Article

Immunomonitoring of tacrolimus in healthy volunteers: the first step from PK- to PD-based therapeutic drug monitoring

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Abstract: In transplantation patients, therapeutic drug monitoring is routinely performed to maintain optimal tacrolimus concentrations. Nonetheless, toxicity and rejection still occur in patients within an acceptable concentration-range. To have a better understanding of the relationship between tacrolimus dose, tacrolimus concentration, and its effect on the target cell, we developed functional immune tests for the quantification of the tacrolimus effect. Twelve healthy volunteers received a single dose of tacrolimus, after which intracellular and whole blood tacrolimus concentrations were measured, and were related to T cell functionality. A significant correlation was found between tacrolimus concentrations in T cells and whole blood concentrations (r=0.71, p=0.009), while there was no correlation found for tacrolimus concentrations in PBMCs and whole blood (r=0.35, p=0.27). PHA-induced IL-2 and IFNγ production, and expression of CD71 and CD154 on T cells showed a clear inhibition at 1.5h post-dose, when tacrolimus levels were peaking. Moreover, the *in vitro* tacrolimus effect of these markers corresponded well to the *ex vivo* effect after dosing. In conclusion, our results show that intracellular tacrolimus concentrations mimic whole blood concentrations, and that PHA-induced cytokine production (IL-2 and IFNγ) and activation marker expression (CD71 and CD154) are suitable readout measures to measure the immunosuppressive effect of tacrolimus on the T cell.

Keywords: immunosuppressive drugs, transplantation, pharmacodynamics, pharmacokinetics, tacrolimus, therapeutic drug monitoring, immunomonitoring

1. Introduction

A combined regimen of tacrolimus, mycophenolate mofetil (MMF) and glucocorticoids is the standard treatment for kidney transplantation patients, preventing rejection of the transplanted organ. Although this therapy results in an excellent survival rate (>90%) in the first year after transplantation, long-term clinical outcome remains poor [1, 2]. Calcineurin inhibitors (CNIs), like tacrolimus, suffer from large intra- and interpatient variability in pharmacodynamics (PD) activity, complicating optimization of an individual dosing strategy [3]. Too little exposure leads to a risk of acute organ rejection and formation of donor-specific antibodies, while too much exposure leads to an increased risk of infection and toxicity.

The calcineurin inhibitor tacrolimus (FK506) is used after allogeneic organ transplantation mainly, but also in other T cell-mediated diseases such as eczema and psoriasis. Therapeutic drug

monitoring (TDM) of pre-dose trough levels (C_0) in whole blood is routinely performed in kidney transplantation patients to maintain an optimally effective tacrolimus concentration, and to minimize the risk of overexposure. Nonetheless, toxicity and rejection still occur in patients with an C_0-range within target, indicating that the relationship between C_0 measurements and the occurrence of rejection or tacrolimus-related toxicity is debatable [4, 5]. Intracellular drug concentrations in PBMCs have been studied as an alternative measure for TDM, but also for this pharmacokinetic (PK) parameter the correlation with clinical outcome remains suboptimal [6].

Tacrolimus exerts its function by forming a complex with the intracellular FK506 binding protein (FKBP12). The complex binds and inhibits calcineurin phosphatase activity, which causes a reduction in NFAT (nuclear factor of activated T cells) mediated pro-inflammatory gene expression, such as interleukin 2 (IL-2) and interferon gamma (IFNγ) [7]. The quantitative relationship between tacrolimus concentration and effect on T cell functionality has been studied extensively, also in primary human cells [8-11]. However, the effect of tacrolimus in fresh human whole blood samples has been studied incompletely. Since the availability of a whole blood-based PD assay could be the missing link in TDM for tacrolimus, this is a shortcoming. A better understanding of the relationship between tacrolimus dose, whole blood tacrolimus concentration, concentration in the target cell, and effect on target cell functionality could enable a PD- rather than PK-based approach for future TDM of tacrolimus.

In the current study we aimed to assess these relationships. We developed functional immune tests for quantification of tacrolimus effects in human whole blood samples. In an open label study, healthy volunteers received a single dose of tacrolimus, after which tacrolimus concentrations were measured in blood and isolated cells (PBMCs and T cells), and were related to T cell functionality (*ex vivo* cytokine production and flow cytometry-based cell activation). This study may be a first step in the identification of functional PD readout measures for future immunomonitoring of transplantation patients, allowing adjustment of treatment regimens according to the needs of individual patients.

2. Results

2.1. Subject characteristics

Twelve healthy volunteers, six men and six women, participated in this clinical study. The average age was 31.5 years (range 18-54). Treatment was well tolerated and there were no treatment related adverse events.

2.2 Whole blood and intracellular pharmacokinetics

The pharmacokinetic profile of tacrolimus in whole blood, PBMCs and T cells is shown in **Figure 1.** For all matrices, the highest tacrolimus levels were observed at 1.5h post-dose, showing considerable variation between subjects (WB: 21.5 ± 6.2 μg/L, PBMCs: 76.8 ± 37.3 pg/10^6 cells, T cells: 14.5 ± 4.9 pg/10^6 cells). At 48 hours after administration, tacrolimus concentrations were almost back at baseline levels. No major differences were observed in the shape of the pharmacokinetic profile between whole blood, PBMCs and T cells. Intracellular tacrolimus concentrations, however, differed largely between T cells and PBMCs. The tacrolimus concentration in T cells was on average 5.3x lower compared to PBMCs at 1.5h post-dose, even though PBMCs consist for 60% of T cells [12].

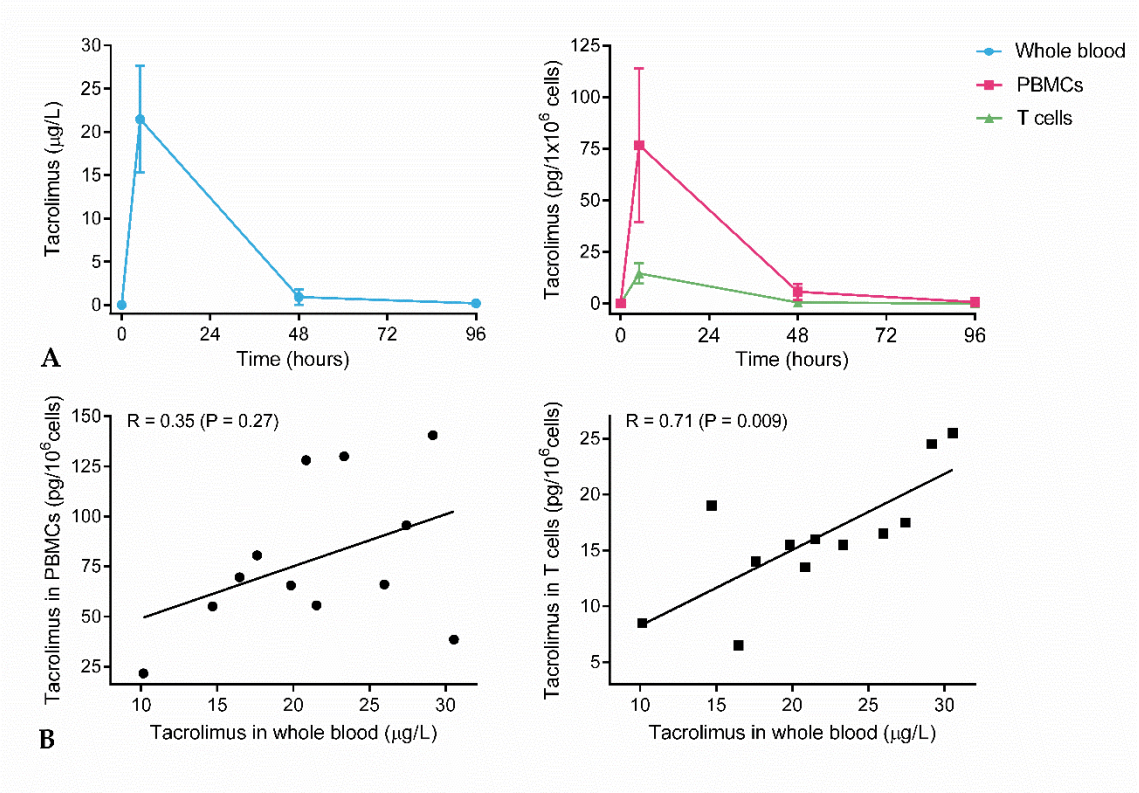

Figure 1. (a) Mean tacrolimus concentration over time in whole blood, PBMCs and T cells. Samples were taken at 0h, 1.5h, 48h and 96h post-dose. (b) Correlation between tacrolimus concentrations at 1.5h post-dose in whole blood and PBMCs, and between whole blood and T cells.

Whole blood concentrations are generally used for therapeutic drug monitoring in renal transplantation patients. To determine whether whole blood levels may serve as proxy for drug concentrations that enter the target cell, whole blood concentrations were correlated to the intracellular concentrations. Interestingly, tacrolimus levels in PBMCs did not seem to correlate with whole blood levels, whereas T cell concentration did show a significant correlation ($r = 0.71$, $p = 0.009$).

2.3. Cytokine production

To study the immunosuppressive effect of tacrolimus, cytokine production was measured after 24h whole blood stimulation with phytohaemagglutinin (PHA). **Figure 2** shows the *in vitro* tacrolimus concentration-response curve that was generated pre-dose for each individual subject, and the *ex vivo* tacrolimus effect on cytokine production after dosing. A clear concentration-response relationship of tacrolimus was found *in vitro* for IL-2 and IFNγ (IC50 of 5.6 µg/L and 18.6 µg/L respectively), with a maximum inhibition of 95% for both cytokines at a tacrolimus concentration of 100 µg/L. *Ex vivo*, tacrolimus strongly inhibited cytokine production at 1.5h post-dose (10.0% IL-2 and 36.3% IFNγ production remaining). Comparing *in vitro* and *ex vivo* pharmacodynamic activities, the observed cytokine inhibition *ex vivo* corresponds well to the observed cytokine inhibition *in vitro* (**Figure 2**, left panels versus right panels, for tacrolimus concentration of 21.5 µg/L and 1.5h time point).

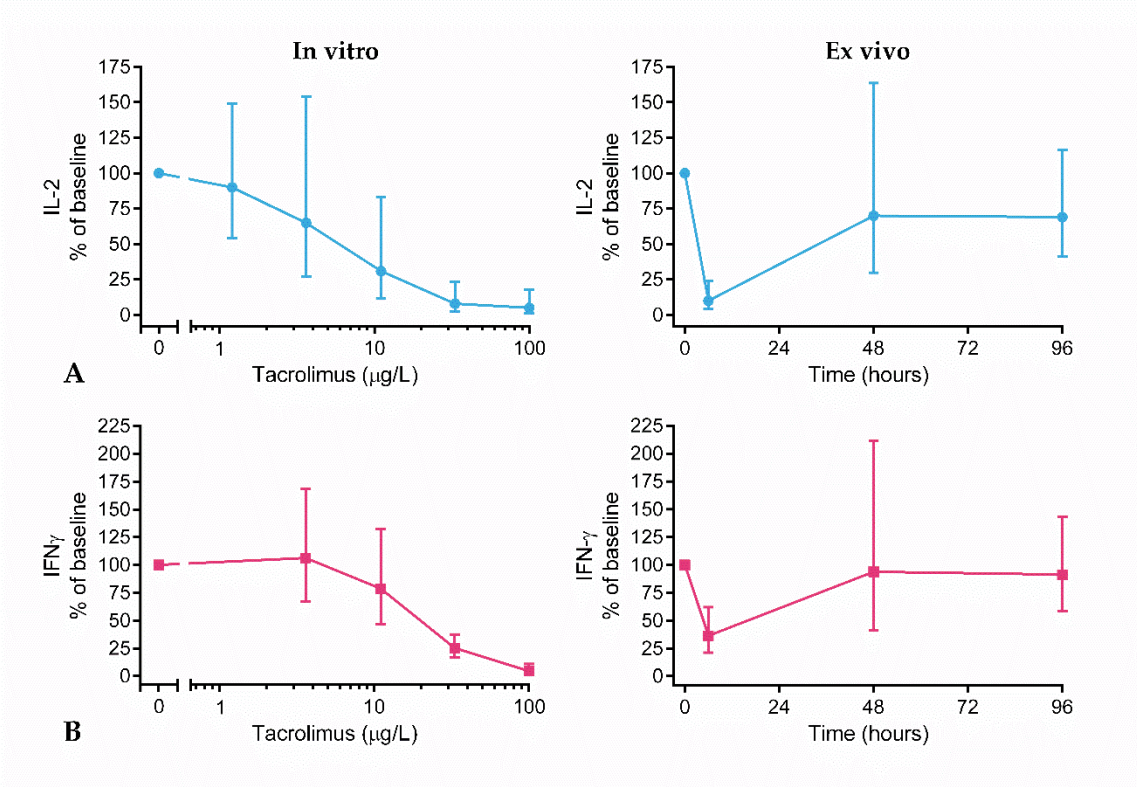

Figure 2. *In vitro* and *ex vivo* tacrolimus effect on cytokine production. (**a**) IL-2 production and (b) IFNγ production in PHA-stimulated whole blood. *In vitro* tacrolimus effect: pre-dose cytokine production after incubation with a dose range of tacrolimus (100-33-11-3.7-1.2 μg/L). *Ex vivo* tacrolimus effect: cytokine production at 0h, 1.5h, 48h and 96h after dosing. The cytokine production is calculated as percentage of baseline, and is displayed as mean ±SD.

In **Figure 3** the *in vitro* and *ex vivo* effect of tacrolimus for a single subject is displayed. At the individual subject level, the *in vitro* concentration-effect curve is a good predictor of the *ex vivo* tacrolimus effect. Cytokine production and tacrolimus concentrations at 1.5h were correlated, and relative IL-2 production was found to correlate significantly with whole blood tacrolimus concentration (**Figure 3B**, r = -0.73, p = 0.0085). T cell tacrolimus concentration also showed a clear correlation with relative IL-2 production (r = -0.51, p = 0.09), while this was not found for tacrolimus concentration in PBMCs (r = -0.03, p = 0.92). Subjects with a higher tacrolimus level at 1.5h post-dose showed a stronger inhibition of IL-2 production compared to subjects with lower tacrolimus levels. This correlation was not found for IFNγ production, indicating that the inter-subject variability in IFNγ production at 1.5h cannot be explained by the difference in tacrolimus concentrations.

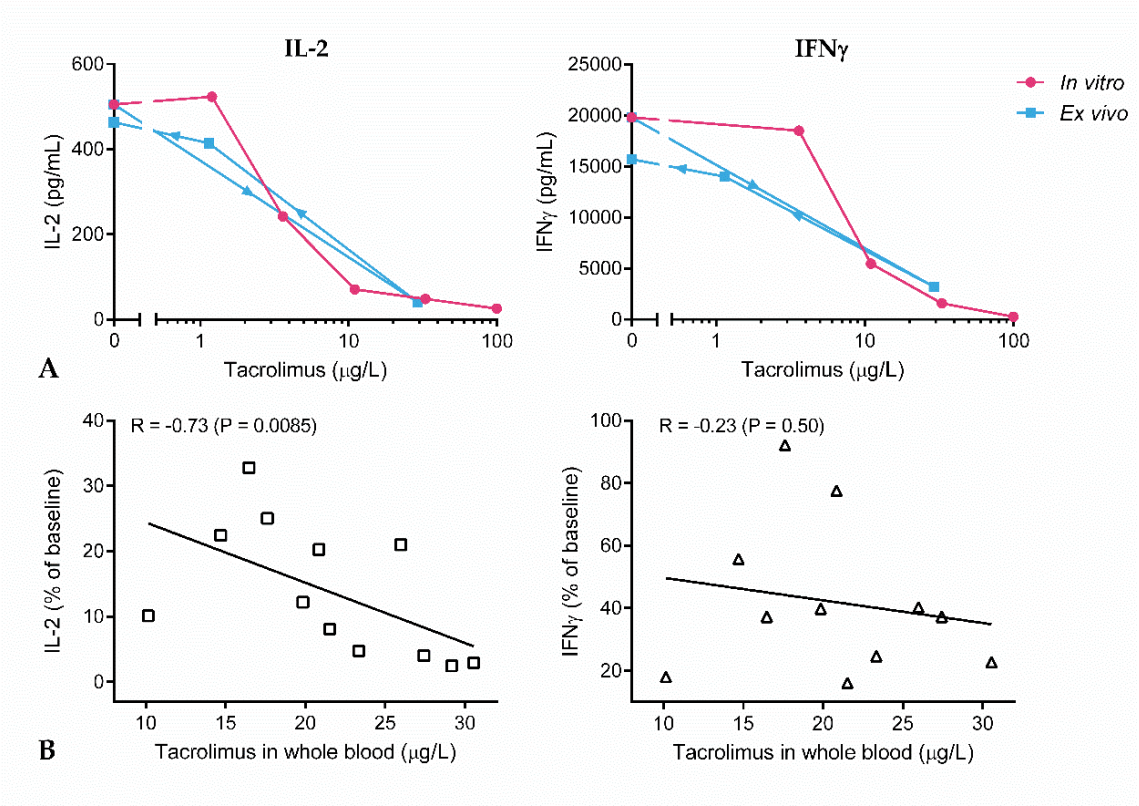

Figure 3. (a) Overlay of *in vitro* and *ex vivo* tacrolimus effect on cytokine production after 24h whole blood stimulation with PHA for one subject. *In vitro* tacrolimus effect: pre-dose cytokine production after incubation with a dose range of tacrolimus (100-33-11-3.7-1.2 µg/L). *Ex vivo* tacrolimus effect: cytokine production at 0h, 1.5h, 48h and 96h after dosing. Arrows indicate time lapse. (b) Correlation between tacrolimus concentrations at 1.5h post-dose in whole blood and relative IL-2 and IFNγ production.

2.3. Surface marker expression

Similar as for the cytokine production, tacrolimus concentration-effect curves were generated for surface marker expression in pre-dose blood samples (*in vitro* drug effect), while whole blood PHA stimulation was also performed in samples collected over time to assess *ex vivo* drug effect. **Figure 4** shows that tacrolimus substantially and concentration-dependently suppressed the expression of CD154 and CD71 *in vitro*. In contrast to PHA-induced cytokine release of Il-2 and IFNγ, tacrolimus did not fully inhibit PHA-driven surface marker expression of CD154 and CD71. Even at a concentration of 100 µg/L tacrolimus, a concentration that is never achieved in patients, a remaining surface marker expression of ~50% was found. When comparing this to the average expression that was found in unstimulated samples (15% for CD71, 17% for CD154, and 24% for CD25), there is still surface marker expression remaining that could not be suppressed.

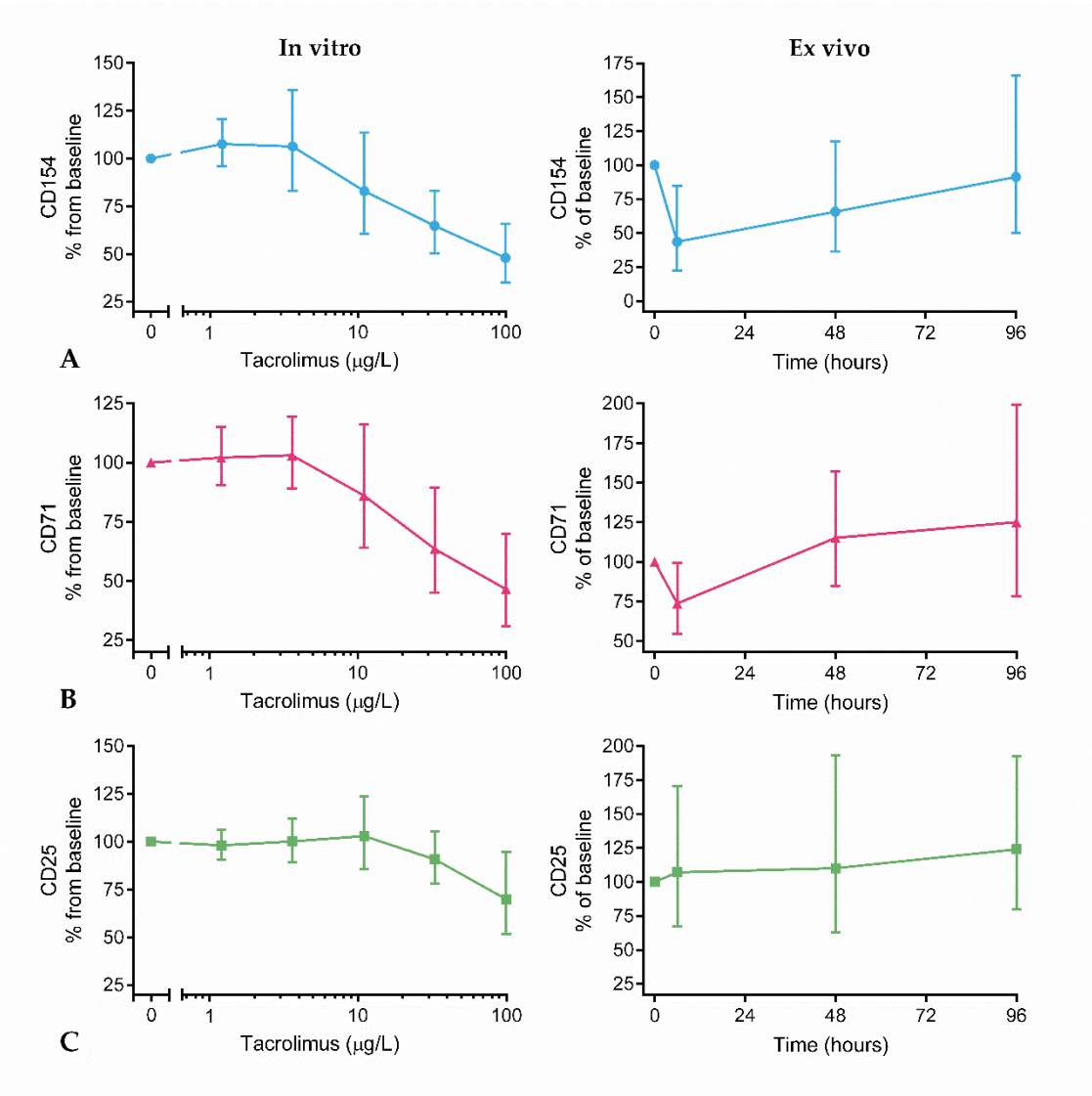

Figure 4. *In vitro* and *ex vivo* tacrolimus effect on expression of (**a**) CD154, (b) CD71, and (c) CD25 on CD3+ T cells after 48h whole blood stimulation with PHA. *In vitro* tacrolimus effect: pre-dose surface marker expression after co-incubation with a tacrolimus concentration range (100-33-11-3.7-1.2 µg/L). *Ex vivo* tacrolimus effect: surface marker expression over time, after PHA stimulation of whole blood samples collected from tacrolimus-exposed subjects. The expression was calculated as percentage of baseline, and is displayed as mean ±SD.

Of all measured activation markers, the expression of CD154 and CD71 on T cells were most strongly inhibited after dosing (44% and 73% remaining respectively). The maximal drug effect *ex vivo* was observed at a whole blood concentration of 21.5 µg/L tacrolimus at 1.5h post-dose, which was in line with the observed effect size *in vitro* at the corresponding drug concentration. CD25 expression, on the other hand, was not affected by tacrolimus, which is in line with the minor drug effect that was found *in vitro*. For CD95 and CD69 only a very small tacrolimus effect was found *in vitro*, and *ex vivo* tacrolimus did not significantly alter the expression levels (data not shown).

As for the cytokine production, the correlation between tacrolimus whole blood levels and CD154 and CD71 expression was assessed at 1.5h, when tacrolimus levels are peaking. For both activation markers, no correlation was found (data not shown).

2.4. Calcineurin activity

To have a PD readout measure more proximal to the drug target, calcineurin phosphatase activity was measured as described by Sellar et al [12]. However, this method was not feasible in this

setting, most due to laborious and time-consuming sample handling before analysis. The data was highly variable and did not show any dose-response relationship. The spectrophotometric readout of calcineurin activity seemed to have worked properly (CV of duplicates < 20%).

3. Discussion

Even though therapeutic drug monitoring (TDM) is routinely performed, transplant rejection, infection and (nephron)toxicity still occur in many patients that do have tacrolimus concentrations within the target range. Tacrolimus trough concentrations in whole blood are roughly targeted between 5-15 μg/L, which is based on a few randomized clinical trials that correlated target concentration to clinical outcome [13]. To improve the understanding of the relationship between tacrolimus concentration in the blood and the ultimate clinical effect, readout measures that link these parameters are required. Such intermediate readout measures may comprise drug concentrations in the target cells and functional cell-based measures quantifying drug effect on the target cell. We conducted a clinical study in healthy volunteers receiving a single dose of tacrolimus. Tacrolimus concentrations were quantified in whole blood, PBMCs, and T cells, and correlated with proximal drug effects (i.e. effects on the target cell). These effects were quantified by cell-based assays, and included cytokine production and expression of activation surface markers after stimulation of T cells.

At 1.5h after drug administration, the highest tacrolimus concentrations were detected in whole blood, PBMCs and T cells. At the next time point, 48h after administration, tacrolimus concentrations had almost reduced to baseline levels again for all three matrices. This is in line with the reported PK profile of tacrolimus in healthy volunteers [14]. Based on our data, there is no indication that tacrolimus remains present within the target cell significantly longer than freely circulating in blood. The intracellular tacrolimus concentrations differed largely between the PBMCs and T cells, even though the majority of PBMCs consist of T cells (60%) [15]. Washing steps during PBMC isolation are known to diminish the effect of tacrolimus [16], but the number of washing steps were kept similar for both isolations. This suggests that the difference in tacrolimus concentrations between PBMCs and T cells is not because of the isolation procedure, but might be caused by another PBMC subpopulation with significant tacrolimus uptake. Since intracellular tacrolimus concentrations have never been measured in cell populations other than PBMCs, the identity of this cell population remains unknown. Tacrolimus concentrations in whole blood and PBMCs at 1.5h post-dose did not correlate significantly. Literature on this is scarce and conflicting, some papers reporting a correlation in transplantation patients [17, 18], whereas other papers do not [19, 20]. Tacrolimus levels in T cells, on the other hand, did correlate significantly with whole blood concentrations. This finding supports the current whole blood-based TDM strategy, that tacrolimus concentration in whole blood is a good representation of the concentration in the target cell.

However, clinical practice shows that whole blood tacrolimus concentration is far from ideal as primary measure for TDM. We hypothesized that drug activity rather than drug concentration may be more suitable for future TDM. As a first step to this future perspective, we selected, optimized and qualified functional assays for quantification of tacrolimus effect on the T cell, and implemented these assays in the current clinical study. PHA-induced IL-2 and IFNγ production, and expression of CD71 and CD154 on T cells showed to be the most promising pharmacodynamic readout measures for quantification of tacrolimus effect *in vitro* and *ex vivo*. The PHA-induced cytokine release was almost completely inhibited at a concentration of 100 μg/L tacrolimus *in vitro*. At a concentration of 20-25 μg/L *in vitro*, which is the peak tacrolimus whole blood concentration in healthy volunteers, the estimated inhibition of cytokine production was 80% for IL-2 production and 50% for IFNγ production (25% and 50% cytokine release remaining, respectively). At this peak concentration, 1.5h after administration, tacrolimus inhibited IL-2 production by 90% and IFNγ production by 64% *ex vivo* (10% and 36% cytokine release remaining, respectively). These data show that the *in vitro* and *ex vivo* tacrolimus effect on cytokine production correspond decently.

Tacrolimus significantly reduced the PHA-induced expression of CD71 and CD154 on T cells. For surface marker CD25, the tacrolimus effect was less apparent. Transferrin receptor (CD71), co-

stimulatory molecule CD40 ligand (CD154), and IL-2 receptor (CD25) are all upregulated upon T cell activation [21], and their expression on lymphocytes has been associated to clinical outcome in transplantation patients [22-24]. All of these surface markers were significantly expressed by non-stimulated T cells (expression levels of 15%, 17%, and 24%, for CD71, CD154 and CD25, respectively, compared to PHA-stimulated condition, set as 100%). At a concentration of 100 μg/L, tacrolimus reduced CD71 and CD154 expression to approximately 50% *in vitro*, which means that at this very high tacrolimus concentration, T cell activation was still not fully inhibited. The tacrolimus effect *ex vivo*, in drug-exposed volunteers, was 27% and 56% for CD71 and CD154 expression, respectively (73% and 44% remaining). The *in vitro* and *ex vivo* tacrolimus effect on activation marker expression corresponded well, as observed when comparing drug effect at 1.5h (*ex vivo*; tacrolimus peak) with drug effect at a tacrolimus concentration of 20-25 μg/L (*in vitro*).

Due to logistical restrictions, the number of data points with significant tacrolimus concentrations and substantial T cell inhibition is limited (namely, only the samples that were collected at 1.5h). Therefore, quantitative correlation or systematic PK/PD integration was not possible based on the collected data set. However, the *ex vivo* tacrolimus effects in drug-exposed volunteers corresponded well with *in vitro* tacrolimus effects quantified in baseline samples from the same volunteers, which underlines the validity of the generated data. Since the *in vitro* concentration-effect curve "predicts" the tacrolimus effect *ex vivo*, tacrolimus dose optimization for an individual transplant patient based on an *in vitro* concentration-effect curve may be a possibility for future TDM. A critical next step will be to investigate the correlation between these functional T cell measures and clinical outcome measures (allograft survival/rejection and side effects) in a patient-based study.

Despite the explorative character of this study, it is tempting to speculate about the theoretical T cell activity profile over time in tacrolimus-treated transplantation patients, based on the current study outcomes. The PK profile of tacrolimus is highly variable between transplantation patients. Trough concentrations are measured 12 hours after dosing, and can vary between 0.6 – 50 μg/L, with an average of 5-10 μg/L [25-28]. Based on the *in vitro* tacrolimus effect that we observed (which corresponded well with *ex vivo* drug effect after treatment of healthy volunteers), such concentrations would translate into a far from maximal inhibition of T cell activity: at a tacrolimus whole blood concentration of 5 μg/L, even none of the PD T cell measures was inhibited, except for IL-2 production (~40% inhibition). These findings suggest that with the conventional tacrolimus dosing regimen, some patients may experience time intervals in which their T cell activity is not inhibited (at least not by tacrolimus). On the other hand, in this study the effect of a single dose of tacrolimus was studied, while in patients the responsiveness of T cells might be different because of the long-term repeated tacrolimus dosing. Moreover, tacrolimus is usually combined with MMF and corticosteroid treatment, drugs that also suppress the T cell, but that were not included in the current study. For these drugs, and combinations of these drugs, an investigational approach is suggested that resembles the one followed in the current study.

In conclusion, this study shows that intracellular tacrolimus concentrations mimic the time course of whole blood concentrations, and that PHA-induced cytokine production (IL-2 and IFNγ) and activation marker expression (CD71 and CD154) are suitable PD readout measures for quantification of the immunosuppressive effect of tacrolimus on the T cell. Though the effect of tacrolimus on T cell activity has been studied before [8-11], this is the first study in which whole blood and intracellular tacrolimus concentrations are related to *ex vivo* drug effect, and in which *in vitro* and *ex vivo* tacrolimus effects are compared. As such, the current study may serve as the first step from PK- to PD-based therapeutic drug monitoring for tacrolimus.

4. Materials and Methods

4.1 Study design

In this open label study, 12 healthy volunteers received a single oral dose of 0.05 mg/kg Prograf®, rounded up to the available dosage forms (0.5 mg, 1 mg, and 5 mg Prograf®). The dosage was based on the recommended dose for renal transplant patients receiving both tacrolimus and

mycophenolate mofetil treatment. The healthy volunteers were both male and female, between 18 and 55 years of age and non-smoking. All subjects gave written informed consent and did not have any disease associated with immune system impairment or evidence of any other active or chronic disease. Volunteers were excluded when taken any other drugs within 21 days prior to study start. This study was approved by the independent medical ethics committee 'Medisch Ethische Toetsingscommissie van de Stichting Beoordeling Ethiek Biomedisch Onderzoek' (Assen, the Netherlands), and is registered with the Dutch Trial Registry (Nederlands Trial Register) under study number NTR7420.

4.2 Whole blood and intracellular PK

Blood samples were drawn pre-dose and 1.5, 48, 96 and 192 hours after drug administration. The samples for whole blood PK measurement were collected in K2EDTA tubes (Becton Dickinson, Franklin Lakes, NJ, US) and stored at -80°C. PBMCs were collected using sodium heparin CPT (Becton Dickinson) and T cells were isolated from heparinized whole blood by negative magnetic cell sorting using RoboSep (StemCell Technologies). After PBMC and T cell isolation, the cells were washed, and the remaining red blood cells were removed using RBC lysis buffer (Thermo Fisher Scientific, Waltham, MA, US). PBMCs and T cells were counted with a MacsQuant 10 analyzer (Miltenyi Biotec, Bergisch Gladbach, Germany) and stored in PBS at 20 x 10^6 cells/mL at -80°C. The purity of the isolated T cell population was assessed with an anti-CD45-FITC and anti-CD3-VioGreen staining (Miltenyi Biotec).

Whole blood and intracellular tacrolimus concentrations were measured using a Waters Acquity UPLC–MS/MS system by the Department of Hospital Pharmacy, Erasmus Medical Center, as described previously [17].

4.3 In vitro and ex vivo whole blood culture

On the same time points as the PK samples, blood was drawn for PD assessments, which entailed whole blood challenges. All incubations were started within one hour after blood withdrawal. For measurement of cytokine production and surface marker expression, heparinized whole blood (Becton Dickinson) was stimulated with 10 μg/mL phytohaemagglutinin (PHA) (Sigma Aldrich, St. Louis, MO, US). To generate an *in vitro* tacrolimus concentration-effect curve for every individual subject, pre-dose samples were incubated with PHA and a concentration range of 100-33-11-3.7-1.2 μg/L tacrolimus (Prograf® for injection). To study the immunosuppressive effect of tacrolimus *ex vivo*, whole blood samples were incubated with PHA only.

Samples for measurement of calcineurin activity were collected in EDTA tubes (Becton Dickinson). Pre-dose samples were first incubated 1 hour at 37°C, 5% CO_2 with a concentration range of tacrolimus. For post-dose samples the analysis was started directly (within one hour) after the blood sample was taken.

4.4 Cytokine production

Whole blood samples were cultured for 24h, after which supernatant was collected and stored at -80°C until analysis. IFNγ and IL-2 concentrations were measured by Meso Scale Discovery Vplex-2 method by Ardena Bioanalytical Laboratory in Assen, the Netherlands.

4.5 Surface marker expression

Following 48h whole blood incubation, RBC lysis buffer (Thermo Fisher Scientific) was used to lyse the red blood cells. After washing with PBS, cells were stained with anti-CD3-Viogreen, anti-CD69-APCVio770, anti-CD95-PEVio770, anti-CD25-PE, anti-CD71-FITC, and anti-CD154-VioBlue (Miltenyi Biotec). The samples were measured after a final washing step, using a MaqsQuant10 analyzer. Before measurement, propidium iodide (PI) (Miltenyi Biotec) was added to assess viability. Analysis of the cell populations was performed with Flowlogic software (Inivai Technologies,

Mentone VIC, Australia). For each time point the unstimulated samples was used to set the correct gating. The gating strategy is shown in **Figure A1** in Appendix A.

4.6 Calcineurin activity

The method described by Sellar et al. was used for the measurement of calcineurin activity [12]. Red blood cells were lysed using ACK lysis buffer (Thermo Fisher Scientific), after which two million cells were lysed in freshly prepared lysis buffer (50 mM Tris-HCL, 1.0 mM 1,4-Dithiothreitol, 5.0 mM L-ascorbic acid, 0.02% Igepal CA-630, 50 mg/L soybean trypsin inhibitor, 50 mg/L phenylmethylsulfonyl fluoride, 5.0 mg/L leupeptin and 5.0 mg/L aprotinin). After lysis, the samples underwent three freeze-thaw cycles and were stored at -80°C until use.

A calcineurin phosphatase activity kit (Enzo Life Sciences, Brussels, Belgium) was used to measure calcineurin activity according to the protocol described by Sellar et al. Phosphatase activity of calcineurin (pmol min^{-1} x 10^6 cells) was calculated by subtracting the phosphate activity that was measured in the presence of Ca^{2+} and calmodulin from the phosphatase activity measured in the presence of EGTA.

4.7 Data analysis

Data are presented as mean value ± standard deviation (SD). Correlation between tacrolimus concentrations, surface marker expression and cytokine production was analyzed by Spearman's rank-order correlation with SAS 9.4 (SAS Institute Inc., Cary, NC, USA). IC50 of *in vitro* cytokine production and surface marker expression was calculated using GraphPad Prism 6.05 (GraphPad software Inc., San Diego, CA, US).

Supplementary Materials: Supplementary materials can be found at www.mdpi.com/xxx/s1.

Author Contributions: conceptualization, A.E.V., A.F.C., and M.M.; methodology, A.E.V. H.W.G., and M.M.; investigation, A.E.V., M.S., F.S., B.C.M.W., J.B., and M.M.; writing—original draft preparation, A.E.V. writing—review and editing, A.E.V., H.W.G., M.S., A.P.J.V., F.S., M.K., B.C.M.W., J.B., A.C., and M.M.; visualization, M.K.; supervision, M.M., and J.B.; project administration, A.E.V., M.S., and F.S.

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Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

CNI	Calcineurin inhibitor
C_0	Pre-dose trough concentration
FK506	Tacrolimus
FKBP	FK506 binding protein
IC50	Half maximal inhibitory concentration
IFNγ	Interferon gamma
IL-2	Interleukin 2
MMF	Mycophenolate mofetil
NFAT	Nuclear factor of activated T cells
PBMCs	Peripheral blood mononuclear cells
PD	Pharmacodynamics
PHA	Phytohaemagglutinin
PI	Propidium iodide
PK	Pharmacokinetics
SD	Standard deviation
TDM	Therapeutic drug monitoring
WB	Whole blood

Appendix A

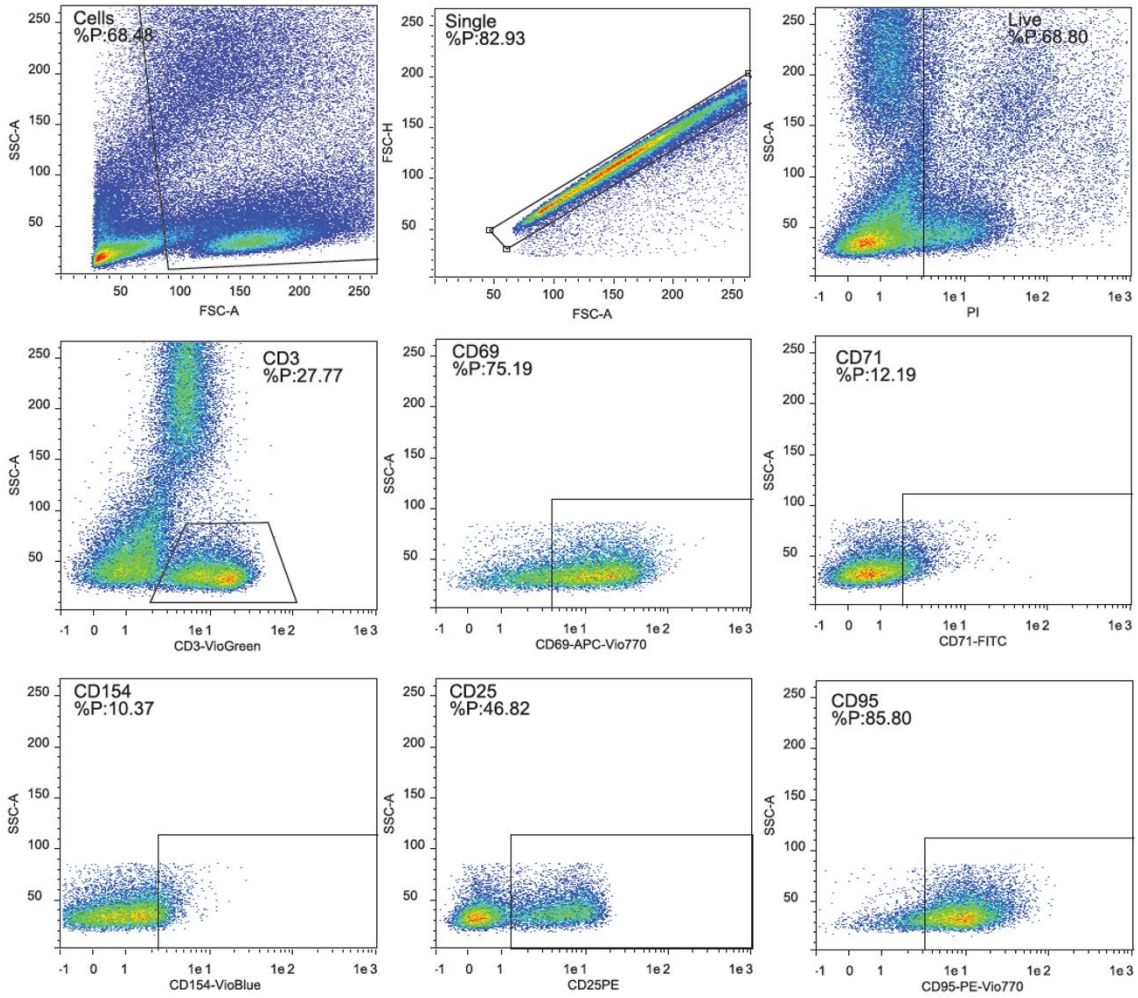

Figure A1. Gating strategy for surface marker expression on CD3+ T cells.

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