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Pharmacodynamic Monitoring of Tacrolimus-based Immunosuppression in CD14$^+$ Monocytes after Kidney Transplantation

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Conflicts of interest

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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.

Key Words

CD14+ monocytes, immunosuppression, signaling pathways, therapeutic drug monitoring, transplantation

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 1. 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, respectively [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 see Table, Supplemental Digital Content 1, which represents the absolute monocyte counts and medication overview). 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 see Table, Supplemental Digital Content 1, which represents the absolute monocyte counts and medication overview). 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 (see Table, Supplemental Digital Content 1, which represents the absolute monocyte counts and medication overview).

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. (See Figure, Supplemental Digital Content 2A, depicting a typical gating example for the selection of $CD14^+$ monocytes and Figure, Supplemental Digital Content 2B, showing an example of p-p38MAPK, p-ERK, and p-Akt measurements on a log scale, in which each dot represents one monocyte.)

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 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 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. (See Figure, Supplemental Digital Content 3, showing the phosphorylation measurements for a patient diagnosed with a BPAR on day 152 after transplantation (red arrows))

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.

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) (see Figure, Supplemental Digital Content 4, which shows the correlation between p-p38MAPK and p-Akt and TAC pre-dose concentrations at day 4 after transplantation). 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 (see Table, Supplemental Digital Content 5, which shows the univariate analysis of the association between patient demographic characteristics and signaling protein phosphorylation). 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 (see Table, Supplemental Digital Content 5).

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

278 variability, respectively [44]. Furthermore, these pharmacodynamic assays were developed to
279 study the effect on T-cells and it is unknown whether these methods can also be used for
280 studying the effect of immunosuppressants on monocytes.

281 A correlation between reduction of p-p38MAPK levels in T-cells and TAC blood
282 concentrations after kidney transplantation was previously found with phospho-specific flow
283 cytometry [37, 51]. In the present study, $CD14^+$ monocytes from kidney transplant patients
284 showed a decrease in p-p38MAPK and p-Akt with a maximum of 36% and 20%, respectively, as
285 compared to pre-transplant levels. These results are in line with previous *in vitro* findings:
286 healthy control blood samples spiked with TAC showed a maximum p-p38MAPK and p-Akt
287 inhibition in monocytes of 33% and 14%, respectively [30]. In the *in vitro* study, spiking with
288 TAC did not affect the production of IL-1β or the phagocytosis by monocytes. Only a slight
289 change in monocyte differentiation toward an M2-like phenotype was measured in the presence
290 of high TAC concentrations [30]. In contrast to the reduction of p-p38MAPK and p-Akt,
291 phosphorylation of the other MAPK member, ERK, was only significantly reduced within the
292 first month after transplantation. The maximum decrease at day 4 and day 30 was 39%,
293 indicating a stronger reduction of p-ERK than for p-p38MAPK and p-Akt. Of note, the
294 expression of p-ERK after stimulation increased with time after transplantation in the one patient
295 suffering from an acute rejection, indicating a potential role for this signaling molecule in acute
296 rejection.

297 The decreased phosphorylation found for the signaling molecules indicates that the innate
298 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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Figure Legends

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

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

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

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.

Table II: Reduction of signaling molecule phosphorylation

% inhibition (mean ± SD)	**p38MAPK**		**ERK**		**Akt**	
	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

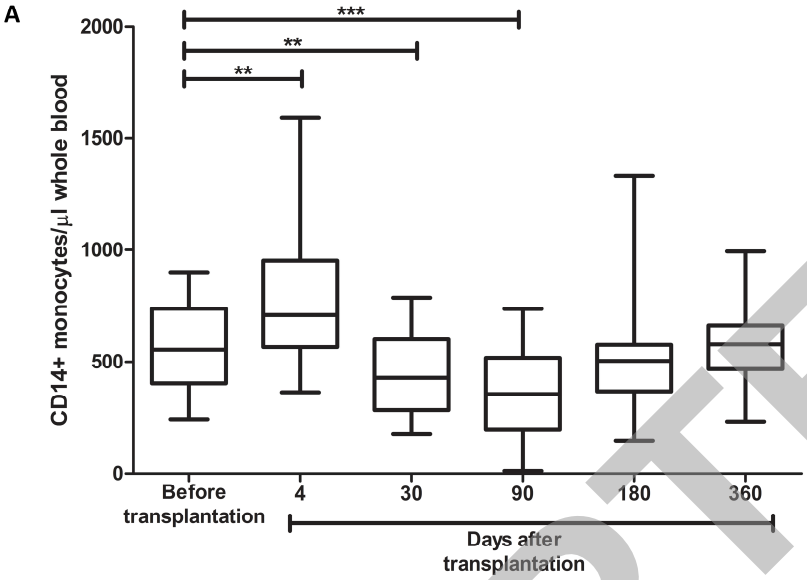
Significant difference with baseline phosphorylation: *) $p < 0.05$; **) $p < 0.01$; ***) $p < 0.001$

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

Correlation		p-p38MAPK		p-ERK		p-Akt	
		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

Figure 1



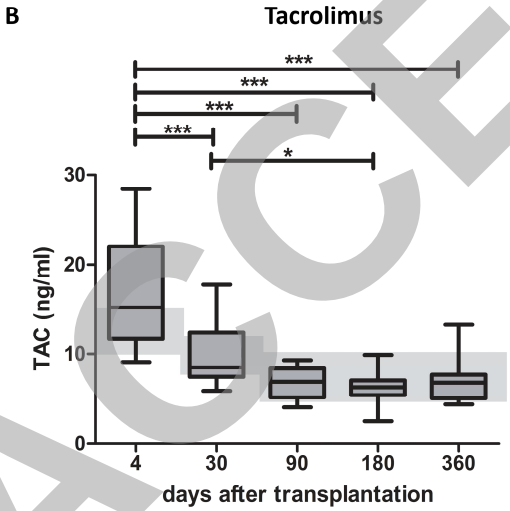


Figure 2

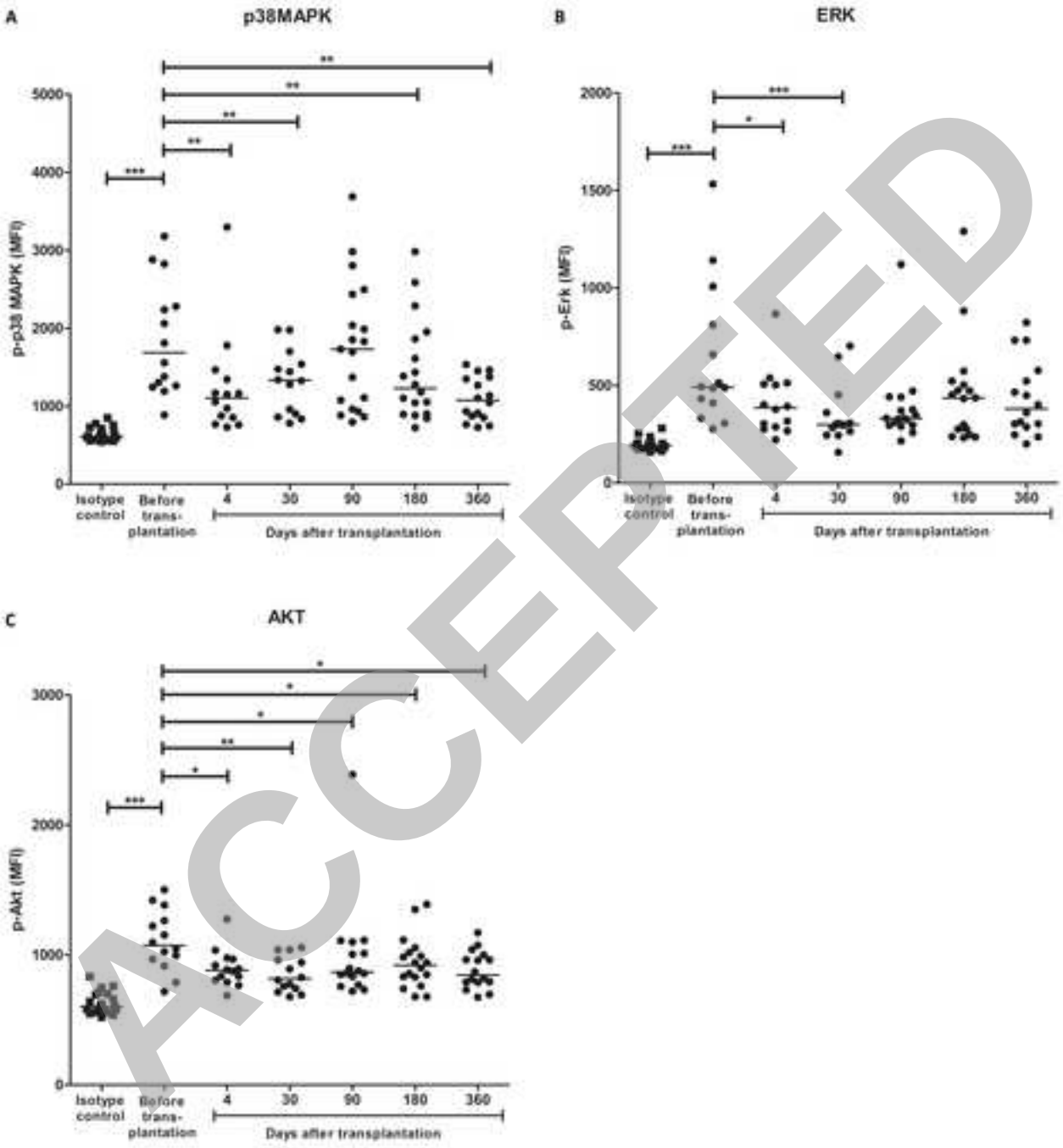


Figure 3