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Tacrolimus Pharmacodynamics and Pharmacogenetics along the Calcineurin Pathway in Human Lymphocytes

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BACKGROUND: Although therapeutic drug monitoring has improved the clinical use of immunosuppressive drugs, there is still interpatient variability in efficacy and toxicity that pharmacodynamic monitoring may help to reduce. To select the best biomarkers of tacrolimus pharmacodynamics, we explored the strength and variability of signal transduction and the influence of polymorphisms along the calcineurin pathway.

METHODS: Peripheral blood mononuclear cells from 35 healthy volunteers were incubated with tacrolimus (0.1–50 ng/mL) and stimulated ex vivo. Inhibition of NFAT1 (nuclear factor of activated T cells 1) translocation to the nucleus and intracellular expression of interleukin-2 in $CD4^+$ and $CD8^+$ T cells and the surface activation marker CD25 in $CD3^+$ cells were measured by flow cytometry. We sequenced the promoter regions of immunophilins and calcineurin subunits and characterized selected single nucleotide polymorphisms in the genes of the calcineurin pathway with allelic discrimination assays.

RESULTS: All responses closely fitted an I/I_{max} sigmoid model. Large interindividual variability ($n = 30$) in I_0 and IC_{50} was found for all biomarkers. Moreover, strong and statistically significant associations were found between tacrolimus pharmacodynamic parameters and polymorphisms in the genes coding cyclophilin A, the calcineurin catalytic subunit α isoenzyme, and CD25.

CONCLUSIONS: This study demonstrates the consistency and large interindividual variability of signal transduction along the calcineurin pathway, as well as the strong influence of pharmacogenetic polymorphisms in the calcineurin cascade on both the physiological activity of this route and tacrolimus pharmacodynamics.

The clinical management of calcineurin inhibitors (CNIs)[7] in organ transplantation is complex owing to their narrow therapeutic range and poor correlation between trough blood concentrations and clinical response. Efficacy and toxicity are influenced by multiple factors and require tailoring the dose to each patient. Pharmacodynamic (PD) monitoring is expected to contribute to such fine tuning, as it focuses on drug biological effects. It must be based on biomarkers capable of quantitatively reflecting the effects of a given drug on its target, whether it is administered alone or in combination, as well as of predicting the clinical response to the drug.

Different PD markers of calcineurin (CaN) inhibition have been examined *(1, 2)*, sometimes showing association with CNI exposure and/or clinical outcome, but none have been fully validated in clinical practice yet, and the measurement of most requires long and cumbersome workup *(1, 3)*. However, no study has investigated all the possible PD biomarkers along the calcineurin/nuclear factor of activated T cells (NFAT) pathway. The knowledge of the strength and variability of signal translation along this pathway, as well as of the steps where sources of internal (genetic) or external variability are the most influential, would help to select the best PD biomarkers, i.e., those with high specificity for CaN inhibition and most affected by interindividual variability. On the other hand, very little is known about the influence on CNI effects of

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[7] Nonstandard abbreviations: CNI, calcineurin inhibitor; PD, pharmacodynamic; CaN, calcineurin; NFAT, nuclear factor of activated T; TAC, tacrolimus; CHU, Centre Hospitalier Universitaire; 3PIGREF, Pharmacodynamic Relationships of Calcineurin Inhibitors Cyclosporine and Tacrolimus in Liver Transplant Recipients; PBMC, peripheral blood mononuclear cell; PMA, phorbol 12-myristate 13-acetate; IL-2, interleukin 2; I_0, basal level in the absence of inhibitor; IC_{50}, half-maximal inhibitory concentration; I_{max}, maximal response; SNP, single nucleotide polymorphism; Treg, regulatory T cell; NK, natural killer.

polymorphisms in the genes coding the different proteins involved.

The present study aimed at finding clues for the rational choice of a specific biomarker or set of biomarkers to monitor tacrolimus (TAC) immunosuppressive activity, reflecting its interindividual variability, and at exploring the potential pharmacogenetic sources of such variability.

Materials and Methods

STUDY DESIGN AND SUBJECTS

In this noninterventional study approved by the Centre Hospitalier Universitaire (CHU) Limoges ethics review board, 30 healthy adult volunteers who were not on chronic treatment (except for oral contraceptive agents) and had not taken drugs in the days preceding blood sampling gave their written informed consent to provide a 50-mL blood sample to the CHU Limoges biobank.

In addition, 5 healthy volunteers from Montevideo gave their written informed consent to provide a 50-mL blood sample for additional experiments regarding analytical and intraindividual variability, as part of the Pharmacodynamic Relationships of Calcineurin Inhibitors Cyclosporine and Tacrolimus in Liver Transplant Recipients (3PIGREF) noninterventional study (ClinicalTrials.gov ref. no. NCT01760356), approved by the Scientific and Ethics Committee for Medical Research of the Hospital Central de las Fuerzas Armadas; the Ethics Committee of the School of Chemistry of the Universidad de la República; and the Uruguayan Directorate of Medical Technologies of Health of the Ministry of Health, in Montevideo, Uruguay.

All investigations complied with the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use guidelines for Good Clinical Practice and Good Laboratory Practice and with the Declaration of Helsinki.

PHARMACODYNAMIC INVESTIGATIONS

A more detailed description of each of the following procedures is provided in the online Supplemental Methods, which accompanies the online version of this article at http://www.clinchem.org/content/vol60/issue10.

In vitro peripheral blood mononuclear cell stimulation. Fifty milliliters of blood was drawn under fasting conditions into sodium heparin vacutainer tubes (Becton Dickinson), and the fresh peripheral blood mononuclear cell (PBMC) fraction was obtained by use of lymphocyte density-gradient separation. Cells were resuspended in 1× supplemented RPMI 1640. For each set of markers, aliquots of 1×10^6 PBMC/mL were incubated ex vivo for 30 min at 37 °C in a humidified atmosphere at 5% CO_2 (in 96-well sterile plates) with 0–50 ng/mL TAC.

Intracellular interleukin-2 expression in $CD4^+$ and $CD8^+$ T cells. Cells were further stimulated with 50 ng/mL phorbol 12-myristate 13-acetate (PMA), 2.5 μg/mL calcium ionophore, and 1 μg/mL GolgiPlug for 5 h at 37 °C in a humidified atmosphere with 5% CO_2. Cells were labeled with anti-CD3, anti-CD4, and anti-CD8 antibodies; after fixation and permeabilization, they were stained with a labeled anti–interleukin-2 (anti–IL-2) antibody.

IL-2Rα expression at the surface of $CD3^+$ T cells. Cells were incubated for at least 72 h at 37 °C and 5% CO_2 in the presence of 7.5 μg/mL concanavalin A. PBMCs were washed and stained with labeled anti-CD3, anti-CD4, anti-CD8, and anti-CD25 antibodies.

NFAT1 translocation to the nucleus of PBMC. To assess the degree of NFAT1 translocation to the nucleus, we used an indirect antibody staining method. PBMC aliquots were then incubated in 50 ng/mL PMA and 2.5 μg/mL calcium ionophore for another 30 min at 37 °C and 5% CO_2. Cells were washed with cold 1× PBS, further incubated for 30 min on ice with PIPES lysis buffer, pH 7.4, washed again, incubated with 2 anti-NFAT1 antibodies, and stained with an IgG-phycoerythrin antibody. We measured the resulting fluorescent signal of labeled PBMC nuclei by flow cytometry and used the mean NFAT1 fluorescence intensity in the separated nuclei for statistical analysis.

We measured the inhibition of NFAT1 nuclear translocation, the intracellular expression of IL-2 in $CD4^+$ and $CD8^+$ subsets, and the percentage of T lymphocytes expressing CD25 using a LSRII Fortessa® flow cytometer equipped with 3 lasers (Becton Dickinson). All determinations were performed by use of 10^6 white blood cells. Cell populations were gated to acquire at least 50 000 $CD4^+$ T lymphocytes, or 50 000 PBMC nuclei for NFAT1 measurement. For each healthy volunteer, we used unstimulated, labeled PBMC aliquots as controls.

PHARMACOGENETIC INVESTIGATIONS

Promoters sequencing. DNA from the 30 healthy volunteers of the main study was sequenced for the promoter region of *PPIA* [peptidylprolyl isomerase A (cyclophilin A)],[8] *PPP3R1* (protein phosphatase 3, regulatory subunit B, α), *PPP3CA* (protein phosphatase 3, catalytic subunit, α isozyme), *PPP3CB* (protein phospha-

[8] Human genes: *PPIA*, peptidylprolyl isomerase A (cyclophilin A); *PPP3R1*, protein phosphatase 3, regulatory subunit B, α; *PPP3CA*, protein phosphatase 3, catalytic subunit, α isozyme; *PPP3CB*, protein phosphatase 3, catalytic subunit, β isozyme; *FKBP1A*, FK506 binding protein 1A, 12 kDa; *CALM1*, calmodulin 1 (phosphorylase kinase, δ); *IL2*, interleukin-2; *IL2RA*, interleukin 2 receptor, α; *JUN*, jun proto-oncogene; *NFATC1*, nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 1.

Table 1. Analytical and intraindividual variability of biomarker measurements.

	Analytical variability		Intraindividual variability	
PD biomarker	I_0 (log_{10} transformed)	Effect at 5 ng/mL TAC (log_{10} transformed)	I_0 (log_{10} transformed)	IC_{50} (log_{10} transformed)
NFAT1 (MFI)	1.1	1.5	6.3	50
IL-2$^+$CD4$^+$ (%)	2.2	8.6	4.7	8.6
IL-2$^+$CD8$^+$ (%)	8.9	62	6.5	17
CD25$^+$CD3$^+$ (%)	6.3	11	3.2	14

[a] Data are CV values in percent.
[b] MFI, mean fluorescence intensity.

tase 3, catalytic subunit, β isozyme), and *FKBP1A* (FK506 binding protein 1A, 12 kDa) to investigate polymorphisms that could modulate DNA transcription or mRNA stability (see online Supplemental Methods).

Genotyping assays. We selected polymorphisms in genes involved in the calcineurin pathway if their minor allele frequency (http://www.1000genomes.org) was at least 10% and they had been significantly associated with clinical effects in at least 2 independent published reports. Accordingly, *CALM1* [calmodulin 1 (phosphorylase kinase, δ)] rs12885713; *IL2* (interleukin-2) rs2069762; *IL2RA* (interleukin 2 receptor, α) rs7090530, rs10795791, rs11594656, rs35285258; *JUN* (jun proto-oncogene) rs2760501, rs4646999; and *NFATC1* (nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 1) rs754093 were genotyped by means of Taqman® allelic discrimination assays with a Rotor-Gene Q 2plex HRM (Qiagen), according to the manufacturer's protocol. We designed customized assays when required.

STATISTICAL METHODS

We tested distribution normality using the Shapiro–Wilk test and, when needed, data were log-transformed. Flow cytometry results were analyzed vs the logarithm of TAC concentration by use of sigmoid inhibition models (GraphPad PRISM®, version 5.02), to derive basal activity (I_0), 50% inhibitory concentration (IC_{50}), and maximal inhibition (I_{max}). IL-2 in CD4$^+$ and CD8$^+$ cells and CD25 in CD3$^+$ cells were studied as a function of NFAT1 expression by use of a large variety of models classically used for enzyme inhibition or receptor response, as well as simpler linear and nonlinear regression models. In all cases, we evaluated goodness of fit using nonparametric tests.

The influence of gene polymorphisms was tested by use of R version 2.15.1 (R foundation for statistical computing, http://www.r-project.org). Conformity of genotyping data with Hardy–Weinberg equilibrium was verified by use of the Fisher exact test with the "SNPassoc" package. Linkage disequilibrium was investigated for rs72174030, rs4347819, and rs4519508 (*PPP3R1*, chromosome 2) and for rs10795791, rs11594656, and rs35285258 (*IL2RA*, chromosome 10), and the most probable haplotypes were inferred with the "haplo.stat" package. The relations between single nucleotide polymorphisms (SNPs) or haplotypes and pharmacodynamic parameters (I_0, IC_{50}, and I_{max}) of the respective phenotypes were investigated by use of multiple linear regression. We compared recessive, dominant, and log-additive models on the basis of the Akaike information criterion. SNPs or haplotypes characterized by $P < 0.05$ were included in an intermediate multivariate model and retained in the final multivariate models only if they survived backward selection on the basis of the Akaike information criterion. The Bonferroni correction was applied to the final models to account for multiple testing. We estimated the percentage of variability explained by the SNPs or haplotypes in these final models through their R^2 coefficients.

Results

Thirty-five healthy volunteers [15 men and 20 women, ages 36 (10) years] were enrolled. None was taking medications (except possibly oral contraceptive agents), and all blood was collected under fasting conditions.

ANALYTICAL AND INTRAINDIVIDUAL VARIABILITY

ANOVA was used to avoid the effect of interindividual variability. Results are expressed as mean and CV of log-transformed data (Table 1).

For the intraassay variability, CVs at physiological levels (I_0) were always <9%, whereas those at 5 ng/mL TAC (i.e., approximately at the IC_{50} concentration) were somewhat higher, ranging from 1.5% to 11%, with the notable exception of IL-2 in CD8$^+$ cells, which apparently peaked at 62%. This last result probably resulted from complete or almost complete inhibition of

IL-2^+CD8+ expression at 5 ng/mL TAC (<2% and often undetectable).

The intraindividual variability estimated in 3 different healthy volunteers ranged from 3.2% to 6.5% for I_0 and from 8.6% to 17% for IC_{50}, except for NFAT1 (IC_{50} CV 50%).

PHARMACODYNAMIC PARAMETERS AND VARIABILITY

The mean fluorescence intensity of PBMC nuclei expressing NFAT1 after ex vivo incubation with stimulants clearly showed the inhibitory effect of TAC. Online Supplemental Fig. 1 presents a typical histogram at 0 (blank) and 5 ng/mL TAC, respectively.

NFAT1 in PBMC nuclei, as well as IL-2 and CD25 responses in each T cell subpopulation, were adequately fitted by I/I_{max} sigmoid models. The best and worst fits obtained among the 35 healthy volunteers are displayed in Fig. 1, showing that reliable PD parameters could be derived from these inhibition curves. The diversity of physiological (I_0) levels and PD responses to TAC is illustrated in Fig. 2 for NFAT1 and in online Supplemental Figs. 2 and 3 for IL-2 and CD25.

After log transformation of all parameters (as most were not normally distributed), mild to moderate interindividual variability (CV 11% to 20%) was found for the physiological level (I_0) of all the biomarkers tested, as well as for NFAT1 and CD25 I_{max} values (CV 14.3% and 13.9%, respectively), whereas much larger interindividual variability was found for IL-2 I_{max} in $CD4^+$ and $CD8^+$ cells (CV 70.6% and 195%, respectively) (Table 2).

At I_{max}, 17%–83% of PBMC nuclei still expressed NFAT1, 0%–18% $CD4^+$ and 0%–62% $CD8^+$ T lymphocytes expressed IL-2, and 29%–71% of $CD3^+$ T cells expressed $CD25^+$ on their membrane.

The IC_{50} of each biomarker increased along the CaN pathway, from a geometric mean of 1.3 ng/mL for NFAT1 to 4.8 ng/mL for CD25 (Table 3). Individual IC_{50} values varied in a 1000-fold range for NFAT1 and CD25, 2000-fold for IL-2 in $CD4^+$ cells, and 10 000-fold for IL-2 in $CD8^+$ lymphocytes.

Among all the models tested (I/I_{max}, Michaelis–Menten, Hill, linear, exponential, and many others) to describe the relationships between the expression of NFAT1 in PBMC nuclei on the one hand and the response of IL-2 in $CD4^+$ and $CD8^+$ cells and CD25 in $CD3^+$ cells on the other, only the allosteric sigmoidal model showed very good fits (Spearman test) in most volunteers (see online Supplemental Fig. 4).

PHARMACOGENETIC–PHARMACODYNAMIC RELATIONSHIPS

A complete linkage disequilibrium was found between rs72174030, rs4347819, and rs4519508 (chromosome 2, *PPP3R1*) and between rs10795791, rs11594656, and rs35285258 (chromosome 10, *IL2RA*) (see online Supplemental Table 1).

Associations between SNPs or haplotypes on the one hand, and phenotypes on the other ($NFAT1^+$PBMC $Nucleus^+$, IL-2^+CD4^+, IL-2^+CD8^+, and $CD25^+$ $CD3^+$), are presented in Table 4. As 12 multivariate models were constructed, Bonferroni correction was applied. Interestingly, the *IL2RA* haplotype accounted for 45% of IL-2^+CD4^+ I_0 variability and for 84% of $CD25^+CD3^+$ I_0 variability (corrected $P = 0.0119$ for both). *PPP3CA* rs45441997 explained 23% of IL-2^+CD4^+ I_{max} variability (corrected $P = 0.0280$), and *PPIA* rs8177826 C>G explained 37% of NFAT1 IC_{50} variability (corrected $P = 0.0420$).

Discussion

This ex vivo PD study in PMBCs from healthy volunteers showed that NFAT1, IL-2, and CD25 all responded to TAC CaN inhibition following I/I_{max} models, in line with previous reports *(4–12)*, with moderate interindividual variability of physiological levels (I_0) but much higher variability of the PD parameters IC_{50} and I_{max}.

IL-2 and CD25 response to the inhibition of NFAT1 expression in PBMC nuclei followed an allosteric sigmoidal model, which is consistent with tight signal translation along the calcineurin pathway. This is the first time this signaling cascade has been fully explored dynamically in humans and that a strong influence of pharmacogenetic polymorphisms is shown on both the physiological activity of this pathway and TAC pharmacodynamics.

Contrary to most previous studies on PD markers of CNIs, we simultaneously investigated the different steps of the CaN pathway to evaluate the reliability and variability of signal transduction. Indeed, PD is a continuum between drug concentration at its site of action and clinical outcomes, going through drug effect on its target protein, target cell response, modifications of cell–cell interactions, pathophysiological response (such as the diminution of the immune response in the present case), and finally therapeutic effects, body dysfunctions, or acquired diseases (e.g., organ preservation, malignancies, or infectious diseases, respectively) *(2)*. A satisfactory PD biomarker would probably be a good intermediate between target site drug concentrations and at least 1 of the clinical outcomes, integrating drug intrinsic activity, pathophysiological regulation, and environmental influences. To integrate the regulation factors or environmental influences in addition to drug effect, such ideal biomarkers should show higher interindividual variability than the measurement of the direct drug–target protein interaction. Thus this study focused not only on the drug concentration–biomarker relationships, but also on the interindividual physiological and pharmacodynamic

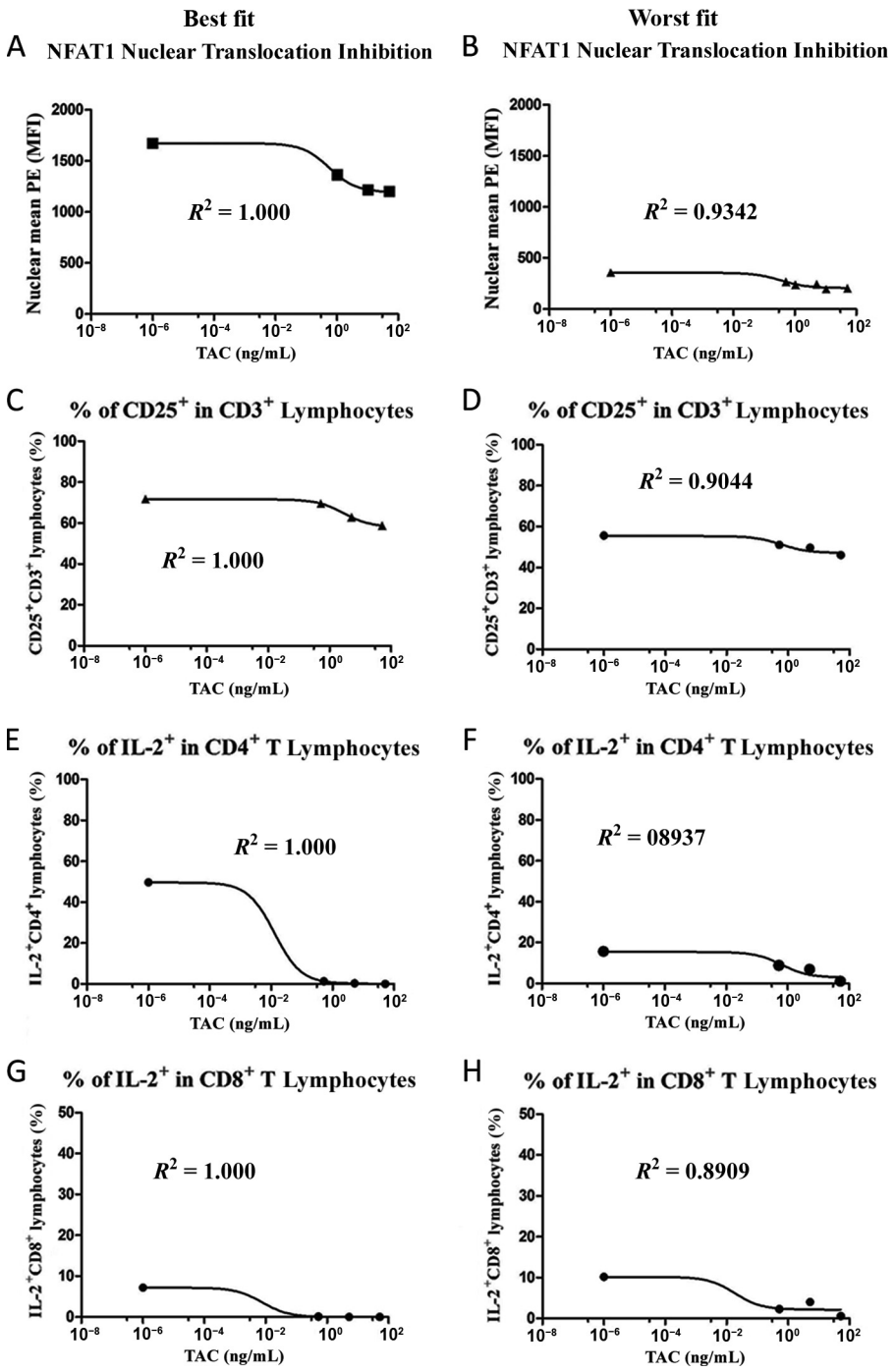

Fig. 1. Modeling of the fluorescence intensity of biomarkers vs TAC concentration with the sigmoid I/I_{max} model. The best (A, C, E, G) and worst (B, D, F, H) fits obtained are shown for NFAT1 (A, B); CD25 (C, D); and IL-2 (E, F, G, H). MFI, mean fluorescence intensity.

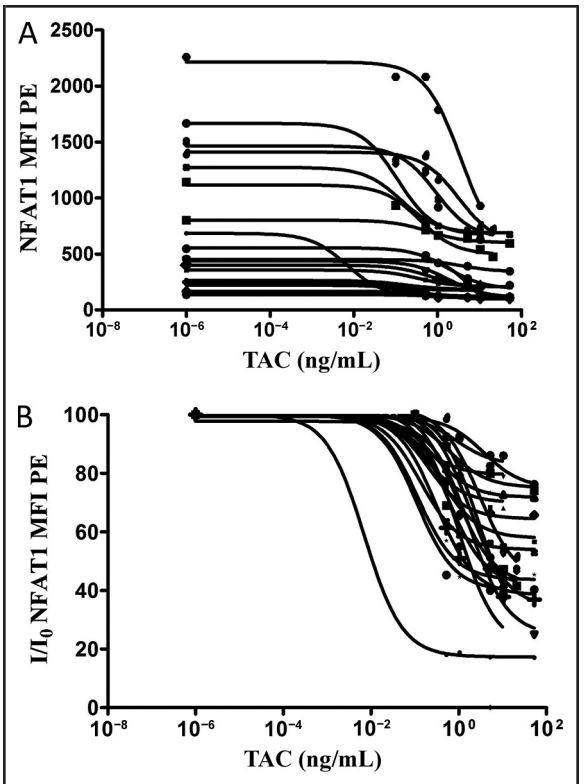

Fig. 2. Nonnormalized and I_0-normalized concentration–response curves of mean fluorescence intensity of NFAT1 in PBMC nuclei.

(A), NFAT1 nuclear translocation inhibition in PBMCs. (B), I/I_0 NFAT1 nuclear translocation inhibition in PBMCs. MFI, mean fluorescence intensity.

variability of these potential biomarkers, as well as on the potential pharmacogenetic sources of such variability.

The specificity of our markers was ensured by the design of our experiments, cell subsets, antibodies and fluorochromes, and gating strategies. The limits of detection, determined by the principle of fluorescence-minus-1 controls, were 0.18% for IL-2^+CD4^+, 0.25% for IL-2^+CD8^+, and 0.25% for NFAT1.

Previous articles have studied NFAT translocation to the nucleus by means of multispectral image stream flow cytometer *(13, 14)*, but this is the first time that NFAT1 nuclear translocation was measured by conventional flow cytometry, with overall low to moderate intraassay variability, consistent with previous reports *(4, 6, 7, 9, 15, 16)*, although comparisons are difficult owing to different markers, cell types, and experimental schemes. The other biomarkers were also measured with good intraassay precision, except for IL-2 in $CD8^+$ cells at 5 ng/mL TAC. However, the very high analytical CV found in this situation resulted from

Table 2. Physiological expression of biomarkers along the calcineurin pathway and response variability to tacrolimus inhibition.

		I_0 (log_{10}-transformed)		I_{max} (log_{10}-transformed)	
PD biomarker	n	Geometric mean	CV (%)	Geometric mean	CV (%)
NFAT1 (MFI)[a]	24[b]	794.3	16.5	251.2	14.3
IL-2^+CD4^+ (%)	32	39.8	11.0	0.3	70.6
IL-2^+CD8^+ (%)	32	12.6	20.0	0.5	195
$CD25^+CD3^+$ (%)	12[b]	39.8	11.2	31.6	13.9

[a] MFI, mean fluorescence intensity.
[b] Reduced n due to technical failures.

measures close to or below the detection limit, because this concentration corresponds to approximately 5 times the IC_{50} for this marker (0.98 ng/mL). A limitation of this study is that the method interassay performance could not be evaluated. We considered it unacceptable to collect enough blood (i.e., 3 × 50 mL) during a single collection from a healthy individual to run the whole assay at least 3 times, and PBMCs cannot be kept over 3 days without cell culture, which artificially changes the expression of our markers and increases interassay imprecision. Instead, we measured intrapatient variability by sampling 3 healthy volunteers weekly over 3 weeks, which is ethically more acceptable and encompasses both physiological and analytical time-dependent variability. With the exception of NFAT1 at IC_{50} TAC concentration (CV 50%), intrapatient variability was <20% for all the pharmacodynamic parameters, suggesting that the basal concentrations of these biomarkers, as well as the residual values obtained at maximal inhibition, are rather stable

Table 3. Pharmacodynamic response to increasing concentrations of TAC and interindividual variability.

	IC_{50}		
PD biomarker	Geometric mean (ng/mL)	Range (ng/mL)	CV of log_{10} transformed data (%)
NFAT1 (MFI)[a]	0.49	<0.25[b]–7.24	226
IL-2^+CD4^+ (%)	0.56	<0.18[b]–13.49	449
IL-2^+CD8^+ (%)	0.98	<0.25[b]–16.60	7446
$CD25^+CD3^+$ (%)	1.74	0.25–21.38	270

[a] MFI, mean fluorescence intensity.
[b] Limit of detection of the corresponding assay.

Table 4. Polymorphism influence on PD markers and parameters.

PD parameter and marker	Associated SNP[a]	Genetic model	Modulation	β (SD)	*P* (univariate model)	Bonferroni-corrected *P* (multivariate model)[b]
I_0						
NFAT1$^+$PBMC nuclei$^+$	None	Not applicable		NS	NS	NS
IL-2$^+$CD4$^+$	Haplotype *IL2RA*, rs4646999 *JUN*	AAT vs ATC	↑	0.35 (0.10)	0.001	**0.0119**
		GTC vs ATC	↑	0.29 (0.09)	0.005	0.0600
		AG-AA vs GG	↑	0.28 (0.12)	0.033	0.3960
IL-2$^+$CD8$^+$	rs4646999 *JUN*	AG-AA vs GG	↑	0.44 (0.18)	0.021	0.3180
CD25$^+$CD3$^+$	Haplotype *IL2RA*	AAT vs ATC	↓	−0.48 (0.06)	0.001	**0.0119**
		GTC vs ATC	↓	−0.27 (0.07)	0.015	0.1850
IC_{50}						
NFAT1$^+$PBMC nuclei$^+$	rs8177826 *PPIA*	G vs C	↓	−1.44 (0.43)	0.004	**0.0420**
IL-2$^+$CD4$^+$	None	Not applicable		NS	NS	NS
IL-2$^+$CD8$^+$	rs2760501 *JUN*	G vs T	↓	−1.51 (0.66)	0.006	0.0734
CD25$^+$CD3$^+$	rs2760501 *JUN*	G vs T	↑	2.51 (0.59)	0.035	0.5259
I_{max}						
NFAT1$^+$PBMC nuclei$^+$	rs12885713 *CALM1*	C vs CT-TT	↑	1.00 (0.39)	0.018	0.2214
IL-2$^+$CD4$^+$ (non–log-transformed)	rs45441997 *PPP3CA*	(GCC)10/10 vs (GCC)8/8	↑	0.25 (0.07)	0.002	**0.0280**
IL-2$^+$CD8$^+$ (non–log-transformed)	None	Not applicable		NS	NS	NS
CD25$^+$CD3$^+$	Haplotype *IL2RA*, rs8177826 *PPIA*	AAT vs ATC	↓	−0.75 (0.16)	0.006	0.0720
		GTC vs ATC	↓	−0.26 (0.13)	0.105	1.0000
		G vs C	↑	0.43 (0.13)	0.024	0.2880

[a] Haplotype *IL2RA*: rs10795791, rs11594656, and rs35285258.
[b] Values in bold are significant at $P < 0.05$.

with time in healthy volunteers. The results are also in favor of even lower interassay imprecision.

The interindividual variability of I_0 in PBMCs is quite similar to what other authors reported for IL-2$^+$ *(4, 14–16)*, and that for CD25$^+$ in CD3$^+$ was almost 3 times less *(4)*. However, our data were log-transformed to comply with normal distribution, which was apparently not the case in previous reports.

I_{max} interindividual variability was low for NFAT1 and CD25, the 2 incompletely inhibited biomarkers, but much higher for IL-2 in CD4$^+$ or CD8$^+$ T cells, whose concentrations were very close to the flow cytometry detection limit. Tacrolimus potency measured as the IC_{50}, a determinant of drug effect, was much more variable with time and between subjects. However, this parameter can be derived only from full concentration–response profiles and hence cannot be measured in patients already on CNIs.

Another important finding is the increasing IC_{50} mean values along the CaN cascade. Moreover, NFAT1 nuclear translocation and CD25 expression could not be fully inhibited *(4, 17)*, contrary to what was previously seen for IL-2 expression *(5)*. This partial inhibition of NFAT1 nuclear translocation (I_{max} range 17%–83%) suggesting incomplete signal transduction is in line with previous reports of incomplete CaN activity inhibition in vivo, even at TAC peak concentrations (40%–80% of residual enzyme activity) *(5, 16, 18, 19)*. Furthermore, Sommerer et al. reported that in kidney transplant recipients on cyclosporine, the inhibition of NFAT gene expression ranged from nearly complete to approximately 50%, whereas in patients on TAC the I_{max} response was always incomplete despite doses ensuring clinical rejection prophylaxis *(7)*, suggesting the influence of other factors in TAC immunomodulation activity *(4, 5, 7, 16, 17)*.

Higher CNI doses are required to suppress the CD8$^+$ than the CD8$^-$ T cell subset *(16, 20)*. Furthermore, IL-2 production may be constitutively higher, or less sensitive to CNIs in patients with rejection *(4)*, in

accordance with the contributory role of $CD8^+$ lymphocytes in the cytotoxic response during acute allograft rejection *(21)*. Boleslawski et al. demonstrated that the frequency of IL-2–expressing $CD4^+$ and $CD8^+$ cells correlated positively with CaN activity and reported that IL-2 expression in >25% of $CD8^+$ T cells predicted the onset of acute rejection *(16)*. In the present study, 5 healthy volunteers (14%), i.e., individuals with no major immunological conflict, had (stimulated) I_0 levels of IL-2^+CD8^+ >25%, together with almost complete inhibition of IL-2^+CD8^+ by TAC (I_{max} 0.1%–2.6%). On the other hand, 2 other individuals with I_0 = 19.2 and 23.2% yielded I_{max} values of 6.4% and 6.1%, respectively. Interestingly, the NFAT1 I_{max} values in the 5 patients with high IL-2^+CD8^+ expression were up to 2-fold higher than those reached in the rest of the cohort (37% vs 28% on average, $P = 0.0019$ Kruskal–Wallis test).

Unlike NFAT1 and CD25, IL-2 was strongly inhibited by TAC, and most individual I_{max} levels were close to the limit of detection (i.e., 0.18% for IL-2^+CD4^+ and 0.25% for IL-2^+CD8^+), hence poorly precise. This is a limitation of flow cytometry in measuring IL-2^+CD8^+ expression as a drug effect biomarker *(16, 21)*. However, augmented values are expected in transplant patients because of the immunological conflict. Despite this limitation, we observed changes in IL-2 response to TAC concentrations as low as 0.1 ng/mL (data not shown) in both T cell subsets, as for the other biomarkers studied here.

Other important findings of this study are the significant associations between polymorphisms in genes coding proteins of the calcineurin pathway and responses along this pathway to increasing levels of TAC. Moreover, the percentage of variability explained by these polymorphisms was quite high, reaching 84% for CD25 on $CD3^+$ cells in stimulated conditions without TAC (I_0). This is the first time that linkage disequilibrium between *IL2RA* (*CD25*) rs10795791, rs11594656, and rs35285258 is reported *(22–24)*. This mutated *IL2RA* haplotype, where rs10795791 is located in the upstream region of the *IL2RA* gene and the other 2 SNPs are intergenic, was associated with lower membrane expression of IL-2Rα on T cells and higher IL-2 expression in memory $CD4^+$ T cells. IL-2Rα increases IL-2 affinity for its receptor, allowing cellular response to the low physiological concentrations of IL-2 in vivo. IL-2Rα signaling also induces IL-2 secretion, which in turn increases and prolongs IL-2Rα expression, upregulating its signaling. In unstimulated conditions, IL-2 is mainly produced by activated $CD4^+$ T helper cells, whereas during the immune response, activated $CD4^+$ and $CD8^+$ T cells produce large amounts of IL-2, which then acts on $CD25^+$ T effector and regulatory cells. Furthermore, the promoter region of the *IL2RA* gene has 2 positive regulatory regions involved in the regulation of *IL2* transcription, and mutations in 1 or both of these responsive elements lead to the diminution or abrogation of *IL2* transcription, respectively *(25)*. The haplotype elements reported here do not belong to the *IL2RA* promoter region, and contrary to the above mutations, were associated with augmented IL-2 expression in $CD4^+$ T helper cells, which are recruited first during the cellular immune response. This increase in IL-2 expression despite decreased IL-2Rα expression seems paradoxical. However, it should be noted that the former concerns $CD4^+$ T helper cells and the latter all $CD3^+$ cells, which might explain this apparent discrepancy. Indeed, high IL-2 expression should be associated with increased activity of regulatory T cells (Tregs). Tregs express CD25 constitutively, but they depend on exogenous sources of IL-2 as they are unable to produce this cytokine. *IL2RA* haplotypes might also regulate the level of expression of CD25 on Tregs or other immunologically relevant cells *(25, 26)*; hence, decreased expression of CD25 would be associated with less robust Treg function. As Tregs prevent the expansion of $CD4^+$, $CD8^+$, and natural killer (NK) lymphocytes, decreased CD25 expression on Tregs would be consistent with higher activation of $CD4^+$ cells in our stimulated conditions (I_0). Interestingly, *IL2RA* polymorphisms and haplotypes (1 including rs10795791 and the other rs11594656, although each associated with 2 different SNPs) have been associated with several autoimmune diseases *(27–37)*. Of course, this hypothesis deserves further exploration.

PPP3CA rs45441997 (GCC)10/10 is a 5′ untranslated region variation located in the promoter region of the catalytic subunit of calcineurin. It was associated here with higher IL-2^+CD4^+ I_{max} (i.e., weaker maximal inhibitory activity) of TAC and accounted for 23% of its pharmacodynamic variability. The CaN catalytic subunit α exerts a critical role in signal transduction along the pathway. It structurally binds $Ca2^+$-calmodulin and the calcineurin regulatory subunit during enzyme activation, a process that triggers NFAT1 dephosphorylation. This mutation in the promoter region of *PPP3CA* may enhance its transcription, inducing higher NFAT dephosphorylation and translocation to the nucleus, thus resulting in an augmentation of the cytokines produced downstream. As $CD4^+$ T cells are the starters of the immune response, they may be more sensitive to signal transduction variations than $CD8^+$ cells.

The *PPIA* rs8177826 C>G SNP located in the promoter region of the gene coding cyclophilin A, the intracellular receptor of cyclosporine (and not tacrolimus), decreases NFAT1 IC_{50} and explains 37% of its interindividual variability. It has been previously demonstrated that 1 of the physiological roles of cyclophilin A is to pro-

mote the assembly of calcineurin regulatory and catalytic subunits *(38–40)*. Interestingly, CNIs intercalate with this complex, causing allosteric restriction and impeding NFAT dephosphorylation. This mutation may alter the assembly process of the enzyme subunits, making it less effective and more responsive to tacrolimus inhibition. Consistently here, the concentration–response curve showed a shift to the left.

A limitation of this pharmacogenetic investigation is the rather small number of healthy volunteers involved. We used nonparametric statistical tests and the rather drastic Bonferroni correction for multiple testing, meaning that the significant associations found here are statistically reliable (i.e., <5% false-positive rate). Moreover, ethics committees are reluctant to authorize the enrollment of large numbers of healthy volunteers. Clearly, this investigation mainly aimed at raising hypotheses that need to be explored further.

In summary, this study demonstrates the consistency of signal transduction along the calcineurin pathway: increasing doses of TAC inhibit NFAT1 translocation and IL-2 and CD25 expression following I/I_{max} models; and NFAT1 translocation to the nucleus is linked to IL-2 and CD25 expression following allosteric sigmoidal models. This study also suggests strong associations between tacrolimus pharmacodynamic parameters and polymorphisms in the genes coding cyclophilin A, calcineurin catalytic subunit, and CD25.

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References

1. Marquet P. Pretransplantation calcineurin activity: towards individualized therapy? Liver Int 2009;29:1454–6.
2. Marquet P. Counterpoint: Is pharmacokinetic or pharmacodynamic monitoring of calcineurin inhibition therapy necessary? Clin Chem 2010;56:736–9.
3. Carr L, Gagez AL, Essig M, Sauvage FL, Marquet P, Gastinel LN. Calcineurin activity assay measurement by liquid chromatography-tandem mass spectrometry in the multiple reaction monitoring mode. Clin Chem 2014;60:353–60.
4. Böhler T, Nolting J, Kamar N, Gurragchaa P, Reisener K, Glander P, et al. Validation of immunological biomarkers for the pharmacodynamic monitoring of immunosuppressive drugs in humans. Ther Drug Monit 2007;29:77–86.
5. Fukudo M, Yano I, Masuda S, Fukatsu, S, Katsura T, Ogura Y, et al. Pharmacodynamic analysis of TAC and CsA in living-donor liver transplant patients. Clin Pharmacol Ther 2005;78:168–81.
6. Giese T, Zeier M, Schemmer P, Uhl W, Schoels M, Dengler T, et al. Monitoring of NFAT-regulated gene expression in the peripheral blood of allograft recipients: a novel perspective toward individually optimized drug doses of cyclosporine A. Transplantation 2004;77:339–44.
7. Zhan A, Schott N, Hinz U, Stremmel W, Schmidt J, Ganten T, et al. Immunomonitoring of nuclear factor of activated t cells–regulated gene expression: the first clinical trial in liver allograft recipients. Liver Transplant 2011;17:466–73.
8. Sommerer C, Konstandin M, Dengler T, Schmidt J, Meuer S, Zeier M, et al. Pharmacodynamic monitoring of cyclosporine A in renal allograft recipients shows a quantitative relationship between immunosuppression and the occurrence of recurrent infections and malignancies. Transplantation 2006;82:1280–5.
9. Kung L, Batiuk T, Palomo-Pinon S, Noujaim J, Helms L, Halloran P. Tissue distribution of calcineurin and its sensitivity to inhibition by cyclosporine. Am J Transplant 2001;1:325–33.
10. Halloran P. Immunosuppressive drugs for kidney transplantation. N Engl J Med 2004;351:2715–29.
11. Hirano T. Cellular pharmacodynamics of immunosuppressive drugs for individualized medicine. Int Immunopharmacology 2007;7:3–22.
12. Kurata Y, Kato M, Kuzuya T, Miwa Y, Iwasaki K, Haneda M, et al. Pretransplant pharmacodynamic analysis of immunosuppressive agents using CFSE-based T-cell proliferation assay. Clin Pharmacol Ther 2009;86:285–9.
13. George TC, Harmon I, Peterson EJ, Burbach BJ, Shimizu Y, Matsuda JL, et al. Measurement of nuclear translocation in primary cells using correlation analysis of images obtained on the ImageStream imaging flow cytometer [Abstract]. J Immunol 2007;178:13.
14. Maguire O, Tornatore KM, O'Loughlin KL, Venuto RC, Minderman H. Nuclear translocation of nuclear factor of activated T cells (NFAT) as a quantitative pharmacodynamic parameter for tacrolimus. Cytometry A 2013;83A:1096–104.
15. Brandt C, Liman P, Bendfeldt H, Mueller K, Reinke P, Radbruch A, et al. Whole blood flow cytometric measurement of NFATc1 and IL-2 expression to analyze cyclosporine A-mediated effects in T cells. Cytometry A 2010;77A:607–13.
16. Boleslawski E, Conti F, Sanquer S, Podevin P, Chouzenoux S, Batteux F, et al. Defective inhibition of peripheral $CD8^+$ T cell IL-2 production by anti-calcineurin drugs during acute liver allograft rejection. Transplantation 2004;77:1815–20.
17. Sommerer C, Meuer S, Zeier M, Giese T. Calcineurin inhibitors and NFAT-regulated gene expression. Clin Chimica Acta 2012;413:1379–86.
18. Sommerer C, Giese T, Meuer S, Zeier M. Pharmacodynamic monitoring of calcineurin inhibitor therapy: is there a clinical benefit? Nephrol Dial

Transplant 2009;24:21–7.
19. Blanchet B, Hulin A, Duvoux C, Astier A. Determination of serine/threonine protein phosphatase type 2B (PP2B) in lymphocytes by HPLC. Anal Biochem 2003;312:1–6.
20. Ahmed M, Venkataraman R, Logar AJ, Rao AS, Bartley GP, Robert K, et al. Quantitation of immunosuppression by TAC using flow cytometric analysis of interleukin-2 and interferon-γ inhibition in CD8- and CD8+ peripheral blood T cells. Ther Drug Monit 2001;23:354–62.
21. Millan O, Benitez C, Guillén D, López A, Rimola A, Sánchez-Fueyo A, et al. Biomarkers of immunoregulatory status in stable liver transplant recipients undergoing weaning of immunosuppressive therapy. Clin Immunol 2010;137:337–46.
22. rs10795791 SNP. http://www.ensembl.org/Homo_sapiens/Variation/HighLD?db=core;r=10:6107840-6108840;v=rs10795791;vdb=variation;vf=7149216 (Accessed November 2013).
23. rs11594656 SNP. http://www.ensembl.org/Homo_sapiens/Variation/HighLD?db=core;r=10:6121509-6122509;v=rs11594656;vdb=variation;vf=7853446 (Accessed November 2013).
24. rs35285258 SNP. http://www.ensembl.org/Homo_sapiens/Variation/HighLD?db=core;r=10:6118270-6119270;v=rs35285258;vdb=variation;vf=11583910 (Accessed November 2013).
25. Dendrou CA, Plagnol V, Fung E, Yang JHM, Downes K, Cooper JD, et al. Cell-specific protein phenotypes for the autoimmune locus *IL2RA* using a genotype-selectable human bioresource. Nat Genet 2009;41:1011–7.
26. Gregersen PK. Closing the gap between genotype and phenotype. Nat Genet 2009;41:958–9.
27. Cavanillas M, Alcina A, Nuñez C, de las Heras V, Fernandez-Arquero M, Bartolomé M, et al. Polymorphisms in the IL2, IL2RA and IL2RB genes in multiple sclerosis risk. Eur J Hum Genet 2010;18:794–9.
28. Alcina A, Fedetz M, Ndagire D, Fernandez O, Leyva L, Guerrero M, et al. IL2RA/CD25 gene polymorphisms: uneven association with multiple sclerosis (MS) and Type 1 diabetes (T1D). PLoS One 2009;4:e4137.
29. Maier LM, Lowe CE, Cooper J, Downes K, Anderson DE, Severson C, et al. IL2RA genetic heterogeneity in multiple sclerosis and type 1 diabetes susceptibility and soluble interleukin-2 receptor production. PLoS Genet 2009;5:e1000322.
30. Chistiakov DA, Chistiakova EI, Voronova NV, Turakulov RI, Savost'anov KV. A variant of the *Il2ra/Cd25* gene predisposing to Graves' disease is associated with increased levels of soluble interleukin-2 receptor. Scand J Immunol 2011;74:496–501.
31. Hinks A, Ke X, Barton A, Eyre S, Bowes J, Worthington J, et al. Association of the IL2RA/CD25 gene with juvenile idiopathic arthritis. Arthritis Rheum 2009;60:251–7.
32. Stahl EA, Raychaudhuri S, Remmers EF, Xie G, Eyre S, Thomson BP, et al. Genome-wide association study meta-analysis identifies seven new rheumatoid arthritis risk loci. Nat Genet 2010;42:508–16.
33. Lowe CE, Cooper JD, Brusko T, Walker NM, Smyth DJ, Bailey R, et al. Large-scale genetic fine mapping and genotype phenotype associations implicate polymorphism in the IL2RA region in type 1 diabetes. Nat Genet 2007;39:1074–82.
34. Fichna M, Zurawek M, Fichna P, Januszkiewicz D, Nowak J. Polymorphic variants of the IL2RA gene and susceptibility to type 1 diabetes in the Polish population. Tissue Antigens 2012;79:198–203.
35. Klinker MW, Schiller JJ, Magnuson VL, Wang T, Basken J, Veth K, et al. Single-nucleotide polymorphisms in the IL2RA gene are associated with age at diagnosis in late-onset Finnish Type 1 Diabetes subjects. Immunogenetics 2010;62:101–7.
36. Hollis-Moffatt JE, Chen-Xu M, Topless R, Dalbeth N, Gow PJ, Harrison AA, et al. Only one independent genetic association with rheumatoid arthritis within the *KIAA1109-TENR-IL2-IL21* locus in Caucasian sample sets: confirmation of association of rs6822844 with rheumatoid arthritis at a genome-wide level of significance. Arthritis Res Therapy 2010;12:R116.
37. Garg G, Tyler JR, Yang JHM, Cutler AJ, Downes K, Pekalski M, et al. Type 1 diabetes-associated *IL2RA* variation lowers IL-2 signaling and contributes to diminished $CD4^+CD25^+$ regulatory T-cell Function. J Immunol 2012;188:4644–53.
38. Göthel SF, Marahiel MA. Peptidyl-prolyl cis-trans isomerases, a superfamily of ubiquitous folding catalysts. Cell Mol Life Sci 1999;55:423–36.
39. Wang P, Heitman J. The cyclophilins. Genome Biol 2005;6:226.
40. Cardenas ME, Hemenway C, Muir RS, Ye R, Fiorentino D, Heitman J. Immunophilins interact with calcineurin in the absence of exogenous immunosuppressive ligands. EMBO J 1994;13:5944–57.