases 1 and 2 (ERK1/2) and AKT8 virus oncogene cellular homolog (Akt) (**Supplementary Figure 1**) [10-14].

A promising approach to determine the biological effects of immunosuppressive drugs may be the measurement of intracellular signaling pathway activation [4,15-19]. Several research groups have tried to find suitable biomarkers for pharmacodynamic immunosuppressive drug monitoring, such as NFAT-regulated gene expression [20-22]. Up until now, these methods have not found their way into routine clinical practice [23-26].

Phospho-specific flow cytometry of the intracellular signaling molecules p38MAPK, ERK and Akt is a promising technique to monitor the pharmacodynamic effects of immunosuppressive drugs in whole-blood of kidney transplant patients [27]. Previous studies on phospho-specific flow cytometry have demonstrated that TAC can inhibit p38MAPK in a dose-dependent manner in kidney transplant patients [28]. Moreover, mycophenolic acid (MPA) was been found to decrease the phosphorylation of p38MAPK and ERK1/2 ***in vitro*** [29]. However, these previous studies lacked an appropriate control group that did not receive TAC and could therefore not exclude an effect of other, concomitantly used immunosuppressive drugs on T cell activation in these kidney transplant patients.

Belatacept (BELA), a fusion protein consisting of the Fc-fragment of human immunoglobulin G1 linked to the extracellular domain of human cytotoxic T-lymphocyte antigen (CTLA)-4, was approved in 2011 for the prevention of acute rejection after kidney transplantation [30-32]. It blocks the co-stimulation signal between CD80/86 and CD28 molecules (on APCs and T cells, respectively) and prevents T cell activation. In contrast to TAC, BELA is not nephrotoxic and has less metabolic side effects, although the incidence of acute rejection with a BELA-based treatment is relatively high [33,34]. The higher risk of acute rejection of

patients who receive treatment with BELA has been associated with a more aggressive T cell-mediated allogeneic response [33,35-37]. One of the explanations for this phenomenon is the fact that memory $CD8^+$ T cells lack CD28 expression and are not dependent on the co-stimulatory signal from CD80/86 [35,38-40].

Here, we expand on our previous work and investigated the phosphorylation status of 3 signaling proteins involved in T cell activation, namely p38MAPK, ERK and Akt, by means of phospho-specific flow cytometry to investigate whether this technique can be used as a tool for pharmacodynamic monitoring of TAC-based immunosuppression after kidney transplantation [28]. Phosphorylation was measured in blood samples of kidney transplant patients treated with either a TAC-based or a BELA-based immunosuppressive regimen as a part of a randomized controlled clinical trial [34]. Both groups received MPA and prednisolone. Because BELA only indirectly inhibits T cell activation, the BELA-treated group served as a control group in this study, circumventing a limitation of previous studies [28,29]. In contrast to previous studies, the effect of TAC and BELA was also determined in different $CD3^+$ T cell subsets and at multiple time points after transplantation.

Material and Methods

Kidney transplant patients

Between January 21th, 2014 and February 19th, 2016 peripheral blood samples were collected from renal transplant recipients to determine the effect of a TAC- or BELA-based treatment on $CD3^+$ T cell subset activation. This study was part of a randomized-controlled clinical trial that was approved by the Medical Ethical Committee of the Erasmus MC, University Medical Center Rotterdam (MEC-2012-421, EudraCT # 2012-003169-16, http://www.trialregister.nl/trialreg/index.asp number NTR4242, registered October 17th 2013) [34]. Detailed information about study design, interventions, outcomes, sample size and randomization was previously reported by de Graav ***et al***. [34]. All patients (≥ 18 years) described in this single center study received a kidney from a living donor at our institution (Erasmus MC, Department of Internal Medicine, Rotterdam, the Netherlands) [34]. Kidneys were either allocated directly or as part of the Dutch national kidney exchange program [41]. Recipients of a deceased donor kidney were not included in this study and no organs were procured from (executed) prisoners. All experiments were performed in accordance with relevant guidelines and regulations. Forty renal transplant patients were included and randomized to receive a BELA-based treatment (n = 20) or TAC-based treatment (n = 20). In- and exclusion criteria were described previously [34]. All participants gave written informed consent for participation in this study and for collecting their blood samples. On the day of transplantation and on day 4 after transplantation, patients were treated with 20 mg basiliximab intravenously (Simulect®, Novartis, Basel, Switzerland). During the first three post-operative days prednisolone was administered intravenously in a dosage of 100 mg/

day. Afterwards, prednisolone was given orally in a dose of 20 mg and tapered to 5 mg/day by month 3 and was then continued throughout the first post-transplant year. Mycophenolate mofetil (MMF; Cellcept®; Roche, Basel, Switzerland) was given in a starting dose of 2000 mg/day equally divided in two doses, and then adjusted to pre-dose concentrations (target 1.5 – 3.0 mg/L). Patients received TAC (Prograf®, Astellas Pharma Inc., Tokyo, Japan) twice a day from the day of transplantation with a starting dose of 0.2 mg/kg/day. Thereafter, TAC was adjusted to pre-dose concentrations: 10-15 ng/mL (week 1-2), 8-12 ng/mL (week 3-4), and 5-10 ng/mL (from week 5 onwards). BELA-treated patients received 10 mg/kg/day BELA (Nulojix®, Bristol-Myers Squibb, New York, USA) intravenously on the day of transplantation and on days 4, 15, 30, 60, and 90 after transplantation. From month 4 onwards, patients received monthly infusions of 5 mg/kg BELA (according to the so-called less intensive regimen) [33]. Heparinized blood samples were collected pre-transplantation and 4, 30, 90, 180 and 360 days post-transplantation or before anti-rejection therapy was started when an acute rejection was suspected (usually on the day of the kidney biopsy).

Immunosuppressive drug whole–blood pre–dose concentrations

TAC and MPA whole-blood or plasma pre-dose concentrations, respectively, were determined in EDTA blood using the antibody-conjugated magnetic immunoassay on a Dimension Xpand analyzer (Siemens HealthCare Diagnostics Inc., Newark, DE) according to the manufacturer's instructions. The lower and upper limits of detection of TAC were 1.5 and 30 ng/mL and for MPA 0.5 µg/ml and 15 µg/ml, respectively. For TAC, the coefficient of variations (CV) was 15.0%, 8.9% and 11.2% for the low, middle and high control samples, respectively. For MPA, the CV was 3.9% and 3.7%, for the low and high controls, respectively. Proficiency samples were obtained from the UK Quality Assessment Scheme (Analytical Services International Ltd, London, UK) and the laboratory successfully participates in international proficiency testing schemes.

Whole–blood phospho–specific flow cytometry

Whole-blood samples were monitored for the expression of phosphorylated (p-) p38MAPK, ERK and Akt according to the manufacturer's instructions for phospho-protein analysis (BD Biosciences, San Jose, CA) and as described previously.[42] The CV for phospho-specific flow cytometry was 5.6%.[43] In short, 200 µl heparinized blood was stained with Brilliant Violet (BV) 510-labeled mouse anti-human CD3 (Biolegend, San Diego, CA), peridinin chlorophyll (PERCP)-labeled mouse anti-human CD4 (BD Biosciences), allophycocyanin(APC)-Cy7-labeled mouse anti-human CD8 (Biolegend), BV421-labeled mouse anti-human CD28 (BD Biosciences) and Fluorescein Isothiocyanate (FITC)-labeled mouse anti-human CD14 (Serotec, Oxford, UK) for 30 minutes at 37˚C. After 15 minutes of staining, PMA/ionomycin (Sigma-Aldrich, Steinheim, Germany) was added to activate T cells in the remaining 15 minutes. A final optimized concentration of 500 ng/ml and 5 µg/ml of PMA and ionomycin, respectively was used for samples stained for p38MAPK and Akt. 100 ng/ml PMA and 1

µg/ml ionomycin was used for ERK. From here, unstimulated and stimulated cells with PMA/ionomycin were considered as two different samples and analyzed separately. Cells were then fixed and lysed for 10 minutes with Lyse/Fix buffer (BD Biosciences) and permeabilized with 90% methanol for 30 minutes at -20˚C. Phycoerythrin (PE)-labeled mouse anti-p-p38MAPK (clone pT180/pY182), PE-labeled mouse anti-p-Akt (clone pS473) or AlexaFluor647 (AF647)-labeled mouse anti-p-ERK1/2 (pT202/pY204) mAB (all from BD Biosciences) were used for 30 minutes at room temperature to stain for the intracellular signaling pathway activation. Samples were analyzed on a FACS Canto II flow cytometer (BD Biosciences). Isotype controls; mouse IgG1-PE (p38MAPK and Akt, Biolegend) and mouse IgG1-AF647 (ERK; Biolegend); served as negative controls and were included in separated tubes. Cytocalbeads (Thermo Scientific, Fremont, CA) were used to correct for interday-variability of the flow cytometer according to the manufacturer's instructions. Absolute numbers of $CD3^+$, $CD4^+$ and $CD8^+$ cells were measured with BD multi-test 6-colour in BD TruCount Tubes (BD Biosciences, San Jose, CA).

Cytokine production

Heparinized blood samples were activated with a final concentration of 0.5 µg/ml PMA and 10 µg/ml ionomycin for four hours at 37°C. Golgiplug (BD Biosciences) was added during this incubation to accumulate cytokines intracellularly. Subsequently, EDTA was added for 15 minutes. Cells were then stained with BV510-labeled mouse anti-human CD3 (Biolegend), BV421-labeled mouse anti-human CD4 (Biolegend), APC-Cy7-labeled mouse anti-human CD8 (Biolegend) and PERCP-Cy5-labeled mouse anti-human CD28 (BD Biosciences) for 30 minutes at room temperature, fixed twice for 10 minutes with FACS lysing solution (BD Biosciences) and treated with permeabilization buffer II (BD Biosciences) for 10 min. FITC-labeled mouse anti-human IFN-γ (BD Biosciences) was used for intracellular cytokine staining for 30 minutes at room temperature.

Statistical Analysis

The Median Fluorescence Intensity (MFI) was measured for the phosphorylation of p38MAPK, ERK and Akt and data-analysis was performed with Diva-version 6.0 software (BD Bioscience). Negative values measured with flow cytometry can be explained by the compensation settings of the FACS and are displayed via hyperlog transformation.[44] MFI values were normalized using Cytocalbeads (Thermo Scientific). Statistical analysis was performed with Graph Pad Prism 5.0 (Graph Pad Software Inc., La Jolla, CA) by using paired and unpaired t-tests (after finding a p-value > 0.05 with the Kolmogorov-Smirnov test for normality for the study population). Correlations between drug concentrations and phosphorylation were calculated as the Pearson correlation coefficient. Associations between phosphorylation levels and co-variates were tested by linear regression with IBM SPSS statistics software (version 21; IBM Analytics, Chicago, Illinois, USA). Bonferroni correction was used to correct for multiple testing. A two-sided p-value < 0.05 was considered statisti-

cally significant and for the association calculations, two-sided p-values of < 0.0042 were considered statistically significant after Bonferroni correction.

Results

Patient demographics and graft survival

Baseline patient characteristics and information regarding patient and graft survival are shown in **Supplementary Tables I and II** [34]. In summary, three patients in the BELA group lost their graft at day 12 (Banff type IIB acute rejection), day 59 (Banff type III acute rejection) and day 161 (Banff type IIB acute rejection) after transplantation. The total incidence of biopsy-proven acute rejection (BPAR) in this group was 55% (n = 11, median time to rejection 81 days). The incidence of BPAR was lower in the TAC-treated group (n = 2, 10%, median time to rejection 56 days). In the TAC group, one patient died 294 days after transplantation, due to a traumatic head injury. For the present study, patients with a BPAR were censored from the moment of rejection onwards, because measurements of intracellular signaling pathways after the occurrence of rejection were likely to be heavily influenced by the anti-rejection therapy.

T cell subset counts and immunosuppressive drug pre–dose concentrations

Figure 1A represents a gating strategy example of the phospho-specific flow cytometry measurements and the selection of the $CD3^+$ T cell subsets. Absolute numbers of $CD3^+$, $CD4^+$, $CD8^+$, $CD4^+CD28^+$, $CD4^+CD28^-$, $CD8^+CD28^+$ and $CD8^+CD28^-$ T cells were constant over time after transplantation (**Figure 1B**). There were no differences between the absolute T cell counts of TAC- and BELA-treated patients (**Figure 1B**).

TAC pre-dose concentrations decreased over time and followed the targeted pre-dose concentration range (**Supplementary Table III**). Likewise, MPA pre-dose concentrations and prednisolone doses were in the range specified by the protocol. BELA was administered according to the less-intensive regimen (10 mg/kg at days 0, 4, 30 and 90 and 5 mg/kg at days 180 and 360.[34] CD86 saturation of monocytes was complete at all time points of all BELA-treated patients. [34]

Whole blood phospho–specific flow cytometry of p38MAPK, ERK and Akt

Phosphorylation of intracellular signaling molecules was measured with or without PMA/ionomycin stimulation to monitor the effects of the immunosuppressive drug combination therapy, consisting of either TAC or BELA in combination with prednisolone and MMF. An example of the phosphorylation measurements is depicted in **Figure 2A**. In the total CD3+ T cell population, p-p38MAPK, p-ERK and p-Akt showed significantly higher MFI values after stimulation (before transplantation; mean MFI: 1308, 718 and 813, respectively) compared to the isotype controls (mean MFI: 394, 177 and 387, respectively; p < 0.001) and unstimu-

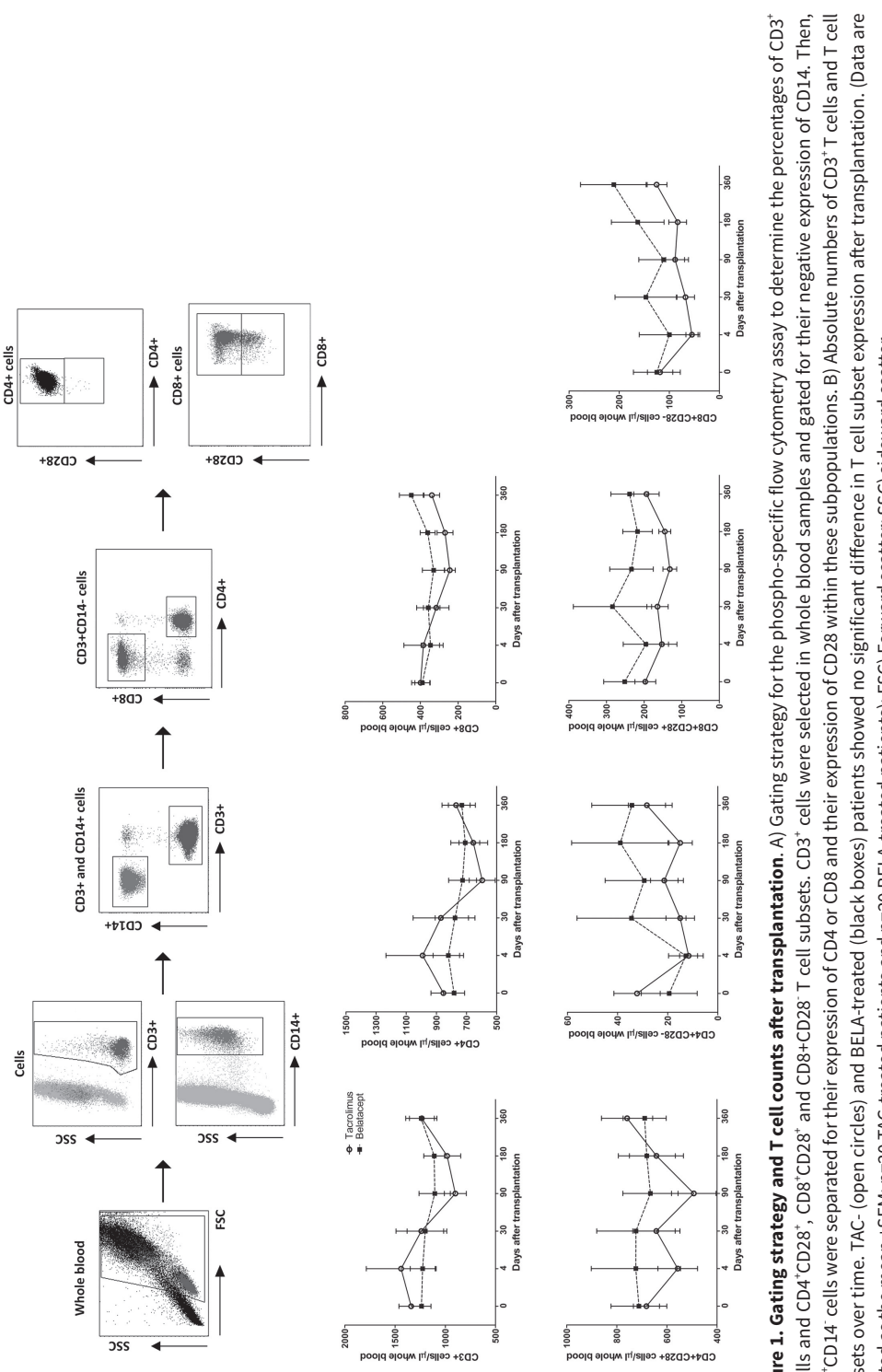


Figure 1. Gating strategy and T cell counts after transplantation. A) Gating strategy for the phospho-specific flow cytometry assay to determine the percentages of $CD3^+$ T cells and $CD4^+CD28^+$, $CD8^+CD28^+$ and $CD8^+CD28^-$ T cell subsets. $CD3^+$ cells were selected in whole blood samples and gated for their negative expression of CD14. Then, $CD3^+CD14^-$ cells were separated for their expression of CD4 or CD8 and their expression of CD28 within these subpopulations. B) Absolute numbers of $CD3^+$ T cells and T cell subsets over time. TAC- (open circles) and BELA-treated (black boxes) patients showed no significant difference in T cell subset expression after transplantation. (Data are plotted as the mean ±SEM; n=20 TAC-treated patients and n=20 BELA-treated patients); FSC) Forward scatter; SSC) sideward scatter

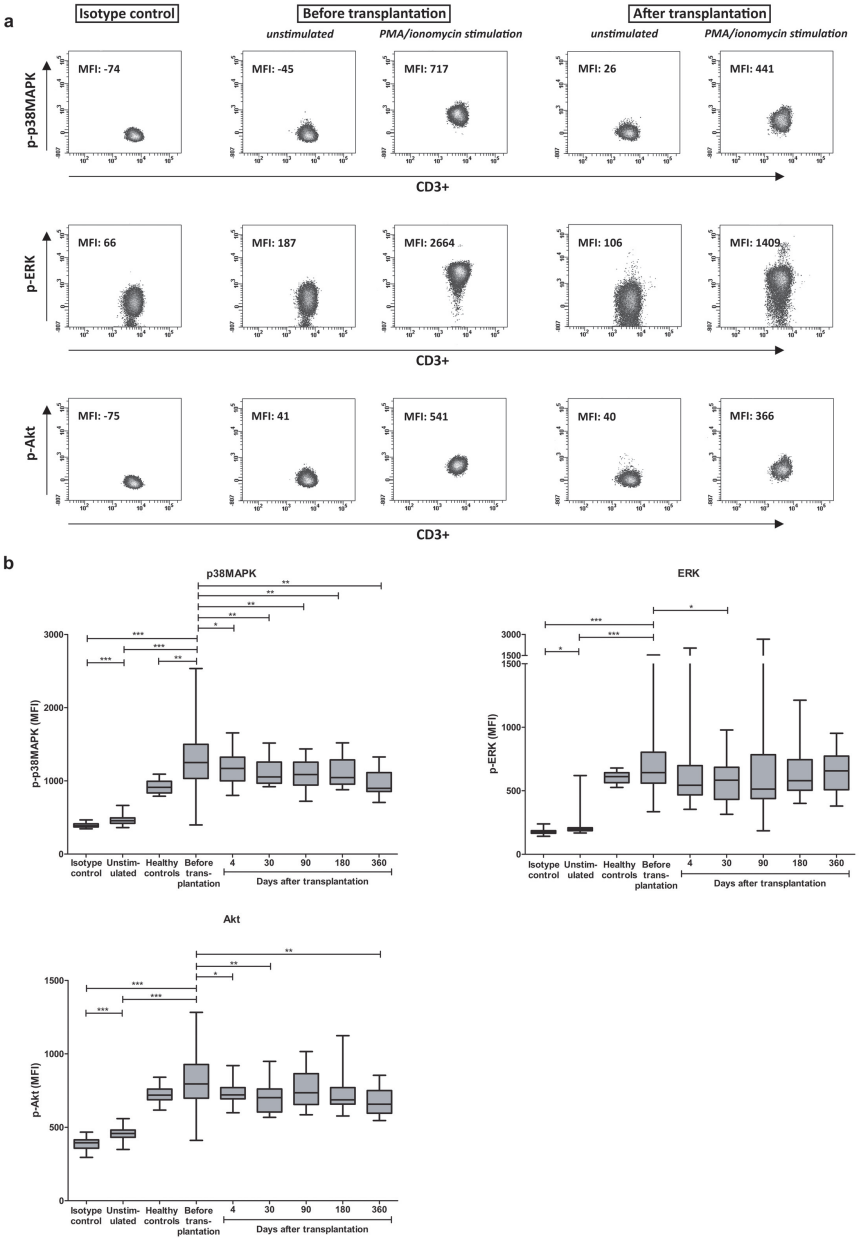


Figure 2. Phosphorylation of p38MAPK, ERK and Akt in CD3$^+$ T cells. A) Example of measured median fluorescence intensities in CD3$^+$ T cells for p-p38MAPK, p-ERK and p-Akt before and after transplantation (in both unstimulated and PMA/ionomycin stimulated samples) compared to their isotype control measurements. Negative values measured with flow cytometry can be explained by the compensation settings of the FACS. B) Total phosphorylation (after stimulation with PMA/ionomycin) of p38MAPK (upper left), ERK (right) and Akt (lower left) before and after transplantation compared to isotype controls, unstimulated samples and stimulated samples of healthy controls. Data are plotted as box and whiskers indicating total range. In contrast to p-ERK and p-Akt, p-p38MAPK is inhibited in CD3$^+$ T cells at all time points after transplantation (n=40); *) $p < 0.05$, **) $p < 0.01$, ***) $p < 0.001$

lated samples (mean MFI: 466, 222 and 458, respectively; $p < 0.001$) (**Figure 2B**). Expression of p-p38MAPK was higher in patients before transplantation than in healthy controls (mean MFI: 1308 ***vs.*** 916; $p < 0.01$). P-p38MAPK expression in $CD3^+$ cells significantly decreased in the total study population and at all measured time points after transplantation, in contrast to p-ERK and p-Akt.

Signaling protein phosphorylation in T cell subsets of TAC– and BELA–treated kidney transplant patients

To assess the effect of TAC on T cell subset activation, phosphorylation of p38MAPK, ERK and Akt was measured before and throughout the first year after transplantation in $CD4^+CD28^+$, $CD8^+CD28^+$ and $CD8^+CD28^-$ T cell subsets of TAC-treated patients (**Figure 3 and S5 Figure**). Samples of BELA-treated patients were included as controls.

In unstimulated blood samples, p-ERK was significantly inhibited in $CD4^+CD28^+$ T cells when TAC was given, but not in the presence of BELA ($p < 0.01$) (**Supplementary Figure 2**). This phenomenon was not observed in $CD8^+CD28^+$ and $CD28^-$ cells (**Supplementary Figure 2**).

After PMA/ionomycin stimulation, inhibition of signaling protein phosphorylation was found for p-p38MAPK and p-Akt, but not for p-ERK and only during TAC-based treatment (**Figure 3**). In $CD4^+CD28^+$ cells, TAC-based treatment showed a decreased expression of p-p38MAPK at all time points ($p < 0.05$) and a reduced p-Akt expression at most tested time points ($p < 0.01$) (**Figure 3A**). In the presence of BELA, reduced expression of p-p38MAPK in $CD4^+CD28^+$ was measured only at day 90 ($p < 0.05$). A comparable effect of a TAC-based immunosuppressive regimen was seen in $CD8^+CD28^+$ and CD8+CD28- T cells, although p-p38MAPK and p-Akt were not inhibited at day 90 and 180, respectively (**Figure 3B and 3C**). Notably, at day 360, BELA-based treatment increased the expression of p-ERK in $CD8^+CD28^+$ T cells (**Figure 3B**).

Pharmacodynamic–pharmacokinetic interrelationship between immunosuppressive drug concentrations, clinical outcomes and signaling protein phosphorylation

After stimulation with PMA/ionomycin, no correlations were found between TAC pre-dose concentrations or MPA pre-dose concentrations and the phosphorylation of intracellular signaling proteins (**Table I**). Also no associations were found between the phosphorylation of p38MAPK, ERK and Akt and patient characteristics pre-, 4 and 360 days after transplantation (**Supplementary Table IV**).

Association between phosphorylation patterns and acute rejection episodes of BELA–treated patients

The association between TAC-based therapy and the incidence of BPAR could not be analyzed since only two out of 20 TAC-treated patients suffered from an acute rejection. These patients suffered from a Banff type 1 and 2 rejection, respectively [34]. To investigate

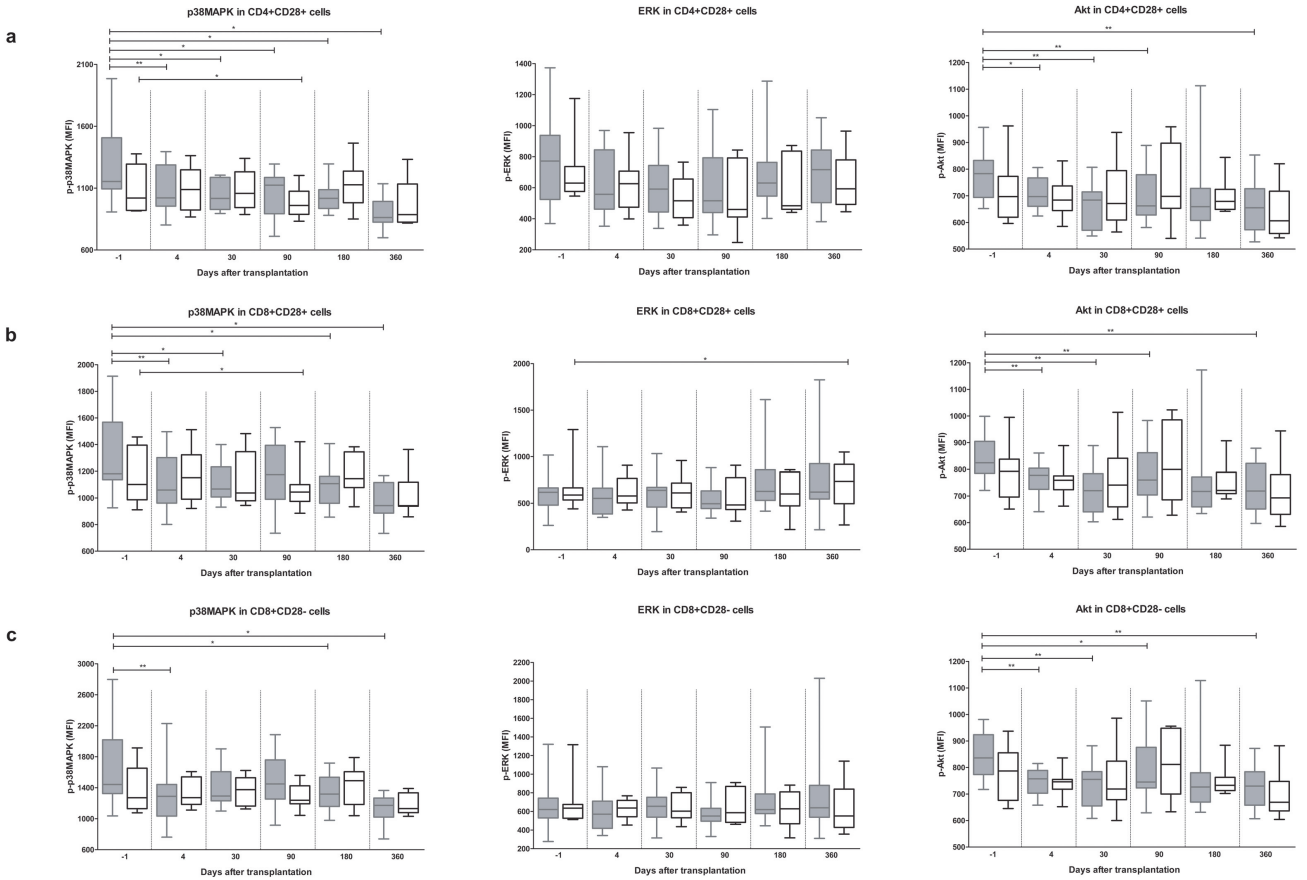


Figure 3. **P-p38MAPK, p-ERK and p-Akt in T cell subsets of TAC- (*grey*) and BELA- (*white*) treated patients after stimulation with PMA/ionomycin.** A) p-p38MAPK (left), p-ERK (middle) and p-Akt (right) within $CD4^+CD28^+$ T cells. P-p38MAPK and p-Akt, but not p-ERK, were significantly decreased in TAC-treated patients at different time points after transplantation, in contrast to BELA-treated patients. B) p-p38MAPK (left), p-ERK (middle) and p-Akt (right) within $CD8^+CD28^+$ T cells. P-p38MAPK and p-Akt, but not p-ERK, were inhibited when TAC was given. BELA caused an increase in p-ERK at day 360. **c**) p-p38MAPK (left), p-ERK (middle) and p-Akt (right) within $CD8^+CD28^-$ T cells. Phosphorylation inhibition by TAC is comparable with $CD8^+CD28^+$ T cells. (Data are plotted as box and whiskers indicating total range; n=20 TAC-treated patients and n=20 BELA-treated patients) *) $p < 0.05$, **) $p < 0.01$, ***) $p < 0.001$

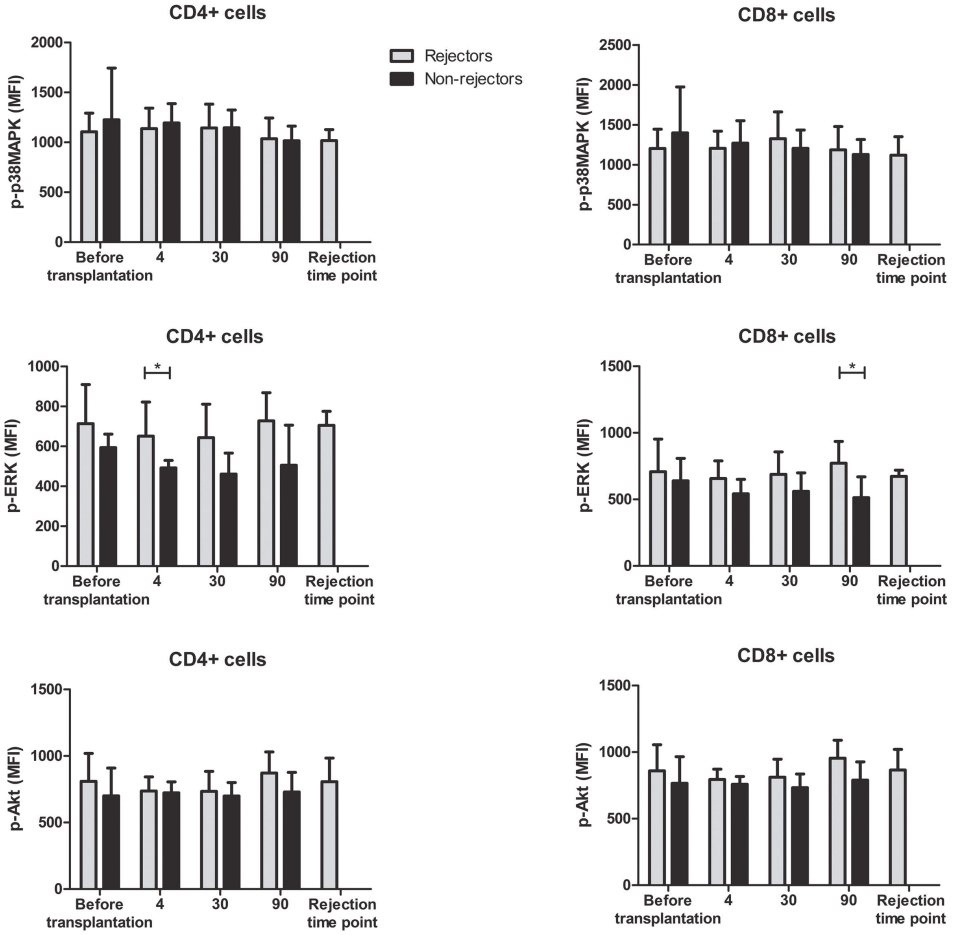


Figure 4. **Signaling protein phosphorylation in BELA-treated patients with and without BPAR.** Phosphorylation of p38MAPK (upper graphs), ERK (middle graphs) and Akt (lower graphs) in $CD4^+$ cells (left) and $CD8^+$ cells (right) after stimulation with PMA/ionomycin is shown. BELA-treated patients suffering from an acute rejection (*grey*) episode showed a significantly higher expression of p-ERK before their rejection time point compared to patients without a rejection within 90 days after transplantation (*black*). (Data are plotted as mean ±SD; n=11 rejectors and n=9 non-rejectors) *) $p < 0.05$

whether a rejection episode was associated with a change in phosphorylation status, blood samples of BELA-treated patients suffering from BPAR were compared with those who remained rejection-free. Eleven out of 20 BELA-treated patients experienced BPAR [34]. Of these, 1 patient was graded Banff type 1, 8 patients were graded Banff type 2, 1 patient was graded Banff type 3, and 1 patient suffered from a mixed type rejection. Both $CD4^+$ and $CD8^+$ T cells of these patients revealed an increase in p-ERK expression at day 4 and day 90, respectively, after stimulation with PMA/ionomycin ($p < 0.05$) (**Figure 4,** middle). In addition, p-ERK expression at the time of rejection was high compared to patients without a

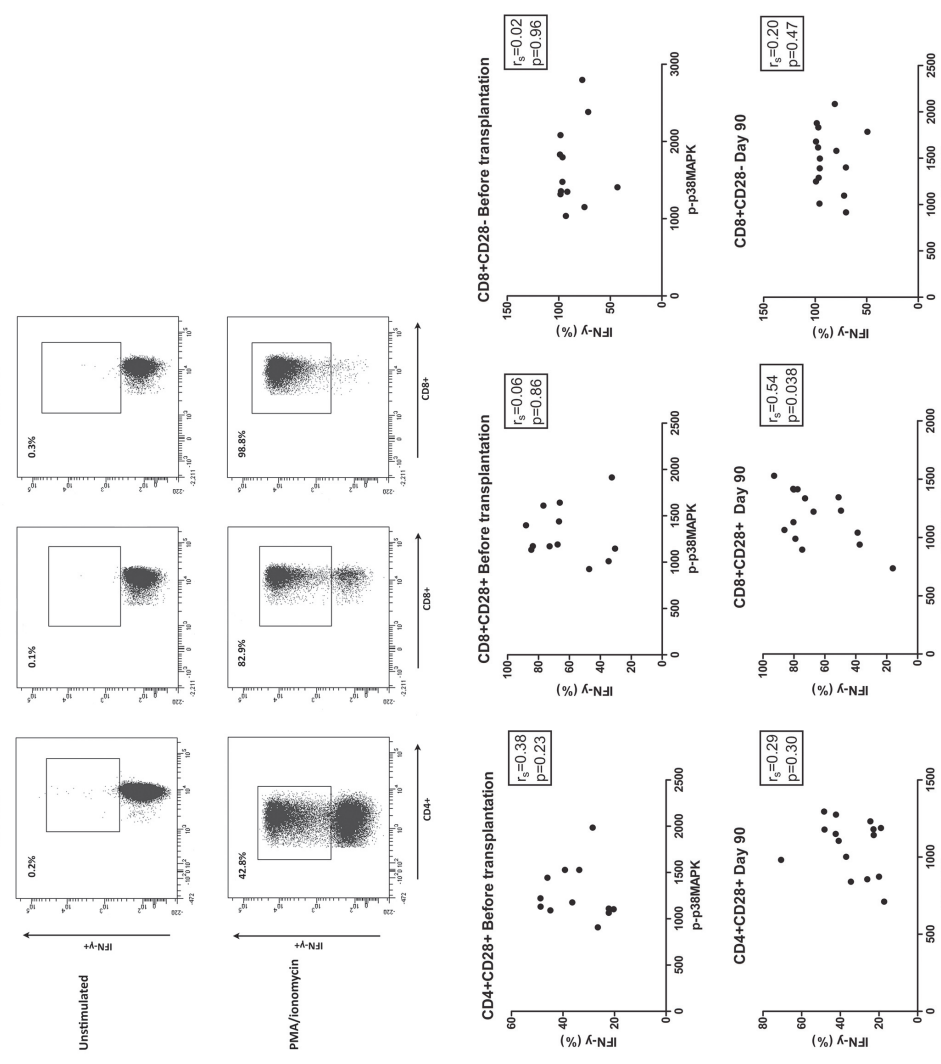


Figure 5. Correlation between signaling protein phosphorylation and IFN-γ production by T cell subsets. A) Gating strategy for the production of IFN-γ by $CD4^+CD28^+$ (left), $CD8^+CD28^+$ (middle) and $CD8^+CD28^-$ (right) cells, in both unstimulated and stimulated samples. B) Correlations between p-p38MAPK (upper graphs), p-ERK (middle graphs) and p-Akt (lower graphs) and the production of IFN-γ by $CD4^+CD28^+$ (left), $CD8^+CD28^+$ (middle) and $CD8^+CD28^-$ (right) cells after stimulation with PMA/ionomycin. Correlations were calculated before transplantation and 90 days after transplantation. P-p38MAPK significantly correlated with the production of IFN-γ in $CD8^+CD28^+$cells at day 90 after transplantation. (n=12 before transplantation and n=15 90 days after transplantation); *) $p < 0.05$, **) $p < 0.01$, ***) $p < 0.001$

Table I. Correlation between signaling molecule phosphorylation (stimulated) and immunosuppressive drug pre–dose blood concentrations at day 4 after transplantation

		p-p38MAPK		p-ERK		p-Akt	
	T cell subset	r_p	*p-value*	r_p	*p-value*	r_p	*p-value*
TAC	CD4+CD28+	-0.09	0.79	0.11	0.73	-0.08	0.82
	CD8+CD28+	0.09	0.78	-0.14	0.66	0.20	0.54
	CD8+CD28-	-0.02	0.96	-0.15	0.64	0.20	0.53
MPA (TAC-group)	CD4+CD28+	-0.40	0.20	0.46	0.13	0.31	0.33
	CD8+CD28+	-0.37	0.24	0.32	0.31	0.37	0.23
	CD8+CD28-	-0.26	0.41	0.31	0.32	0.36	0.25
MPA (BELA-group)	CD4+CD28+	-0.51	0.16	-0.38	0.32	-0.28	0.47
	CD8+CD28+	-0.60	0.09	-0.17	0.66	-0.32	0.39
	CD8+CD28-	-0.29	0.44	-0.08	0.85	-0.35	0.35

r_p) Pearson correlation coefficient

rejection. There was no difference in p38MAPK and Akt phosphorylation between rejectors and non-rejectors in both cell subsets.

Cytokine production and phosphorylation of p38MAPK

To determine whether the pharmacodynamic drug effects also correlated with T cell subset function, correlations were calculated between p-p38MAPK expression and the production of IFN-γ. Before transplantation, no correlation existed between p-p38MAPK expression and IFN-γ production in all tested T cell subsets. At day 90 after transplantation, a significant positive correlation was found between p-p38MAPK expression and IFN-γ production in both $CD4^+CD28^+$ and $CD8^+CD28^+$ T cells (**Figure 5**).

Discussion

Pharmacokinetic monitoring of immunosuppressive drug therapy is routinely performed in most transplant centers. However, the TAC pre-dose concentration does not accurately predict the occurrence of acute rejection after kidney transplantation [2,6]. Here, phospho-specific flow cytometry, a promising technique with a short turnaround time for the pharmacodynamic measurement of immunosuppressive drug effects, was investigated [15,16,45]. The technique has been used previously for testing immunosuppressive drug responses in patients with rheumatoid arthritis and for the monitoring of mTOR inhibitor therapy. This technique was also studied with regard to TDM of TAC by our group [28]. However, and in contrast to the present work, in that study, only p38MAPK was investigated and only in $CD4^+$ and $CD8^+$ T cells of patients treated with TAC-based immunosuppression. Here, three major signaling molecules involved in T cell activation, p38MAPK, ERK and Akt, were tested as potential biomarkers for detecting biological drug effects of TAC in different T cell subsets and compared to a group of patients treated with BELA-based treatment.

In directly measured blood samples of patients treated with a TAC-based immunosuppressive regimen, expression of p-ERK in $CD4^+$ T cells was decreased at almost all measured time points after transplantation, whereas no effect was found on p38MAPK and Akt phosphorylation. In contrast, $CD8^+$ T cells showed no decrease in p-ERK but showed a lower expression of p-p38MAPK and p-Akt at day 30.

There was no decrease in p-ERK induced by TAC-based treatment after stimulation with PMA/ionomycin, suggesting that the ERK pathway can still be activated after transplantation, despite the inhibited expression in unstimulated samples. A TAC-based-treatment also affected the expression of p-p38MAPK and p-Akt in stimulated $CD4^+CD28^+$, $CD8^+CD28^+$ and $CD8^+CD28^-$ cells, but not to the same extent. For example, $CD8^+$ cells did not show an inhibition of p38MAPK at day 90, while this molecule was significantly inhibited at all measured time points in $CD4^+$ cells. P-Akt showed no significant inhibition at day 180 in all T cells subsets.

BELA blocks the co-stimulation signal between the CD80/86 molecule on APC's and the CD28 molecule on T cells. This indicates that BELA only indirect affects the phosphorylation of signaling pathways in T cells. Indeed, no effects of BELA on signaling protein phosphorylation were noticed in both unstimulated and stimulated samples. Only at day 90, when blood samples were stimulated with PMA/ionomycin, a decrease of p-p38MAPK was found in $CD28^+$ cells after treatment with BELA. This effect was not noticed in $CD8^+CD28^-$ cells, indicating that BELA did not affect these cells. However, this effect was not observed at other time points, indicating that any difference between the effect of BELA on $CD28^+$ and on the more aggressive $CD28^-$ T cells could not be detected with phospho-specific flow cytometry. Notably and in contrast to the TAC-treated study population, expression of p-ERK was increased at day 360 in the presence of BELA, reflecting immune activation.

The absence of decreased signaling protein phosphorylation during BELA-based therapy also suggests that p-p38MAPK and p-Akt are mainly affected by TAC. However, the significant reductions that were found during TAC-based treatment were small and no correlations were found between TAC pre-dose concentrations and p-p38MAPK. However, there could be a correlation between phosphorylation expression and peak drug concentrations or area under the concentration-***versus*** time curve, but these were not measured in this study. No other patient baseline characteristic showed an association with the expression of p-p38MAPK, p-ERK or p-Akt, suggesting that the decrease in phosphorylation was not influenced by these parameters. Downstream of the MAPK pathway, p-p38MAPK will initiate the transcription of the IFNG gene, which in turn will lead to the production of IFN-γ by T cells [46,47]. Here, the expression of p-p38MAPK, measured by phospho-specific flow cytometry, significantly correlated with IFN-γ production in $CD8^+CD28^+$ T cells but only after transplantation when there is less variation in p-p38MAPK expression due to the presence of immunosuppressive drugs. Unfortunately, IFN-γ production was not measured at day 4 and 30 after transplantation, but the immunosuppressive drug concentrations were higher at day 90 compared to day 180 and 360 after transplantation suggesting that a TAC-based

immunosuppressive drug therapy is involved in the regulation of p38MAPK phosphorylation. Altogether, the correlation between p-p38MAPK and the production of IFN-γ suggests that measuring phosphorylation of p38MAPK could be an effective manner to monitor $CD8^+CD28^+$ T cell function after transplantation.

Eleven out of 20 BELA-treated patients in this study suffered from an acute rejection [34]. BELA-treated patients suffering from a BPAR showed an increased phosphorylation of ERK after stimulation with PMA/ionomycin in both $CD4^+$ and $CD8^+$ T cells, in contrast to patients without an acute rejection episode. This observation was made at day 4 in $CD4^+$ and at day 90 in $CD8^+$ cells before the rejection episode was detected. p-ERK could be inhibited in the presence of a TAC-based regimen, as was measured in unstimulated samples. In contrast, a BELA-based treatment even increased the expression of p-ERK at day 360 after stimulation of the samples. Previously, a weak correlation was also found between the expression of p-ERK and antibody-mediated rejection biopsies of heart transplant patients [48]. Moreover, graft survival could be prolonged by the use of an ERK1/2 inhibitor as was shown in a mouse model [49]. Altogether, these results indicate an important role for ERK in both $CD4^+$ and $CD8^+$ T cells before an acute rejection episode is diagnosed clinically. Unfortunately, it could not be verified in this study whether the expression of p-ERK during a rejection episode was also increased in the presence of a TAC-based treatment, since only two patients in this treatment group suffered from acute rejection.

This study has other limitations, such as the small sample size and the lack of correlations between the expression of p-p38MAPK, p-ERK and p-Akt and clinical outcomes. Larger prospective cohort studies should focus on the phosphorylation of ERK in $CD4^+$ and $CD8^+$ T cells after transplantation to prove that p-ERK is a reliable biomarker for rejection in the presence of either a BELA- or TAC-based therapy. Such a study should also measure the total expression of ERK to ensure that any effects are due to the phosphorylation inhibition and not to the changed expression of the total protein. Moreover, in order to use phospho-specific flow cytometry for daily clinical diagnostics, the technique should be validated by using area-under the concentration ***versus*** time-curve values instead of predose concentrations. These values could also give an explanation for the low inhibitory effects on signaling molecule phosphorylation at specific time points and the increase in p-ERK in BELA-treated patients suffering from a rejection.

In conclusion, phospho-specific flow cytometry is a promising technique to monitor the pharmacodynamic effects of a TAC, but not BELA, -based immunosuppressive therapy after transplantation. In contrast to TAC-based treatment, BELA-based immunosuppression does not inhibit key T cell activation pathways, despite the expression of CD28, which may contribute to the high rejection incidence observed among BELA-treated kidney transplant recipients.

References

1. Saint-Marcoux F, Woillard JB, Jurado C, Marquet P. Lessons from routine dose adjustment of tacrolimus in renal transplant patients based on global exposure. Ther Drug Monit 2013;35:322-7.
2. Matas AJ, Smith JM, Skeans MA, et al. OPTN/SRTR 2012 Annual Data Report: kidney. Am J Transplant 2014;14 Suppl 1:11-44.
3. Hon YY, Chamberlain CE, Kleiner DE, et al. Evaluation of tacrolimus abbreviated area-under-the-curve monitoring in renal transplant patients who are potentially at risk for adverse events. Clin Transplant 2010;24:557-63.
4. Wallemacq P, Armstrong VW, Brunet M, et al. Opportunities to optimize tacrolimus therapy in solid organ transplantation: report of the European consensus conference. Ther Drug Monit 2009;31:139-52.
5. Shuker N, van Gelder T, Hesselink DA. Intra-patient variability in tacrolimus exposure: causes, consequences for clinical management. Transplant Rev (Orlando) 2015;29:78-84.
6. Bouamar R, Shuker N, Hesselink DA, et al. Tacrolimus predose concentrations do not predict the risk of acute rejection after renal transplantation: a pooled analysis from three randomized-controlled clinical trials(dagger). Am J Transplant 2013;13:1253-61.
7. Whalen HR, Glen JA, Harkins V, et al. High Intrapatient Tacrolimus Variability Is Associated With Worse Outcomes in Renal Transplantation Using a Low-Dose Tacrolimus Immunosuppressive Regime. Transplantation 2017;101:430-6.
8. Halloran PF. Immunosuppressive drugs for kidney transplantation. N Engl J Med 2004;351:2715-29.
9. Nakayama T, Yamashita M. The TCR-mediated signaling pathways that control the direction of helper T cell differentiation. Semin Immunol 2010;22:303-9.
10. Cantrell DA. T-cell antigen receptor signal transduction. Immunology 2002;105:369-74.
11. Rincon M, Flavell RA, Davis RJ. Signal transduction by MAP kinases in T lymphocytes. Oncogene 2001;20:2490-7.
12. Smith-Garvin JE, Koretzky GA, Jordan MS. T cell activation. Annu Rev Immunol 2009;27:591-619.
13. Matsuda S, Moriguchi T, Koyasu S, Nishida E. T lymphocyte activation signals for interleukin-2 production involve activation of MKK6-p38 and MKK7-SAPK/JNK signaling pathways sensitive to cyclosporin A. J Biol Chem 1998;273:12378-82.
14. Zhang W, Liu HT. MAPK signal pathways in the regulation of cell proliferation in mammalian cells. Cell Res 2002;12:9-18.
15. Krutzik PO, Nolan GP. Intracellular phospho-protein staining techniques for flow cytometry: monitoring single cell signaling events. Cytometry A 2003;55:61-70.
16. Baan C, Bouvy A, Vafadari R, Weimar W. Phospho-specific flow cytometry for pharmacodynamic monitoring of immunosuppressive therapy in transplantation. Transplant Res 2012;1:20.

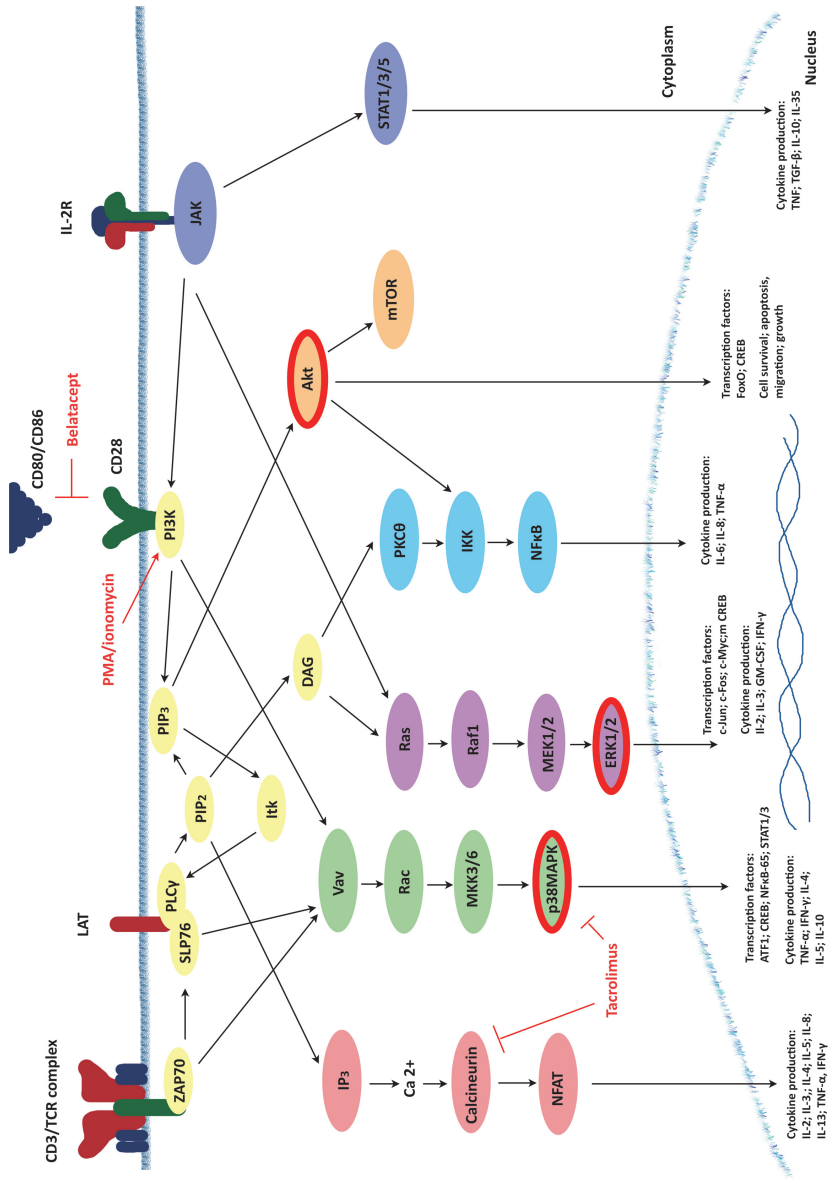
17. Bouvy AP, Klepper M, Kho MM, et al. T cells exhibit reduced signal transducer and activator of transcription 5 phosphorylation and upregulated coinhibitory molecule expression after kidney transplantation. Transplantation 2015;99:1995-2003.
18. Liu X, Wu X, Cao S, et al. B7-H1 antibodies lose antitumor activity due to activation of p38 MAPK that leads to apoptosis of tumor-reactive CD8+ T cells. Sci Rep 2016;6:36722.
19. Wang H, Zhang X, Zheng X, et al. Prevention of allograft rejection in heart transplantation through concurrent gene silencing of TLR and Kinase signaling pathways. Sci Rep 2016;6:33869.
20. Sommerer C, Meuer S, Zeier M, Giese T. Calcineurin inhibitors and NFAT-regulated gene expression. Clin Chim Acta 2012;413:1379-86.
21. Maguire O, Tornatore KM, O'Loughlin KL, Venuto RC, Minderman H. Nuclear translocation of nuclear factor of activated T cells (NFAT) as a quantitative pharmacodynamic parameter for tacrolimus. Cytometry A 2013;83:1096-104.
22. Giese T, Zeier M, Meuer S. Analysis of NFAT-regulated gene expression in vivo: a novel perspective for optimal individualized doses of calcineurin inhibitors. Nephrol Dial Transplant 2004;19 Suppl 4:iv55-60.
23. Sommerer C, Giese T, Meuer S, Zeier M. Pharmacodynamic monitoring of calcineurin inhibitor therapy: is there a clinical benefit? Nephrol Dial Transplant 2009;24:21-7.
24. Steinebrunner N, Sandig C, Sommerer C, et al. Pharmacodynamic monitoring of nuclear factor of activated T cell-regulated gene expression in liver allograft recipients on immunosuppressive therapy with calcineurin inhibitors in the course of time and correlation with acute rejection episodes--a prospective study. Ann Transplant 2014;19:32-40.
25. Keller F, Sommerer C, Giese T, Zeier M, Schroppel B. Correlation between pharmacokinetics of tacrolimus and pharmacodynamics on NFAT-regulated gene expression in stable kidney transplant recipients. Clin Nephrol 2017;87 (2017):93-9.
26. Albring A, Wendt L, Harz N, et al. Relationship between pharmacokinetics and pharmacodynamics of calcineurin inhibitors in renal transplant patients. Clin Transplant 2015;29:294-300.
27. Maguire O, Tario JD, Jr., Shanahan TC, Wallace PK, Minderman H. Flow cytometry and solid organ transplantation: a perfect match. Immunol Invest 2014;43:756-74.
28. Vafadari R, Hesselink DA, Cadogan MM, Weimar W, Baan CC. Inhibitory effect of tacrolimus on p38 mitogen-activated protein kinase signaling in kidney transplant recipients measured by whole-blood phosphospecific flow cytometry. Transplantation 2012;93:1245-51.
29. Olejarz W, Bryk D, Zapolska-Downar D, Malecki M, Stachurska A, Sitkiewicz D. Mycophenolic acid attenuates the tumour necrosis factor-alpha-mediated proinflammatory response in endothelial cells by blocking the MAPK/NF-kappaB and ROS pathways. Eur J Clin Invest 2014;44:54-64.
30. Wojciechowski D, Vincenti F. Belatacept in kidney transplantation. Curr Opin Organ Transplant 2012;17:640-7.
31. de Graav GN, Bergan S, Baan CC, Weimar W, van Gelder T, Hesselink DA. Therapeutic drug monitoring of belatacept in kidney transplantation. Ther Drug Monit 2015;37:560-7.

32. Rostaing L, Malvezzi P. Costimulation Blockade in Kidney Transplantation: Beyond Belatacept. Transplantation 2016;100:2516-8.
33. Vincenti F, Charpentier B, Vanrenterghem Y, et al. A phase III study of belatacept-based immunosuppression regimens versus cyclosporine in renal transplant recipients (BENEFIT study). Am J Transplant 2010;10:535-46.
34. de Graav G, Baan CC, Clahsen-van Groningen MC, et al. A randomized controlled clinical trial comparing belatacept with tacrolimus after de novo kidney transplantation. Transplantation 2017.
35. de Graav GN, Hesselink DA, Dieterich M, Kraaijeveld R, Weimar W, Baan CC. Down-regulation of surface CD28 under belatacept treatment: an escape mechanism for antigen-reactive T-cells. PLoS One 2016;11:e0148604.
36. Newell KA, Mehta AK, Larsen CP, et al. Lessons learned: Early termination of a randomized trial of calcineurin inhibitor and corticosteroid avoidance using Belatacept. Am J Transplant 2017.
37. de Graav GN, Hesselink DA, Dieterich M, et al. An acute cellular rejection with detrimental outcome occurring under belatacept-based immunosuppressive therapy: an immunological analysis. Transplantation 2016;100:1111-9.
38. Arosa FA. CD8+CD28- T cells: certainties and uncertainties of a prevalent human T-cell subset. Immunol Cell Biol 2002;80:1-13.
39. Vallejo AN. CD28 extinction in human T cells: altered functions and the program of T-cell senescence. Immunol Rev 2005;205:158-69.
40. Ashokkumar C, Ganguly B, Townsend R, et al. Alloreactive CD154-expressing T-cell subsets with differential sensitivity to the immunosuppressant, belatacept: potential targets of novel belatacept-based regimens. Sci Rep 2015;5:15218.
41. de Klerk M, Keizer KM, Claas FH, Witvliet M, Haase-Kromwijk BJ, Weimar W. The Dutch national living donor kidney exchange program. Am J Transplant 2005;5:2302-5.
42. Kannegieter NM, Hesselink DA, Dieterich M, et al. The effect of tacrolimus and mycophenolic acid on CD14+ monocyte activation and function. PLoS One 2017;12:e0170806.
43. Vafadari R, Weimar W, Baan CC. Phosphospecific flow cytometry for pharmacodynamic drug monitoring: analysis of the JAK-STAT signaling pathway. Clin Chim Acta 2012;413:1398-405.
44. Bagwell CB. Hyperlog-a flexible log-like transform for negative, zero, and positive valued data. Cytometry A 2005;64:34-42.
45. Landskron J, Tasken K. Phosphoprotein detection by high-throughput flow cytometry. Methods Mol Biol 2016;1355:275-90.
46. Wang X, Zhang F, Chen F, et al. MEKK3 regulates IFN-gamma production in T cells through the Rac1/2-dependent MAPK cascades. J Immunol 2011;186:5791-800.
47. Rincon M, Enslen H, Raingeaud J, et al. Interferon-gamma expression by Th1 effector T cells mediated by the p38 MAP kinase signaling pathway. Embo J 1998;17:2817-29.

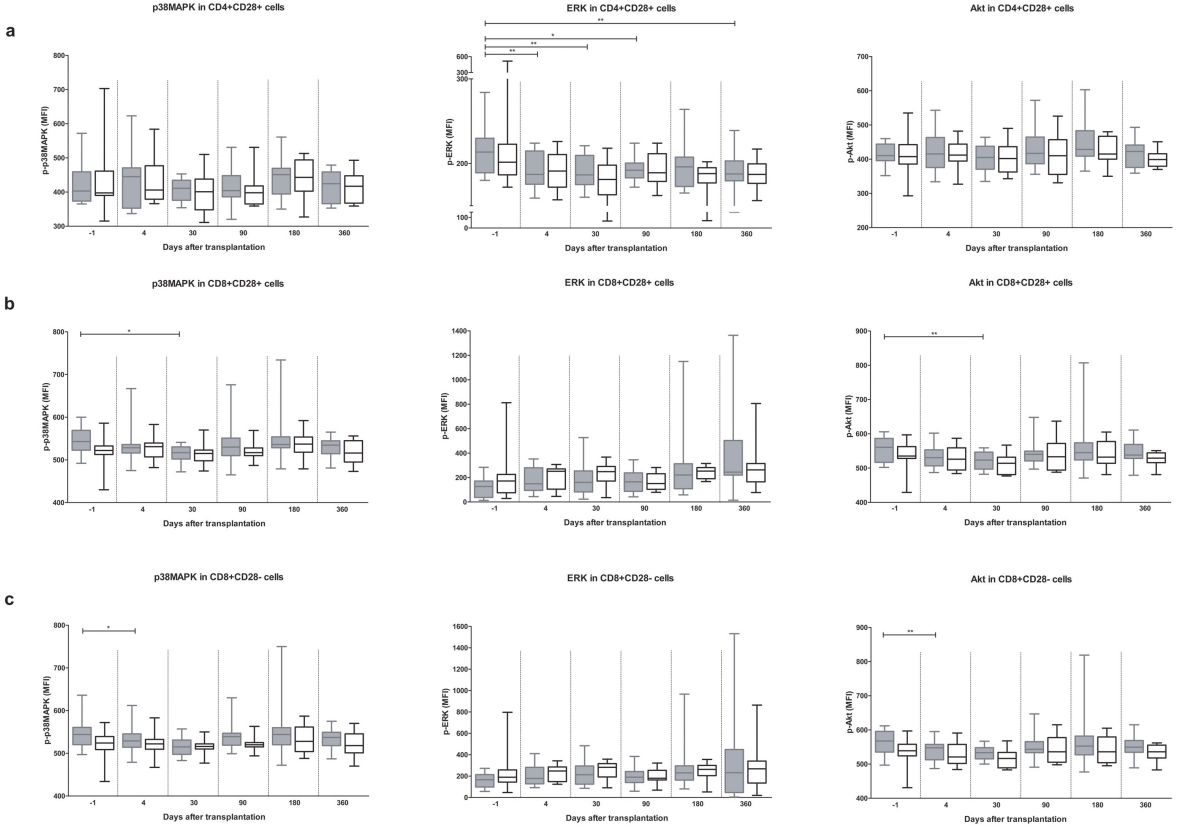
48. Li F, Wei J, Valenzuela NM, et al. Phosphorylated S6 kinase and S6 ribosomal protein are diagnostic markers of antibody-mediated rejection in heart allografts. J Heart Lung Transplant 2015;34:580-7.

49. Wang S, Guan Q, Diao H, et al. Prolongation of cardiac allograft survival by inhibition of ERK1/2 signaling in a mouse model. Transplantation 2007;83:323-32.

Supplementary Figures and Tables



Supplementary Figure 1. Schematic overview of T cell signaling pathways and their interaction downstream of the TCR. After antigen recognition by the TCR and after receiving a co-stimulatory signal via CD28, several intracellular signaling pathways become activated via phosphorylation. These pathways include the signaling molecules p38MAPK, ERK1/2 and Akt. Downstream of the signaling pathways, this activation will initiate gene-transcription and the control of T cell functions, such as cytokine production, cell survival, cell differentiation and cell apoptosis. Activation of the signaling pathways can be mimicked by the stimulation of the T cell with PMA/ionomycin. TAC is known to inhibit the calcineurin and p38MAPK pathway, while BELA blocks the co-stimulatory signal between the CD80/86 molecule on APC's and the CD28 molecule on T cells.



Supplementary Figure 2. **P-p38MAPK, p-ERK and p-Akt in unstimulated T cell subsets of TAC (*grey*)- and BELA (*white*)-treated patients before and after transplantation.** A) p-p38MAPK (left), p-ERK (middle) and p-Akt (right) within $CD4^+CD28^+$ T cells. P-ERK was significantly decreased in TAC-treated patients, but not in BELA-treated patients.B) p-p38MAPK (left), p-ERK (middle) and p-Akt (right) within $CD8^+CD28^+$ T cells. P-p38MAPK and p-AKT were only inhibited at day 30 after transplantation when TAC was given.C) p-p38MAPK (left), p-ERK (middle) and p-Akt (right) within $CD8^+CD28^-$ T cells. Phosphorylation inhibition is comparable with $CD8^+CD28^+$ T cells. (Data are plotted as box and whiskers indicating total range; n=20 TAC-treated patients and n=20 BELA-treated patients) *) $p < 0.05$, **) $p < 0.01$, ***) $p < 0.001$

Supplementary Table I: Baseline characteristics at time of transplantation

	Belatacept group (n = 20)	Tacrolimus group (n = 20)	p
Age (years)	57 (25-76)	55 (21-76)	0.88
Male / female	14 (70%) / 6 (30%)	16 (80%) / 4 (20%)	0.72
Ethnicity			1.00
• Caucasian	17 (85%)	16 (80%)	
• African	2 (10%)	2 (10%)	
• Asian	1 (5%)	2 (10%)	
Body weight (kg)	79.0 (56.6-111.4)	93.6 (51.4-120.0)	0.06
HLA A mismatch (mean ± SD)	1.1 (± 0.7)	1.4 (± 0.5)	0.13
HLA B mismatch (mean ± SD)	1.3 (± 0.5)	1.5 (± 0.5)	0.51
HLA DR mismatch (mean ± SD)	1.1 (± 0.4)	1.3 (± 0.4)	0.70
Current PRA (%)	0 (0-5)	0 (0-17)	0.30
Peak PRA (%)	4 (0-6)	4 (0-21)	0.78
CMV status			0.80
• Donor + / Recipient -	3 (15%)	2 (10%)	
• Donor + / Recipient +	4 (20%)	7 (35%)	
• Donor - / Recipient -	7 (35%)	6 (30%)	
• Donor - / Recipient +	6 (30%)	5 (25%)	
Donor age (years)	59 (24-71)	51 (22-80)	0.18
Related / unrelated donor	6 (30%) / 14 (70%)	5 (25%) / 15 (75%)	1.00
Cause of end-stage renal disease			0.09
• Diabetes mellitus	3 (15%)	7 (35%)	
• Hypertension	2 (10%)	5 (25%)	
• IgA nephropathy	1 (5%)	3 (15%)	
• Polycystic kidney disease	3 (15%)	3 (15%)	
• Obstructive nephropathy	3 (15%)	1 (5%)	
• Unknown	5 (25%)	0 (0%)	
• Other	3 (15%)	1 (5%)	
Renal replacement therapy			0.91
• None (pre-emptive)	10 (50%)	12 (60%)	
• Hemodialysis	7 (35%)	6 (30%)	
• Peritoneal dialysis	3 (15%)	2 (10%)	
Time on dialysis (days)	425 (123-2782)	605 (465-1519)	0.41
Number of kidney transplantation			1.00
• First	19 (95%)	20 (100%)	1.00
• Second	1 (5%)	-	

Continuous variables are presented as medians (plus ranges) and categorical variables as numbers (plus percentages), unless otherwise specified

BPAR, biopsy-proven acute rejection; CMV, cytomegalovirus; HLA, human leukocyte antigen; PRA, panel reactive antibodies (current = PRA at time of transplantation, peak = historically highest measured PRA); SD, standard deviation.

Supplementary Table II: Incidence of rejection according to the treatment group

	Belatacept group (n = 20)	Tacrolimus group (n = 20)	p
Borderline	0 (0%)	0 (0%)	-
Type 1			1.00
• 1A	0 (0%)	0 (0%)	
• 1B	1 (5%)	1 (5%)	
Type 2			0.004
• 2A	2 (10%)	1 (5%)	
• 2B	6 (30%)	0 (0%)	
Type 3	1 (5%)	0 (0%)	1.00
Mixed	1 (5%)	0 (0%)	1.00
Total BPAR	11 (55%)	2 (10%)	0.006

The incidence of the first rejection episodes is given. The highest Banff score is depicted if sequential biopsies were performed.
BPAR, Biopsy-proven acute rejection

Supplementary Table III: Immunosuppressive drugs dose and pre–dose concentrations

	TAC-treated patients					*BELA-treated patients*			
	TAC dose (mg)	**TAC C_0** (ng/ml)	**MMF dose** (mg)	**MPA C_0** (µg/ml)	**PRED dose** (mg)	**BELA dose** (mg)	**MMF dose** (mg)	**MPA C_0** (µg/ml)	**PRED dose** (mg)
Day 4	16.7 (± 2.5)	16.8 (± 6.2)	2000 (± 0)	3.35 (± 1.59)	20.0 (± 0)	792 (± 153)	2000 (± 0)	3.85 (± 1.55)	20.0 (± 0)
Day 30	8.9 (± 4.6)	9.8 (± 3.3)	1750 (± 546)	2.69 (± 1.57)	12.5 (± 3.3)	767 (± 106)	1682 (± 603)	3.39 (± 1.74)	10.5 (± 2.7)
Day 90	4.7 (± 1.4)	6.8 (± 1.7)	1342 (± 473)	2.83 (± 1.62)	5.4 (± 1.3)	774 (± 130)	1364 (± 393)	3.27 (± 1.57)	5.0 (± 0)
Day 180	4.3 (± 1.1)	6.3 (± 1.5)	1181 (± 499)	2.17 (± 0.98)	4.7 (± 1.2)	383 (± 63)	1222 (± 441)	2.15 (± 1.34)	5.0 (± 0)
Day 360	4.2 (± 1.3)	7.0 (± 2.3)	1103 (± 493)	2.02 (± 0.92)	4.3 (± 1.5)	382 (± 58)	1139 (± 377)	1.99 (± 0.63)	5.0 (± 0)

mean (±SD)

Supplementary Table IV. Univariate regression analysis between patient demographic characteristics and signaling protein phosphorylation

		p-p38MAPK		p-ERK		p-Akt	
	Predictor	*β*	*p value*	*β*	*p value*	*β*	*p value*
Before transplantation	Age (years)	0.381	*0.045*	-0.175	*0.374*	0.126	*0.523*
	Gender (male vs female)	-0.267	*0.169*	0.267	*0.169*	-0.273	*0.160*
	Ethnicity (non caucasian vs caucasian)	-0.370	*0.053*	-0.016	*0.934*	-0.324	*0.092*
	Bodyweight before transplantation (kg)	0.222	*0.257*	0.321	*0.096*	0.210	*0.284*
	Highest PRA (%)	0.068	*0.730*	0.049	*0.805*	0.069	*0.727*
	HLA total mismatches (4 or more vs 3 or less)	-0.046	*0.815*	0.102	*0.607*	-0.075	*0.704*
	HLA DR mismatches (2 vs 1)	-0.228	*0.243*	0.197	*0.315*	0.147	*0.457*
	CMV (negative vs positive)	0.234	*0.230*	-0.363	*0.058*	0.142	*0.470*
	Renal replacement therapy (no vs yes)	-0.067	*0.735*	-0.023	*0.906*	-0.099	*0.616*
	CD86 molecules/monocyte pre-transplantation	-0.065	*0.741*	-0.038	*0.847*	-0.101	*0.607*
	Donor age (years)	0.217	*0.267*	-0.100	*0.613*	0.044	*0.823*
	Donor gender (male vs female)	-0.032	*0.873*	-0.072	*0.715*	-0.029	*0.884*
Day 4	Age (years)	0.423	*0.031*	0.049	*0.806*	-0.252	*0.205*
	Gender (m vs f)	-0.346	*0.084*	0.021	*0.915*	-0.033	*0.872*
	Ethnicity (non caucasian vs caucasian)	-0.285	*0.159*	-0.006	*0.975*	-0.152	*0.448*
	treatment group (tacrolimus vs belatacept	0.118	*0.566*	-0.134	*0.505*	0.242	*0.225*
	Bodyweight before transplantation (kg)	-0.026	*0.900*	0.253	*0.203*	0.087	*0.667*
	Highest PRA (%)	0.029	*0.890*	-0.203	*0.310*	0.231	*0.246*
	HLA total mismatches (4 or more vs 3 or less)	-0.084	*0.689*	-0.261	*0.188*	-0.247	*0.214*
	HLA DR mismatches (2 vs 1)	-0.349	*0.087*	-0.054	*0.789*	-0.430	*0.025*
	CMV (negative vs positive)	0.270	*0.191*	0.112	*0.577*	0.054	*0.788*
	Renal replacement therapy (no vs yes)	0.087	*0.681*	-0.081	*0.686*	0.057	*0.779*
	CD86 molecules/monocyte pre-transplantation	-0.061	*0.771*	-0.027	*0.895*	-0.144	*0.473*
	Donor age (years)	0.442	*0.027*	-0.239	*0.230*	0.133	*0.510*
	Donor gender (male vs female)	-0.047	*0.823*	-0.092	*0.649*	0.112	*0.577*
Day 360	Age (years)	0.388	*0.055*	0.213	*0.307*	0.049	*0.816*
	Gender (m vs f)	-0.213	*0.307*	-0.090	*0.669*	-0.115	*0.586*
	Ethnicity (non caucasian vs caucasian)	-0.007	*0.974*	-0.211	*0.312*	-0.054	*0.797*
	treatment group (tacrolimus vs belatacept	0.180	*0.389*	-0.099	*0.639*	-0.092	*0.662*
	Bodyweight before transplantation (kg)	-0.162	*0.438*	0.161	*0.442*	-0.045	*0.829*
	Highest PRA (%)	-0.131	*0.533*	-0.157	*0.453*	-0.148	*0.481*
	HLA total mismatches (4 or more vs 3 or less)	-0.148	*0.481*	-0.136	*0.518*	-0.281	*0.173*
	HLA DR mismatches (2 vs 1)	0.020	*0.923*	-0.106	*0.613*	0.154	*0.462*
	CMV (negative vs positive)	0.285	*0.167*	0.316	*0.124*	0.346	*0.090*
	Renal replacement therapy (no vs yes)	0.080	*0.705*	0.198	*0.344*	-0.046	*0.828*
	CD86 molecules/monocyte pre-transplantation	0.279	*0.176*	0.171	*0.414*	0.303	*0.141*
	Donor age (years)	0.424	*0.035*	0.369	*0.070*	0.155	*0.458*
	Donor gender (male vs female)	-0.330	*0.107*	0.060	*0.774*	-0.275	*0.184*

8

Targeting JAK/STAT Signaling to Prevent Rejection After Kidney Transplantation: A Reappraisal

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Abstract

The profound involvement of cytokines in allograft rejection makes the molecules that control their actions, members of the Jak-Stat pathway, ideal targets for pharmacological intervention. Numerous studies have demonstrated that Jak3 is widely involved in the activation cascade and function of most immune cells. Tofacitinib, an oral Janus kinase inhibitor that targets Jak1/Jak3 dependent Stat activation, has been assessed as a substitute for calcineurin inhibitor therapy after low-to-moderate risk kidney transplantation in 3 randomized trials. Results using fixed-dose regimens showed a low incidence of rejection and better renal function with less interstitial fibrosis/tubular atrophy versus calcineurin inhibitor therapy. However, the safety profile of tofacitinib was poor, including increased incidences of cytomegalovirus disease, herpes zoster, BK virus, and nephropathy, which led to the discontinuation of its development for transplantation. High tofacitinib concentrations were independently associated with serious infection. Dosing according to exposure levels, coupled with pharmacodynamic monitoring based on phosphorylation of Stat5, could improve safety compared to the early fixed-dose regimens. Future studies could assess individualized dosing based on pharmacokinetic and pharmacodynamic monitoring. Additionally, because the increase of viral infections under tofacitinib may have been influenced by overlapping toxicity with concomitant mycophenolic acid, exploration of alternative adjunctive therapies (***e.g.***, a mammalian target of rapamycin inhibitor or belatacept) may demonstrate a better efficacy/safety profile. We believe that Jak inhibitors are a good and useful addition to the immunosuppressive armentarium for kidney transplant patients, and that new studies with personalized drug dosing, improved immune monitoring, and better patient selection should be performed.

Introduction

Drugs that target protein kinases have become popular. The benefit of treatment with this class of therapeutics is clear: a high level of selectivity. The Food and Drug Administration has now approved several agents that inhibit cellular signaling of pathways activated by receptors of the platelet-derived growth factor, epidermal growth factor, vascular endothelial growth factor, hepatocyte growth factor pathway, and cytokines of the IL-2 family [1-5]. These agents are given for the treatment of a wide range of diseases, that is, cancer, macular degeneration, auto immune diseases and for the prevention of allograft rejection after organ transplantation.

Allograft rejection is characterized by the production of a wide variety of cytokines including members of the IL-2, IL-12, IL-27 families, interferons, and growth factors. These soluble factors exert their biological functions through janus tyrosine kinases (Jaks) and signal transduction and activators of transcription (Stats), which enable direct communication from the transmembrane cytokine receptor to the nucleus to interact with regulatory elements for gene expression (**Figure 1**) [6]. In human cells, 4 different cytoplasmic tyrosine kinases have been identified: Jak1, Jak2, Jak3 and Tyk2, and 7 Stat proteins: Stat1, Stat2, Stat3, Stat4, Stat5a, Stat5b, and Stat6 [7]. The profound involvement of cytokines in allograft rejection makes the molecules that control their actions, members of the Jak-Stat pathway, ideal targets for pharmacological intervention. *In vitro* studies using human cells and cell lines have been performed with the small drug molecule named tofacitinib, an oral Janus kinase inhibitor (formerly known as tasocitinib and CP-690,550) [3]. These have demonstrated that this agent targets Jak1/Jak3-dependent Stat activation and can be used as a substitute for toxic calcineurin inhibitors (CNIs) in a murine model of heart transplantation and in cynomolgus monkeys receiving kidney transplants [3,8-10].

For organ transplant patients in whom alloreactivity must be controlled by immunosuppressive medication, blockade of Jak3 signaling has a huge therapeutic potential as it inhibits allogeneic T, B, and natural killer (NK) cell–mediated antidonor responses in a nonredundant way. In this overview article, we will discuss the outcomes of clinical trials of tofacitinib in kidney transplantation, and put these data in a broader context to understand why these studies were relatively unsuccessful and had to be discontinued. In addition, we will discuss what we have learned from our mistakes, what immune monitoring has shown, and how a new trial in kidney transplantation could be planned because we believe this class of immunosuppressive drugs deserves a second chance using an improved trial design guided by immune monitoring.

JAK3 Signaling

Numerous studies have investigated the role of Jak3 in immune cell development and function, demonstrating that it is widely involved in the activation cascade and function of most immune cells [11]. These studies demonstrate that the biology of Jak3 is not restricted to T, B, and NK cells and that Jak3 plays a functional role in the activation of

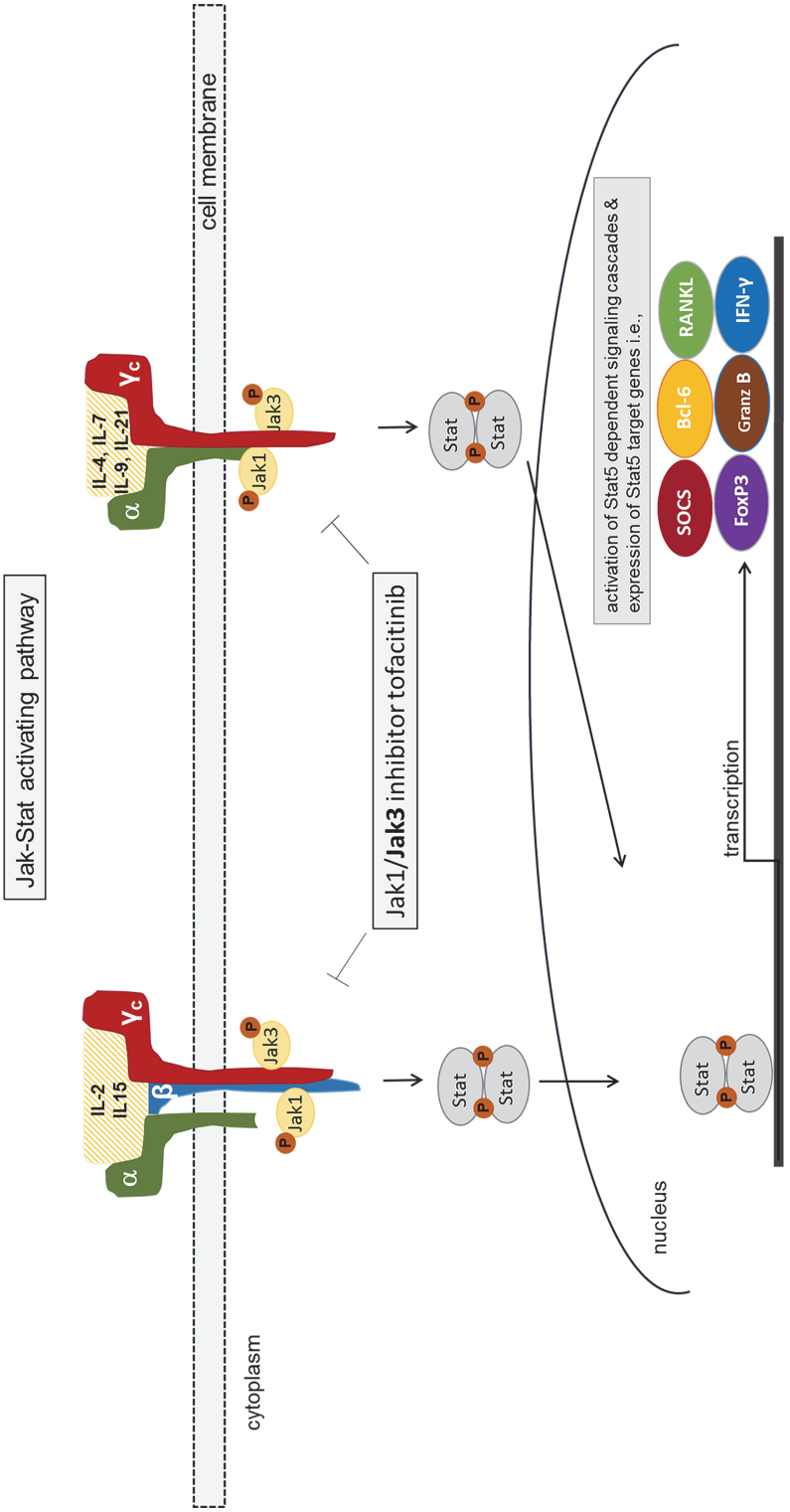


Figure 1. Cytokine receptor Jak-Stat activation pathway. The IL-2 receptor consists of three chains: IL-2Rα, IL-2Rß, and γc. Binding of a ligand activates the receptor Janus-activated kinase 3 (Jak3), which associates with the γc chain, while JAK1 associates with IL-2Rβ. This in turn creates docking sites for Stat molecules that are then phosphorylated by Jak. Activated STATs translocate to the nucleus to interact with regulatory elements for gene expression, for example, nuclear factor kappa-B ligand, IFN-γ, granzyme B, FasL, suppressor of cytokine signaling, and FoxP3.

all hematopoietic cells including cells of the myeloid lineage. Jak3 is expressed at low levels in resting monocytes and can be induced by lipopolysaccharide and in response to [gamma]$_c$ cytokines [12] and during granulocytic differentiation Jak3 is phosphorylated as it is a response gene for granulocyte colony-stimulating factor [13,14]. A mouse study by Ghoreschi ***et al.*** [15] reported that blocking Jak 3 phosphorylation also modulates the innate immune responses. The IL-2/IL-2receptor-triggered Jak1/Jak3/Stat5 signaling pattern in both conventional and regulatory T cells has been characterized [16,17] and involvement of the Jak1/Jak3/Stat5 signaling pathway in B cell development has also been firmly established [18,19]. Binding of IL-7 to its receptor activates this signaling pathway, which interacts with the regulatory elements of genes essential for B-cell development. Typical examples of the key role of Jak3 function in B cells are the reports showing that mutations in the Jak3 gene result in abnormal B-cell numbers and B-cell function [18,19]. Recently, the role of Jak3 was further acknowledged by the work of Cattaneo ***et al.*** [19] who demonstrated abolished B-cell differentiation to plasmablasts in response to the CD40 ligand and IL-21.

Experience With Jak3 Inhibitors in Kidney Transplant Patients

Tofacitinib is licensed by the Food and Drug Administration as a disease-modifying treatment for rheumatoid arthritis, and has been assessed in a placebo-controlled randomized trial for the management of moderate-to-severe psoriasis [20]. Clinical trials are also exploring the use of tofacitinib in psoriatic arthritis [21], inflammatory bowel disease (Crohn disease and ulcerative colitis) [22], ankylosing spondylitis [23]. In kidney transplantation, 3 randomized trials have been undertaken in progressively larger populations, between 2005 and 2012.The first study was performed in 28 stable kidney transplant recipients, evaluating the pharmacokinetics, pharmacodynamics, safety and tolerability of 3 dose levels of tofacitinib (5, 15 and 30 mg, twice a day [BID]) [3]. It was a double-blind study comprising 28 days of treatment with a subsequent 28-day follow up period. Patients between 1.1 and 11.7 years after transplantation were included but there was no mention of baseline renal function or any exclusion criteria. Patients were randomized (3:1) to tofacitinib or placebo in 4 sequential dose escalation cohorts. Compared with first dose, measures of drug exposure (Cmax, area under the curve [AUC]$_{0–12}$, C_{12}) remained at steady state after 28 days for the 5- and 15-mg doses but not for 30-mg BID. Higher mean dose-normalized $AUC_{0–12}$values were observed after 5 mg BID (15%) and 15 mg BID (54%), groups in that all but 1 patient was receiving CNI therapy, compared with the 30 mg BID group in that all patients were CNI-free. The lack of dose linearity suggests that coadministration with CNIs may moderately increase tofacitinib exposure. Importantly, although good correlations were observed between early time points (1, 2, and 4 hours) after drug administration and $AUC_{0–12}$, the correlation with trough concentrations was poor, limiting its utility for therapeutic drug monitoring.

There were no deaths, malignancies, systemic opportunistic infections or acute rejection episodes during the study. No consistent changes were observed in vital signs, including

electrocardiogram evaluations. Infections and gastrointestinal disorders were the most frequent adverse events reported. A dose-dependent reversible reduction in hemoglobin and reticulocyte counts was observed primarily after the administration of 15 mg BID and 30 mg BID. No significant changes in CD3, CD4, and CD8 lymphocytes were observed, but a mean 50% decrease in absolute NK cell counts (CD16 and CD56) were observed after the administration of 15 or 30 mg BID. A mean 130% increase in absolute CD19+ B lymphocytes was observed after the administration of 30 mg BID [3].

Subsequently, a randomized 2-stage 6-month study comparing the efficacy of tofacitinib with tacrolimus in de novo kidney allograft recipients receiving IL-2 receptor antagonist induction, mycophenolate mofetil and corticosteroids was undertaken [24]. In stage 1 (pilot trial), 61 patients were randomized to receive tofacitinib 15 mg BID (n = 20) or 30 mg BID (n = 20), or tacrolimus (n = 21). Patients completing 6 months of treatment were enrolled in an extension study to month 12 and doses of tofacitinib were reduced to 10 mg BID and 15 mg BID, respectively. The numbers of biopsy proven acute rejection (BPAR) at 6 months were 1, 4 and 1, respectively with no further episodes by month 12. At 12 months' mean estimated glomerular filtration rates (GFRs) were 83.6, 77.6 and 73.3 mL/min, respectively. Patients receiving tofacitinib presented higher incidences of serious adverse events and clinically significant infections including cytomegalovirus (CMV) disease, herpes zoster, and BK viremia and nephropathy (**Table I**). Due to a high incidence of BK virus nephropathy only in patients receiving tofacitinib 30 mg BID, mycophenolate mofetil was discontinued in this group. In addition, patients receiving tofacitinib showed modest lipid elevations and a trend toward more frequent anemia and neutropenia during the first 6 months. Again, NK cells were reduced by 77% or less in tofacitinib-treated patients. Altogether these data indicated that tofacitinib at 15 mg BID showed an efficacy/safety profile that was comparable to the tacrolimus control group, except for a higher rate of viral infection. Stage 2, anticipating enrollment of an additional 195 patients, was not implemented due to signs of overimmunosuppression in the 30-mg BID dose group [24].

Based on the results of this pilot study, a phase 2b prospective study was conducted, enrolling 331 low-to-moderate risk de novo kidney transplant patients [25]. Patients were randomized to receive tofacitinib 15 mg BID from months 1 to 6, reduced to 10 mg BID (more intensive [MI] regimen, n = 110), or 15 mg BID from months 1 to 3, reduced to 10 mg BID (less intensive (LI) regimen, n = 111) or cyclosporine (CsA) (n = 110). All patients received basiliximab induction, mycophenolic acid (MPA) and corticosteroids (**Table I**). There were no differences in the incidences of first clinical BPAR at 6 or 12 months (**Table I**). African American patients receiving tofacitinib showed a higher incidence of acute rejection compared to CsA (30.2%, 29.5%, and 8.3%). Patients receiving the LI regimen showed only vascular rejection (>IIA) and 2 of these patients eventually lost their graft. More patients in the CsA group were diagnosed with antibody mediated rejection (1.9%, 0.9%, and 4.6%).

At month 12, iohexol-measured GFRs were higher for MI and LI versus CsA and fewer patients in the MI or LI groups developed chronic allograft lesions (**Table I**). Drug discon-

Table I. Dosing regimens, efficacy and safety parameters observed in 2 tofacitinib clinical trials[24,25]

Groups	n	Dosing regimen	Discon-tinuations	BCAR	Severity of BCAR	GFR, mL/min	CAN	Neutrophil <1000/mm^3 (months 1-6)	Hemoglobulin <10 g/dL (months 1-6)	SAE	CMV[a]	HZV	BKV	BKN	Malig-nancy	Graft loss	Death
Busque et al[24]																	
Tofacitinib 15 mg BID	20	15 mg BID (months 1-6) 10 mg BID (months 7-12)	3	1	IIA	83.6[b]		1	4	50%	3[c]	4	2	0			
Tofacitinib 30 mg BID	20	30 mg BID (months 1-6) 15 mg BID (months 7-12)	8	4	IA IIA(2) IIB	77.6[b]		2	3	50%	4[d]	0	0	4[e]	2[f]	1[g]	1[g]
Tacrolimus	21	7-14 ng/mL (months 1-3) 5-12 ng/mL (months 4-12)	2	2	IB IIA	73.3[b]		1	2	38%	1	1	3	0			
Vincenti et al[25]																	
Tofacitinib MI	106	15 mg BID (months 1-6) 10 mg BID (months 7-12)	46	11[h]18[i]	IA(2) IB(3) IIA(7) IIB(3) III(1) AMR(2)	64.6[j]	25	19	68	NR	16		15	2	6 (4 PTLD)	2	3
Tofacitinib less intensive (LI)	107	15 mg BID (months 1-3) 10 mg BID (months 4-12)	48	7[h]14[i]	IIA(11) IIB(2) AMR(1)	64.7[j]	23.9	4	68	NR	11		19	3	1 (1 PTLD)	5	2
CsA	109	125-400 ng/mL (months 1-3) 100-300 ng/mL (months 4-12)	31	9[h]23[i]	IA(1) IB(4) IIA(8) IIB(5) AMR (5)	53.9[j]	48.3	4	55	NR	4		6	1	1	1	3

[a] CMV prohylaxis was required for at least 3 months in D+R- patients. [b] LSM. [c] 1 CMV D+R-. [d] 1 tissue invasive and 2 CMV DR+R-. [e] 2 coinfections with CMV. MMF was discontinued after the last BKV case. [f] Basal cell carcinoma and mixed carcinoma of the appendix, both surgically excised. [g] Graft loss due to refractory IIB rejection and death 8 months after returning to dialysis due to hyperkalemia associated arrhythmia. [h] First clinical BCAR. [i] Total BCAR. [j] GFR was measured by iohexol serum clearance. BCAR, biopsy-confirmed acute rejection; CAN, chronic allograft nephropathy; SAE, serious adverse event; HZV, herpes zoster infection; BKV, polyomavirus viremia; BKN, polyomavirus nephropathy; AMR, antibody-mediated rejection.

tinuations, serious infections, CMV disease, anemia, neutropenia, and posttransplant lymphoproliferative disease (PTLD) occurred more frequently in the MI and LI groups compared with CsA. A lower frequency of posttransplant diabetes mellitus or impaired glucose fasting glucose was observed in patients receiving tofacitinib compared to CsA (24.2%, 17.8%, and 38%) [25]. It was also noted that in patients receiving tofacitinib, MPA exposure was 37.4% higher compared with patients receiving CsA [25].

When patients were analyzed according to tofacitinib plasma concentrations, those below the median level of the whole group showed a similar incidence of serious infection or CMV disease compared to CsA. Univariate analysis showed that tofacitinib exposure, as measured by C_0 and C_2, donor and recipient age, and use of CMV prophylaxis were associated with occurrence of serious infection [26]. Using multiple regression analysis, only tofacitinib C_2 concentrations were independently associated with serious infection (C_0 was not entered into the multiple regression analysis). Interestingly, MPA C_0 concentrations were not associated with serious infections. Serious infections occurred more frequently in patients with tofacitinib exposure above median (AME, 53.0%) than below median exposure (BME, 28.4%) or in those given cyclosporine (25.5%). PTLD only occurred in the AME subgroup. In terms of efficacy, no differences were observed in the incidence of first BPAR at 6 and 12 months comparing BME and CsA groups, but patients in the AME group tended to show lower rates compared to the BME and CsA groups. Among black patients, first BPAR was observed more frequently in the BME group compared to the AME and CsA groups (0%, 30.6%, and 8.3%), respectively. Measured GFR was higher in both the AME and BME groups versus CsA (61.2 and 67.9 vs 53.9 mL/min) at month 12. Fewer patients developed interstitial fibrosis and tubular atrophy at month 12 in the AME (20.5%) and BME (27.8%) groups versus CsA (48.3%). These data suggest that monitoring of plasma concentrations of tofacitinib may preserve the overall benefits including low rates of acute rejection, improved renal function, and a lower incidence of interstitial fibrosis and tubular atrophy with similar rates of serious infection and no PTLD [26].

Pharmacokinetics, Pharmacodynamics, and Drug–Drug Interactions

In healthy individuals, pharmacokinetic characterization revealed that about 74% of the oral dose of tofacitinib is rapidly absorbed reaching peak plasma concentrations after 0.5 to 1 hour [27]. After intravenous administration, tofacitinib shows moderate tissue distribution, with a volume of distribution of 87 L. In plasma, about 70% is parent drug, and the protein binding is about 40%. Clearance mechanisms appear to be 70% through hepatic metabolism and 30% renal excretion of the parent drug. The metabolism of tofacitinib is mediated by CYP3A4 with a minor contribution from CYP2C19. Thus, inhibitors and inducers of CYP3A4 are likely to alter the disposition of tofacitinib. Steady-state pharmacokinetics in healthy subjects are predictable from single-dose data, with no evidence of accumulation. The elimination half-life (t½) is about 3 hours [28].

Due to inter-individual variability, it is difficult to determine the optimal therapeutic regimen for each patient. Individual variations in drug sensitivity can be determined by pharmacodynamic monitoring, which focuses on measuring the biological effects of a drug. For tofacitinib monitoring, the phosphorylation of Stat5 can be used as a measure guiding dosing [29]. IL-2–induced pStat5, the key substrate of Jak3, was reduced in the presence of serum collected from 8 patients receiving 30 mg BID of tofacitinib for 29 days, an effect observed in both CD4 and CD8 T cells. This effect was associated with the plasma concentration of tofacitinib and with reduced expression of several Stat5 target genes, including FoxP3, granzyme B, FAS ligand, and IFN-[gamma]. IL-2–induced pStat5 predominated in regulatory CD4 T cells (71%) compared with effector T cells (42%). ***Ex vivo*** addition of tofacitinib blocked the IL-2 induced pStat5, while the pStat5 of regulatory T cells was barely inhibited. In addition, tofacitinib preserved the suppressive activity of these patient-derived regulatory T cells [17].

It is possible that drug-drug interactions between tofacitinib and MPA may have contributed to the relatively high incidence of infections and malignancies observed in clinical studies. Therefore, the pharmacokinetics of MPA were evaluated in patients receiving tofacitinib or tacrolimus. Plasma MPA concentrations were obtained from 17 adult patients who received either 15 mg or 30 mg tofacitinib BID (8 patients) or tacrolimus (9 patients) after kidney transplantation. All patients also received concomitant mycophenolate mofetil, prednisone, and basiliximab induction. The median mycophenolate mofetil dose was 1000 mg BID. Based on individual estimates, oral clearance from the population pharmacokinetic model, mean steady-state area under the concentration-time curve values for a mycophenolate mofetil dose of 1000 mg BID were 63 mg/h per liter (22%) and 59 mg/h per liter (36%) for the tofacitinib and tacrolimus groups, respectively, that is, tofacitinib does not influence systemic MPA exposure [30].

It is well recognized that pharmacokinetic and pharmacodynamic characteristics are influenced by other drugs, such as antibiotics and cardiovascular drugs, factors related to the underlying disease, and age of the recipient. These factors, combined with a highly complex immune system that is specific to each patient, makes it extremely difficult to predict the efficacy and safety of immunosuppressive drugs, such as tofacitinib. Ideally, the effects of Jak3 blockade should be studied on graft-infiltrating cells attacking the transplant by single-cell analysis. Apart from the limited availability of tissue, this is technically challenging and currently cannot be used for diagnostic purposes. The ultimate goal would be to have a set of pharmacodynamic and immunological parameters to establish an immunological and safety risk profile. This would be a valuable pharmacodynamic tool kit for clinicians to help with transplantation-related risk assessment and tailoring of immunosuppressive medication, for example, in vulnerable groups of patients such as the frail and older adults because aging and immune senescence have an impact on treatment and outcomes [31].

JAK 3 Inhibitors

Initial studies suggested that tofacitinib was a selective inhibitor of JAK3 kinase [8]. Subsequent studies showed that tofacitinib also inhibits JAK1, JAK2 and Tyk2 kinases at nanomolar concentrations [32]. This finding may account for the occurrence of adverse events observed in clinical studies beyond those which would be anticipated based on selective inhibition of [gamma]$_c$/Jak3-dependent cytokines. Selective JAK3 inhibitors could improve the safety profile of tofacitinib. Several compounds were tested but none showed sufficient selectivity in ***in vitro*** kinase and cellular assays [33]. Nevertheless, a series of elegant studies has indicated that Jak1 has a dominant role over Jak3 in [gamma]$_c$-dependent signal transduction. This suggests that selective pharmacological inhibition of the catalytic activity of Jak3 is not enough to achieve efficient immunosuppression, as demonstrated in severe combined immunodeficiency patients with Jak3 mutations [41]. These observations show that Jak1 activation by the IL-2 family of cytokines, or other Jak1-dependent cytokines like IL-6, can compensate for the impaired Jak3 responsiveness that results in the activation of immune competent cells. Cytokines also have the ability to activate multiple signaling pathways which contribute to cellular functions. These "backup" elements of the immune system make it impossible for immunosuppressive drugs to provide complete suppression of immune competent cells.

Future Investigation in Kidney Transplantation

Studies conducted thus far in kidney transplant recipients and in patients with autoimmune diseases have confirmed that Jak3 inhibition is a good target for immunosuppression and that tofacitinib is a potent inhibitor of Jak3 [20,28,34]. Tofacitinib, in combination with mycophenolate, is efficacious for the prevention of acute rejection after kidney transplantation [24,25]. Various questions, however, remained unexplored.

First, combination of tofacitinib with mycophenolate has been associated with higher incidence of viral infections and perhaps viral-associated malignancies. Whether this safety profile is due to the drug combination or tofacitinib alone is not known. Mycophenolate has been associated with a higher incidence of viral infection [35]. Tofacitinib possibly shares the same characteristic [36]. Although no rigorous studies have been done so far, the consistent and reproducible reduction in NK cells under tofacitinib may be associated with this observation. On the other hand, the effect of tofacitinib on NK, T, and B cells may reduce the incidence of chronic antibody mediated rejection [37]. Also, 1 experimental study in mice demonstrated that JAK3/Stat6 stimulates bone marrow-derived fibroblast activation in renal fibrosis. Treatment with tofacitinib significantly reduced myofibroblast transformation, matrix protein expression, interstitial fibrosis development, and apoptosis [38]. Follow-up of patients in both phase 2 trials may also provide important information on the long-term efficacy/safety profile of tofacitinib.

Second, the pharmacokinetics of tofacitinib based on serum concentration, together with pharmacodynamic monitoring, provide tools for the optimization of this compound

in individual transplant patients. It is unfortunate that due to the adverse event profile under a fixed dosing regimen, tofacitinib clinical development for transplantation was prematurely terminated. Tofacitinib has a narrow therapeutic index, with a 5- to 6-fold intersubject variability in drug exposure among patients receiving the same dose, and efficacy/safety appear to be associated with plasma concentration rather than with oral dose.26 The relatively short half-life may hamper trough plasma concentration monitoring, suggesting that an earlier time point (C_2) may be preferable. Also, because tofacitinib is metabolized by CYP3A4 and CYP2C19, there is potential for drug-to-drug interaction with drugs commonly used to treat comorbidities. As for sirolimus [39], everolimus [40], and belatacept [41], the chosen higher-dose groups tested in clinical trials were not associated with increased efficacy but did incur increased toxicity. Therefore, concentration-controlled studies are needed to evaluate the efficacy/safety profile of tofacitinib, and it could be anticipated that therapeutic drug monitoring—possibly with reduced exposure over time posttransplant—may improve outcomes and reduce serious infections and PTLD.

Third, as has been observed previously in CNI-free immunosuppressive regimens using mammalian target of rapamycin (mTOR) inhibitors [42], the combination of tofacitinib with mycophenolate has been challenging due to overlapping toxicities, primarily infections and bone marrow toxicities. Whether a combination with an mTOR inhibitor or belatacept would result in a better efficacy/safety profile is not known.

Proposed Study Designs

Considering the rational for use to tofacitinib and the studies performed to date, the next trial would compare tofacitinib, using concentration-controlled dosing based on C2 monitoring versus tacrolimus. All patients would receive induction with basiliximab, and maintenance therapy with mycophenolate and steroids. Two target tofacitinib concentration ranges would be tested, with minimal overlap between them, to further explore concentration-effect relationships. Prophylaxis for viral infections would be mandatory for at least 6 months. Serial measurements of viral replication, including Ebstein-Barr Virus and polyomavirus viremia, would also assist dose adjustments as indicators of over immunosuppression. After determination of the therapeutic concentrations of tofacitinib, early conversion trials could be explored, whereby patients receiving tacrolimus would be converted to tofacitinib at 3 to 6 months after transplantation, a period when the risk of acute rejection and viral infections is lower.

Another possible study design would combine tofacitinib and an mTOR inhibitor. This approach offers 2 advantages. First, it has been demonstrated that patients receiving mTOR inhibitors have a lower incidence of viral infections. Second, based on ***in vitro*** and ***ex vivo*** studies, this combination could enhance the number and function of regulatory T cells. However, the trial would require therapeutic drug monitoring of 2 drugs and the concerns associated with de novo use of mTOR inhibitors regarding wound healing and recovery of renal function would have to be taken into account.

References

1. Rosell R, Carcereny E, Gervais R, et al. Erlotinib versus standard chemotherapy as first-line treatment for European patients with advanced EGFR mutation-positive non-small-cell lung cancer (EURTAC): a multicentre, open-label, randomised phase 3 trial. Lancet Oncol. 2012;13:239-246.
2. Shaw AT, Kim DW, Nakagawa K, et al. Crizotinib versus chemotherapy in advanced ALK-positive lung cancer. N Engl J Med. 2013;368: 2385-2394.
3. van Gurp E, Weimar W, Gaston R, et al. Phase 1 dose-escalation study of CP-690 550 in stable renal allograft recipients: preliminary findings of safety, tolerability, effects on lymphocyte subsets and pharmacokinetics. Am J Transplant. 2008;8:1711-1718.
4. Bachelez H, van de Kerkhof PC, Strohal R, et al. Tofacitinib versus etanercept or placebo in moderate-to-severe chronic plaque psoriasis: a phase 3 randomised non-inferiority trial. Lancet. 2015;386:552-561.
5. Boyle DL, Soma K, Hodge J, et al. The JAK inhibitor tofacitinib suppresses synovial JAK1-STAT signalling in rheumatoid arthritis. Ann Rheum Dis. 2015;74:1311–1316.
6. Rochman Y, Spolski R, Leonard WJ. New insights into the regulation of T cells by gamma(c) family cytokines. Nat Rev Immunol. 2009;9:480-490.
7. Levy DE, Darnell JE Jr. Stats: transcriptional control and biological impact. Nat Rev Mol Cell Biol. 2002;3:651-662.
8. Changelian PS, Flanagan ME, Ball DJ, et al. Prevention of organ allograft rejection by a specific Janus kinase 3 inhibitor. Science. 2003;302:875-878.
9. Borie DC, Changelian PS, Larson MJ, et al. Immunosuppression by the JAK3 inhibitor CP-690,550 delays rejection and significantly prolongs kidney allograft survival in nonhuman primates. Transplantation. 2005;79: 791-801.
10. Paniagua R, Si MS, Flores MG, et al. Effects of JAK3 inhibition with CP-690,550 on immune cell populations and their functions in nonhuman primate recipients of kidney allografts. Transplantation. 2005;80: 1283-1292.
11. Ghoreschi K, Laurence A, O'Shea JJ. Janus kinases in immune cell signaling. Immunol Rev. 2009;228:273-287.
12. Musso T, Johnston JA, Linnekin D, et al. Regulation of JAK3 expression in human monocytes: phosphorylation in response to interleukins 2, 4, and 7. J Exp Med. 1995;181:1425-1431.
13. Mangan JK, Reddy EP. Activation of the Jak3 pathway and myeloid differentiation. Leuk Lymphoma. 2005;46:21-27.
14. Mangan JK, Tantravahi RV, Rane SG, et al. Granulocyte colony-stimulating factor-induced upregulation of Jak3 transcription during granulocytic differentiation is mediated by the cooperative action of Sp1 and Stat3. Oncogene. 2006;25:2489-2499.
15. Ghoreschi K, Jesson MI, Li X, et al. Modulation of innate and adaptive immune responses by tofacitinib (CP-690,550). J Immunol. 2011;186: 4234-4243.
16. Bensinger SJ, Walsh PT, Zhang J, et al. Distinct IL-2 receptor signaling pattern in CD4 + CD25+ regulatory T cells. J Immunol. 2004;172: 5287-5296.

17. Sewgobind VD, Quaedackers ME, van der Laan LJ, et al. The Jak inhibitor CP-690,550 preserves the function of CD4CD25FoxP3 regulatory T cells and inhibits effector T cells. Am J Transplant. 2010;10:1785-1795.
18. Cacalano NA, Migone TS, Bazan F, et al. Prevention of organ allograft rejection by a specific Janus kinase 3 inhibitor. Science. 2003;302:875-878.
19. Cattaneo F, Recher M, Masneri S, et al. Hypomorphic Janus kinase 3 mutations result in a spectrum of immune defects, including partial maternal T-cell engraftment. J Allergy Clin Immunol. 2013;131:1136-1145.
20. Hsu L, Armstrong AW. JAK inhibitors: treatment efficacy and safety profile in patients with psoriasis. J Immunol Res. 2014;2014:283617.
21. Hansen RB, Kavanaugh A. Novel treatments with small molecules in psoriatic arthritis. Curr Rheumatol Rep. 2014;16:443.
22. Lowenberg M, D'Haens G. Next-generation therapeutics for IBD. Curr Gastroenterol Rep. 2015;17:21.
23. Braun J, Kiltz U, Heldmann F, et al. Emerging drugs for the treatment of axial and peripheral spondyloarthritis. Expert Opin Emerg Drugs. 2015; 20:1-14.
24. Busque S, Leventhal J, Brennan DC, et al. Calcineurin-inhibitor-free immunosuppression based on the JAK inhibitor CP-690,550: a pilot study in de novo kidney allograft recipients. Am J Transplant. 2009; 9:1936-1945.
25. Vincenti F, Tedesco Silva H, Busque S, et al. Randomized phase 2b trial of tofacitinib (CP-690,550) in de novo kidney transplant patients: efficacy, renal function and safety at 1 year. Am J Transplant. 2012; 12:2446-2456.
26. Vincenti F, Silva HT, Busque S, et al. Evaluation of the effect of tofacitinib exposure on outcomes in kidney transplant patients. Am J Transplant. 2015;15:1644-1653.
27. Dowty ME, Lin J, Ryder TF, et al. The pharmacokinetics, metabolism, and clearance mechanisms of tofacitinib, a janus kinase inhibitor, in humans. Drug Metab Dispos. 2014;42:759-773.
28. Wojciechowski D, Vincenti F. Tofacitinib in kidney transplantation. Expert Opin Investig Drugs. 2013;22:1193-1199.
29. Quaedackers ME, Mol W, Korevaar SS, et al. Monitoring of the immunomodulatory effect of CP-690,550 by analysis of the JAK/STAT pathway in kidney transplant patients. Transplantation. 2009;88:1002-1009.
30. Lamba M, Tafti B, Melcher M, et al. Population pharmacokinetic analysis of mycophenolic acid coadministered with either tasocitinib (CP-690, 550) or tacrolimus in adult renal allograft recipients. Ther Drug Monit. 2010;32:778–781.
31. Exterkate L, Slegtenhorst BR, Seyda M, et al. Frailty and transplantation. Transplantation. 2015;4:727-733.
32. Thoma G, Drückes P, Zerwes HG. Selective inhibitors of the Janus kinase Jak3—are they effective? Bioorg Med Chem Lett. 2014;24:4617-4621.
33. Clark JD, Flanagan ME, Telliez JB. Discovery and development of Janus kinase (JAK) inhibitors for inflammatory diseases. J Med Chem. 2014; 57:5023-5038.

34. Tanaka Y. Recent progress and perspective in JAK inhibitors for rheumatoid arthritis: from bench to bedside. J Biochem. 2015;158:173-179.

35. Brennan DC, Aguado JM, Potena L, et al. Effect of maintenance immunosuppressive drugs on virus pathobiology: evidence and potential mechanisms. Rev Med Virol. 2013;23:97-125.

36. Egli A, Kumar D, Broscheit C, et al. Comparison of the effect of standard and novel immunosuppressive drugs on CMV-specific T-cell cytokine profiling. Transplantation. 2013;95:448-455.

37. Schinstock CA, Stegall M, Cosio F. New insights regarding chronic antibody-mediated rejection and its progression to transplant glomerulopathy. Curr Opin Nephrol Hypertens. 2014;23:611-618.

38. Yan J, Zhang Z, Yang J, et al. JAK3/STAT6 stimulates bone marrowderived fibroblast activation in renal fibrosis. J Am Soc Nephrol. 2015; 26:3060-3071.

39. Kahan BD. Efficacy of sirolimus compared with azathioprine for reduction of acute renal allograft rejection: a randomised multicentre study. The Rapamune US Study Group. Lancet. 2000;356:194-202.

40. Tedesco Silva H, Jr, Cibrik D, Johnston T, et al. Everolimus plus reducedexposure CsA versus mycophenolic acid plus standard-exposure CsA in renal-transplant recipients. Am J Transplant. 2010;10:1401-1413.

41. Vincenti F, Charpentier B, Vanrenterghem Y, et al. A phase III study of belatacept-based immunosuppression regimens versus cyclosporine in renal transplant recipients (BENEFIT study). Am J Transplant. 2010;10: 535-546.

42. Sharif A, Shabir S, Chand S, et al. Meta-analysis of calcineurin-inhibitor– sparingregimens in kidney transplantation. J Am Soc Nephrol. 2011;22: 2107-2118.

9

Summary and general discussion

Summary

The introduction of calcineurin inhibitors (CNIs) and mycophenolic acid (MPA) to the transplantation clinic improved the outcomes of kidney transplantation [1-3]. Unfortunately, the use of the CNI tacrolimus is associated with side effects such as nephrotoxicity, diabetes mellitus, neurotoxicity and dyslipidemia and there is thus an unmet need for an alternative drug with comparable efficacy yet less side effects [4]. The most recently approved immunosuppressive drug belatacept, a co-stimulation blocker, is non-nephrotoxic and more selective than tacrolimus, but its use is associated with an increased incidence of rejection [5-7].

Therapeutic drug monitoring (TDM) of tacrolimus is based on a pharmacokinetic (PK) method that measures whole-blood (pre-dose) concentrations. Unfortunately, this method has a limited predictive power with regard to the occurrence of acute rejection episodes or toxicity. One explanation for this observation may be that whole-blood concentrations do not correlate with the amount of drug acting on its receptor. Furthermore, tacrolimus displays considerable intra-patient variability limiting the predictive power of a drug concentration measurement at a single time point [8-11]. A better or complementary approach to traditional PK TDM may be the measurement of the biological effects of tacrolimus, also called pharmacodynamic (PD) monitoring [12-15]. In our studies, we aimed to assess novel assays for PD monitoring of immunosuppressive drugs within their main target: the T cell. Additionally, we have investigated the efficacy of currently prescribed immunosuppressive drugs to inhibit monocyte activation, a cell type that also plays an important role in rejection processes after kidney transplantation [16-18].

The role of cells from the monocyte-macrophage cell lineage in acute and chronic rejection processes after SOT has been increasingly recognized but the knowledge of the effect of currently prescribed immunosuppressive drugs on monocyte activation is relatively limited. In **chapter 2**, we reviewed the effects that are currently known and discussed the potential use of other available immunosuppressive drugs in- and outside the field of transplantation. We conclude that tacrolimus, MPA and mTOR inhibitors have limited inhibitory effects on monocyte functions, including the inhibition of cytokine production, co-stimulation blockade and the inhibition of monocyte migration to the allograft. Monoclonal antibodies, such as canakinumab (interleukin-1β inhibitor), tocilizumab (interleukin-6 receptor blocker) and infliximab (tumor necrosis factor-α inhibitor) are effective in inhibiting monocyte functions in autoimmune diseases. We speculated that after organ transplantation new immunosuppressive therapies, possibly including these monoclonal antibodies, should also target cells of the monocyte-macrophage lineage to prevent activation and inhibit the function of these cells.

Because of the limited knowledge about biological effects of the currently prescribed immunosuppressive drugs on other signal transduction pathways besides nuclear factor of activated T cells (NFAT) in monocytes, the individual drug effects of tacrolimus and MPA on monocyte intracellular activation pathways were studied (**chapter 3**). By use of phospho-specific flow cytometry, the phosphorylation patterns of three signaling proteins involved

in monocyte activation, ***i.e.*** p38MAPK, ERK and Akt, were measured in blood samples of healthy volunteers spiked with therapeutic concentrations of either tacrolimus or MPA. A limited effect of both drugs on monocyte signaling pathway activation and the functions of monocytes, such as cytokine production and phagocytosis, was found.

In **chapter 4**, we further investigated the PD effects of tacrolimus-based immunosuppression on monocyte activation. In contrast to the previous chapter, where we studied the individual drug effects, we determined the inhibition of monocyte activation in the presence of combination immunosuppressive drug therapy in blood samples of kidney transplant patients. Phospho-specific flow cytometry analysis of p38MAPK, ERK and Akt phosphorylation not only revealed that this technique is a potential tool for measuring the biological drug effect of tacrolimus-based immunosuppression, but also showed that even combination therapy only partially inhibited monocyte activation *in vivo*.

In the second part of the thesis, we investigated potential PD assays for improving TDM of tacrolimus after transplantation. The main target of tacrolimus in T cells is the calcineurin pathway, of which NFAT is an important signaling molecule. In **chapter 5**, we determined the PD effects of tacrolimus and other immunosuppressive drugs by measuring the expression of the inducible isoform of NFAT, NFATc1, in T cells, as no tools are available to measure dephosphorylation of NFAT. The study described here showed that the amplification of NFATc1 is reduced in $CD4^+CD28^+$ and $CD8^+CD28^+$ T cells upon treatment with tacrolimus-based immunosuppression. Belatacept-based treatment was used as a negative control. We confirmed that belatacept-based immunosuppression does not inhibit NFATc1 amplification. Furthermore, tacrolimus-based immunosuppression had no effect on NFATc1 amplification in $CD8^+CD28^-$ T cells, indicating that measuring NFATc1 amplification is a promising tool for PD monitoring of tacrolimus in $CD28^+$ T cells.

Another intracellular pathway in T cells which is inhibited by tacrolimus is the MAPK pathway. Measuring p-p38MAPK, a member of this pathway, with phospho-specific flow cytometry can be a promising tool to monitor the effects of conversion from the twice-daily tacrolimus formulation to the once-daily, prolonged-release tacrolimus formulation, as is described in **chapter 6**. Once-daily tacrolimus has been developed to overcome adherence problems associated with the use of twice-daily tacrolimus but blood pre-dose concentrations of this formulation may decrease on average by 15% after 1:1 (mg for mg basis) conversion. We showed that after conversion no difference was measured in the pre-dose concentrations of tacrolimus, whereas the p38MAPK phosphorylation significantly increased in $CD4^+$ T cells and $CD8^+$ T cells, indicating T cell activation. Therefore, phospho-specific flow cytometry analysis of p38MAPK phosphorylation may be a more sensitive tool for monitoring subtle changes in tacrolimus exposure after conversion and should be assessed for its suitability as an additional tool in transplantation diagnostics.

In **chapter 7**, we determined the PD drug effects of a tacrolimus-based therapy compared to belatacept-based therapy (which served as a negative control) in T cells of kidney transplant recipients. Before and during the first year after transplantation, blood samples

of kidney transplant recipients were measured for their phosphorylation of p38MAPK, ERK and Akt in T cell subsets to see whether phospho-specific flow cytometry can be used for TDM of tacrolimus-based immunosuppression. Phosphorylation of p38MAPK and Akt, but not ERK, was inhibited in $CD4^+$ and $CD8^+$ T cells during tacrolimus-based treatment. Moreover, in belatacept-treated patients suffering from an acute rejection episode, ERK phosphorylation was higher immediately prior to the diagnosis of rejection compared to patients without rejection. This suggests that p-ERK is involved in rejection processes and that belatacept-based immunosuppression does not inhibit key T cell activation pathways, which may contribute to the high rejection incidence observed among belatacept-treated kidney transplant recipients. These findings suggest also that phospho-specific flow cytometry is a promising tool to PD monitor tacrolimus-based therapy in kidney transplant recipients.

Rejection of the transplant is characterized by the production of a wide variety of cytokines that activate Janus tyrosine kinases (JAKs) and signal transduction and activators of transcription (STATs) intracellularly within T cells to induce the immune response. In **chapter 8,** we reviewed the use of the JAK inhibitor tofacitinib as an immunosuppressive drug in kidney transplantation. The outcomes of several clinical trials where this drug was assessed as a substitute for CNI therapy were discussed. Although the results were promising, the high fixed-dose regimens of tofacitinib caused an increase in infections and, as a consequence, the discontinuation of the development of the drug for transplantation. PD monitoring in combination with PK monitoring for dose adjustment could improve the safety profile of tofacitinib. Therefore, new studies should be performed that focus on personalized tofacitinib dosing, improved immune monitoring and a better patient selection.

General Discussion

TDM of tacrolimus is currently based on a PK approach that measures whole-blood (predose) drug concentrations. Unfortunately, these measurements cannot accurately predict clinical outcomes after transplantation, such as rejection, and have a poor correlation with long-term outcomes after transplantation. PD monitoring is an alternative and possibly complimentary way for TDM that measures the biological effects of the drug at its target site. The research described in this thesis assessed novel techniques for PD monitoring of immunosuppressive therapy after transplantation.

New strategies for pharmacodynamic therapeutic drug monitoring of tacrolimus

Two promising methods for the optimization of TDM are the phospho-specific flow cytometry assay and the intracellular monitoring of NFATc1 amplification. Phospho-specific flow cytometry measures the phosphorylation of molecules involved in T cell activation, such as p-p38MAPK and p-ERK (both members of the MAPK pathway) and p-Akt (a member of the PI3k/Akt/mTOR pathway). Advantages of this assay are the short turn-around time, its

sensitivity, the material that can be used (small volume whole-blood samples) and the detection at the single-cell level [12,19]. The assay can be applied to monitor therapeutic drug effects on T cells (**chapter 6 and 7**) and monocytes (**chapter 3 and 4**). Phospho-specific flow cytometry is a promising tool for TDM as it measures drug-related effects upstream in the T cell signaling cascade. This assay can be done in several hours which makes it an appropriate tool for clinical purposes. In T cells, phospho-specific flow cytometry analysis shows a clear inhibition of p38MAPK and Akt phosphorylation during tacrolimus-based therapy, while no effect was measured on the phosphorylation of ERK (**chapter 6 and 7**). Moreover, the phosphorylation of p38MAPK correlated significantly with the pre-dose concentrations of the twice-daily formulation of tacrolimus in T cells [20].

Next, we studied whether immunosuppressive drugs inhibited the expression of the activation molecules p-p38MAPK, p-ERK and p-Akt in monocytes, because up until now little is known about the effects of immunosuppressive drug therapy on monocyte function. From our studies described in this thesis it is clear that the currently prescribed immunosuppressive drugs also inhibit monocyte function but only to a limited extent and that new therapies are needed for targeting monocytes (**chapter 2, 3 and 4**). Furthermore, these studies show that the phospho-specific flow cytometry assay is not only suitable for monitoring the direct biological effects of tacrolimus on cells of the adaptive immune system, but that it can also be used to study effects on the intracellular signaling pathway activation in cells of the innate immune system. In addition, our studies show for the first time that immunosuppressive effects of tacrolimus can be routinely monitored in whole-blood samples of transplant patients. A combination between PK and PD monitoring of immunosuppressive drugs will give additional information about the drug's effects on monocyte activation. At the same time, it is clear that validation of our findings is warranted. Phospho-specific flow cytometry might be a useful tool to further optimize immunosuppression and to achieve optimal clinical outcome in the future [21].

Another promising method for PD monitoring of tacrolimus in T cells is the analysis of NFATc1 amplification (**chapter 5**). This assay does not measure dephosphorylation of NFAT but measures the production of NFATc1 after T cell activation. NFATc1 is the only NFAT member that can be enhanced upon antigenic stimulation and maintained by positive autoregulation in T cells and its expression is regulated by the calcineurin pathway. As a consequence, its amplification will be directly affected by tacrolimus. Like phospho-specific flow cytometry, this assay can also detect the amplification of NFATc1 in different T cell subsets each with its own specific role in the rejection process. For example, $CD8^+CD28^-$ T cells are known to be potentially more harmful than $CD8^+CD28^+$ T cells and it is important to monitor this $CD8^+CD28^-$ T cell subset intensively to determine whether these cells can still contribute to rejection after transplantation. NFATc1 analysis might be helpful to determine the immunosuppressive effects of tacrolimus and other agents on this T cell population [22-24]. Our data suggest that $CD8^+CD28^-$ T cells might be less sensitive to immunosuppressive drug therapy than the other T cell subsets, which was not known before

(**chapter 5**). In addition, we showed that this PD assay can be measured in whole blood samples of kidney transplant patients. However, measuring NFATc1 amplification includes a 7h procedure, which requires further optimization to use it for monitoring patients in routine clinical practice. We here present the first data and in line with our phosphorylation studies, this assay also needs validation in other cohorts to prove its robustness for routine use.

Measuring NFATc1 amplification has not yet been validated for monitoring monocyte activity, although these cells also express this molecule [25]. Future studies should also determine whether this assay can be used to monitor the immunosuppressive effects of tacrolimus on NFATc1 amplification of monocytes. A first approach would be to spike blood samples of healthy volunteers with different immunosuppressive drugs and to test for the amplification of NFATc1. Thereafter, samples of a large group of kidney transplant patients should be tested before and after transplantation in order to get more sustained information about the effects of a combined immunosuppressive drug therapy on monocytes.

In addition to the above described assays, other PD assays have also been developed, such as the measurement of calcineurin phosphatase activity, IL-2 production, and NFAT-regulated gene expression. Evaluation of all these read-outs and parameters is performed in mostly small patient cohorts and it is clear that more and larger patient numbers are needed to demonstrate if these tools are sensitive and accurate enough to contribute to better diagnostics of tacrolimus therapy [26-28].

A way to move forward with the PD measuring of tacrolimus is standardization of the assays, such as the cytokine production assay [21,29,30]. Currently, no threshold level of cytokine production for adjusting drug doses exist and the assay is still being evaluated in ongoing multicenter clinical trials [29]. Moreover, this method has a lack of specificity, since the production of cytokines may also be influenced by other factors, such as infection, which affects the interpretation of the cytokine measurements [27].

Analyzing NFAT-regulated gene expression for PD monitoring of CNI's is under investigation in ongoing multicenter clinical trials and the benefit of the method for monitoring tacrolimus-treated patients will be evaluated in these studies. These studies show that monitoring residual NFAT-regulated gene expression may be helpful to identify transplant recipients at higher risk of rejection or infection. Hopefully these studies will also reveal the additive contribution of the assay in TDM [27]. For the interpretation of the data it is of course important to realize that, apart from tacrolimus, also other immunosuppressive drugs can influence the expression of NFAT-regulated genes. For example, NFAT molecules are known to cooperate with the AP-1 proteins that are regulated by prednisolone [31,32].

Lessons learned and the road ahead

Although the results are promising, both the phospho-specific flow cytometry and NFATc1 amplification assay are not yet ready for routine clinical use. Both assays were tested in a single center study and in a small patient cohort. A next step is to perform larger (multi-

center) studies aiming to confirm our findings. Such studies should also determine the phosphorylation threshold associated with drug-related complications of under- and overdosing [33]. The threshold will be helpful for standardized clinical protocols for TDM of tacrolimus.

For the validation of phospho-specific flow cytometry and the NFATc1 amplification assay, a correlation between the outcomes of those assays and pre-dose tacrolimus concentrations is required. On top of that, validation of both assays can also be done by the calculation of the area-under the concentration ***versus*** time-curve values. These values give a better reflection of tacrolimus exposure than pre-dose concentrations. Eventually, the validation will most likely lead to a complementary tool that supports conventional TDM of immunosuppressive drugs. Phospho-specific flow cytometry can also be combined with other PK approaches, such as measuring the intra-cellular immunosuppressive drug levels in the target cell, which might give a better reflection of drug efficacy [34-36]. Recent studies showed that intra-lymphocytic concentrations of tacrolimus are better correlated to drug efficacy and toxicity than pre-dose whole-blood concentrations and would therefore be a better form of PK monitoring of CNIs.

Next to their role in optimizing TDM of tacrolimus, PD assays may be also useful in predicting clinical outcomes after transplantation, such as acute rejection [37,38]. However, we were not able to associate the phosphorylation profiles of p38MAPK, ERK and Akt with the occurrence of acute rejection, one reason being the low number of patients suffering from a rejection episode in our study population. The use of a larger study population will reveal the power of the assay to determine if p-p38MAPK, p-Akt or p-ERK levels are associated with rejection responses and perhaps also with other clinical outcomes. Our first studies suggest that p-ERK might be a good marker to identify patients at risk for acute rejection (**Chapter 7**). In monocytes, we showed that phosphorylation of ERK was only limited affected by immunosuppressive drugs (**Chapter 3 and 4**). This observation shows that these cells are still active and can contribute to rejection processes. The important role of ERK phosphorylation was also noticed by other research groups, describing the association between p-ERK and the occurrence of antibody-mediated rejection in heart allografts and the relation between targeting p-ERK and the suppression of allo-immune responses in a cardiac allograft mouse model [39,40].

Future perspectives and recommendations

In the future, when phospho-specific flow cytometry is ready for routine use we recommend that the assay should be used in combination with PK measurements. In our view, this will improve TDM and will lead to improved dosing of immunosuppressive drugs for organ transplant patients. For this, multiple signaling proteins should be monitored, because measuring a single biomarker will not reflect the wide range of complications and events patients are suffering from. In addition, in monocytes, tacrolimus inhibits p-p38MAPK, while mycophenolate mofetil (MMF) shows the strongest effect on p-Akt (**chapter 3**).

Thus, for a more specific monitoring of tacrolimus both p-p38MAPK and p-Akt should be measured in monocytes and T cells, while p-ERK should be monitored in T cells to assess the risk for rejection.

Both the phospho-specific flow cytometry assay and the measurement of NFATc1 amplification are specific for tacrolimus and are not influenced by the use of MMF or prednisone in T cells. This was found since in belatacept-based therapy, no effect on any of the studied parameters was found. Therefore, we recommend the assay only for TDM of tacrolimus or a tacrolimus-based therapy and not for the monitoring of other immunosuppressive drug effects.

Conclusions

- The currently prescribed immunosuppressive drugs have only a limited effect on intracellular monocyte activation pathways, demonstrating the need for new drugs that target monocytes after solid organ transplantation.
- Phospho-specific flow cytometry is a potential pharmacodynamic tool for TDM of tacrolimus in kidney transplant recipients.
- Measuring NFATc1 amplification is a specific whole-blood test to monitor the biological effects of tacrolimus in T cells of kidney transplant recipients.
- Phosphorylation of ERK in both monocytes and T cells is a promising parameter for risk assessment of acute rejection.
- Conventional pharmacokinetic measurements should be combined with the pharmacodynamic phospho-specific flow cytometry assay to optimize TDM of tacrolimus.
- Both the phospho-specific flow cytometry assay and the NFATc1 amplification assay can measure the activation of signaling molecules in the different $CD4^+CD28^+$, $CD8^+CD28^+$ and $CD8^+CD28^-$ T cell subsets.
- Phospho-specific flow cytometry after transplantation is a relatively new tool to assess the efficacy and safety of the once-daily formulation of tacrolimus and JAK inhibitors.

References

1. Matas AJ, Smith JM, Skeans MA, et al. OPTN/SRTR 2011 Annual Data Report: kidney. Am J Transplant 2013;13 Suppl 1:11-46.
2. Taylor AL, Watson CJ, Bradley JA. Immunosuppressive agents in solid organ transplantation: Mechanisms of action and therapeutic efficacy. Crit Rev Oncol Hematol 2005;56:23-46.
3. Halloran PF. Immunosuppressive drugs for kidney transplantation. N Engl J Med 2004;351:2715-29.
4. Malvezzi P, Rostaing L. The safety of calcineurin inhibitors for kidney-transplant patients. Expert Opin Drug Saf 2015;14:1531-46.
5. Vincenti F, Charpentier B, Vanrenterghem Y, et al. A phase III study of belatacept-based immunosuppression regimens versus cyclosporine in renal transplant recipients (BENEFIT study). Am J Transplant 2010;10:535-46.
6. de Graav GN, Baan CC, Clahsen-van Groningen MC, et al. A Randomized Controlled Clinical Trial Comparing Belatacept With Tacrolimus After De Novo Kidney Transplantation. Transplantation 2017;101:2571-81.
7. Larsen CP, Pearson TC, Adams AB, et al. Rational development of LEA29Y (belatacept), a high-affinity variant of CTLA4-Ig with potent immunosuppressive properties. Am J Transplant 2005;5:443-53.
8. Shuker N, van Gelder T, Hesselink DA. Intra-patient variability in tacrolimus exposure: causes, consequences for clinical management. Transplant Rev (Orlando) 2015;29:78-84.
9. Bouamar R, Shuker N, Hesselink DA, et al. Tacrolimus predose concentrations do not predict the risk of acute rejection after renal transplantation: a pooled analysis from three randomized-controlled clinical trials(dagger). Am J Transplant 2013;13:1253-61.
10. Whalen HR, Glen JA, Harkins V, et al. High Intrapatient Tacrolimus Variability Is Associated With Worse Outcomes in Renal Transplantation Using a Low-Dose Tacrolimus Immunosuppressive Regime. Transplantation 2017;101:430-6.
11. Wallemacq P, Armstrong VW, Brunet M, et al. Opportunities to optimize tacrolimus therapy in solid organ transplantation: report of the European consensus conference. Ther Drug Monit 2009;31:139-52.
12. Baan C, Bouvy A, Vafadari R, Weimar W. Phospho-specific flow cytometry for pharmacodynamic monitoring of immunosuppressive therapy in transplantation. Transplant Res 2012;1:20.
13. Dieterlen MT, Eberhardt K, Tarnok A, Bittner HB, Barten MJ. Flow cytometry-based pharmacodynamic monitoring after organ transplantation. Methods Cell Biol 2011;103:267-84.
14. Noceti OM, Woillard JB, Boumediene A, et al. Tacrolimus pharmacodynamics and pharmacogenetics along the calcineurin pathway in human lymphocytes. Clin Chem 2014;60:1336-45.
15. Dambrin C, Klupp J, Morris RE. Pharmacodynamics of immunosuppressive drugs. Curr Opin Immunol 2000;12:557-62.
16. Girlanda R, Kleiner DE, Duan Z, et al. Monocyte infiltration and kidney allograft dysfunction during acute rejection. Am J Transplant 2008;8:600-7.

17. Hancock WW, Thomson NM, Atkins RC. Composition of interstitial cellular infiltrate identified by monoclonal antibodies in renal biopsies of rejecting human renal allografts. Transplantation 1983;35:458-63.
18. Rowshani AT, Vereyken EJ. The role of macrophage lineage cells in kidney graft rejection and survival. Transplantation 2012;94:309-18.
19. Krutzik PO, Trejo A, Schulz KR, Nolan GP. Phospho flow cytometry methods for the analysis of kinase signaling in cell lines and primary human blood samples. Methods Mol Biol 2011;699:179-202.
20. Vafadari R, Hesselink DA, Cadogan MM, Weimar W, Baan CC. Inhibitory effect of tacrolimus on p38 mitogen-activated protein kinase signaling in kidney transplant recipients measured by whole-blood phosphospecific flow cytometry. Transplantation 2012;93:1245-51.
21. Fernandez-Ruiz M, Kumar D, Humar A. Clinical immune-monitoring strategies for predicting infection risk in solid organ transplantation. Clin Transl Immunology 2014;3:e12.
22. Vallejo AN. CD28 extinction in human T cells: altered functions and the program of T-cell senescence. Immunol Rev 2005;205:158-69.
23. Mou D, Espinosa J, Lo DJ, Kirk AD. CD28 negative T cells: is their loss our gain? Am J Transplant 2014;14:2460-6.
24. de Graav GN, Hesselink DA, Dieterich M, Kraaijeveld R, Weimar W, Baan CC. Down-Regulation of Surface CD28 under Belatacept Treatment: An Escape Mechanism for Antigen-Reactive T-Cells. PLoS One 2016;11:e0148604.
25. Komano Y, Nanki T, Hayashida K, Taniguchi K, Miyasaka N. Identification of a human peripheral blood monocyte subset that differentiates into osteoclasts. Arthritis Res Ther 2006;8:R152.
26. Caruso R, Perico N, Cattaneo D, et al. Whole-blood calcineurin activity is not predicted by cyclosporine blood concentration in renal transplant recipients. Clin Chem 2001;47:1679-87.
27. Brunet M, Shipkova M, van Gelder T, et al. Barcelona Consensus on Biomarker-Based Immunosuppressive Drugs Management in Solid Organ Transplantation. Ther Drug Monit 2016;38 Suppl 1:S1-20.
28. Sanquer S, Amrein C, Grenet D, et al. Expression of calcineurin activity after lung transplantation: a 2-year follow-up. PLoS One 2013;8:e59634.
29. Millan O, Brunet M. Cytokine-based immune monitoring. Clin Biochem 2016;49:338-46.
30. van Gelder T. Biomarkers in solid organ transplantation. Br J Clin Pharmacol 2017.
31. Barnes PJ. Molecular mechanisms and cellular effects of glucocorticosteroids. Immunol Allergy Clin North Am 2005;25:451-68.
32. Herrlich P. Cross-talk between glucocorticoid receptor and AP-1. Oncogene 2001;20:2465-75.
33. Beland MA, Lapointe I, Noel R, et al. Higher calcineurin inhibitor levels predict better kidney graft survival in patients with de novo donor-specific anti-HLA antibodies: a cohort study. Transpl Int 2017;30:502-9.

34. Capron A, Haufroid V, Wallemacq P. Intra-cellular immunosuppressive drugs monitoring: A step forward towards better therapeutic efficacy after organ transplantation? Pharmacol Res 2016;111:610-8.
35. Han SS, Yang SH, Kim MC, et al. Monitoring the Intracellular Tacrolimus Concentration in Kidney Transplant Recipients with Stable Graft Function. PLoS One 2016;11:e0153491.
36. Lemaitre F, Blanchet B, Latournerie M, et al. Pharmacokinetics and pharmacodynamics of tacrolimus in liver transplant recipients: inside the white blood cells. Clin Biochem 2015;48:406-11.
37. Staatz CE, Tett SE. Clinical pharmacokinetics and pharmacodynamics of tacrolimus in solid organ transplantation. Clin Pharmacokinet 2004;43:623-53.
38. Sommerer C, Giese T, Meuer S, Zeier M. Pharmacodynamic monitoring of calcineurin inhibitor therapy: is there a clinical benefit? Nephrol Dial Transplant 2009;24:21-7.
39. Li F, Wei J, Valenzuela NM, et al. Phosphorylated S6 kinase and S6 ribosomal protein are diagnostic markers of antibody-mediated rejection in heart allografts. J Heart Lung Transplant 2015;34:580-7.
40. Wang S, Guan Q, Diao H, et al. Prolongation of cardiac allograft survival by inhibition of ERK1/2 signaling in a mouse model. Transplantation 2007;83:323-32.

10

Nederlandse samenvatting

Nederlandse samenvatting

Niertransplantatie is voor veel patiënten met nierfalen de beste behandeling. In de eerste plaats, omdat de kwaliteit van leven sterk verbetert na een succesvolle transplantatie. Daarnaast stijgt de levensverwachting na niertransplantatie met drie tot vijftien jaar, waarbij zelfs oudere niertransplantatiepatiënten tot wel 5 jaar levensverlenging kunnen behalen [1-3]. Sinds de eerste succesvolle niertransplantatie in 1954 zijn de uitkomsten na niertransplantatie sterk verbeterd. Dit is mede te danken aan de ontwikkeling van afweerremmende geneesmiddelen waardoor ook transplantatie met niet-verwante donoren of een transplantatie door de bloedgroep- of HLA barrière mogelijk zijn geworden. Deze geneesmiddelen zorgen ervoor dat de donornier niet wordt afgestoten en worden ook wel immunosuppressiva genoemd.

Afstoting kent vele vormen en pathofysiologische mechanismen. Al voor de transplantatie zelf kan er schade in de donornier ontstaan doordat de donor hersendood is en door de onderbreking van de circulatie (bloeddoorstroming) van de nier na de uitname. Als gevolg van deze schade, de zogenaamde ischemie-reperfusie schade, zal het aangeboren afweersysteem van de ontvanger reageren waarbij verschillende soorten immuun cellen geactiveerd raken. Hieronder vallen dendritische cellen, natural killer cellen en monocyten [4-6]. Deze cellen raken geactiveerd door signaalmoleculen die worden uitgescheiden door de apoptotische cellen in de donornier. Daarnaast herkennen de cellen van het aangeboren afweersysteem de lichaamsvreemde eiwitten (antigenen) die behoren tot het witte bloedgroepensysteem (Humane Leukocyten Antigenen-systeem, HLA) en voorkomen op het oppervlak van cellen van de donornier. De activatie van het aangeboren immuunsysteem draagt bij aan het ontstaan van een ontstekings- en afstotingsreactie. Deze reactie leidt vervolgens tot activatie van de cellen van het verworven immuunsysteem, waartoe T en B lymfocyten worden gerekend. Antigeen-presenterende cellen van het aangeboren immuunsysteem presenteren antigenen gebonden aan een HLA molecuul aan de T cel receptor (TCR; signaal-ontvangend molecuul) die aanwezig is op het oppervlak van iedere T cel (**Figuur 1**). Dit gebeurt in zogenaamde secundaire lymfoïde organen, zoals lymfeklieren en de milt. Wanneer een T cel een TCR op zijn oppervlakte heeft die in staat is te binden aan het antigeen-HLA complex, zal de T cel geactiveerd raken, zich gaan delen en vermenigvuldigen en migreren naar het niertransplantaat. Daar aangekomen, veroorzaken T cellen een ontstekingsreactie waarbij zij direct via een mechanisme genaamd cytotoxie, cellen van het transplantaat vernietigen. Dit staat bekend als de klassieke, acute T cel-gemedieerde afstoting.[7] Daarnaast kunnen T cellen ook de productie van antilichamen door B cellen activeren. Deze antilichamen binden eveneens aan cellen van het transplantaat en kunnen via verschillende mechanismen cellen van de donornier vernietigen. Dit proces staat bekend als antilichaam-gemedieerde afstoting, en kan zowel chronisch als acuut optreden. Chronische, antilichaam-gemedieerde rejectie is een van de belangrijkste oorzaken van laat transplantaat verlies [7-9].

T cellen worden geactiveerd door 3 verschillende signalen (**Figuur 1**): het eerste signaal, het zogenaamde signaal 1, is de hierboven genoemde presentatie van een lichaamsvreemd antigeen in een HLA molecuul door een antigeen-presenterende cel (APC); Het tweede of co-stimulatoire signaal is eveneens noodzakelijk en bestaat uit de interactie tussen diverse receptoren op het oppervlak van de APC en de T cel. Tot slot zorgt het derde signaal er voor dat de activatie van T cellen versterkt wordt. Dit signaal wordt gevormd door de binding van diverse signaalstoffen (cytokines) aan receptoren op het T cel oppervlak [10,11]. Zodra een T cel deze signalen heeft ontvangen, zullen er binnenin de cel verschillende signaaltransductieroutes worden aangezet. Deze signaaltransductieroutes bestaan uit allerlei soorten signaalmoleculen die elkaar kunnen activeren. Zo wordt het signaal versterkt en doorgegeven tot aan het DNA in de celkern. Deze signaaltransductieroutes beheersen de functie van de T cel en zorgen ervoor dat er uiteindelijk cytokines worden geproduceerd. Er bestaan verschillende soorten T cellen. Elke groep T cellen heeft een aantal karakteristieke signaalmoleculen (bijvoorbeeld CD4, CD8 en CD28) op het cel oppervlak, waardoor hun functie kan worden herkend. Het bekendste onderscheid is dat tussen cytotoxische T cellen (CD8) en T helper cellen (CD4).

Monocyten (cellen van het aangeboren immuunsysteem) kunnen, zodra zij geactiveerd zijn, antigenen presenteren aan T cellen. Monocyten worden geactiveerd via verschillende moleculen op hun oppervlakte, zoals de CCR2 en de Fc-γ receptor, waarna zij differentieren in macrofagen of dendritische cellen [12,13]. Monocyten spelen een belangrijke rol in het afstotingsproces maar er is weinig bekend over de effecten van immunosuppressieve geneesmiddelen op deze cellen [14-16]. Vanwege de centrale rol van monocyten in het afstotingsproces, is dieper inzicht in de effecten van immunosuppressiva op deze cellen van belang.

Met de huidige immunosuppressieve therapie is het aantal acute afstotingen in het eerste jaar na niertransplantatie teruggebracht tot zo'n 20% [17]. De meest voorgeschreven immunosuppressieve geneesmiddelen na niertransplantatie zijn tacrolimus, mycofenolaat mofetil (MMF) en prednison. Deze geneesmiddelen worden meestal in combinatie voorgeschreven [18]. Tacrolimus is een calcineurine-remmer en blokkeert de calcineurine signaaltransductieroute in T cellen (**Figuur 1**) [19]. Hierdoor wordt het T cel-activatie signaal geblokkeerd en wordt de vermeerdering van T cellen en de productie van cytokines geremd.

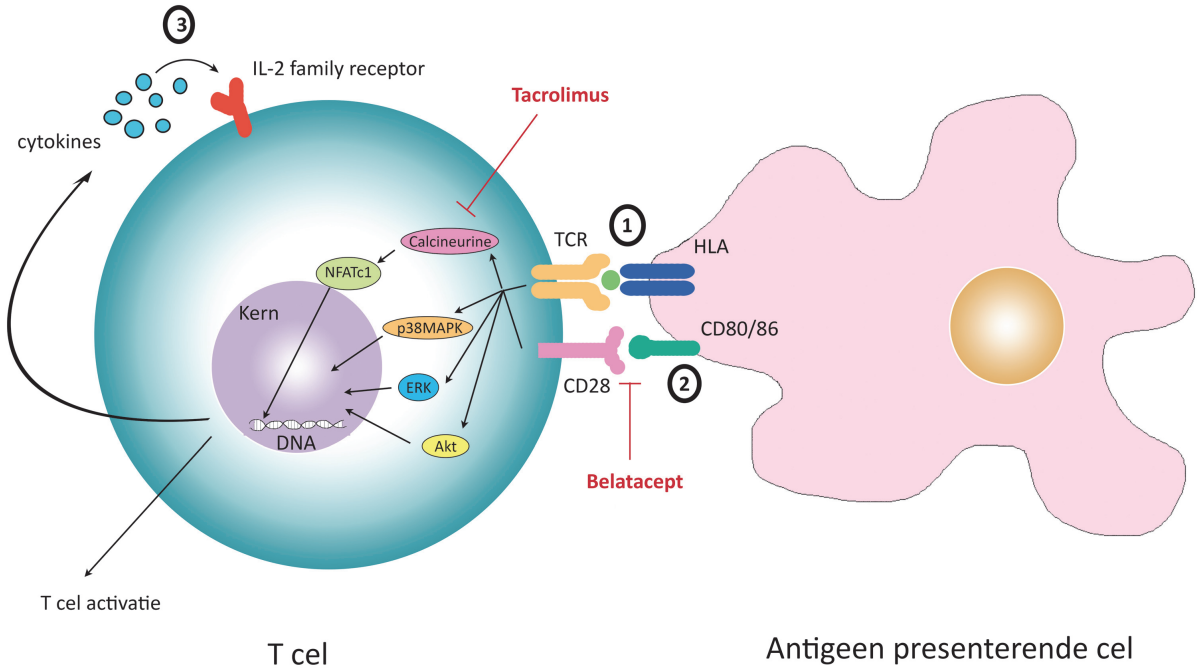
Er kleven echter ook nadelen aan het gebruik van tacrolimus. Zo moeten patiënten dit geneesmiddel iedere dag gebruiken en bovendien is het therapeutisch venster van tacrolimus is erg smal [20]. Dit laatste betekent dat het verschil tussen de minimale effectieve dosering en de dosering waarbij bijwerkingen (toxiciteit) ontstaan, klein is. Daarnaast zal eenzelfde dosering tacrolimus bij verschillende patiënten leiden tot sterk verschillende concentraties in het bloed. Dit staat bekend als de inter-patiënt variabiliteit [21]. Het is daarom essentieel dat de tacrolimus dosering wordt bijgesteld op geleide van de (vol) bloed concentraties.

Een alternatief immunosuppressief geneesmiddel voor tacrolimus is belatacept, wat in 2012 is geregistreerd voor het gebruik bij niertransplantatie patiënten. Belatacept blokkeert het co-stimulatie signaal tussen een APC en een T cel, waardoor de activatie van T cellen wordt geremd (**Figuur 1**) [22]. Belatacept heeft minder bijwerkingen dan tacrolimus maar het gebruik van dit geneesmiddel is ook geassocieerd met het optreden van meer en ernstiger afstotingen in de eerste maanden na niertransplantatie.

Het is essentieel dat de huidige immunosuppressieve therapie wordt verbeterd. Aangezien er de laatste jaren weinig nieuwe immunosuppressieve geneesmiddelen voor transplantatie-doeleinden op de markt zijn gekomen, moet de huidige standaardbehandeling na niertransplantatie worden verbeterd. In de meeste transplantatie-klinieken wordt de dosering van tacrolimus bijgesteld op geleide van de gemeten concentraties tacrolimus in het bloed. Dit staat ook bekend als farmacokinetisch therapeutic drug monitoring [23]. Helaas is er slechts een beperkte correlatie tussen deze volbloed concentraties en het optreden van een afstoting of het optreden van bijwerkingen zoals bijvoorbeeld een hoge bloeddruk en het ontstaan van een maligniteit, een infectie of diabetes mellitus [24-26]. Wellicht is het beter om de biologische effecten (farmacodynamiek) van tacrolimus te meten en hier de dosering op aan te passen [27-29]. Hierbij valt te denken aan het monitoren van de T cel activatie en het meten van ontstekingseiwitten in het bloed. In verschillende onderzoeken werden dergelijke farmacodynamische methodes ontwikkeld en getoetst maar tot op heden is geen een van deze methoden goed genoeg gebleken om in de kliniek te worden toegepast.

In dit proefschrift worden twee nieuwe farmacodynamische methodes getest voor het monitoren van immunosuppressiva bij niertransplantatie patiënten, namelijk I) de fosfo-specifieke flowcytometrie assay en II) het meten van NFATc1 amplificatie. Het doel van dit onderzoek was om deze relatief nieuwe farmacodynamische methodes te testen en te bestuderen of deze potentie hebben voor het doseren van tacrolimus en eventuele andere immunosuppressiva. Daarnaast hebben we onderzocht of deze nieuwe testen gebruikt kunnen worden voor het monitoren van de effecten van immunosuppressieve geneesmiddelen op zowel cellen van het verworven immuunsysteem (T cellen en de verschillende subgroepen) als ook op cellen van het aangeboren immuunsysteem (monocyten).

Fosfo-specifieke flowcytometrie meet de fosforylering (activatie) van signaalmoleculen in een cel die een rol spelen bij het overbrengen van activatie signalen naar het DNA [28,30-32]. Immunosuppressiva zullen deze fosforylering beïnvloeden en met behulp van de assay kan worden bepaald in welke mate dit gebeurt [33]. Naast fosfo-specifieke flowcytometrie kan ook het meten van de hoeveelheid NFATc1, een signaalmolecuul uit de calcineurine route, een indicatie geven over de biologische effecten van tacrolimus. Tacrolimus remt deze route en de hypothese was dat de productie van NFATc1 in de celkern door dit geneesmiddel wordt geremd [34]. Deze en andere onderzoeksvragen zijn in de volgende hoofdstukken bestudeerd:



Figuur 1. T cel activatie signalen. Voor T cel activatie zijn 3 signalen nodig. (1) Het eerste signaal wordt gegeven via het HLA molecuul op de antigeen presenterende cel (bijvoorbeeld monocyt). Dit molecuul presenteert een antigeen aan de T cel receptor (TCR) op de T cel. (2) Het tweede signaal wordt ook wel co-stimulatie genoemd en wordt gegeven tussen onder andere het CD80 of CD86 molecuul op de antigeen presenterende cel en het CD28 molecuul op de T cel. (3) Het derde signaal bestaat uit het binden van cytokines (onder andere geproduceerd door de geactiveerde T cel) aan een specifieke cytokine receptor. Hierdoor wordt het activatie signaal versterkt. Zodra signaal 1 en 2 door de T cel zijn ontvangen zal er een binnenin de cel een signaal worden doorgegeven richting de celkern. Dit gebeurt door middel van signaalmoleculen. Geactiveerde signaalmoleculen zijn gefosforyleerd, dat wil zeggen dat er een fosfaatgroep aan het molecuul is gebonden. De mate van fosforylering correleert met de mate van signaalmolecuul activatie. Voorbeelden van signaalmoleculen zijn p38MAPK, ERK, Akt en NFATc1. Zodra het signaal het DNA in de celkern bereikt, vindt er productie van signaalstoffen (cytokines) plaats. Deze vormen signaal 3. Tacrolimus is een calcineurine-remmer. Hierdoor kan calcineurine het signaalmolecuul NFATc1 niet langer activeren en wordt er geen activatie signaal doorgegeven naar de celkern. Dit leidt tot een remming van T cel activatie. Belatacept is immunosuppressivum met een ander werkingsmechanisme. Het is een alternatief voor tacrolimus en remt het co-stimulatie signaal tussen het CD80/86 molecuul en het CD28 molecuul.

In **hoofdstuk 2** wordt de rol beschreven van monocyten in de afweerreactie na transplantatie, alsmede wat de effecten zijn van de meest voorgeschreven immunosuppressiva op deze cellen. Daarnaast wordt er een overzicht gegeven van de effecten van andere immunosuppressiva die (nog) niet worden gebruikt in de transplantatie-kliniek. Literatuuronderzoek laat zien dat tacrolimus, MMF en mTOR remmers slechts een beperkt effect hebben op de functie van monocyten, terwijl medicaties zoals canakinumab, tocilizimab en infliximab wel in staat zijn om de functies van monocyten effectief te remmen. Desondanks worden deze medicijnen nog niet gebruikt na transplantatie. Het is daarom belangrijk dat in nieuw pre-klinisch onderzoek wordt onderzocht of deze afweerremmende geneesmiddelen ook transplantaat-afstoting kunnen tegen gaan. Daarnaast is het belangrijk dat eventuele nieuwe afweerremmende therapieën ook een remmend effect hebben op monocyten.

In **hoofdstuk 3** hebben we bestudeerd wat de individuele effecten zijn van tacrolimus en MMF op activering van verschillende signaalmoleculen in de monocyt. Met behulp van fosfo-specifieke flowcytometrie en bloedmonsters van gezonde vrijwilligers werd de fosforylering van de signaalmoleculen p38MAPK, ERK en Akt bestudeerd. Beide geneesmiddelen blijken slechts een beperkt effect te hebben op de fosforylering (activatie) van deze moleculen. Daarnaast werden ook de functies van monocyten, zoals cytokineproductie en fagocytose, nauwelijks geremd.

In **hoofdstuk 4** hebben we verder onderzocht wat de farmacodynamische effecten zijn van een op tacrolimus gebaseerde immunosuppressieve combinatie-therapie op monocyt activatie. Fosfo-specifieke flowcytometrie werd gebruikt om de fosforylering van p38MAPK, ERK en Akt te meten in bloedmonsters van niertransplantatiepatiënten. Dit liet niet alleen zien dat de techniek een grote potentie heeft om gebruikt te worden voor het meten van de biologische effecten van een op tacrolimus gebaseerde therapie op monocyten, maar ook dat deze therapie slechts ten dele de activatie van monocyten in patiënten remt.

In **hoofdstuk 5** hebben we de farmacodynamische effecten van tacrolimus en andere immunosuppressiva getest door middel van het meten van NFATc1 amplificatie in T cellen. Tacrolimus kan deze amplificatie remmen, maar niet in alle T cel typen. Alleen $CD4^+$ en $CD8^+$ T cellen die het co-stimulatie molecuul CD28 op hun celoppervlak tot expressie brengen toonden een afgenomen amplificatie van NFATc1. Als negatieve controle werden bloedmonsters gebruikt van patiënten die behandeld werden met belatacept, het geneesmiddel dat geen direct effect heeft op signaaltransductieroutes in T cellen. Zoals verwacht, liet belatacept geen effect zien op NFATc1 amplificatie. De gevonden effecten berusten dus op de werking van tacrolimus en niet zozeer op die van de andere geneesmiddelen die met tacrolimus werden gecombineerd (MMF en prednisolon).

In **hoofdstuk 6** hebben we de fosfo-specifieke flowcytometrie assay verder uitgetest in T cellen en bestudeerd of de assay een verschil kon detecteren in de mate van immunosuppressie in patiënten die werden behandeld met de tweemaal-daagse of de eenmaal-daagse formulering van tacrolimus. De eenmaal-daagse tacrolimus formulering is ontwikkeld om de therapietrouw van patiënten te vergroten. Echter, wanneer een pati-

ent 1:1 wordt geconverteerd van de tweemaal-daagse naar de eenmaal-daagse variant zal dit gemiddeld leiden tot een daling van de tacrolimus dal-concentratie van zo'n 15%. Uit de studie blijkt dat, ondanks dat er geen significant verschil werd gevonden in tacrolimus dal-concentratie voor en na conversie, de fosforylering van p38MAPK was verhoogd in T cellen tijdens het gebruik van de eenmaal-daagse tacrolimus formulering. Dit betekent dat T cellen meer geactiveerd zijn tijdens het gebruik van de eenmaal-daagse formulering dan bij de tweemaal-daagse en dat dit waarschijnlijk berust op een verminderde blootstelling aan tacrolimus die niet wordt gedetecteerd met behulp van een dal-concentratie. Dit suggereert dat fosfo-specifieke flowcytometrie een gevoelige methode is voor het monitoren van minimale verschillen in de blootstelling aan tacrolimus. Wij zijn van mening dat er verder moet worden onderzocht of de assay een toegevoegde waarde heeft in de transplantatie diagnostiek.

In **hoofdstuk 7** hebben we met behulp van de fosfo-specifieke flowcytometrie assay de farmacodynamische effecten in T cellen vergeleken tussen een op tacrolimus gebaseerde en een op belatacept gebaseerde (negatieve controle) therapie. Bloedmonsters van niertransplantatie-patiënten werden afgenomen voor en gedurende het eerste jaar na transplantatie. De expressie van gefosforyleerd p38MAPK, ERK en Akt in verschillende T cel populaties werd vervolgens gemeten. De fosforylering van p38MAPK en Akt was geremd in zowel $CD4^+$ als in $CD8^+$ T cellen wanneer patiënten werden behandeld met een op tacrolimus gebaseerde therapie. Deze verschillen werden niet gevonden wanneer patiënten werden behandeld met een op belatacept gebaseerde therapie, hetgeen suggereert dat de gevonden effecten veroorzaakt werden door het gebruik van tacrolimus. Ook hadden beide therapieën geen effect op de fosforylering van ERK, wat erop duidt dat de T cellen nog steeds geactiveerd kunnen worden via dit signaalmolecuul. Tot slot bleek de fosforylering van dit molecuul verhoogd in patiënten die, ondanks dat zij belatacept kregen, een afstotingsreactie doormaakten. Dit alles bij elkaar toont aan dat de fosfo-specifieke flowcytometrie assay een veelbelovende methode is voor het monitoren van tacrolimus.

In **hoofdstuk 8** wordt het gebruik van het immunosuppressivum tofacitinib na niertransplantatie besproken. Dit is een afweerremmend medicijn dat de JAK-STAT signaaltransductieroute in T cellen remt. Deze signaaltransductieroute is vooral belangrijk voor het doorgeven van het derde T cel activatie-signaal: het binden van cytokines (ontstekingseiwitten) aan de oppervlakte-receptoren op T cellen. Tofacitinib is getest als vervanging voor calcineurine-remmers in verschillende klinische studies. Het gebruik van tofacitinib werd geassocieerd met een verhoogde incidentie van virale infecties. Dit wordt waarschijnlijk verklaard door het feit dat de patiënten in deze studies relatief hoge doseringen tofacitinib kregen voorgeschreven. Desondanks werd besloten om tofacitinib niet verder voor transplantatie doeleinden te ontwikkelen. Het gebruik van tofacitinib zou mogelijk veiliger kunnen worden wanneer een combinatie van farmacodynamische en farmacokinetische assays zou worden gebruikt om dit geneesmiddel te monitoren. Er zullen daarom nieuwe studies moeten worden opgezet met tofacitinib waarbij rekening wordt gehouden met

gepersonaliseerde medicijndosering, een verbeterde immuun monitoring en een betere selectie van patiënten.

Uit het onderzoek zoals beschreven in dit proefschrift blijkt dat het meten van de fosforylering van intracellulaire signaalmoleculen veel voordelen met zich meebrengt. Zo is de tijd die nodig is voor deze meting relatief kort, kan de assay worden uitgevoerd in volbloedmonsters van patiënten en kan de fosforylering van signaalmoleculen per individueel cel type worden bepaald [28,35]. Ook kunnen de biologische effecten van immunosuppressiva op zowel het verworven immuunsysteem (T cellen, **hoofdstuk 6 en 7**) als op het aangeboren immuunsysteem (monocyten, **hoofdstuk 3 en 4**) worden onderzocht. De fosfo-specifieke flowcytometrie assay laat een duidelijke remming zien van p38MAPK en Akt fosforylering in T cellen wanneer deze met tacrolimus zijn behandeld. Ook laat deze assay de remmende effecten van tacrolimus in verschillende T cel typen zien. $CD8^+CD28^-$ T cellen staan er bekend om dat zij snel en agressief op een lichaamsvreemd antigeen kunnen reageren [36]. Deze cellen spelen waarschijnlijk een sleutelrol in het ontstaan van afstoting na transplantatie, doordat de signaaltransductieroutes in deze cellen nog steeds actief zijn ondanks een behandeling met tacrolimus of belatacept. Het is daarom van belang dat de eventuele activatie van deze cellen na transplantatie goed wordt vervolgd. Van eventuele nieuwe immunosuppressieve therapieën zal moeten worden onderzocht of deze ook een voldoende remmende werking hebben op $CD8^+CD28^-$ T cellen.

Het gebruik van tacrolimus leidt ook tot een remming van zowel p38MAPK als Akt fosforylering in monocyten. Dit effect van tacrolimus is echter niet volledig en de signaaltransductieroutes worden niet volledig geremd in monocyten. Ook de functies van monocyten wordt niet volledig geremd. Hierdoor bestaat de kans dat monocyten, ondanks het gebruik van immunosuppressiva, nog steeds verantwoordelijk kunnen zijn voor een afweerreactie na transplantatie. Ons onderzoek toont ook aan dat de assay gebruikt kan worden voor het meten van de biologische effecten van tacrolimus op zowel het verworven immuunsysteem (T cellen) als op het aangeboren immuunsysteem (monocyten). Een assay die dit kan meten bestond tot op heden nog niet, maar is wel nodig om de klinische resultaten na transplantatie te verbeteren [37]. Daarnaast is fosfo-specifieke flowcytometrie zeer geschikt als methode voor geneesmiddelenbewaking, omdat het de biologische effecten van immunosuppressiva op de signaaltransductieroutes meer proximaal detecteert dan wanneer eindproducten, zoals cytokines, worden gemeten.

Het meten van NFATc1 amplificatie is een tweede methode die geschikt lijkt te zijn voor het monitoren van de biologische effecten van tacrolimus op verschillende T cel populaties (**hoofdstuk 5**). Deze assay is nog niet getest voor het monitoren van de effecten van immunosuppressieve therapie op monocyten, ook al brengen deze cellen NFATc1 tot expressie [38]. De assay vertoont een correlatie met de bloedconcentraties van tacrolimus en kan, net als fosfo-specifieke flowcytometrie, ook gemeten worden in bloedmonsters van

patiënten. Het kost alleen wel 7 uur om de test procedure te doorlopen en bovendien is de techniek nog niet gevalideerd voor het gebruik in de kliniek.

Andere farmacodynamische assays die de afgelopen jaren zijn bestudeerd, hebben vooralsnog geen toepassing in de kliniek gevonden [39]. Voorbeelden hiervan zijn het meten van de calcineurine activiteit, interleukine-2 productie en NFAT-gereguleerde gen expressie. Tot nu toe zijn er voor deze assays nog geen sterke correlaties gevonden met de bloedconcentraties van de gebruikte calcineurine-remmers [40]. Ook ontbreekt er een gestandaardiseerd protocol voor deze assays, waardoor resultaten uit verschillende medische centra lastig vergeleken kunnen worden. De uitslagen van deze assays kunnen bovendien worden beïnvloed door andere factoren, zoals infecties en het gebruik van andere immunosuppressiva [41]. De fosfo-specifieke flowcytometrie en NFATc1 amplificatie assay laten wel een correlatie zien met tacrolimus bloedconcentraties en lijken dus meer potentie te hebben om in de kliniek toepassing te vinden.

Een beperking is echter wel dat voor zowel de fosfo-specifieke flowcytometrie en NFATc1 amplificatie assays als voor de andere farmacodynamische assays geldt dat zij tot op heden alleen zijn bestudeerd in kleine studiepopulaties en in individuele transplantatiecentra. Voor vervolgstudies is het belangrijk dat er grotere studiepopulaties worden geselecteerd, bij voorkeur afkomstig uit verschillende medische centra [40-42]. Ook zal een dergelijk groter patiënt cohort gebruikt kunnen worden om drempelwaardes te bepalen voor de fosfo-specifieke en NFATc1 amplificatie assays, zodat duidelijker wordt wanneer de dosis van het geneesmiddel moet worden aangepast [43]. Wellicht dat de uitslagen van beide assays beter correleren met de klassieke farmacokinetische monitoring wanneer de expositie aan tacrolimus wordt gemeten middels de oppervlakte onder de concentratie-tijd-curve in plaats van de dal-concentratie.

Wanneer de assays gevalideerd zijn, kunnen zij als farmacodynamische test gecombineerd worden met de huidige farmacokinetische methode. Meerdere signaalmoleculen zullen daarbij gemeten moeten worden, omdat één enkel signaalmolecuul niet alle therapeutische effecten van immunosuppressiva kan weergeven. De beide assays zijn alleen geschikt voor het monitoren van tacrolimus, omdat er geen correlaties zijn gevonden tussen de uitkomsten van deze assays en het gebruik van andere immunosuppressiva. Voor een optimale monitoring van geneesmiddelen zou ook een alternatieve farmacokinetische assay gebruikt kunnen worden, zoals het meten van de intra-lymfocytaire tacrolimus concentratie [44-46].

Daarnaast zou een groter patiënten-cohort een tweede toepassing van de fosfo-specifieke en NFATc1 amplificatie assay kunnen valideren: het voorspellen van klinische uitkomsten na transplantatie, zoals acute afstoting [39,47]. Daarbij moet vooral worden gelet op de fosforylering van ERK. In T cellen is de fosforylering van ERK geassocieerd met afstoting na transplantatie, maar immunosuppressiva blijken weinig effect te hebben op ERK in monocyten.

Conclusies en aanbevelingen

- De huidige immunosuppressiva hebben slechts een beperkt effect op signaaltransductieroutes die leiden tot monocyt activatie. Er is derhalve behoefte aan nieuwe afweerremmende medicijnen die aangrijpen op monocyten.
- Fosfo-specifieke flowcytometrie is een veelbelovende farmacodynamische methode voor het monitoren van tacrolimus therapie in niertransplantatiepatiënten en kan zowel voor T cellen als monocyten worden gebruikt.
- Het meten van NFATc1 amplificatie is een specifieke volbloed test voor het monitoren van de biologische effecten van tacrolimus in T cellen van niertransplantatie patiënten.
- De fosforylering van ERK in zowel monocyten als T cellen is een veelbelovende marker voor de risico-inschatting van afstoting na transplantatie.
- De gebruikelijke farmacokinetische manier van tacrolimus monitoren kan het beste worden gecombineerd met de farmacodynamische fosfo-specifieke flowcytometrie, zodat het aanpassen van de tacrolimus dosis kan worden geoptimaliseerd.
- De fosfo-specifieke flowcytometrie assay en de NFATc1 amplificatie assay kunnen beiden de activatie van signaalmoleculen in de verschillende $CD4^+CD28^+$, $CD8^+CD28^+$ en $CD8^+CD28^-$ T cel groepen meten.
- Het farmacodynamisch monitoren van immunosuppressieve therapie na transplantatie kan de effectiviteit en de veiligheid van (nieuwe) immunosuppressiva, zoals de JAK-remmer tofacitinib verbeteren.
- Fosfo-specifieke flowcytometrie is een relatief nieuwe methode om de effectiviteit en de veiligheid van (nieuwe) immunosuppressiva, zoals de JAK-remmer tofacitinib, te beoordelen.

Literatuur

1. Heldal K, Hartmann A, Grootendorst DC, et al. Benefit of kidney transplantation beyond 70 years of age. Nephrol Dial Transplant 2010;25:1680-7.
2. Laupacis A, Keown P, Pus N, et al. A study of the quality of life and cost-utility of renal transplantation. Kidney Int 1996;50:235-42.
3. Wong G, Howard K, Chapman JR, et al. Comparative survival and economic benefits of deceased donor kidney transplantation and dialysis in people with varying ages and comorbidities. PLoS One 2012;7:e29591.
4. Farrar CA, Kupiec-Weglinski JW, Sacks SH. The innate immune system and transplantation. Cold Spring Harb Perspect Med 2013;3:a015479.
5. LaRosa DF, Rahman AH, Turka LA. The innate immune system in allograft rejection and tolerance. J Immunol 2007;178:7503-9.
6. Wood KJ, Goto R. Mechanisms of rejection: current perspectives. Transplantation 2012;93:1-10.
7. Nankivell BJ, Alexander SI. Rejection of the kidney allograft. N Engl J Med 2010;363:1451-62.
8. Sellares J, de Freitas DG, Mengel M, et al. Understanding the causes of kidney transplant failure: the dominant role of antibody-mediated rejection and nonadherence. Am J Transplant 2012;12:388-99.
9. Kim MG, Kim YJ, Kwon HY, et al. Outcomes of combination therapy for chronic antibody-mediated rejection in renal transplantation. Nephrology (Carlton) 2013;18:820-6.
10. Esposito P, Grosjean F, Rampino T, et al. Costimulatory pathways in kidney transplantation: pathogenetic role, clinical significance and new therapeutic opportunities. Int Rev Immunol 2014;33:212-33.
11. Halloran PF. Immunosuppressive drugs for kidney transplantation. N Engl J Med 2004;351:2715-29.
12. Zuidema MY, Zhang C. Ischemia/reperfusion injury: The role of immune cells. World J Cardiol 2010;2:325-32.
13. Wasowska BA. Mechanisms involved in antibody- and complement-mediated allograft rejection. Immunol Res 2010;47:25-44.
14. Rogacev KS, Zawada AM, Hundsdorfer J, et al. Immunosuppression and monocyte subsets. Nephrol Dial Transplant 2015;30:143-53.
15. Vereyken EJ, Kraaij MD, Baan CC, et al. A shift towards pro-inflammatory CD16+ monocyte subsets with preserved cytokine production potential after kidney transplantation. PLoS One 2013;8:e70152.
16. Sekerkova A, Krepsova E, Brabcova E, et al. CD14+CD16+ and CD14+CD163+ monocyte subpopulations in kidney allograft transplantation. BMC Immunol 2014;15:4.
17. Taylor AL, Watson CJ, Bradley JA. Immunosuppressive agents in solid organ transplantation: Mechanisms of action and therapeutic efficacy. Crit Rev Oncol Hematol 2005;56:23-46.

18. Matas AJ, Smith JM, Skeans MA, et al. OPTN/SRTR 2011 Annual Data Report: kidney. Am J Transplant 2013;13 Suppl 1:11-46.
19. Thomson AW, Bonham CA, Zeevi A. Mode of action of tacrolimus (FK506): molecular and cellular mechanisms. Ther Drug Monit 1995;17:584-91.
20. Bouamar R, Shuker N, Hesselink DA, et al. Tacrolimus predose concentrations do not predict the risk of acute rejection after renal transplantation: a pooled analysis from three randomized-controlled clinical trials(dagger). Am J Transplant 2013;13:1253-61.
21. Shuker N, van Gelder T, Hesselink DA. Intra-patient variability in tacrolimus exposure: causes, consequences for clinical management. Transplant Rev (Orlando) 2015;29:78-84.
22. Larsen CP, Pearson TC, Adams AB, et al. Rational development of LEA29Y (belatacept), a high-affinity variant of CTLA4-Ig with potent immunosuppressive properties. Am J Transplant 2005;5:443-53.
23. Schiff J, Cole E, Cantarovich M. Therapeutic monitoring of calcineurin inhibitors for the nephrologist. Clin J Am Soc Nephrol 2007;2:374-84.
24. Meier-Kriesche HU, Schold JD, Srinivas TR, Kaplan B. Lack of improvement in renal allograft survival despite a marked decrease in acute rejection rates over the most recent era. Am J Transplant 2004;4:378-83.
25. Tang IY, Meier-Kriesche HU, Kaplan B. Immunosuppressive strategies to improve outcomes of kidney transplantation. Semin Nephrol 2007;27:377-92.
26. Malvezzi P, Rostaing L. The safety of calcineurin inhibitors for kidney-transplant patients. Expert Opin Drug Saf 2015;14:1531-46.
27. Dieterlen MT, Eberhardt K, Tarnok A, Bittner HB, Barten MJ. Flow cytometry-based pharmacodynamic monitoring after organ transplantation. Methods Cell Biol 2011;103:267-84.
28. Baan C, Bouvy A, Vafadari R, Weimar W. Phospho-specific flow cytometry for pharmacodynamic monitoring of immunosuppressive therapy in transplantation. Transplant Res 2012;1:20.
29. Noceti OM, Woillard JB, Boumediene A, et al. Tacrolimus pharmacodynamics and pharmacogenetics along the calcineurin pathway in human lymphocytes. Clin Chem 2014;60:1336-45.
30. Maguire O, Tario JD, Jr., Shanahan TC, Wallace PK, Minderman H. Flow cytometry and solid organ transplantation: a perfect match. Immunol Invest 2014;43:756-74.
31. Landskron J, Tasken K. Phosphoprotein Detection by High-Throughput Flow Cytometry. Methods Mol Biol 2016;1355:275-90.
32. Krutzik PO, Nolan GP. Intracellular phospho-protein staining techniques for flow cytometry: monitoring single cell signaling events. Cytometry A 2003;55:61-70.
33. Nakayama T, Yamashita M. The TCR-mediated signaling pathways that control the direction of helper T cell differentiation. Semin Immunol 2010;22:303-9.
34. Macian F. NFAT proteins: key regulators of T-cell development and function. Nat Rev Immunol 2005;5:472-84.

35. Krutzik PO, Trejo A, Schulz KR, Nolan GP. Phospho flow cytometry methods for the analysis of kinase signaling in cell lines and primary human blood samples. Methods Mol Biol 2011;699:179-202.

36. Mou D, Espinosa J, Lo DJ, Kirk AD. CD28 negative T cells: is their loss our gain? Am J Transplant 2014;14:2460-6.

37. Fernandez-Ruiz M, Kumar D, Humar A. Clinical immune-monitoring strategies for predicting infection risk in solid organ transplantation. Clin Transl Immunology 2014;3:e12.

38. Komano Y, Nanki T, Hayashida K, Taniguchi K, Miyasaka N. Identification of a human peripheral blood monocyte subset that differentiates into osteoclasts. Arthritis Res Ther 2006;8:R152.

39. Sommerer C, Giese T, Meuer S, Zeier M. Pharmacodynamic monitoring of calcineurin inhibitor therapy: is there a clinical benefit? Nephrol Dial Transplant 2009;24:21-7.

40. Caruso R, Perico N, Cattaneo D, et al. Whole-blood calcineurin activity is not predicted by cyclosporine blood concentration in renal transplant recipients. Clin Chem 2001;47:1679-87.

41. Brunet M, Shipkova M, van Gelder T, et al. Barcelona Consensus on Biomarker-Based Immunosuppressive Drugs Management in Solid Organ Transplantation. Ther Drug Monit 2016;38 Suppl 1:S1-20.

42. Sanquer S, Amrein C, Grenet D, et al. Expression of calcineurin activity after lung transplantation: a 2-year follow-up. PLoS One 2013;8:e59634.

43. Beland MA, Lapointe I, Noel R, et al. Higher calcineurin inhibitor levels predict better kidney graft survival in patients with de novo donor-specific anti-HLA antibodies: a cohort study. Transpl Int 2017;30:502-9.

44. Capron A, Haufroid V, Wallemacq P. Intra-cellular immunosuppressive drugs monitoring: A step forward towards better therapeutic efficacy after organ transplantation? Pharmacol Res 2016;111:610-8.

45. Han SS, Yang SH, Kim MC, et al. Monitoring the Intracellular Tacrolimus Concentration in Kidney Transplant Recipients with Stable Graft Function. PLoS One 2016;11:e0153491.

46. Lemaitre F, Blanchet B, Latournerie M, et al. Pharmacokinetics and pharmacodynamics of tacrolimus in liver transplant recipients: inside the white blood cells. Clin Biochem 2015;48:406-11.

47. Staatz CE, Tett SE. Clinical pharmacokinetics and pharmacodynamics of tacrolimus in solid organ transplantation. Clin Pharmacokinet 2004;43:623-53.

Appendices

Curriculum Vitae Auctoris

PhD portfolio

List of Publications

Abbreviations

Acknowledgements

Curriculum Vitae Auctoris

Nynke Marise Kannegieter was born on July 10th 1990 in Zwijndrecht, the Netherlands. From 2002 to 2008 she attended the VWO at Walburg College in Zwijndrecht, the Netherlands. In September 2008 she started studying Bio-Pharmaceutical Sciences at Leiden University and obtained her master's degree in August 2013. In January 2014 she started with her PhD project at the Transplantation Laboratory of the Internal Medicine Department, Division of Nephrology and Transplantation, at the Erasmus Medical Center, under the supervision of prof. dr. Carla Baan and dr. Dennis Hesselink. In this project she investigated new pharmacodynamic assays for monitoring signaling pathways after transplantation. This thesis is a representation of this research. At this moment she lives with Mark Buitendijk in Hendrik-Ido-Ambacht.

PhD Portfolio

Name PhD student	Nynke Marise Kannegieter
Erasmus MC department	Internal Medicine, section Nephrology and Transplantation
Research school	Postgraduate School Molecular Medicine
PhD period	January 2014 – December 2017
Promotor	Prof.dr. C.C. Baan
Co-promotor	Dr. D.A. Hesselink

General courses and workshops

2014	Hesperis Course (ESOT)
2014	Biomedical English Writing course (Molmed)
2015	Advanced Immunology (Molmed)
2015	Biostatistical Methods I: Basic Principles (Nihes)
2015	Research Integrity Course (Erasmus MC)
2017	Adobe InDesign Course (Molmed)

(Inter)national conferences and presentations

2014	Annual meeting Dutch Transplantation Society (NTV Bootcongres), Leiden, the Netherlands	
2014	Molecular Medicine Day, Rotterdam, the Netherlands	
2014	Dutch Society for Immunology Congress (NVVI), Kaatsheuvel, the Netherlands	Poster
2015	Science Days, Antwerp Belgium	
2015	Molecular Medicine Day, Rotterdam the Netherlands	Pitch presentation + Poster
2015	European Society for Organ Transplantation congress (ESOT), Brussels, Belgium	Oral presentation + Poster
2015	International Congress of Therapeutic Drug Monitoring & Clinical Toxicology (IATDMCT), Rotterdam, the Netherlands	Oral presentation
2016	Science Days, Antwerp, Belgium	Poster
2016	Molecular Medicine Day, Rotterdam, the Netherlands	Oral presentation
2016	Annual meeting Dutch Transplantation Society (NTV Bootcongres), Groningen, the Netherlands	Poster
2016	The Transplantation Society congress (TTS), Hong Kong, China	Oral presentation
2017	Science Days, Antwerp Belgium	Poster

2017	Molecular Medicine Day, Rotterdam, the Netherlands	Poster
2017	Annual meeting Dutch Transplantation Society (NTV Bootcongres), Zeist, the Netherlands	2x Poster
2017	European Society for Organ Transplantation congress (ESOT), Barcelona Spain	Brief Presentation + Poster

Seminars and workshops

2014-2017	Journal club, Department of Internal Medicine, Erasmus MC, Rotterdam
2014	New kids on the block meeting, Nierstichting/Academisch Medisch Centrum, Amsterdam
2016	Young professionals day, Nederlandse Transplantatie Vereniging, Utrecht
2017	Vesicles symposium, Department of Internal Medicine, Erasmus MC, Rotterdam
2017	Monocyte symposium, Erasmus MC, Rotterdam

Travel grants

2015	Travel grant Vereniging Trustfonds, Erasmus Universiteit Rotterdam
2016	Travel grant Vereniging Trustfonds, Erasmus Universiteit Rotterdam
2016	Scholingsbeurs Nederlandse Transplantatie Vereniging
2017	Travel grant Vereniging Trustfonds, Erasmus Universiteit Rotterdam

Teaching activities

2014 – 2017	1 day per year, supervision and teaching students of the master Infection and Immunity
2015	Counseling and teaching a Master student from Aarhus University, Denmark in labtechniques and setting up a study protocol

Memberships

2014 – present	Nederlandse Transplantatie Vereniging (NTV)
2014 – present	The European Society of Organ Transplantation (ESOT)
2014 – 2016	Nederlandse Vereniging voor Immunologie (NVVI)

List of publications

ter Braak B, Siezen CL, **Kannegieter N**, Koedoot E, van de Water B, van der Laan JW. Classifying the adverse mitogenic mode of action of insulin analogues using a novel mechanism-based genetically engineered human breast cancer cell panel. *Archives of Toxicology* 2014 Apr; 88(4):953-966

Kannegieter NM*, Shuker N*, Vafadari R, Weimar W, Hesselink DA, Baan CC. Conversion to Once-Daily Tacrolimus Results in Increased p38MAPK Phosphorylation in T Lymphocytes of Kidney Transplant Recipients. *Therapeutic Drug Monitoring* 2016 Apr; 38(2):280-284
* Both authors equally share first authorship

Doedée AM, **Kannegieter N**, Öztürk K, van Loveren H, Janssen R, Buisman AM. Higher numbers of memory B-cells and Th2-cytokine skewing in high responders to hepatitis B vaccination. *Vaccine* 2016 Apr 27; 34(19):2281-2289

Baan CC, **Kannegieter NM**, Felipe CR, Tedesco Silva H Jr. Targeting JAK/STAT Signaling to Prevent Rejection After Kidney Transplantation: A Reappraisal. *Transplantation* 2016 Sep; 100(9):1833-1839

Kannegieter NM, Hesselink DA, Dieterich M, Kraaijeveld R, Rowshani AT, Leenen PJ, Baan CC. The Effect of Tacrolimus and Mycophenolic Acid on CD14+ Monocyte Activation and Function. *PLoS One* 2017 Jan 25; 12(1):e0170806

Kannegieter NM*, van den Bosch TP*, Hesselink DA, Baan CC, Rowshani AT. Targeting the Monocyte-Macrophage Lineage in Solid Organ Transplantation. *Frontiers in Immunology* 2017 Feb 16; 8:153
* Both authors equally share first authorship

Kannegieter NM, Hesselink DA, Dieterich M, de Graav GN, Kraaijeveld R, Rowshani AT, Leenen PJM, Baan CC. Pharmacodynamic Monitoring of Tacrolimus-Based Immunosuppression in CD14+ Monocytes After Kidney Transplantation. *Therapeutic Drug Monitoring* 2017 Oct; 39(5):463-471

Huang L, Litjens NHR, **Kannegieter NM**, Klepper M, Baan CC, Betjes MGH. pERK-dependent defective TCR-mediated activation of CD4+ T cells in end-stage renal disease patients. *Immunity and Ageing* 2017 Jun 19; 14:14

Kannegieter NM, Hesselink DA, Dieterich M, de Graav GN, Kraaijeveld R, Baan CC. Differential T Cell Signaling Pathway Activation by Tacrolimus and Belatacept after Kidney

Transplantation: Post Hoc Analysis of a Randomised-Controlled Trial. *Scientific Reports* 2017 Nov 9; 7(1):15135

Manuscript submitted or under review

Kannegieter NM, Hesselink DA, Dieterich M, de Graav GN, Kraaijeveld R, Baan CC. Analysis of NFATc1 amplification in T Cells for pharmacodynamic monitoring of tacrolimus in kidney transplant recipients. *Submitted*

Abbreviations

ABMR	Antibody-mediated rejection
ACMIA	Antibody-conjugated magnetic immunoassay
ACR	Acute cellular rejection
AF647	AlexaFluor 647
Akt	AKT8 virus oncogene cellular homolog
AME	Above median
APC	Antigen presenting cell
APC	Allophycocyanin
AUC	Area under the concentration ***versus*** time curve
BELA	Belatacept
BET	Bromodomain and extra-terminal motif
BID	Twice a day
BME	Below median
BPAR	Biopsy-proven acute rejection
BV	Brilliant violet
C_0	Pre-dose concentration
cABMR	Chronic antibody-mediated rejection
CD	Cluster of differentiation
CMV	Cytomegalovirus
CNI	Calcineurin inhibitor
CsA	Cyclosporin A
CSF1R	Colony stimulating factor 1 receptor
CTLA	Cytotoxic T-lymphocyte antigen
CV	Coefficient of variation
DAMP	Damage-associated molecular pattern
DC	Dendritic cell
DSA	Donor-specific antibodies
ERK	Extracellular signal-regulated kinase
ESRD	End-stage renal disease
FITC	Fluorescein isothiocyanate
GFR	Glomerular filtration rate
GRE	Glucocorticoid response element
GSK3	Glycogen synthase kinase 3
HLA	Human leucocyte antigen
ICAM	Intercellular adhesion molecule
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IMPDH	Inosine monophosphate dehydrogenase

IP_3	Inositol 1,4,5-trisphosphate
IRI	Ischemia-reperfusion injury
JAK	Janus activated kinase
JNK	c-Jun N-terminal kinase
LI	Less intensive
MAPK	Mitogen-activated protein kinase
M-CSF	Macrophage colony-stimulating factor
MCP	Monocyte chemotactic protein
MFI	Median fluorescence intensity
MHC	Major histocompatibility complex
MMF	Mycophenolate mofetil
MPA	Mycophenolic acid
Mreg	Regulatory macrophage
mTOR	Mammalian target of rapamycin
NFAT	Nuclear factor of activated T-cells
NFκB	Nucelar factor kappa-light-chain enhancer of activated B cells
NK	Natural killer
OD	Once a day
PBMC	Peripheral blood mononuclear cells
PD	Pharmacodynamic
PE	Phycoerythrin
PE-Cy7	Phycoerythrin-cyanine 7
PERCP	Peridinin-chlorophyll-protein
PK	Pharmacokinetic
PLC	Phospholipase C
PMA	Phorbol myristate acetate
PTLD	Posttransplant lymphoproliferative disease
RA	Rheumatoid arthritis
rATG	Rabbit anti-thymocyte globulin
r_s	Spearman correlation coefficient
SD	Standard deviation
SEB	Staphylococcal enterotoxin B
SOT	Solid organ transplantation
STAT	Signal transducer and activator of transcription
TAC	Tacrolimus
TAT	Tyrosine aminotransferase
TCR	T cell receptor
TDM	Therapeutic drug monitoring
Th	T helper cell
TLR	Toll-like receptor

TNF	Tumor necrosis factor
Treg	Regulatory T cell

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