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Sensitivity of Whole-Blood T Lymphocytes in Individual Patients to Tacrolimus (FK 506): Impact of Interleukin-2 mRNA Expression as Surrogate Measure of Immunosuppressive Effect

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Background: **To optimize immunosuppressive treatment in individual transplant patients, functional measurements of the effects of tacrolimus (FK 506) are of clinical importance. Previous investigations have demonstrated the occurrence of tacrolimus-resistant production of interleukin-2 (IL-2) in vitro, which may explain in part why rejection episodes are still a frequent problem despite attainment of therapeutic blood concentrations and HLA matching. However, an adequate surrogate marker to define the tacrolimus response in individual patients has not been established.**

Methods: **We investigated the immunosuppressive effects of tacrolimus on anti-CD3/anti-CD28 T-cell costimulation in a human whole-blood assay, analyzing T-cell proliferation, activation marker expression (CD25, CD69), IL-2 protein expression, and cytokine mRNA expression in vitro (n = 11 healthy individuals). We also quantified IL-2 mRNA expression in patients undergoing tacrolimus (n = 4) or cyclosporin A (CsA; n = 4) monotherapy before ex vivo living-donor kidney transplantation.**

Results: **T-cell proliferation; CD25, CD69, and IL-2 concentrations; and IL-4 mRNA were significantly decreased in vitro. In contrast, cytokine mRNA profiles revealed variable tacrolimus sensitivity. Whole-blood samples from 3 of 11 healthy individuals demonstrated marked suppression of IL-2 mRNA expression (>50%) when tacrolimus was administered in vitro. When CsA was added to whole-blood cultures, the influence on IL-2 mRNA expression was comparable to that of tacrolimus in 9 of 11 individuals. Two individuals responded conversely, indicating that differences in the in vitro response to tacrolimus and CsA among individuals may be attributable to potential heterogeneity in the involvement of the CD28 pathway. Kinetic profiles of IL-2 mRNA expression also revealed individually distinct degrees of calcineurin inhibitor sensitivity in patients undergoing tacrolimus or CsA monotherapy before living-donor kidney transplantation.**

Conclusions: **Our results suggest an individual degree of calcineurin inhibitor sensitivity of activated whole-blood lymphocytes based on IL-2 mRNA expression. Our approach is potentially valuable for identifying transplant patients in whom IL-2 mRNA expression is unaffected or even enhanced after initiation of immunosuppressive therapy. Such individuals may be less sensitive to the immunosuppressive agent and therefore at increased risk of transplant rejection. Prospective studies are necessary to determine the correlation of IL-2 mRNA expression with the clinical risk of transplant rejection.**

Tacrolimus (FK 506) is a potent macrolide immunosuppressive agent that has been used as a primary immunosuppressant for the prevention of allograft rejection *(1, 2)*. It has also been successfully used as a treatment for steroid- and antibody-resistant rejection *(3)* and as an alternative agent for patients who are cyclosporin A

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(CsA)[4] resistant *(4)* or experience severe CsA side effects *(5)*. The mode of immunosuppressive action is well documented: Tacrolimus binds to its intracellular receptor, the immunophilin FK-binding protein, forming inhibitory complexes that block the phosphatase activity of calcineurin. The effect of this block is inhibition of the translocation of nuclear factor-ATc from the cytoplasm to the nucleus *(6)*, which is demonstrated by a lack of cytokine gene expression and T-cell activation *(7–9)*. Full activation of T lymphocytes requires engagement of the T-cell receptor–CD3 complex as well as a second signal induced by stimulation of coreceptors such as CD2, CD4, CD8, or CD28 *(10)*.

Despite the differences in tacrolimus and CsA as chemical substances, the mechanism of action—the blockade of T-cell receptor-stimulated transcription of interleukin-2 (IL-2)—is similar (calcineurin inhibitors) *(11)*. Tacrolimus, however, has a 10- to 100-fold greater in vitro immunosuppressive activity than CsA *(11–13)*. Previous studies have demonstrated that stimulation of T cells by CD28 may be highly resistant to tacrolimus in vitro *(12, 14–16)*. In clinical transplantation, the occurrence of tacrolimus resistance in vitro might explain in part why rejection episodes are still a frequent problem despite the attainment of therapeutic blood concentrations and HLA matching. However, an adequate surrogate marker to define the tacrolimus response in individual transplant patients has not been established. The aim of our study was to investigate the potential pharmacodynamic parameters for tacrolimus effects, such as IL-2 mRNA expression and T-lymphocyte proliferation, in a human whole-blood assay in vitro. To further elucidate tacrolimus sensitivity, we also investigated IL-2 protein concentrations and T-cell surface marker expression (CD25 and CD69).

Materials and Methods

WHOLE-BLOOD CULTURE

Human whole-blood cultures were performed in 6-well-cluster tissue culture dishes (35-mm diameter; Costar). For each sample, duplicate 1-mL aliquots were diluted in 9 mL of Iscove's modified Dulbecco's medium supplemented with penicillin (100 000 units/L), streptomycin (100 mg/L), and 10 mmol/L L-glutamine and stimulated with 1 mg/L anti-CD3 monoclonal antibody (mAb; CLBT3/4E) and 1 mg/L anti-CD28 mAb (CLB-CD28/1; Hiss Diagnostics) for 24 or 72 h [ultraviolet-light-induced detection (UVID) technology]. After 24 h of incubation, 2 mL of each supernatant was withdrawn for enzyme-linked immunoassay analysis of IL-2 protein concentrations. Residual culture material was processed for RNA isolation and quantitative PCR.

[4] Nonstandard abbreviations: CsA, cyclosporin A; IL, interleukin; mAb, monoclonal antibody; UVID, ultraviolet-light-induced detection; TNF-α, tumor necrosis factor-α; FITC, fluorescein isothiocyanate; and PE, phycoerythrin.

RNA ISOLATION

Total RNA from whole-blood leukocytes or peripheral blood mononuclear cells was isolated with use of the Purescript RNA isolation reagent set (Gentra Systems) according to the manufacturer's protocol. The resulting RNA was resuspended in 300 μL of diethylpyrocarbonate-treated water and stored at −80 °C until use.

TaqMan REVERSE TRANSCRIPTION-PCR

The reverse transcription-PCR protocol for quantitative detection of cytokine mRNA has been described previously *(17)*. In brief, the PCR mixture contained 25 μL of 2× TaqMan Mastermix (Perkin-Elmer), 100 nM each of the forward and reverse primers, 100 nM fluorogenic probe, 20 units of RNase inhibitor (Life Technologies), 25 U of murine leukemia virus reverse transcriptase (Perkin-Elmer), 1.25 U of AmpliTaq Gold DNA polymerase (Perkin-Elmer), and 20 μL of water control, diluted calibrators, or unknown RNA template in a total volume of 50 μL. Sequence-specific primer pairs and fluorogenic probes were obtained from TIB Molbiol [IL-2, IL-4, tumor necrosis factor-α (TNF-α) *(9)*] or Perkin-Elmer Cetus (β-actin reagent set for cDNA samples). PCR conditions were 2 min at 50 °C and 30 min at 48 °C for reverse transcription, 10 min at 95 °C for DNA polymerase activation, and 40 cycles of 15 s at 95 °C and 1.5 min at 60 °C with a final 25 °C hold. Standardized cytokine mRNA quantities (cytokine copies/10^6 β-actin copies) were determined by dividing interpolated values from the cytokine calibration curve by the normalization factor (β-actin content in test samples) *(17, 18)*.

ENZYME-LINKED IMMUNOASSAY ANALYSIS OF IL-2 PROTEIN SYNTHESIS

Supernatants of whole-blood cultures were costimulated for 24 h with anti-CD3/anti-CD28 mAb and assayed for IL-2 protein concentrations by a sandwich enzyme-linked immunoassay technique using unlabeled and enzyme-coupled mAbs against different IL-2 epitopes according to the manufacturer's instructions (Laboserv).

ANALYSIS OF CD25/CD69 EXPRESSION BY FLOW CYTOMETRY

For analysis of CD25 and CD69 expression on the cell surface, EDTA-anticoagulated whole blood was incubated in duplicate aliquots as described above. After incubation, whole-blood cultures were centrifuged at 300*g* for 10 min, and 100-μL aliquots were labeled with 20 μL of anti-CD4-fluorescein isothiocyanate (FITC; BD PharMingen; clone RPA-T4) and 20 μL of anti-CD25-phycoerythrin (PE; Beckman-Coulter Immunotech; clone 1HT44H3) or 20 μL of anti-CD69-PE (BD PharMingen; clone L78) for 10 min at room temperature. Erythrocytes were lysed automatically by a MultiQprep workstation (Beckman-Coulter Immunotech). Identical IgG isotype mAbs were used as negative controls to determine the degree of nonspecific staining before the percentage of

CD4+ T cells expressing either CD25 or CD69 was determined on an EPICS XL MCL flow cytometer (Beckman-Coulter Immunotech).

ANALYSIS OF T-CELL PROLIFERATION: UVID OF BROMODEOXYURIDINE

The principle of the UVID methodology for detecting halogenated pyrimidines incorporated into DNA of S-phase cells has been described recently *(19, 20)* (UVID proliferation reagent set; SpartaLabs LLC) and was used for analysis of T-cell proliferation (percentage of cells in S phase). Heparinized venous whole-blood specimens were preincubated in duplicate as described above and then stimulated with anti-CD3/anti-CD28 mAb for 72 h. At the end of the incubation, bromodeoxyuridine was added to a final concentration of 30 μmol/L, and the cell culture was incubated for 60 min at 37 °C under 95% air–5% CO_2. Cells were then processed for separation of the mononuclear fraction, UVB irradiation (8 W; 312 nm UV lamp; Herolab), and immunophenotyping according to the manufacturer's instructions *(20)*. For fixation, cells were washed once in phosphate-buffered saline and treated for 5 min with 1000 μL of SpartaLabs fixative freshly prepared at 4 °C from the SpartaLabs reagent set. Cells were washed and then transferred to flow cytometer tubes, after which 1000 μL of distilled water, 50 μL of the UVID flow-activated cell-sorting buffer, 20 μL of the UVID anti-bromodeoxyuridine-FITC antibody, and 20 μL of the SpartaLabs DNA stain were added to the tubes before they were incubated for another 60 min in the dark at ambient temperature. The specimens were then subjected to flow cytometric analysis.

FLOW CYTOMETRY

All flow cytometric analyses were performed on an EPICS XL MCL (Coulter-Immunotech) equipped with a single air-cooled argon laser with an excitation line at 488 nm. Green fluorescence (FITC) was detected through a 525 nm bandpass filter, orange emission (PE) through a 575 nm bandpass filter, and deep red fluorescence from 7-amino-actinomycin D was detected through a 675 nm bandpass filter. All measurements were performed for at least 10 000 cells at low sample pressure.

IN VITRO STUDY

To determine the individual variability of tacrolimus and CsA sensitivity in vitro, we prepared heparinized whole-blood samples from healthy volunteers (n = 11) for culture as described above and preincubated the samples for 2 h with 25 μg/L tacrolimus (Fujisawa) or 1000 μg/L CsA (Novartis Pharma). All healthy individuals (8 females and 3 males) were Caucasian nonsmokers 25–40 years of age and had no evidence of recent infection. In addition, patients undergoing tacrolimus (n = 4) or CsA monotherapy (n = 4; ex vivo study) were tested for tacrolimus or CsA sensitivity in vitro.

EX VIVO STUDY

To investigate the effects of tacrolimus or CsA monotherapy on IL-2 mRNA expression on anti-CD3/anti-CD28 mAb costimulation ex vivo, we selected living-donor kidney transplant patients who had received treatment with Prograf (tacrolimus) or CsA before transplantation (Table 1). Whole blood was drawn at baseline (before patients received tacrolimus/CsA for the first time) and after 96 h of tacrolimus or CsA monotherapy. Tacrolimus and CsA concentrations were determined as trough concentrations (c_0) and in samples collected 2 h post-dose (c_2) and 4 h after dosing (c_4), and assayed with the IMx Tacrolimus II assay (Abbott Diagnostics) *(21)* and the AxSYM cyclosporine assay (fluorescence polarization immunoassay; Abbott Diagnostics) *(22)*, which was kindly performed by Prof. Heiko Iven, Institute of Pharmacology and Toxicology, University of Lübeck Medical School.

All performed ex vivo and in vitro studies were

Table 1. Clinical data for patients receiving monotherapy with tacrolimus or CsA.

Patient	Age, years	Gender	Disease	Antibodies[a]	Mismatch[b]	CMV[c,d]	Acute rejection	CN inhibitor
I	39	F	PN	2%	1, 2, 2	+/+	No	FK 506[e]
II	64	M	PN	No	1, 2, 1	−/+	Yes	FK 506
III	30	M	degNP	No	1, 1, 1	−/+	No	FK 506
IV	38	F	RN	No	1, 1, 1	−/−	No	FK 506
V	26	M	RN	No	1, 1, 1	−/−	Yes	CsA
VI	44	M	mpGN	No	1, 1, 1	−/−	Yes	CsA
VII	22	F	mpGN	No	1, 1, 1	−/−	No	CsA
VIII	42	F	degNP	No	0, 2, 1	+/+	Yes	CsA

[a] Preformed HLA antibodies.

[b] HLA mismatch in HLA A, HLA B, or HLA DR.

[c] CMV, cyotmegalovirus; CN, calcineurin; PN, chronic pyelonephritis; degNP, degenerative nephropathy (nephronophthisis); RN, reflux nephropathy; mpGN, membranoproliferative glomerulonephritis.

[d] Status of recipient/donor.

[e] Tacrolimus.

approved by the Ethics Commission of the Lübeck University School of Medicine.

STATISTICAL ANALYSIS

Statistical analysis was performed with a Wilcoxon rank-sum test or Mann–Whiney *U*-test (SPSS for Windows, Release 6; SPSS). A two-tailed P value <0.05 was the criterion for statistical significance.

Results

IN VITRO STUDY I: EFFECT OF TACROLIMUS CONCENTRATION ON CYTOKINE mRNA EXPRESSION

IL-4 and TNF-α mRNA expression exhibited dose-dependent inhibition after in vitro addition of tacrolimus (0, 12.5, 25, and 100 μg/L) to whole-blood samples from healthy individuals (n = 4) at all time points of anti-CD3/anti-CD28 mAb costimulation (Fig. 1, A and B). In contrast, the in vitro addition of tacrolimus inhibited IL-2 mRNA expression after 4 h of anti-CD3/anti-CD28 mAb costimulation (range of inhibition, 82.2–90.0%) and to a lesser extent after 8 h (range of inhibition, 33.1–71.3%) in a dose-dependent fashion. However, after 24 h, the inhibitory effects of tacrolimus were no longer detectable, demonstrating not only a delay in IL-2 mRNA expression kinetics but also increased IL-2 mRNA expression peaks of up to 147.2% (Fig. 1C).

IN VITRO STUDY II: INVESTIGATION OF DIFFERENT SURROGATE MEASURES OF TACROLIMUS SENSITIVITY IN HEALTHY INDIVIDUALS

The addition of tacrolimus at a concentration of 25 μg/L produced a variable pattern of T-lymphocyte sensitivity after anti-CD3/anti-CD28 mAb costimulation in whole-blood samples from healthy individuals. As can be seen in Fig. 2, IL-2 mRNA expression for 3 of 11 individuals was highly sensitive to tacrolimus (>50% suppression; donors II, X, and XI). In contrast, IL-2 mRNA expression for 8 of 11 individuals was resistant to in vitro addition of tacrolimus or was markedly stimulated (>50% increase; donors I, IV, V, and IX). In 9 of 11 individuals, IL-2 mRNA expression was either highly sensitive or resistant to both tacrolimus and CsA. Notably, IL-2 mRNA expression in whole blood from donor III was sensitive to CsA but resistant to tacrolimus; conversely, IL-2 mRNA concentrations in whole blood from donor X were reduced by tacrolimus but not CsA. The expression of IL-4 mRNA was inhibited in whole-blood samples from all individuals by in vitro addition of tacrolimus (P = 0.003; Fig. 3A).

To evaluate other surrogate measures of in vitro tacrolimus sensitivity, we also assessed IL-2 protein concentrations (Fig. 3B), T-cell surface marker expression (CD25 and CD69; Fig. 3C), and proliferation of T lymphocytes (percentage of bromodeoxyuridine-incorporating cells in S phase; Fig. 3D). In contrast to IL-2 mRNA expression, the secretion of IL-2 protein and IL-4 mRNA

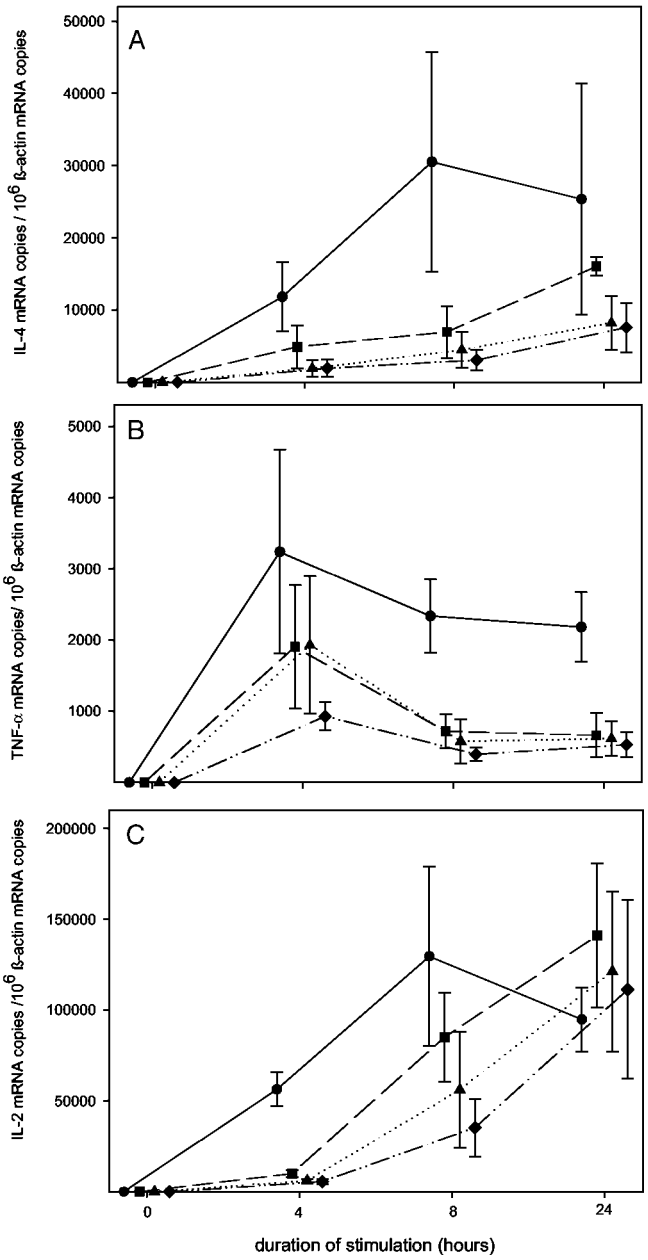

Fig. 1. In vitro study I: Effect of tacrolimus concentration on IL-2 mRNA expression kinetics.

Whole-blood samples from healthy controls (n = 4) were preincubated for 30 min without (●) or with 12.5 μg/L (■), 25 μg/L (▲), or 100 μg/L (◆) tacrolimus and stimulated for 4, 8, and 24 h with 1 mg/L anti-CD3 mAb plus anti-CD28 mAb. IL-2 mRNA copy numbers were determined by real-time reverse transcription-PCR and normalized with respect to β-actin mRNA copy numbers (cytokine mRNA expression/10^6 β-actin mRNA copies). Data are the means (SE; *error bars*).

expression were significantly decreased in anti-CD3/anti-CD28 mAb-costimulated whole-blood cultures when tacrolimus was added (P = 0.003). In addition, the percentages of proliferating CD4+ (P = 0.003) and CD4− cells (P = 0.003) were markedly reduced in all tested individuals receiving tacrolimus. Similarly, activation marker expression was generally diminished in the pres-

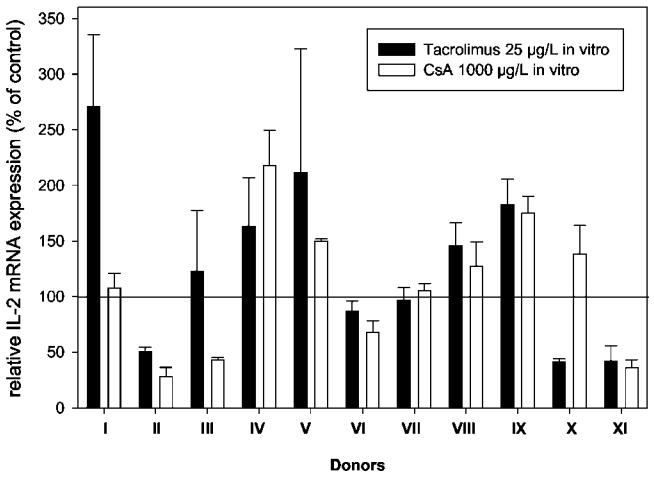

Fig. 2. In vitro study II: Differential IL-2 mRNA expression in whole blood from healthy individuals after addition of tacrolimus/CsA in vitro.

Whole-blood samples from healthy individuals were incubated for 24 h with 1 mg/L anti-CD3 mAb and anti-CD28 mAb, respectively, in the presence or absence of 25 μg/L tacrolimus (■) or 1000 μg/L CsA (□; n = 11). The relative change in IL-2 mRNA expression after the addition of CsA in vitro is given as the percentage of control IL-2 mRNA expression without tacrolimus/CsA added. Data represent the means (SE; *error bars*) of quadruplicate experiments.

ence of 25 μg/L tacrolimus (CD25, $P = 0.01$; CD69, $P = 0.003$).

ex vivo study: effect of tacrolimus or CsA monotherapy on IL-2 mRNA expression in whole-blood samples of kidney transplant patients

To elucidate the effect of tacrolimus or CsA on IL-2 mRNA expression kinetics ex vivo, we studied living-donor kidney transplant patients (n = 8; Table 1) receiving tacrolimus or CsA monotherapy for 5 days before transplantation. In vitro addition of tacrolimus (25 μg/L) to whole blood drawn at baseline (before patients had received the calcineurin inhibitor for the first time) revealed an inhibition of IL-2 mRNA expression of >50% in whole-blood samples from four of four patients after 4 h of anti-CD3/anti-CD28 mAb costimulation and in samples from two of four patients (patients I and IV) after 24 h of costimulation (Fig. 4A). We further assessed the effects of a 5-day monotherapy with tacrolimus at three different time points (trough concentrations, 2 h post-dose, and 4 h post-dose). Fig. 4A shows the interindividual variation in tacrolimus sensitivity in vitro and ex vivo. Patients I and IV (no acute rejection episode) not only had markedly decreased IL-2 mRNA concentrations (4 and 24 h of costimulation) in vitro but also ex vivo at tacrolimus trough and post-dose concentrations. In contrast, IL-2 mRNA concentrations in patient III (no rejection episode) were unaffected after 4 h of costimulation ex vivo but were significantly inhibited after 24 h of costimulation at trough and 4-h post-dose concentrations. Patient II, however, who had suffered an acute rejection episode, was observed to have unaffected or even increased IL-2 mRNA expression after 24 h of costimulation in vitro and ex vivo, whereas IL-2 mRNA concentrations after 4 h of costimulation were markedly decreased in vitro and ex vivo. The individual responses to CsA monotherapy are shown in Fig. 4B. All patients (donors V–VIII) demonstrated diminished IL-2 mRNA expression kinetics when CsA was added in vitro. Of those receiving CsA monotherapy, two of four patients with acute rejection (donors VI and VIII) had decreased post-CsA IL-2 mRNA concentrations after 4 h but not 24 h of anti-CD3/anti-28 mAb costimulation. At CsA trough concentrations, both patients had increased IL-2 mRNA expression compared with baseline. In contrast, the data for patient V, who also had an acute rejection, showed lower IL-2 mRNA concentrations throughout the monotherapy treatment. Interestingly, in patient VII (without acute rejection) IL-2 mRNA was decreased after 4 h of costimulation at all time points of CsA monotherapy but only 2 h after CsA intake when whole blood was costimulated for 24 h. Taken together, these findings indicate that a delayed increase in IL-2 mRNA expression during T-cell costimulation may represent a sensitive effect of tacrolimus or CsA immunosuppression in vitro and ex vivo. Analysis of a single absolute or peak mRNA value could be misleading, because calcineurin inhibitor sensitivity is highly variable on an individual basis.

Discussion

Because acute rejection and side effects of immunosuppressive drugs are frequent problems in organ transplantation *(23, 24)*, better understanding of individual variations would be relevant to clinical care. This study describes the practical assessment of pharmacodynamic responses to tacrolimus in a human whole-blood assay after anti-CD3/anti-CD28 mAb T-cell costimulation *(17, 25, 26)*. Our results show considerable interindividual variation in IL-2 mRNA expression profiles both in vitro and ex vivo, as well as the potential relevance of IL-2 mRNA expression kinetics, i.e., a dose-dependent delay in peak expression, as a predictive marker for sensitivity to the calcineurin inhibitor.

Inhibition of IL-2 production is central to the immunosuppressive action of tacrolimus *(11)*. In the clinical setting, failed IL-2 inhibition may be associated with an increased likelihood of organ rejection *(27–29)*. Further evidence of the clinical impact of IL-2 production has been provided by studies on IL-2 receptor blockade, which was shown to prevent acute rejection in renal transplantation patients *(30)*. Thus, the assessment of tacrolimus-induced IL-2 expression profiles might define a biologically relevant drug effect that would allow development of a pharmacodynamic measure of interindividual variability in responses to tacrolimus therapy.

Conflicting data exist regarding the sensitivity of costimulated T-cell activation to tacrolimus. Several investigators have reported that IL-2 expression in purified T cells or peripheral blood lymphocytes, induced by the costimulatory B7/CD28 pathway, is resistant to inhibition

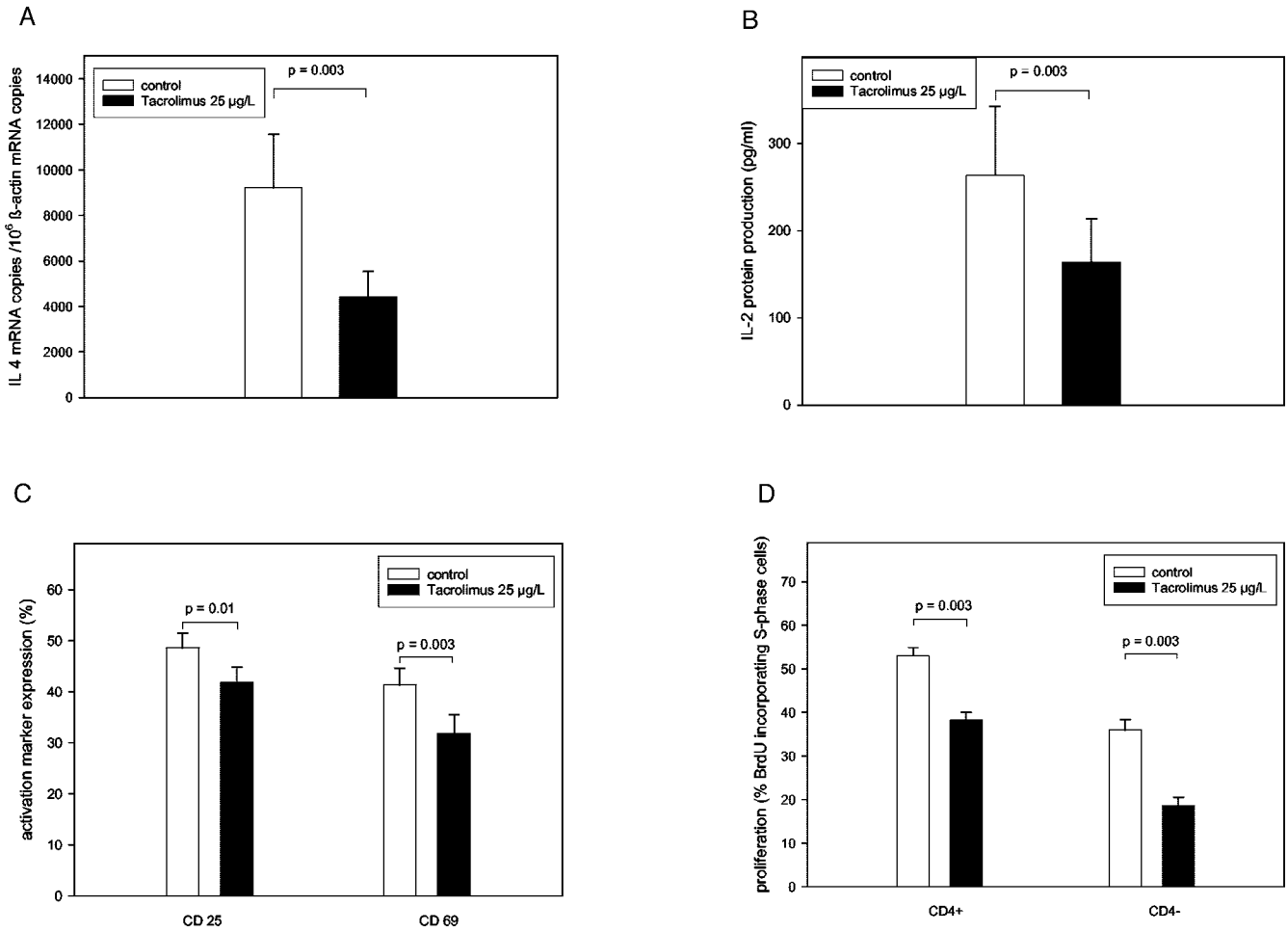

Fig. 3. In vitro study II: Investigation of different surrogate measures of individual tacrolimus sensitivities in healthy individuals.

(*A–C*), whole-blood samples from healthy individuals were incubated for 24 h with 1 mg/L anti-CD3 mAb and anti-CD28 mAb, respectively, in the absence (control; □) or presence of 25 μg/L tacrolimus (■). (*A*), IL-4 mRNA expression; (*B*), IL-2 protein production; (*C*), CD25/CD69 surface marker expression. (*D*), whole-blood samples from healthy individuals were incubated for 72 h with 1 mg/L anti-CD3 mAb and anti-CD28 mAb, and T-cell proliferation was measured. Data are the means (SE; *error bars;* n = 11). Statistical analysis was performed with the Wilcoxon rank-sum test (n = 11). $P < 0.05$ was considered statistically significant. *BrdU*, bromodeoxyuridine.

by tacrolimus in vitro, even with drug concentrations of 100 μg/L *(31–33)*. In contrast, Sakuma et al. *(34)* noted that anti-CD3/anti-CD28-costimulated IL-2 mRNA expression in peripheral blood mononuclear cells is significantly inhibited (IC_{50}) by the addition of low tacrolimus concentrations (0.12 μg/L). In our in vitro study, 3 of 11 healthy individuals had marked suppression (>50%) of IL-2 mRNA concentrations. We also demonstrated a dose-dependent delay in IL-2 mRNA expression during T-cell costimulation. In contrast, IL-4 mRNA expression was significantly decreased independent of the duration of anti-CD3/anti-CD28 costimulation. In accordance with the data of Sakuma et al. *(34)*, who found that TNF-α expression in lymphocytes and monocytes was inhibited by tacrolimus, we observed a dose-dependent inhibition of TNF-α mRNA expression during costimulation. Thus, based on the cytokine mRNA expression data in our whole-blood matrix, we cannot confirm a general resistance to tacrolimus, as suggested by previous studies *(12, 14–16)*, when the CD28 pathway is involved. In line with this, the influence of both calcineurin inhibitors CsA and tacrolimus on IL-2 mRNA expression was comparable in 9 of 11 individuals but differed on an individual basis. Interestingly, two individuals responded conversely, indicating that the differences among in vitro responses to tacrolimus and CsA may be attributable to a potential heterogeneity in the involvement of the CD28 pathway *(35)*. Furthermore, in contrast to CsA, tacrolimus was found to significantly inhibit IL-4 mRNA expression *(26)*. CsA and tacrolimus share the same capacity to inhibit the enzyme calcineurin phosphatase and by that route to suppress the production of a range of cytokines. Tacrolimus, however, significantly decreases the rate of acute rejection episodes in renal transplantation patients and does not up-regulate transforming growth factor-β, as CsA does, which may have an impact on the prevention of chronic graft rejection *(36, 37)*. These differences in efficacy between the two drugs could be related to differences in the inhibition of cytokine expression *(38)*. We therefore propose that investigation of IL-2 mRNA ex-

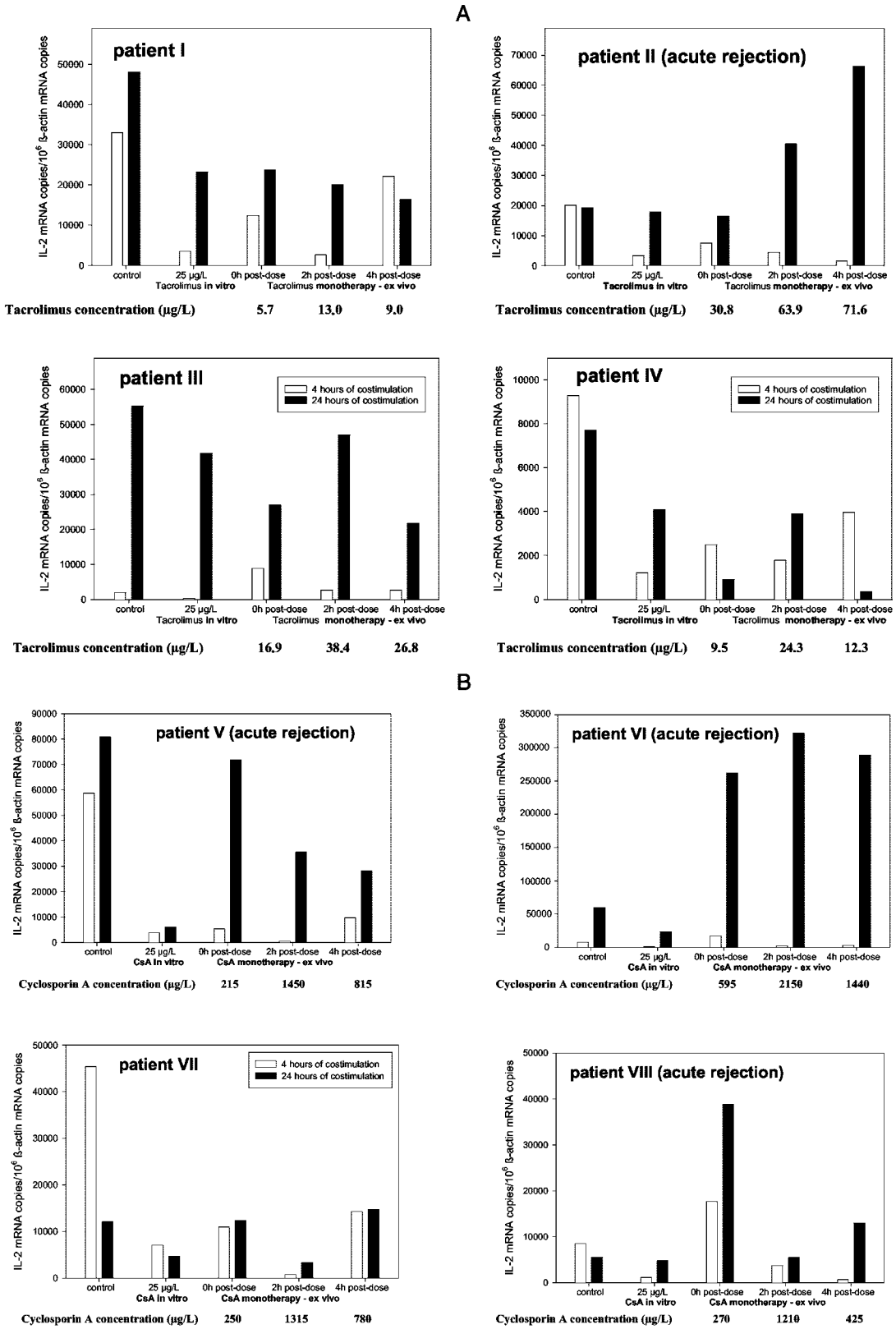

Fig. 4. Ex vivo study: Effects of tacrolimus/CsA on anti-CD3/anti-CD28-induced IL-2 mRNA expression in patients undergoing calcineurin inhibitor monotherapy.

Whole-blood samples from patients I—VIII were incubated for 4 (□) or 24 h (■) with 1 mg/L anti-CD3 mAb and anti-CD28 mAb on the day before first dose of tacrolimus (patients I–IV; *A*) or CsA (patients V–VIII; *B*) for control values and for the assessment of in vitro sensitivity to tacrolimus (addition of 25 µg/L tacrolimus) or CsA (addition of 1000 µg/L CsA) and after a 5-day monotherapy with oral tacrolimus or CsA [trough (*0 h post-dose*), 2 h post-dose, and 4 h post-dose].

pression kinetics may add insight into the degree of T-cell activation in individual patients. This could complement the information obtained from traditional thresholds, such as the EC_{50}, which is the drug concentration associated with a half-maximal effect, or the IC_{50}, which is the drug concentration associated with inhibitory effect (in this case, the prevention of rejection) in 50% of the target population. For example, if rejection occurs despite drug exposure at or above the EC_{50} or IC_{50} thresholds, the kinetics of IL-2 mRNA expression may explain this occurrence based on the relative unresponsiveness of IL-2 in an individual patient to the effect of such agents as CsA or tacrolimus *(34, 39)*.

Notably, in the majority of healthy individuals, IL-2 mRNA after 24 h of costimulation was unaffected or even increased when calcineurin inhibitors were administered in vitro. In clinical investigations we found that patients, after peroral monotherapy with tacrolimus, may be distinguished by their individual tacrolimus responses because two of four patients showed a relevant inhibition of IL-2 mRNA expression kinetics in whole-blood samples in vitro and ex vivo. In addition, three of four patients receiving CsA monotherapy were found to have delayed kinetics of IL-2 mRNA expression after T-cell costimulation ex vivo. In each patient, for measurements made at 0 and 2 h post-dose of CsA or tacrolimus, IL-2 mRNA after 4 h of costimulation was decreased with increasing drug concentration. Similar to our in vitro study, we found in four of eight patients IL-2 mRNA concentrations that were unaffected, or even increased, after 24 h of costimulation for measurements made at 0, 2, and 4 h after the dose of calcineurin inhibitor.

There are several possible explanations for these findings: The first is that the B7/CD28 signaling pathway has been described to be resistant to calcineurin inhibitors in vitro *(12, 14, 35, 40)*. Therefore unaffected or even increased IL-2 mRNA concentrations could be explained by CsA/tacrolimus insensitivity or loss of intracellular drug concentrations. In the transplant situation, however, individual variability but not general resistance to CsA or tacrolimus is evident. Therefore, the immunosuppressive effect may not necessarily be exhibited by a decreased peak IL-2 mRNA concentration but by an enhanced half-life of IL-2 mRNA or a delay in IL-2 gene transcription if the measurements are compared after 4 and 24 h for controls and post-dosage samples.

The second reason is that the failure of immunosuppression and the subsequent transplant rejection are regarded to have a multifactorial pathogenesis. Therefore, the role of other cytokines in the network should be considered. In this line, enhanced IL-2 mRNA expression could be explained by the ability of calcineurin inhibitors to remove a negative regulatory signal, e.g., one that is IL-10 mediated *(41, 42)*. Furthermore, the cytokine IL-7 may increase IL-2 mRNA expression by enhancing the binding activity of the transcription factor activator protein-1, which is CsA-insensitive, in the IL-2 gene promoter region *(43)*. On the other hand, the expression of IL-7 is increased by CD3/CD28 costimulation but not inhibited by CsA *(44)*.

The third reason is that IL-2 transcript accumulation but not T-cell proliferation may be a result of costimulation-dependent stabilization of IL-2 mRNA in which other surface accessory molecules play a crucial role (intercellular adhesion molecule-1, leukotactic factor activity-1). These costimulatory molecules (which are also active in the whole blood system applied) enhance the half-life of IL-2 in a manner that is insensitive to calcineurin inhibitors. Furthermore, costimulatory molecules impact on qualitatively different signaling pathways, e.g., leukotactic factor activity-1 but not CD28 requires the actin-based cytoskeleton for IL-2 mRNA stabilization, which may the explain unaffected/enhanced IL-2 mRNA despite an abundance of CsA or tacrolimus *(45)*. It has also been shown that CsA may inhibit the expression of vascular cell adhesion molecule-1 but not intercellular adhesion molecule-1 *(46)*.

Careful evaluation of the patient's immune status before initiation of immunosuppressive therapy and subsequent clinical documentation, including (*a*) underlying and secondary diseases; (*b*) influence of additional treatment, such as dialysis therapy and surgery *(47)*; (*c*) cytokine mRNA concentrations in the transplanted organ; and (*d*) the functional impact of gene mutations and polymorphisms with a role in the innate or acquired immune system *(48, 49)*, are needed before conclusions from IL-2 mRNA monitoring data (i.e., enhanced IL-2 mRNA expression) can be drawn.

Because the CD28 pathway is mainly influencing mRNA stability and posttranscriptional regulation of IL-2 mRNA expression *(50, 51)*, we also studied anti-CD3/anti-CD28-costimulated IL-2 protein production, IL-2 receptor expression on the cell surface (CD25), CD69 activation marker expression, and T-cell proliferation. Notably, all additional investigated potential biological markers were significantly inhibited by tacrolimus in vitro. Our data suggest that tacrolimus-insensitive IL-2 mRNA expression in some individuals (8 of 11 healthy individuals) may not necessarily lead to tacrolimus-insensitive T-cell growth after anti-CD3/anti-CD28 costimulation. This implies the existence of interindividual variations in mRNA and protein processing after full activation of tacrolimus administered T lymphocytes. It also supports the data of Appleman et al. *(52)*, who proposed an IL-2-independent regulation of T-cell proliferation after costimulation with anti-CD28. Furthermore, a disconnect between unaffected or enhanced IL-2 mRNA but decreased IL-2 protein was observed. This finding raises the question of whether the calcineurin inhibitors have an impact on posttranscriptional modification and translation of IL-2 mRNA into protein. This issue should be studied further.

The existence of individual degrees of sensitivity to the immunosuppressive agents may have potential clinical

relevance for future immunosuppressive strategies, which need to be more effective, safer, and focused on targeted therapy. At a time when newer, non-calcineurin inhibitor agents such as rapamycin (sirolimus) are available or in preclinical testing (sanglifehrin A), the most promising alternative could consist of combination therapies, such as sirolimus plus low-dose tacrolimus/CsA, that provide good renal allograft survival and low rates of side effects *(53)*. Recent advances in safer immunosuppressive therapy also include modifications of currently available agents such as the rapamycin derivative SDZ-RAD and ERL080A, a new formulation of mycophenolic acid. In the arena of more targeted therapies within drug combination strategies, various monoclonal and polyclonal antibodies (anti-IL-2-receptor antibodies, thymoglobulin, anti-CD3 antibodies, and antibodies against adhesion molecules) are now being investigated in preclinical and clinical studies *(54)*. Moreover, the selective inhibition of T-cell costimulation by the B7-specific fusion protein CTLA4-immunoglobulin has been shown to prolong rejection-free survival in primates *(55)*. These advances in immunosuppressive therapy, however, emphasize the need for continued investigations into better monitoring of the pharmacodynamic effects of the administered agents. Although the pharmacodynamic effects of rapamycin are under current investigation in our laboratory, the approach described here may potentially contribute to the development of predictive parameters of hypo- or hyperresponsiveness to immunosuppression, which could allow identification of patients in whom immunosuppressive strategies need to be changed or for whom drug concentrations may be safely lowered without risk of graft rejection. In monitoring patients receiving monotherapy with a calcineurin inhibitor before transplantation, our data indicate that universal tacrolimus or CsA resistance of T cells on anti-CD3/anti-CD28 costimulation is unlikely, but rather that pharmacodynamic monitoring may require more than one parameter for an accurate determination of drug sensitivity in an individual patient *(24)*. However, to date, no single measure of drug effect in transplantation has demonstrated the collective ability to serve as (*a*) a parametric endpoint of drug therapy that satisfies relationships defined by traditional pharmacodynamic equations; (*b*) a parametric endpoint of the severity of posttransplantation clinical states such as rejection; or (*c*) a measure of drug sensitivity. Therefore, the parameter "area of IL-2 mRNA expression over time", which should include absolute cytokine mRNA concentrations at two different time points, i.e., 4 and 24 h of costimulation mRNA kinetics, may potentially complement the monitoring data obtained from traditional pharmacodynamic thresholds such as EC_{50} or IC_{50} *(25)*. From our preliminary investigations on whole-blood samples predose (c_0) and after dosing (c_2 and c_4), we propose a c_2 (2 h post-dose) strategy as the most appropriate for testing calcineurin inhibitor sensitivity. However, prospective studies are required to determine whether individual degrees of calcineurin inhibitor sensitivity in whole blood correlate with drug concentrations determined by mass spectrometry or immunoassays *(56)* and whether they are associated with a low or high risk of transplant rejection. This would require a multicenter study design with recruitment of an adequate number of patients.

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