British Journal of Clinical Pharmacology

Br J Clin Pharmacol (2017) **83** 2494–2502 2494

PHARMACOKINETIC DYNAMIC RELATIONSHIPS

NFAT-regulated cytokine gene expression during tacrolimus therapy early after renal transplantation

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Received 2 March 2017; **Revised** 8 June 2017; **Accepted** 4 July 2017

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Keywords acute rejection, calcineurin inhibitors, NFAT-regulated gene expression, pharmacodynamic drug monitoring, renal transplantation, tacrolimus, viraemia

AIMS

Despite pharmacokinetic monitoring of calcineurin inhibitors, the long-term outcome after transplantation (Tx) is still hampered by the side effects of these drugs. The aim of the present study was to characterize nuclear factor of activated T cells (NFAT)-regulated gene expression as a potential pharmacodynamic biomarker for further individualization of tacrolimus (Tac) therapy.

METHODS

In 29 renal allograft recipients, samples were drawn once pre-Tx, and before and 1.5 h after Tac dosing at approximately 1 week, 6 weeks and 1 year post-Tx. Tac concentrations were measured by immunoassay, while the expression of genes encoding NFAT-regulated cytokines [interleukin 2 (*IL2*), interferon gamma (*IFNG*), colony stimulating factor 2 (*CSF2*)] and cytochrome P450 3A5 (*CYP3A5*) genotyping were determined by real-time polymerase chain reaction.

RESULTS

The cytokine response after Tac dosing varied up to 46-fold between patients and changed significantly with time post-engraftment. Tac concentrations 1.5 h postdose ($C_{1.5}$) >15 μg l^{-1} were associated with strong cytokine inhibition and residual gene expression (RGE) ≤10%, while lower Tac $C_{1.5}$ resulted in more variable responses (RGE 2.5–68.7%). Patients with ongoing subclinical acute rejection ($n = 5$) demonstrated limited cytokine inhibition (RGE 39.7–72.6%), while patients with polyoma virus viraemia ($n = 3$) had relatively strong inhibition of cytokines (RGE 2.5–32.5%). By contrast, there was no association between Tac exposure and rejection or viraemia.

CONCLUSIONS

The findings of our study support the potential of NFAT-regulated gene expression measurements as a pharmacodynamic tool for additional monitoring of Tac therapy, especially in the context of overimmunosuppression and viraemia.

DOI:10.1111/bcp.13367

WHAT IS ALREADY KNOWN ABOUT THIS SUBJECT

- Nuclear factor of activated T cells (NFAT)-regulated gene expression has been reported to be a promising pharmacodynamic biomarker of the calcineurin inhibitor response.
- The majority of studies so far have investigated long-term stable transplant recipients treated with cyclosporine.

WHAT THIS STUDY ADDS

- We have studied NFAT-regulated cytokine expression in patients treated with tacrolimus in the early phase after transplantation, and describe that the cytokine response changed significantly with time.
- Patients with ongoing subclinical acute rejection demonstrated limited inhibition, while patients with polyoma virus viraemia had relatively strong inhibition of NFAT-regulated cytokines.

Introduction

The calcineurin inhibitors (CNIs) **tacrolimus** (Tac) and **cyclosporin A** (CsA) are cornerstone immunosuppressive drugs for solid organ transplantation (Tx). Owing to the narrow therapeutic range and large pharmacokinetic variability of CNIs, therapeutic drug monitoring is necessary to balance sufficient efficacy with minimal toxicity. However, despite concentration-based dosing, CNI-related toxicity is still a considerable challenge limiting the optimal long-term outcome after Tx [1].

The immunosuppressive effect of CNIs is primarily caused by inhibition of the calcineurin–nuclear factor of activated T cells (NFAT) signalling pathway in T helper cells. Distribution of CNIs into T cells and the drug sensitivity varies between individuals. Therefore, measurement of systemic CNI trough concentrations does not necessarily accurately predict the biological activity of the drugs in immune cells, and measurement of pharmacodynamic biomarkers related to the inhibition of calcineurin may better reflect the biological effect of CNIs in individual patients.

A promising biomarker of the clinical response to CNIs is the expression of NFAT-regulated cytokines, **interleukin 2** (*IL2*), **interferon-gamma** (*INFG*) and **colony-stimulating factor 2** (*CSF2*), in *ex vivo* stimulated immune cells [2]. Studies in renal, heart and liver transplant recipients have shown stronger cytokine inhibition among patients with recurrent infections or viraemia [3–8]. Furthermore, strong cytokine suppression after CNI dosing has been associated with an increased risk of malignancies [4, 6, 9, 10]. On the other hand, low cytokine inhibition has been associated with an increased risk of rejection [8, 11, 12]. However, the association between the CNI cytokine response and clinical outcome is not consistent between studies.

The majority of studies so far have characterized the NFAT-regulated gene expression among long-term stable patients receiving CsA-based immunosuppression [2, 13]. The aim of the present study was to characterize NFAT-regulated gene expression during Tac therapy in renal Tx recipients in the early phase after engraftment, and to study the cytokine expression response in relation to Tac concentrations and short-term outcome including renal function, rejection episodes and viraemia.

Materials and methods

Patients and samples

The patients were enrolled at Oslo University Hospital, Rikshospitalet in the period from February 2012 to March 2014. The inclusion criteria were renal Tx with a graft from a living donor, planned Tac-based immunosuppression and recipient age ≥18 years. Patients were excluded if there were any medical limitations of blood sampling. The planned number of subjects for inclusion was 30. However, within the inclusion period, only 29 patients fulfilled the inclusion criteria and were admitted to the hospital at a time when there was staff available for the extra sample handling. The study was approved by the Regional Committee for Medical and Health Research Ethics (2011/1282 D) and performed in accordance with the Declaration of Helsinki and relevant regulations. Prior to inclusion, written informed consent was obtained from all study participants and they were free to withdraw from the study at any time.

Blood samples were drawn in heparin and ethylenediaminetetraacetic acid (EDTA) tubes pre-Tx (0–4 days before Tx) and before and 1.5 h after Tac dosing approximately 1 week (6–9 days), 5–7 weeks (referred to as 6 weeks) and 1 year post-Tx. The postdose sampling time point was based on a previous study demonstrating that the strongest inhibition of NFAT-regulated cytokines occurs approximately 1.5 h after Tac dosing [11]. The exact time points for the sampling and dose administration were recorded.

Immunosuppressive treatment

All patients received induction therapy consisting of intravenous basiliximab (20 mg) on day 0 and day 4 and intravenous methylprednisolone (250 mg) on day 0. The standard immunosuppressive maintenance regimen consisted of Tac combined with mycophenolate mofetil (MMF, 750 mg twice daily) and prednisolone (20 mg day^{-1}, tapered to 10 mg during the first 4 weeks). Tac was initiated at fixed doses of 0.04 mg kg^{-1} twice daily, followed by dose adjustments to reach target predose concentrations (C_0) of 3–7 μg l^{-1}. Five patients received allografts from HLA-identical donors and were treated with a standard regimen without MMF. One patient presented donor-specific antibodies pre-Tx and received a high-risk regimen involving higher Tac target levels (8–12 μg l^{-1} from days 0–30 and 6–10 μg l^{-1} after day 30), and higher starting doses of methylprednisolone (500 mg

on day 0) and prednisolone (80 mg day^{-1}) tapered to 20 mg within 1 week and further tapered to 10 mg by week 8. The high-risk patient also received additional induction therapy with intravenous rituximab (375 mg m^{-2}) 30 days pre-Tx and intravenous human immune globulin (400 mg kg^{-1} day^{-1}) from days 0–4.

Tacrolimus concentration measurements

Tac concentrations were determined in EDTA anticoagulated whole blood using a chemiluminescent microparticle immunoassay on the Architect i2000SR system according to the manufacturer's instructions (Abbott Laboratories, Rungis, France). The validated concentration range of the assay was 1.0–30 μg l^{-1} with coefficients of variation ≤10%. Samples with concentrations above 30 μg l^{-1} were diluted as specified by the manufacturer and reanalysed.

Cytochrome P450 3A5 (CYP3A5) genotyping

CYP3A5 genotyping included analysis of the NM_000777.4: c.219-237A > G (rs776746, A = *1 and G = *3) variant, and was performed using real-time polymerase chain reaction (PCR) and melting curve analysis (LightCycler 480 instrument, Roche Applied Science, Penzberg, Germany) as previously described [14].

Gene expression of NFAT-regulated cytokines

The gene expression of NFAT-regulated cytokines was determined after *ex vivo* immune activation using a method modified after Giese *et al*. [15, 16]. After sampling, 150 μl heparinized whole blood was incubated in equal volumes of Roswell Park Memorial Institute (RPMI) 1640 medium containing 100 ng ml^{-1} phorbol 12-myristate 13-acetate (PMA) and 5 μg ml^{-1} ionomycin (Sigma, St Louis, MO, USA) for 3 h at 37°C. Following *ex vivo* immune activation, samples were lysed with lysis/binding buffer with 1% (w/v) dithiothreitol (Roche Diagnostics, Mannheim, Germany) and frozen at −70°C until extraction of total RNA using the MagNA Pure LC RNA Isolation Kit – High Performance (Roche Diagnostics) on the MagNA Pure LC instrument (Roche Applied Science, Penzberg, Germany). Total RNA was reverse transcribed into complementary DNA using the Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics) and a combination of random hexamer and oligo deoxythymidine primers according to the manufacturer's instructions. MS2 RNA (Roche Diagnostics) was used as carrier RNA in all steps at a final concentration of 10 μg ml^{-1}.

Gene expression measurements were performed by real-time PCR on the LightCycler 480 instrument using the LightCycler® 480 Probes Master Kit (Roche Diagnostics) and hybridization probes for product detection. The target genes *IL2*, *IFNG* and *CSF2* and three previously validated reference genes, aminolevulinate delta synthase 1 (*ALAS1*), β-2-microglobulin (*B2M*) and ribosomal protein L13a (*RPL13A*), were amplified in triplicate in separate reactions [17]. Oligonucleotide sequences, reagent concentrations and amplification conditions are listed in Tables S1 and S2. The PCR results were analysed using the LightCycler 480 Software v.1.5 (Roche Applied Science). Quantification cycles were defined by the second derivative maximum method. Target gene expressions were calculated relative to the geometric mean expression of the reference genes and normalized to a calibrator. The calculations included PCR efficiency correction based on standard curves as previously described [17]. Finally, residual gene expression (RGE) values were calculated by normalizing the relative gene expression 1.5 h after dosing to the gene expression predose.

Performance of the gene expression assay

The general reverse transcription–PCR assay (including the same reagent kits, reference genes and instruments) has been thoroughly validated in previous studies, demonstrating within-run and between-day coefficients of variation (CV) <15% [17]. Furthermore, the between-day CV of the specific NFAT-regulated gene expression assay was 14.8% (PCR step repeated 10 times within a 2-month period).

Clinical data

Clinical data were obtained from the hospital records of each patient. Rejection episodes were biopsy verified and graded according to the Banff classification [18]. Polyoma virus viraemia was defined as BK or JC virus levels ≥10 000 DNA copies ml^{-1} in whole blood. The glomerular filtration rate (eGFR) was estimated from serum creatinine using the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) formula [19].

Statistical methods

The study was exploratory and the sample size was based on practical constraints (e.g. sample handling). Statistical analyses were performed using IBM® SPSS® Statistics version 21.0 (IBM Corporation, NY, USA). Comparisons between independent groups were performed using the chi-squared or Fisher exact test for categorical variables and Mann–Whitney U test for continuous variables. The Wilcoxon Rank Sum test was used for comparisons between related samples. Correlations between continuous variables were characterized using the Pearson's product–moment correlation coefficient (r) and the Spearman's rank correlation coefficient (r_s). All statistical tests were two sided, and $P < 0.05$ was considered statistically significant.

Nomenclature of targets and ligands

Key ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY [20].

Results

Patient characteristics are summarized in Table 1.

Immunosuppressive treatment

Two patients experienced biopsy-proven acute rejection (BPAR) within the first week post-Tx and were receiving rejection therapy with intravenous methylprednisolone (500–125 mg) at the time of the 1-week gene expression measurement. One of these patients received additional rejection

Table 1

Patient characteristics

	Median (range) or number of patients
Age (years)	58 (20–74)
Gender (female/male)	5/24
Body weight at transplant (kg)	82 (54–106)
Height (cm)	178 (160–191)
Underlying diagnosis	
Nephrosclerosis	9
IgA nephropathy	4
Polycystic kidney disease	4
Glomerulonephritis	4
Diabetes mellitus	3
Other conditions	5
Dialysis pretransplant (yes/no)	10/19
Previous solid organ transplantation (yes/no)	0/29
Immunological risk (standard/high)	28/1
HLA-DR mismatches (mean, standard deviation)	0.76 (±0.74)
Total HLA mismatches (mean, standard deviation)	1.7 (±1.3)
***CYP3A5* genotype**[a]	
CYP3A5*1/*3	5
CYP3A5*3/*3	23

CYP, cytochrome P450; HLA, human leukocyte antigen; Ig, immunoglobulin

[a]Data from a patient with previous haematopoietic stem cell transplantation are not included

therapy with antithymocyte globulin (ATG). Furthermore, one of the patients initially receiving an MMF-free regimen experienced subclinical rejection and was started on MMF approximately 6 weeks post-Tx. Another patient had previously received haematopoietic stem cell Tx and was excluded from the genotyping analyses. At 1 year post-Tx, 20 of the 29 patients had been switched from the regular twice-daily formulation of Tac (Prograf®, Astellas Ireland Co. Limited, Killorglin, Kerry, Ireland) to a modified-release once-daily formulation (Advagraf®, Astellas Ireland Co. Limited, Killorglin, Kerry, Ireland).

Tacrolimus exposure

Daily Tac doses among the standard risk patients (n = 28) were median 0.064 mg kg^{-1} (ranging 0.024–0.110 mg kg^{-1}), 0.058 mg kg^{-1} (0.020–0.144 mg kg^{-1}) and 0.045 mg kg^{-1} (0.015–0.117 mg kg^{-1}) at 1 week, 6 weeks and 1 year, respectively, and were significantly higher at 1 week *vs.* 1 year post-Tx (P = 0.014). The corresponding Tac C_0 levels were median 5.0 μg l^{-1} (ranging 2.5–7.8 μg l^{-1}), 6.0 μg l^{-1} (3.5–9.3 μg l^{-1}) and 5.6 μg l^{-1} (3.2–8.8 μg l^{-1}), and the concentrations 1.5 h postdose ($C_{1.5}$) were median 10.3 μg l^{-1} (3.5–25.3 μg l^{-1}), 8.1 μg l^{-1} (5.0–18.7 μg l^{-1}) and 9.3 μg l^{-1} (3.8–19.6 μg l^{-1}). The Tac C_0 levels were significantly higher at 6 weeks *vs.* 1 week (P = 0.005), while the Tac $C_{1.5}$ levels were lower at 6 weeks (P = 0.029). Furthermore, dose-normalized Tac C_0 was also higher at 6 weeks compared with 1 week [median 202 (μg l^{-1})/(mg kg^{-1}) *vs.* 180 (μg l^{-1})/(mg kg^{-1}), P = 0.002]. By contrast, no significant changes were observed in measured Tac concentrations from 1 week or 6 weeks to 1 year post-Tx ($P \geq 0.102$).

There were no significant differences in Tac concentrations between drug formulations. Patients treated with the twice-daily formulation 1 year post-Tx (n = 9, median dose 3.0 mg kg^{-1}) presented a median Tac C_0 of 5.9 μg l^{-1} (3.5–8.8 μg l^{-1}) and $C_{1.5}$ 7.7 μg l^{-1} (4.7–15.1 μg l^{-1}), while the corresponding levels among standard-risk patients treated with the extended-release formulation (n = 19, median dose 4.0 mg kg^{-1}) were 5.4 μg l^{-1} (3.2–8.3 μg l^{-1}) and 9.6 μg l^{-1} (3.8–19.6 μg l^{-1}), respectively ($P \geq 0.56$).

Dose-normalized Tac exposure was lower among patients carrying the *CYP3A5*1/*3* genotype (n = 5), demonstrating a median Tac C_0 of 102 (μg l^{-1})/(mg kg^{-1}) and $C_{1.5}$ of 237 (μg l^{-1})/(mg kg^{-1}) 1 week post-Tx *vs.* a median C_0 of 185 (μg l^{-1})/(mg kg^{-1}) and $C_{1.5}$ of 424 (μg l^{-1})/(mg kg^{-1}) among patients with the *CYP3A5*3/*3* genotype (n = 23, $P \leq 0.033$).

Cytokine gene expression response

In total, 18 of the 203 samples for gene expression measurements (10 at 6 weeks and eight at 1 year post-Tx) were excluded owing to technical problems (failed RNA extraction and immune activation, respectively).

The measured cytokine expression levels varied ≤135-fold before dosing (E_0) and ≤1373-fold 1.5 h after dosing ($E_{1.5}$) (Table 2 and Table 3). There was also a large variability in the normalized cytokine response, demonstrating ≤46-fold differences in RGE between patients (Figure 1). Furthermore, the gene expression response changed significantly with time, showing the strongest inhibition of cytokines 1 week post-Tx (Figure 1).

The RGE correlated inversely with Tac $C_{1.5}$ (Figure 2), as well as with Tac $C_{1.5} - C_0$ (data not shown). At 1 week post-Tx, Tac $C_{1.5}$ levels >15 μg l^{-1} or $C_{1.5} - C_0$ > 10 μg l^{-1} were associated with strong cytokine inhibition and RGE <10%, while lower Tac exposure resulted in more variable inhibition, with RGE ranging from 2.5% to 68.7% (Figure 2A). At 6 weeks and 1 year post-Tx, the majority of patients had a Tac $C_{1.5}$ <15 μg l^{-1} and the gene expression response varied considerably, despite similar Tac levels (Figure 2B and C). The two patients who demonstrated postdose increases in cytokine expression 1 year post-Tx (Figure 2C, RGE of 166% and 220%, respectively) both presented reductions in Tac levels postdose (decline from 4.2 μg l^{-1} to 3.8 μg l^{-1} and from 6.9 μg l^{-1} to 6.4 μg l^{-1}, respectively). Another patient with limited cytokine inhibition 1 year post-Tx despite a Tac $C_{1.5}$ of 19.6 μg l^{-1} (Figure 2C, RGE of 86.9%) demonstrated a Tac C_0 of 8.3 μg l^{-1} and the lowest predose cytokine expression level in the patient population.

The two patients with ongoing rejection therapy 1 week post-Tx demonstrated lower predose cytokine levels, with an E_0 of 0.041 and 0.029 *vs.* a median of 1.47 (0.14–3.90)

Table 2

Nuclear factor of activated T cells-regulated cytokine expression and Tac concentrations among patients with and without subclinical rejection

	6 weeks post-Tx		1 year post-Tx	
BPAR[a]	**Yes (*n* = 2)**	**No (*n* = 22)**	**Yes (*n* = 3)**[b]	**No (*n* = 22)**
RGE, %	40.6 (39.7–41.6)	45.3 (5.65–76.2)	54.3 (44.8–72.6)	48.1 (17.2–220)
E_0	1.46 (0.44–2.48)	1.98 (0.22–4.99)	1.65 (1.55–2.18)	1.80 (0.30–5.64)
$E_{1.5}$	0.60 (0.18–1.03)	0.73 (0.023–2.52)	0.841 (0.74–1.58)	0.98 (0.12–3.45)
Tac C_0, μg l^{-1}	5.7 (5–6.3)	5.9 (3.5–9.3)	4.3 (4.0–7.2)	5.6 (3.2–8.3)
Tac $C_{1.5}$, μg l^{-1}	7.0 (6.5–7.5)	8.7 (5.0–18.7)	7.1 (4.7–12.7)	8.9 (3.8–19.6)
Tac doses, mg kg^{-1} day^{-1}	0.060 (0.057–0.063)	0.048 (0.020–0.107)	0.049 (0.045–0.089)	0.042 (0.021–0.117)

BPAR, biopsy-proven acute rejection; E_0, gene expression before dosing; $E_{1.5}$, gene expression 1.5 h after dosing; RGE, residual gene expression; Tac C_0, predose tacrolimus concentrations; Tac $C_{1.5}$, tacrolimus concentrations 1.5 h postdose; Tx, transplantation

[a]Subclinical biopsy-proven acute rejection. Gene expression and Tac measurements were performed before the initiation of rejection therapy

[b]One patients experienced concurrent viraemia and subclinical rejection

Table 3

NFAT-regulated cytokine expression and Tac concentrations among patients with and without viremia

	1 week post-Tx		6 weeks post-Tx	
Viraemia[a]	**Yes (*n* = 1)**	**No (*n* = 28)**	**Yes (*n* = 2)**	**No (*n* = 22)**
RGE, %	2.5	18.5 (1.5–68.7)	24.0 (15.5–32.5)	45.3 (5.7–76.2)
E_0	0.596	1.28 (0.029–3.90)	0.50 (0.22–0.78)	2.11 (0.22–4.99)
$E_{1.5}$	0.015	0.14 (0.001–1.37)	0.14 (0.033–0.25)	0.97 (0.023–2.52)
Tac C_0	5.2	5.0 (2.5–10.6)	5.4 (4.3–6.4)	5.9 (3.5–9.3)
Tac $C_{1.5}$	16.4	10.3 (3.5–33.4)	6.9 (6.7–7.0)	8.7 (5.0–18.7)
Tac doses, mg kg^{-1} day^{-1}	0.11	0.064 (0.024–0.16)	0.059 (0.042–0.075)	0.054 (0.020–0.107)

E_0, gene expression before dosing; $E_{1.5}$, gene expression 1.5 h after dosing; RGE, residual gene expression; Tac C_0, predose tacrolimus concentrations; Tac $C_{1.5}$, tacrolimus concentrations 1.5 h postdose; Tx, transplantation

[a]Polyoma virus viraemia defined as BK or JC virus levels ≥10 000 DNA copies ml^{-1} in whole blood. Measurements were performed at the closest sampling period preceding the viraemia

among patients without rejection therapy (P = 0.005). Furthermore, both patients showed strong relative inhibition of cytokines after Tac dosing, presenting RGEs of 1.5% and 11.1%, respectively.

There were no significant associations between the gene expression response and Tac C_0, regimens with or without MMF, total leucocyte numbers, age or gender.

NFAT-regulated gene expression and clinical outcome

Five patients demonstrated subclinical BPAR 6 weeks (n = 2) or 1 year (n = 3) post-Tx. The patients with ongoing subclinical rejection all showed limited cytokine inhibition, with an RGE ≥39.7%. The corresponding cytokine response among patients without ongoing rejection was more variable, demonstrating RGE levels from 5.65% to 220% (Table 2). Tac C_0 levels were within, or slightly above, the therapeutic target range in both groups. Furthermore, Tac $C_{1.5}$ was similar between patients with and without subclinical rejection (Table 2).

Patients with polyoma virus viraemia (n = 3) showed relatively strong inhibition of cytokines and RGEs from 2.5% to 32.5% at the closest measurement preceding the viraemia (Table 3). In addition to the strong inhibition of cytokines, patients who later experienced viraemia also demonstrated relatively low cytokine expression levels before dosing (E_0, Table 3). Tac C_0 levels were within the therapeutic target. Furthermore, two of the patients with viraemia demonstrated a Tac $C_{1.5}$ in the lower range of concentrations, while the third patient presented a Tac $C_{1.5}$ above the median concentration (Table 3).

One of the patients experienced persistent BK virus viraemia from 23 weeks post-Tx and throughout the study period. Furthermore, 1 year post-Tx this patient had also developed subclinical cellular rejection. The RGE of this patient increased from 32.5% 6 weeks post-Tx (preceding viraemia) to 54.2% 1 year post-Tx (during concurrent

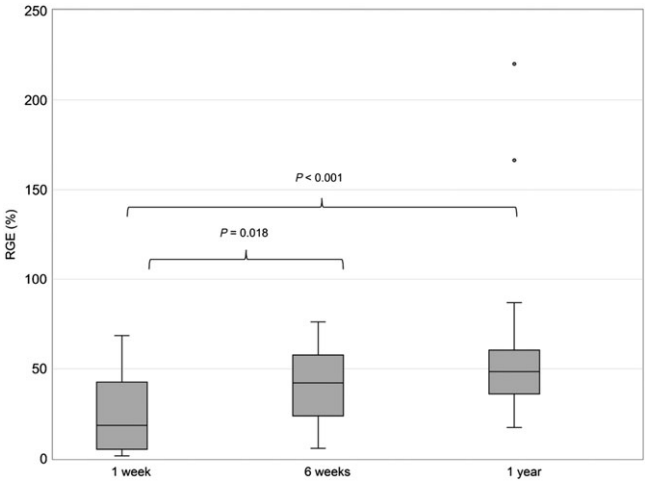

Figure 1

Residual gene expression (RGE) of nuclear factor of activated T cells-regulated cytokines at 1 week (n = 29), 6 weeks (n = 24) and 1 year (n = 24) after transplantation. Data are shown as median ± interquartile range

viraemia and rejection). Despite less cytokine inhibition, Tac levels tended to be higher 1 year post-Tx, with a Tac C_0 of 7.2 μg l^{-1} and a $C_{1.5}$ of 12.7 μg l^{-1} *vs.* 6.4 μg l^{-1} and 6.7 μg l^{-1} 6 weeks post-Tx, respectively.

There were no significant associations between eGFR and cytokine response or Tac levels.

Discussion

The results of the present study indicate that there is a possibility of utilizing NFAT-regulated gene expression measurements as a tool for improvement of current Tac monitoring practice. Several previous studies have reported RGE as a promising pharmacodynamic biomarker of the CNI response [2]. However, the majority of studies so far have focused on long-term stable transplant patients treated with CsA [13]. The present study investigated NFAT-regulated gene expression in 29 renal allograft recipients treated with Tac in the early phase after Tx.

There was a large variability in cytokine gene expression levels between patients before (E_0) and 1.5 h after ($E_{1.5}$) Tac dosing. The observed variability in baseline expression (E_0) may be related to differences in the underlying disease, comorbidities, drug therapy, genetics, immune status and the response to surgery. Importantly, normalization of $E_{1.5}$ to E_0, expressed as RGE, largely corrects for the variability in baseline cytokine levels and allows more accurate measurement of the drug response after dosing [21].

The observed correlation between RGE and Tac $C_{1.5}$ (Figure 2), as well as with Tac $C_{1.5}$ – C_0, indicates that the variability in the cytokine response after dosing is partly explained by differences in Tac levels. However, the considerable variability in RGE in the lower range of Tac concentrations ($C_{1.5}$ <15 μg l^{-1} or $C_{1.5}$ – C_0 <10 μg l^{-1}) suggests that there may be individual differences in the sensitivity to Tac.

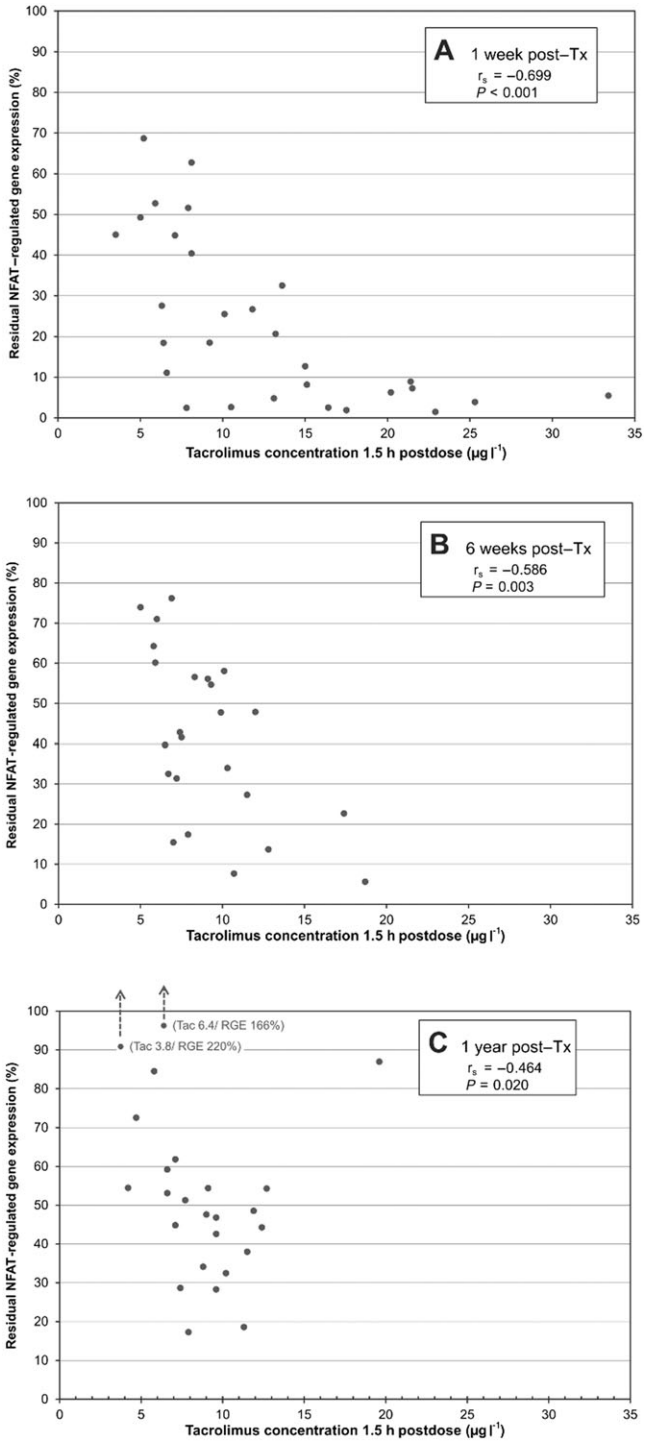

Figure 2

Correlation between tacrolimus (Tac) concentrations 1.5 h postdose ($C_{1.5}$) and the residual gene expression (RGE) of nuclear factor of activated T cells(NFAT)-regulated cytokines at 1 week (A), 6 weeks (B) and 1 year (C) after transplantation (Tx). r_s, Spearman's rank correlation coefficient

Furthermore, the variability in cytokine inhibition may be related to different sensitivities or exposure to other immunosuppressive drugs (e.g. glucocorticoids), or differences in the

relationship between whole blood and intracellular Tac concentrations. Patients with Tac $C_{1.5}$ >15 μg l^{-1}, or $C_{1.5} - C_0$ >10 μg l^{-1}, demonstrated strong cytokine inhibition and RGE <10% (Figure 2). Similar to our findings, Sommerer *et al*. (2010) reported that patients with Tac $C_{1.5} - C_0 \geq 11.4$ μg l^{-1} had strong cytokine inhibition (RGE <30%) [11]. The lack of additional inhibitory effect by increasing Tac levels suggests that in individual patients the total Tac exposure may be reduced, while maintaining sufficient immunosuppressive effect.

The genotyping analysis showed that the dose-normalized Tac concentrations pre- and postdose were 1.8 times higher among homozygous carriers of the nonfunctional *CYP3A5*3* allele than in heterozygous carriers of the *CYP3A5*1* allele. This is in agreement with several previous reports in transplant recipients treated with Tac [22]. Furthermore, the variability in Tac pharmacokinetics (Table 2) may have been related to factors such as differences in body weight, age, gender, haematocrit and drug interactions [14, 23].

The patients demonstrated significantly less inhibition of cytokines (higher RGE) with increasing time since Tx (Figure 1). Similar changes in cytokine response and calcineurin activity have also been observed in previous studies of CNI-treated renal and liver allograft recipients [10–12, 24, 25]. However, other studies have shown relatively unchanged cytokine responses with time after Tx [4, 16, 26]. The difference in findings may be related to the timing of measurements, and the observed increase in RGE with time (Figure 1) may partly have been related to alterations in Tac pharmacokinetics in the early phase post-Tx. The actual and dose-normalized Tac C_0 levels were higher at 6 weeks *vs*. 1 week post-Tx. Previous studies have reported an increase in bioavailability and a reduction in steady-state clearance of CNIs after Tx [27, 28], which in turn may be related to changes in immune status and glucocorticoid exposure [28, 29]. Other studies have shown that the time-dependent increase in Tac C_0 early post-Tx largely can be explained by a simultaneous increase in haematocrit [14]. Importantly, the observed time-dependent changes in gene expression response indicate that single gene expression measurements may not be sufficient to predict the pharmacodynamic response of Tac.

The different Tac formulations may also affect Tac exposure and RGE. One year post-Tx, 19 of the standard-risk patients had been switched to an extended-release formulation. However, Tac concentrations did not tend to differ between formulations, and the higher RGE observed at 1 year *vs*. 1 week post-Tx (Figure 1) is probably not explained by the switch in formulations. Nevertheless, the increasing use of the extended-release formulation emphasizes the need for further investigations of NFAT-regulated gene expression during treatment with the once-daily formulation.

The limited correlation between RGE and Tac levels in the lower range of concentrations (Figure 2) suggests that the change in cytokine response is not explained by Tac exposure alone. Other factors with a potential impact on the cytokine response may be comedication, the immune status of the patient and homeostatic processes counteracting the cytokine inhibition. Giese *et al*. (2004) reported the NFAT-regulated gene expression assay to be relatively CNI specific [16]. However, other studies have shown reduced expression of NFAT-regulated cytokines during treatment with drugs such as glucocorticoids and mycophenolic acid (MPA) [30, 31]. It is likely that the gene expression measurements 1 week post-Tx may have been more influenced by the induction therapy, comprising basiliximab and methylprednisolone, than later measurements. Moreover, the initial prednisolone doses were 20 mg day^{-1} *vs*. 10 mg day^{-1} 6 weeks post-Tx. Glucocorticoids inhibit the binding of transcription factors [e.g. activator protein-1 (AP-1) and nuclear factor kappa B (NF-kB)] that are required for the transcription of proinflammatory cytokines, including *IL2*, *INFG* and *CSF2* [30, 32]. Furthermore, glucocorticoids are reported to inhibit cytokine secretion by reducing mRNA stability [33], and by suppressing the cytokine-producing cell populations [34]. We observed that the two patients with ongoing rejection therapy with methylprednisolone (500–125 mg) presented significantly reduced E_0, as well as strong inhibition of cytokines after dosing (RGE 1.5% and 11.1%). Basiliximab, and specifically ATG, have long-lasting immunosuppressive effects, with a potential impact on both E_0 and $E_{1.5}$ levels. The effect of these drugs on cytokine expression is probably largely corrected by the calculation of remaining gene expression relative to predose expression. However, basiliximab and ATG have dramatic effects on the composition of leukocyte subpopulations [35, 36], which in turn may affect the relative inhibition of cytokine expression after Tac dosing. MPA interferes with proinflammatory signalling, including the p38, Jun N-terminal kinase (JNK) and NF-κB pathways, which in turn interact with the NFAT signalling pathway [37]. In addition to the effects on signalling, MPA inhibits the clonal expansion of activated T lymphocytes, and may thereby also reduce the number of cytokine-producing cells [31]. Millan *et al*. (2003) reported that immunosuppressive regimens with Tac and MMF caused stronger inhibition of IL2 than Tac-based regimens without MMF [31]. Altogether, this emphasizes the need for further studies to characterize the impact of glucocorticoids and other drugs on NFAT-regulated gene expression.

We observed that the patients with ongoing subclinical rejection (n = 5) had weak cytokine inhibition, with RGE ranging from 39.7% to 72.6% (Table 2). The patients without ongoing rejection demonstrated highly variable cytokine inhibition, with an RGE between 5.7% and 220% (Table 2). These results are in line with previous studies in CsA-treated [5, 12, 21, 26], as well as Tac-treated [8, 11, 12], kidney and liver recipients.

Two of the patients in the present study demonstrated postdose increases in cytokine expression and RGEs of 166% and 220%, respectively, 1 year post-Tx (Figure 2C). However, despite the apparent lack of Tac response after dosing, the patients did not experience rejection. Similar findings have also been observed in other studies of long-term stable Tx patients [7, 10]. The increase in cytokine expression after dosing may be partly explained by differences in Tac pharmacokinetics (e.g. delayed absorption or elimination), implying that measurements predose and 1.5 h post-dose might not be sufficient to reflect the Tac response in these patients. Furthermore, Tac has been associated with higher RGE than CsA in long-term stable Tx patients [7, 10, 11, 38]. However, Tac has not been shown to be inferior to CsA [39], and the difference in cytokine responses may be related to

differences in molar concentrations, binding to immunophilins, alterations of cytokine profiles, cytokine receptor blockade or additional mechanisms of action.

In the present study, we observed that the three patients with polyoma virus viraemia (≥10 000 DNA copies ml^{-1}) experienced relatively strong cytokine inhibition and RGEs ranging from 2.55% to 32.5% before the development of viraemia (Table 3). Furthermore, the patients with viraemia demonstrated relatively low E_0, while there was no difference in Tac levels (Table 3). These findings are in agreement with previous reports describing associations between low RGE and infectious complications [3–8, 11]. Additionally, strong cytokine suppression has been reported to be a risk factor for the development of malignancies [4, 6, 9, 10]. Altogether, this indicates that the gene expression of NFAT-regulated cytokines may be used to identify overimmunosuppression, even when the Tac levels are within therapeutic ranges.

The main limitation of the present study was the small number of patients, presenting with different immunological statuses and immunosuppressive regimens. Furthermore, Tac concentration measurements were performed by an immunoassay, which generally has lower analytical sensitivity and specificity than liquid chromatography–tandem mass spectrometry assays. A strength of the study was the single-centre prospective approach, following participants from before Tx and with repeated measurements at predefined time-points post-engraftment.

In conclusion, the findings of our study support the potential of NFAT-regulated gene expression measurements as a tool for the pharmacodynamic monitoring of Tac therapy in the early phase post-Tx, especially in the context of overimmunosuppression and viraemia. However, further knowledge is needed, considering different patient populations, interfering parameters, monitoring strategies and target ranges. The assays for measurement of NFAT-regulated gene expression need further validation and standardization to allow comparisons between studies. Finally, the clinical value of pharmacodynamic monitoring based on NFAT-regulated gene expression should be evaluated in prospective clinical studies.

Competing Interests

There are no competing interests to declare.

The authors are grateful to Lene Pham, Kristine Hole and Rolf Klaasen for their excellent assistance with sample handling, genetic analyses and extraction of clinical data. The authors would also like to acknowledge Laila Gjerdalen, Torunn Skogseth, Marit Hansen Hallberg, May Ellen Lauritsen and their colleagues for organizing laboratory facilities and assistance with pharmacokinetic measurements.

Contributors

S.B., N.T.V. and St.B. participated in research design. M.S. and K.M. recruited patients. S.B., N.T.V., M.K., E.D.J. and St. B. participated in performance of the research. S.B., N.T.V. and St.B. participated in data analysis and wrote the manuscript. All authors were involved in the discussion of results, critical revision of the manuscript and approval of the final version.

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Supporting Information

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Table S1 Oligonucleotides for the nuclear factor of activated T cells-regulated gene expression assay
Table S2 Amplification conditions for the nuclear factor of activated T cells-regulated gene expression assay