Activity of the calcineurin pathway in patients on the liver transplantation waiting list: factors of variability and response to tacrolimus inhibition. Pierre Marquet Institutions and affiliations

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Authors' contributions: Ofelia Noceti and Pierre Marquet conceived research ideas and wrote the manuscript; Ofelia Noceti wrote the study protocol, performed all PD experiments, recruited human subjects, collected samples for the clinical study, supervised the clinical research and analyzed the data; Lucie Pouché designed the calcineurin promoters' strategy and provided technical advice on sequencing; Daniela Lens and Cristina Touriño gave access to cell culture and flow cytometry infrastructure and provided technical advice; Patricia Esperón and Marcelo Vital performed calcineurin promoters sequencing in Montevideo; Jean Baptiste Woillard performed statistical analysis, and Solange Gerona gave clinical hepatology consultancy.

Abstract

Background: To evaluate in patients on a liver transplantation waiting list, potential biomarkers of the base calcineurin pathway activity in a new model of non-stimulated peripheral blood mononuclear cells (PBMC) and ex vivo response to tacrolimus.

Methods: The calcineurin pathway activity was explored *ex vivo* in stimulated and non-stimulated PBMC from 19 patients. The inhibition of $NFAT1^+$ translocation to PBMC, expression of intracellular IL-2 and membrane CD25 in different T cells subsets in basal conditions and after exposure to tacrolimus at pharmacological levels were measured by multiparametric flow cytometry. We also studied the influence on the individual response of polymorphisms in 3 key genes of the calcineurin pathway: *PPIA*, *PPP3CA* and *IL2RA*.

Results: All pharmacodynamics profiles closely fitted an I/I_{max} sigmoid model. Interindividual variability was higher in non-stimulated than in stimulated conditions, as well as in the presence of tacrolimus. $IL\text{-}2^+CD8^+$ cells at tacrolimus I_{max} showed the highest interindividual variability, suggesting its usefulness as a biomarker of individual tacrolimus effects integrating many different sources of regulation and variability. Moreover, in the absence of tacrolimus, patients with end stage liver disease exhibited lower NFAT1 translocation and T cell activation as compared to healthy volunteers from a previous study in similar conditions. Multivariate statistical analysis showed strong and significant associations between tacrolimus pharmacodynamic parameters and two polymorphisms in the gene coding cyclophilin A (rs8177826 and rs6850).

Conclusion: This study showed the feasibility of using non-stimulated PBMCs to explore the calcineurin pathway in more physiologic conditions and points towards potential biomarkers for tacrolimus pharmacodynamics monitoring.

INTRODUCTION

The individual response of solid organ transplant recipients to immunosuppressive drugs (ISD) is highly variable in terms of efficacy and adverse effects, and the factors governing such responses are still largely unknown. Different donor, recipient and graft characteristics, including gene polymorphisms and environmental factors through regulation pathways, may modulate the pharmacokinetics (PK) and pharmacodynamics (PD) of calcineurin inhibitors (CNIs) as well as the innate and adaptive immune responses (1,2).

T cell cytokines and surface activation markers have been investigated as markers of TAC pharmacodynamics in individuals (3,4,5). The recently published consensus on biomarkers to adjust ISD in transplantation encompasses proteins that might predict the risk of rejection and tolerance; biomarkers of the individual response to ISD; pharmacogenetic (PG) polymorphisms influencing ISD PK and PD; as well as BM associated with graft dysfunction or injury (6).

Previous studies by Canivet et al. (7) using whole blood from cirrhotic patients on the waiting list for liver transplantation (LT) found an increased expression of intra-lymphocyte interleukin 2 (IL-2) and tumor necrosis factor alpha (TNFα) and a decreased expression of the transferrin receptor (CD71) and CD25 (also called IL2Rα, the interleukin 2 receptor alpha-chain subunit) as compared to healthy volunteers (HV). However, in patients with hepatitis C virus infection (HCV), both T cell activation and proliferation were upregulated, suggesting that this condition would favor a switch in the immune response. We previously explored TAC PD ex-vivo along the calcineurin (CaN) pathway in HV (8).

The aim of this study in patients on the waiting list for LT was to investigate ex-vivo the basal calcineurin pathway activity in a new model of non-stimulated peripheral blood mononuclear cells (PBMC), its response to increasing concentrations of TAC and the

influence of end-stage liver disease (ESLD) and target gene polymorphisms on the inter-individual pathophysiological and pharmacodynamic variability.

MATERIALS AND METHODS

STUDY DESIGN AND SUBJECTS

This work is the second research step of the 3PIGREF trial (Ref. NCT01760356 ClinicalTrials.gov), which addresses 4 groups of subjects to discover and validate pharmacodynamics biomarkers of tacrolimus effects: healthy volunteers, patients on the waiting list for liver transplantation, and liver transplant patients on CNIs recruited in a trans-sectional study or in a longitudinal cohort. This trial was approved by the Scientific and Ethics Committee for Medical Research of the Hospital Central de las Fuerzas Armadas and the School of Chemistry of the Universidad de la República in Montevideo, Uruguay. All investigations complied with the ICH Guidelines for GCP and GLP and with the Declaration of Helsinki. Patients registered on the waiting list for liver transplantation (WLP) were enrolled if they were free from corticosteroids, immunosuppressive drug or interferon gamma therapies, were not candidates for combined hepatorenal transplantation, and if they gave informed consent in writing. One blood draw was collected from all the patients, on different days over a period of 5 months.

PHARMACODYNAMIC STUDY

PBMC were obtained using lymphocyte density-gradient separation of fresh blood collected on sodium heparin, re-suspended in supplemented RPMI and counted in a cell-counter.

For each set of markers, aliquots of PBMC in supplemented RPMI were incubated ex-vivo for 30min at 37°C in a humidified atmosphere at 5% CO_2 with 0 to 50ng/ml TAC.

NFAT1 translocation to PBMC nuclei, intracellular IL-2 expression in $CD4^+$ and $CD8^+$ T cells and $CD25^{High}$ expression at the surface of $CD3^+$, $CD4^+$, and $CD8^+$ T cells, were determined by multiparametric flow cytometry (8).

For each whole blood sample, one aliquot of 1 million cells in 100 µl of supplemented RPMI was analyzed without mitogen stimulation or tacrolimus addition (so-called "physiological" or "non-stimulated – NS- values" thereafter), while other aliquots were analyzed with and without tacrolimus after incubation with: 50 ng/ml PMA and 2.5 µg/ml calcium ionophore at 37°C in a humidified atmosphere with 5% CO_2, for 30 minutes for NFAT1 and for 5h plus 1 µg/ml of Golgi Plug for IL-2; 7.5 µg/ml Concanavalin A for 72h at 37°C and 5% CO_2 (8) for CD25 expression measurement.

IL-2 and CD25 expression were reported as the percentage of fluorescent positive cells over the $CD4^+$, $CD8^+$ or $CD3^+$ fractions respectively and NFAT1 as mean fluorescence intensity in PBMC $nuclei^+$.

Flow cytometry was performed on a single instrument, standardized according to the Euroflow guidelines and the Human ImmunoPhenotyping Project, and the technique was fully validated for all biomarkers, showing intra-assay variability <9% at TAC I_0 and <11% at IC_{50}. A detailed description is provided in the Supplemental Methods section (S1).

PHARMACOGENETIC STUDY

We analyzed the calcineurin pathway gene variants previously associated with ex vivo TAC PD in HV (8) (i.e., *PPIA* rs8177826 and 6850; *PPP3CA* rs45441997; *IL2RA* rs10795791, 11594656 and 35285258 coding for CD25), as well as two additional key genes of the calcineurin pathway (*PPP3R1 rs4519508; FKBP1A* rs141252617).

In addition, 10ng of genomic DNA were amplified using forward and reverse primers and then sequenced for the upstream and 5'UTR region of *PPIA*, and the 5'UTR regions

of *PPP3R1* and *PPP3CA* to detect polymorphisms that could modulate DNA transcription or mRNA stability. A detailed description is provided in the Supplemental Methods section (S1).

STATISTICAL METHODS

Distribution normality was tested using the Shapiro–Wilk test and, when needed, data were log-transformed. Multiparametric flow cytometry results were analyzed vs. TAC log(concentration) using sigmoidal inhibition models (GraphPad PRISM®, version 5.02), to derive basal activity (NS, I_0), 50% inhibitory concentration (IC_{50}), and maximal inhibition (I_{max}). The responses of IL-2 in $CD4^+$ and $CD8^+$ cells and $CD25^{High}$ in $CD3^+$, $CD4^+$ and $CD8^+$ T cells were studied as a function of $NFAT1^+$ in PBMC $nuclei^+$ expression employing an allosteric sigmoidal model. In all cases, goodness of fit was evaluated using nonparametric tests. ANOVA was used to estimate the inter-individual variability. The influence of gene polymorphisms was analyzed with R version 2.15.1 (R foundation for statistical computing, http://www.r-project.org). The conformity of genotyping results with Hardy–Weinberg equilibrium was verified using the Fisher exact test. Linkage disequilibrium was tested for rs11594656 and rs35285258 (IL2RA, chromosome 10), and the most probable haplotypes were inferred using the "haplo.stat" R package. The relations between single nucleotide polymorphisms (SNPs) or haplotypes and TAC NS, I_0, IC_{50}, and I_{max} on the respective phenotypes were investigated by multiple linear regression. We compared recessive, dominant, and log-additive models using 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 model only if they survived backward selection based on the Akaike information criterion. The Bonferroni correction was applied to account for the 24 multivariate models built. The percent contribution to the overall variability

explained by the SNPs or haplotypes in these final models was calculated using the 'gam' R function, as the ratio of variability associated to the variable divided by the total variability.

RESULTS

Nineteen patients from the liver transplantation waiting list (13 men and 6 women) were enrolled. Their indication for LT and demographic characteristics are summarized in Table I.

The mean TAC PD parameters measured in fresh PBMC samples are shown in Table II, while individual values can be found in Supplemental Table S2. The median and extreme numbers of events detected in non-stimulated and stimulated conditions for the different PD parameters are listed in Supplemental Table S3.

NFAT1 in PBMC nuclei, intracellular IL-2 and membrane CD25 expression in each of the T cell subpopulations studied all adequately fitted I/I_{max} sigmoid models (figure 1). Precise PD parameters could be derived from these well-fitted inhibition curves (figure S4). Large inter-individual variability was found for all biomarkers, in NS (unstimulated, physiological) (CV = 59-265%), I_0 (no TAC exposure, stimulated conditions) (CV= 56-106%), IC_{50} (CV = 122-174%) and I_{max} (CV = 94-230%) conditions. At TAC I_{max}, NFAT1 in PBMC nuclei, IL-2 in $CD8^+$ T cells and $CD25^{High}$ in T cells showed incomplete inhibition (Table II) and huge inter-individual variability, IL-2 exhibiting the largest (CV = 230% and 190% for $CD4^+$ and $CD8^+$ cells, respectively).

TAC IC_{50} augmented along the CaN pathway, from a geometric mean of 0.6 ng/ml for NFAT1 up to 1.3 ng/ml for $CD25^{High}CD8^+$ (Table II). Individual IC_{50} values varied 100-fold for NFAT1, IL-2 in $CD8^+$ and $CD25^{High}$ in T lymphocytes and 200-fold for IL-2 and $CD25^{High}$ in $CD4^+$cells, and CD25 in $CD8^+$ cells.

The response of NFAT1 in PBMC nuclei was linked to those of IL-2 in $CD4^+$ and $CD25^{High}$ in $CD8^+$ T cells by an allosteric sigmoidal relationship (figure 2).

In multivariate analysis, several significant associations were found between SNPs and either the physiological activity of the calcineurin pathway or TAC PD biomarkers (Table III). After multivariate modeling and Bonferroni correction, only two associations reached statistical significance: *PPIA* 6850G upregulates T cell activation in $CD3^+$ and $CD4^+$ cells in NS conditions, accounting for 81% (corrected p=0.00016) and 86% (corrected p=0.00004) of the variability in the levels of $CD25HighCD3^+$ and $CD25HighCD4^+$ NS, respectively; *PPIA* 8177826G downregulates $CD25HighCD4^+$ at TAC I_0, explaining up to 57% of the $CD25HighCD4^+$ inter-individual variability.

DISCUSSION

To the best of our knowledge, this is the first time that the CaN/NFAT signaling cascade, tacrolimus inhibitory signal transmission and the influence of gene variants along this cascade have been investigated in patients. This study follows a similar design as a previous study in healthy volunteers (8), except that herein we also explored the physiological activity of the calcineurin pathway and its variability in non-stimulated conditions, in the absence of tacrolimus. This research confirmed: (i) that NFAT1, IL-2 and CD25 responded to calcineurin inhibition following I/Imax models; (ii) consistent signal transduction from NFAT1 translocation to PBMC nuclei to CD25 expression in $CD8^+$ T cells, with increasing TAC IC_{50} values along the pathway, showing less sensitivity to TAC inhibitory effect of CD25 membrane expression than of NFAT1 translocation. Contrary to the other PD biomarkers, intracellular IL-2 expression in $CD4^+$ T cells was completely suppressed at high TAC concentrations, suggesting complex and diverse signal transduction or regulation along the pathway in the different T cell subsets. Large interindividual variability was found for most of the

BM in these patients with terminal liver diseases and it was larger than in healthy volunteers (CV% in stimulated conditions: $I_0 \leq 20\% < I_{max} < IC_{50}$) (8). Interestingly, in NS conditions (i.e. physiological conditions without ex-vivo stimulation and tacrolimus inhibition) BM exhibited greater variability than in the equivalent stimulated status I_0, except for $CD25^+CD3^+$ and $CD25+CD4^+$. However, overall the largest variability was found at TAC IC_{50}, the parameter of TAC pharmacodynamic potency, consistent with the large interindividual differences in clinical drug effects among patients. At TAC I_{max}, PD biomarkers also exhibited very large variability, especially for IL-2 in $CD8^+$cells. Several groups previously published on $CD8^+IL\text{-}2^+$ T cells as being a promising biomarker of CNI effects (9-11). With the novel hypothesis that a good biomarker would have a high and even the highest inter-individual variability so as to integrate as many sources of regulation and variability of the response as possible, the present study provides a further and independent hint regarding the potential of $CD8^+IL\text{-}2^+$ as a pertinent tacrolimus pharmacodynamics biomarker.

Per se, the determination of the "physiologic" values of the biomarkers is unusual and innovative and it showed that they were all measurable in the absence of stimulation, apart from IL-2 in $CD4^+$ and CD25 in $CD8^+$ T cells whose levels were near or below the detection limit of the flow cytometer technique (LOD = 0.15% for IL-2 in $CD4^+$and 0.10% for CD25 in T cell subsets). In the Supplemental Table S3, we listed the numbers of events analyzed in NS and stim conditions without TAC exposure, which illustrate the feasibility of this novel approach, hence of novel potential biomarkers. "Physiologic" readouts reflect the basal state of the patient and are free from artificial influences brought by mitogenic stimulation, which can modify cell expression, while stimulated biomarkers may better mirror the maximum capacity of the patient immune response. Potential biomarkers obtained in either condition may serve to monitor TAC PD.

Comparisons of I_0 levels measured in identical conditions in HV (8) and WLP are shown in figure 3, showing that they were lower for all BM in the former, except for IL-2^+CD8^+ T cells. For TAC IC_{50}, we found slightly higher mean values in WLP, which means that TAC might be slightly less potent in these patients. The largest difference was found at TAC I_{max} for IL-2 expression in cytotoxic T cells, where WLP seem to be more resistant to TAC inhibition. On the contrary, lower TAC I_{max} values as compared to HV were found for NFAT1 and CD25, suggesting immune depression in patients with terminal liver diseases. In this sense, NFAT1 translocation might reflect the degree of individual immunological competence, in accordance with Sommerer et al.(12) and Zhan et al.(13), who established a correlation between lower levels of NFAT-regulated gene expression and increased frequency of infection episodes in transplant patients. This fact may also partly explain the lower immunosuppressant doses required in liver than in other organ transplantation.

Using whole blood, Canivet et al. (7) reported that patients with liver cirrhosis had decreased ($CD3^+$) T cell activation (membrane expression of CD25 and CD71) but not T cell function (intra-cell expression of IL2 and TNFα) as compared to healthy volunteers, except in HCV-positive patients whose T cells became prime-stimulated and promoted to proliferate. Here, using PBMC from pre-transplant patients, mostly cirrhotic, we found a general decrease in practically all of the pharmacodynamic BM studied in the different T cell subpopulations compared to healthy volunteers, except for the cytotoxic effector activity (IL-2^+CD8^+), pertaining to T cell function following Canivet et al's terms, which was higher. Although not directly comparable, the results of the two studies are compatible. It is well established that in decompensated cirrhotic patients, innate and adaptive immune responses are decreased, fostering a higher incidence of bacterial and viral colonization (14,15,16). Interestingly, our results reveal that despite decreased T-helper cell response (IL-2^+CD4^+), the cytotoxic effector

activity (IL-$2^{+}CD8^{+}$) was higher than that observed in HV; one possible explanation would lay in the continuous effort of the host to get rid of the antigens (16,17), exhausting the immune response capacity.

In the present study, two polymorphisms in the gene coding cyclophilin A exhibited a significant impact on TAC PD in WLP. First, the *PPIA* 8177826G allele was linked with lower expression of $CD25^{High}CD4^{+}$ in the absence of TAC (I_0), explaining 57% of its variability. The same association was previously found in healthy volunteers (8). Secondly, *PPIA* 6850G was associated with upregulated T helper cell activation ($CD25^{High}CD4^{+}$ and $CD3^{+}$ T cells) in NS conditions, accounting for 86% of the inter-patient variability, which was not previously evidenced in HV (8). Interestingly, these two *PPIA* polymorphisms seem to exert opposite effects on patients' T-helper cells. No genome-wide association study in solid organ transplantation has reported statistical associations between these two *PPIA* variants and clinical outcomes; however, one study did in HIV-1 patients (1). The candidate-gene strategy carried out by Pouché et al. did not find significant relationships between *PPIA* rs8177826 or rs6850 and biopsy proven acute rejection, nephrotoxicity or infections in renal transplant patients (2). On the other hand, Vinitha et al. reported increased plasma levels of cyclophilin A in carriers of the rs6850G allele in the context of vascular inflammation (18). These discrepant results might be explained by different study designs, disease conditions and the measured outcome. TAC pharmacodynamics biomarkers may be much more sensitive to genetic variants in the calcineurin pathway than 'hard' clinical outcomes such as graft rejection, which is prone to many other influences that may stimulate the immune response. However, the present results still need to be confirmed in an independent patient group.

This study only enrolled a small number of patients, but the ex-vivo experiments are very long, cumbersome and costly and it is unlikely that a much larger patient group

could be studied to this extent. The methods employed can hardly be considered for the pharmacodynamics monitoring of tacrolimus in routine, but they allowed us to explore signal transduction along the calcineurin pathway rather than a single predefined biomarker, and to compare our results with those previously obtained in healthy volunteers or that we will obtain in transplanted patients, as planned in the 3PIGREF trial. Also, PBMC were incubated with pure tacrolimus, thus the influence of its eight known metabolites was not considered (19); however, their pharmacodynamics, potential therapeutic and adverse effects have only been poorly explored so far.

In summary, non-stimulated measures may be suitable to assess the physiological activity of calcineurin signaling, while measures after PBMC ex vivo stimulation might rather evaluate the pathway maximal capacity. IL-2 in $CD8^+$ cells at TAC I_{max} might be a useful biomarker of tacrolimus effects owing to its greatest variability, while based on the literature, inhibition of NFAT1 translocation to PBMC nuclei might be a better biomarker of immunological competence linked to infections. The influence of polymorphisms in cyclophilin A on the pathway in the absence of TAC was impressive, in both non-stimulated and stimulated conditions. Our patients with ESLD exhibited a decrease in practically all the biomarkers studied (except T cytotoxic function) as compared to healthy subjects. Whether these biomarkers are predictive of graft rejection or other clinical outcomes is the object of an on-going prospective follow-up of transplanted patients as part of the 3PIGREF trial.

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Tables

Table I. Patient demographic characteristics and main clinical data.

Parameter	Occurrence (number of cases)
Age	57 (24-66)*
AB0, n	0+(6); 0-(2); A+(6); A-(1);B+(3)
CMV Status, n	-(4); +(9); NA*(3)
EBV, n	-(3); +(10); NA* (6)
MELD	17 (13-25)
Renal Failure; T2 Diabetes	0
Ulcerative Colitis; Asthma/Allergy; Hypothyroidism	1
Ascites	13
Budd Chiari Syndrome; Wilson's Disease; Primary Sclerosing Cholangitis	1
Cirrhosis	16
NASH, Alcoholic, Alcoholic + Hemochromatosis, Biliary secondary, Hepatitis B Virus + HCV	1
Biliary primary, Alcoholic + Hepatocellularcarcinoma	2
HCV	3
Cryptogenic	4
Infections at monitoring:	7

- Types of microorganisms	Bacteria (n=4), Fungi (n=1),Virus (n=1); Bacteria+Virus (n=1)
- Colonisation site	Peritoneal cavity, biliary tract, lower limbs, nose, esophagus, respiratory tract, systemic
Patients transplanted after the study	15
Patients deceased post-transplantation	2
Patients deceased while on the waiting list	2

*Not available

Table II. Tacrolimus PD parameters in patients on the liver transplantation waiting list (n = 19). Results are expressed as mean and inter-individual CV% of log-transformed data.

PK	***NFAT1 (MFI)*** **(CV%)**	***$IL2^+CD4^+$ (%)*** **(CV %)**	***$IL2^+CD8^+$ (%)*** **(CV%)**	***$CD25^{High}CD3^+$ (%)*** **(CV%)**	***$CD25^{High}CD4^+$ (%)*** **(CV%)**	***$CD25^{High}CD8^+$ (%)*** **(CV%)**
NS*	*126* (59)	*0.1* (187)	*16* (124)	*2.3* (68)	*3.1* (70)	*0.4* (265)
I_0	*195* (99)	*25* (56)	*22* (106)	*11* (102)	*11* (90)	*5.6* (97)
IC_{50}	***0.7*** ***(129)***	***2.7*** ***(161)***	***3.0*** ***(129)***	***3.1*** ***(122)***	***4.4*** ***(147)***	***5.5*** ***(174)***
IC_{50} **geometric *Mean***	***0.6***	***1.1***	***1.2***	***1.1***	***1.3***	***1.3***
I_{max}	*150* (118)	*0,5* (230)	*11* (190)	*4.0* (106)	*4.7* (94)	*1.3* (114)

**NS: non-stimulated, "physiologic" conditions*

Table III. PD/PG relationships in patients on the liver transplantation waiting list.

PD parameter	***PD marker***	***Associated SNP***	***Genetic Model***	***β ± SD (Multivariate Model)***	***Modulation***	***p value* (Multivariate Model)***	***Contributio to variabili (%)***
NS	*NFAT1^{+} PBMC nuclei^{+} (log transformed)*	*rs4519508 PPP3R1*	*C > T MAF =0.46 Tf = 0.05*	*1.2 ± 0.5*	↑	0.0292	39
	CD25^{High}CD3^{+}	***rs6850 PPIA***	***A > G MAF =0.42 Gf = 0.11***	***2.7 ± 0.4***	↑	***0.00016***	***81***
	CD25^{High}CD4^{+}	***rs6850 PPIA***	***A >G MAF = 0.42 Gf = 0.11***	***3.8 ± 0.5***	↑	***0.00004***	***86***
	CD25^{High}CD8^{+}	*rs45441997 PPP3CA*	*(GGC)8/8 >10/10 MAF NA (GGC)10/10f = 0.05*	*- 1.7 ± 0.6*	↓	*0.0228*	*42*
I_0	*IL-2^{+}CD4^{+}*	*rs8177826 PPIA*	*C > G MAF =0.04 Gf = 0.16*	*- 20 ± 6*	↓	*0.0043*	*39*
	CD25^{High}CD3^{+} (log transformed)	*rs8177826 PPIA*	*C > G MAF =004 Gf = 0.16*	*- 1.5 ± 0.6*	↓	*0.0169*	*39*
	CD25^{High}CD4^{+} *(log transformed)*	***rs8177826 PPIA***	***C > G MAF =0.04 Gf = 0.16***	***- 1.7 ± 0.4***	↓	***0.0011***	***57***
IC_{50}	*NFAT1^{+} PBMC nuclei^{+} (log transformed)*	*rs141252617 FKBP1A*	*C > A MAF =0.02 Af = 0*	*1.8 ± 0.6*	↑	*0.0139*	*41*
	IL-2^{+}CD8^{+} (log transformed)	*rs8177826 PPIA*	*C > G MAF =0.04 Gf = 0.16*	*- 1.6 ± 0.7*	↓	0.0350	26
	CD25^{High}CD4^{+} (log transformed)	*rs45441997 PPP3CA*	*(GGC)8/8 >10/10 MAF NA (GGC)10/10f = 0.05*	*- 1.9 ± 0.7*	↓	*0.0202*	37
I_{max}	*NFAT1^{+} PBMC nuclei^{+} (log transformed)*	*rs45441997 PPP3CA*	*(GGC)8/8 > 10/10 MAF NA (GGC)10/10f = 0.05*	*1.1 ± 0.5*	↑	*0.0221*	*29*
	IL-2^{+}CD8^{+} (log transformed)	*rs141252617 FKBP1A*	*C > A MAF =0.02 Af = 0*	*2.9 ± 1.2*	↑	*0.0276*	*27*
	CD25^{High}CD3^{+} (log transformed)	*rs45441997 PPP3CA*	*(GGC)8/8 >10/10 MAF NA (GGC)10/10f = 0.05*	*1.1 ± 1.2*	↑	*0.0214*	*64*
	CD25^{High}CD3^{+} (log transformed)	*rs141252617 FKBP1A*	*C > A MAF =0.02 Af = 0*	*1.3 ± 0.5*	↑	*0.0250*	*64*

	$CD25^{High}CD4^{+}$ (log transformed)	*rs141252617 FKBP1A*	*C > A MAF =0.02 Af = 0*	*2.5 ± 0.7*	↑	*0.0035*	*52*

**Bonferroni correction relies on significativity at $p < 0.0021$.*

f, frequency

MAF values, from https://www.ncbi.nlm.nih.gov/snp, last accessed November 2016.

Figure Legends

Figure 1. The fluorescence intensity of biomarkers *vs.* TAC concentration follows a sigmoid I/I_{max} model in patients on the liver transplantation waiting list.

Figure 2. IL-2 and CD25 expression in $CD4^+$ and $CD8^+$ T cell subsets as a function of NFAT1 expression in PBMC $nuclei^+$, following allosteric sigmoid models, showing the best and worst individual fits obtained in WLP.

Figure 3. I_0 expression of $NFAT1^+$ in PBMC nuclei, IL-2 in $CD4^+$ and $CD8^+$T and CD25 in $CD4^+$, $CD8^+$ and in T lymphocytes, in patients on the liver transplantation waiting list (WLP) as compared to healthy volunteers (HV) [with permission from (8)].

Supporting Information

Supplemental Material S1_PD_PG Procedures.

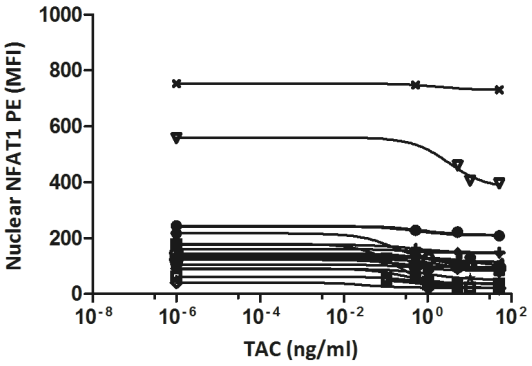
Supplemental Table S2.

Supplemental Table S3.

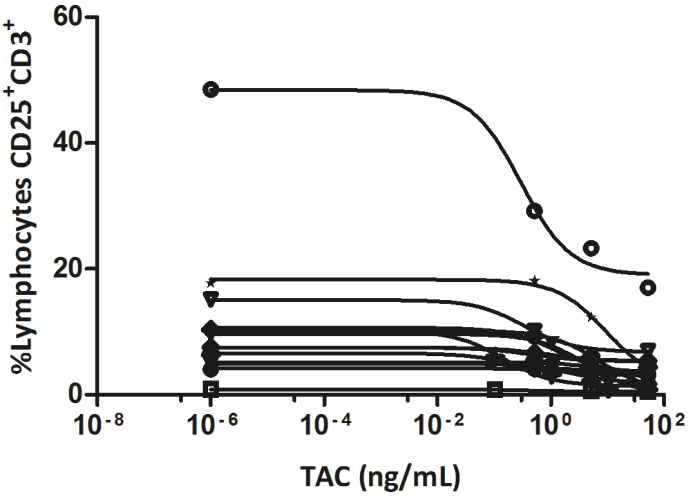
Supplemental Figure S4.

Supplemental Table S5.

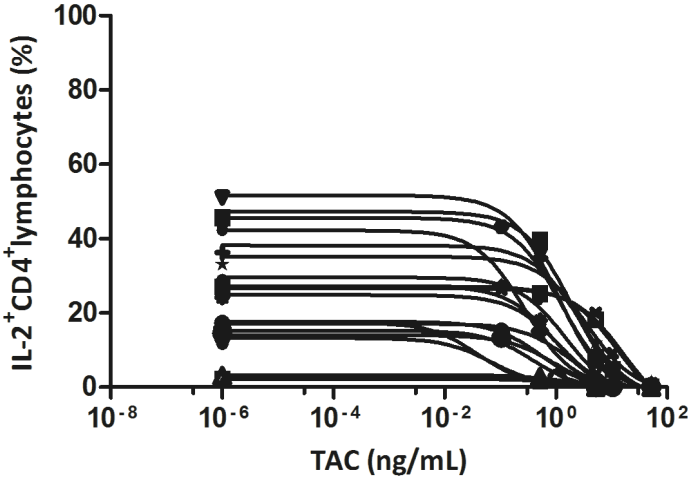
NFAT1 Nuclear Translocation Inhibition in PBMC



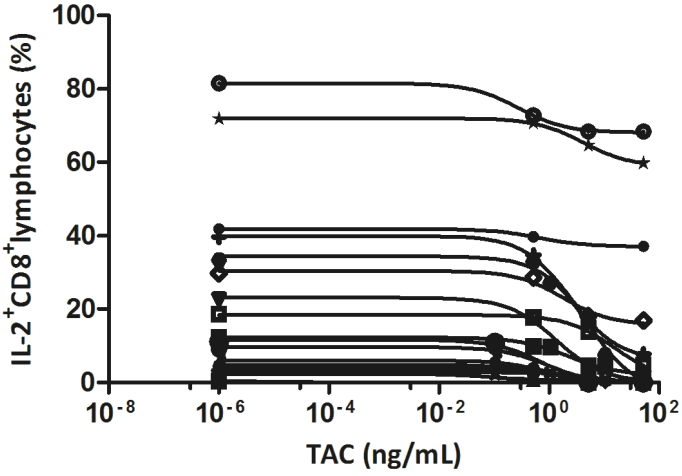
% of $CD25^{+}$ in $CD3^{+}$ Lymphocytes



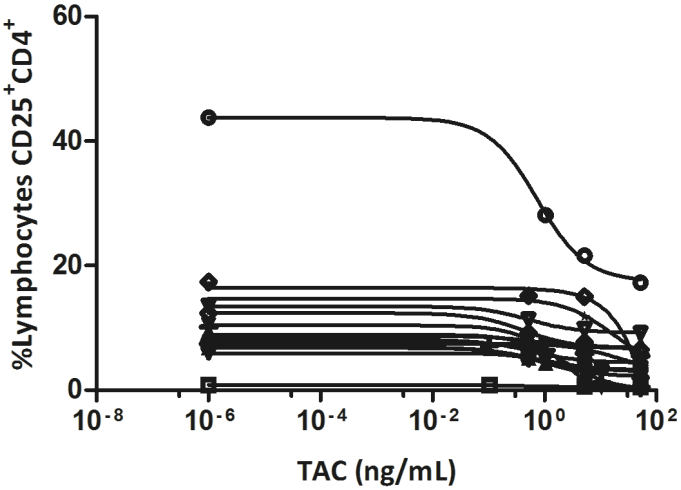
% of $IL2^{+}$ in $CD4^{+}$ T Lymphocytes



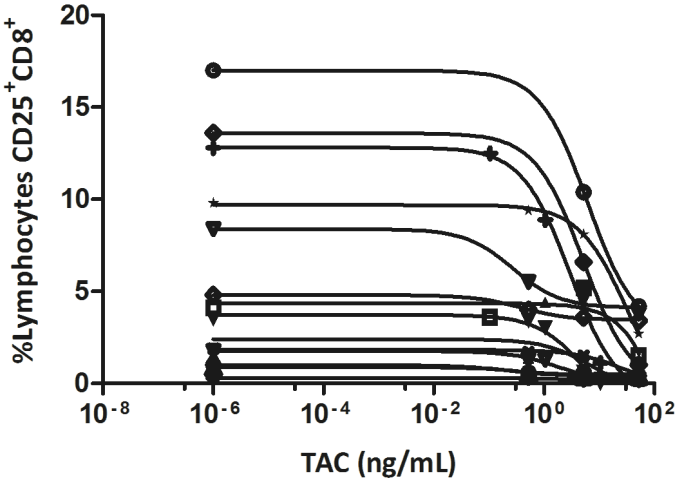
% of $IL2^{+}$ in $CD8^{+}$ T Lymphocytes



% of $CD25^{+}$ in $CD4^{+}$ Lymphocytes

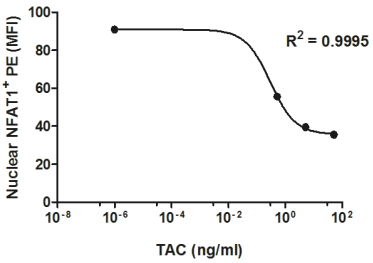


% of $CD25^{+}$ in $CD8^{+}$ Lymphocytes

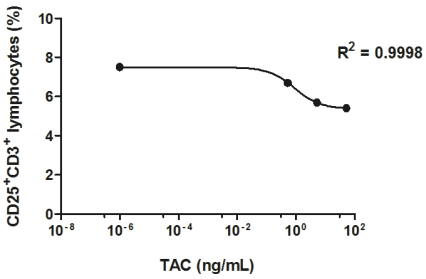


Best Fit

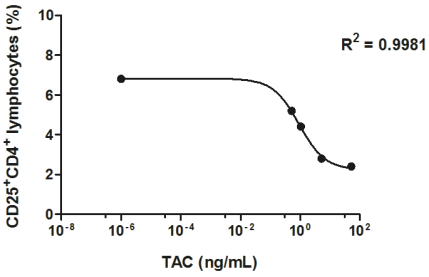
A NFAT1 Nuclear Translocation Inhibition in PBMC



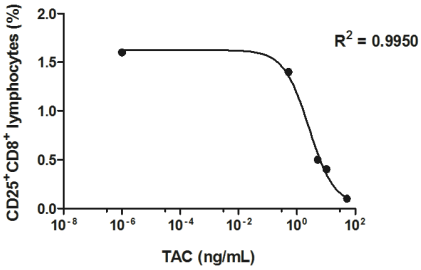
C % of $CD25^+$ in $CD3^+$ Lymphocytes



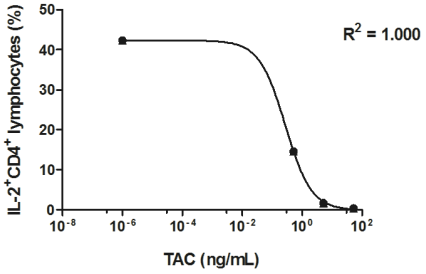
E % of $CD25^+$ in $CD4^+$ Lymphocytes



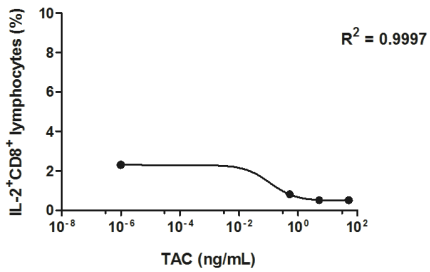
G % of $CD25^+$ in $CD8^+$ Lymphocytes



I % of $IL2^+$ in $CD4^+$ Lymphocytes

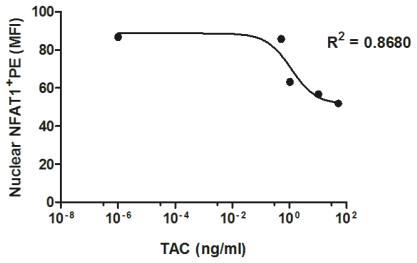


K % of $IL2^+$ in $CD8^+$ Lymphocytes

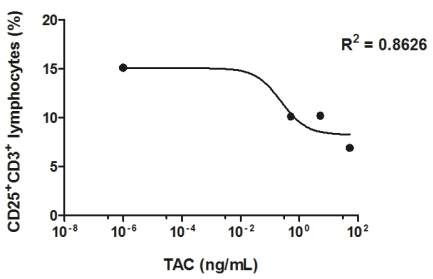


Worst Fit

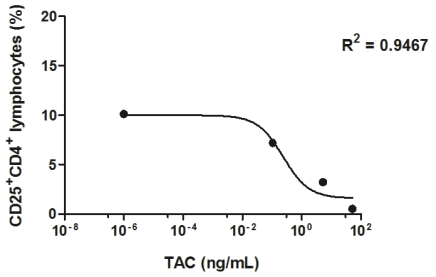
B NFAT1 Nuclear Translocation Inhibition in PBMC



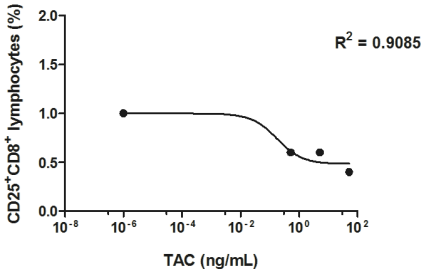
D % of $CD25^+$ in $CD3^+$ Lymphocytes



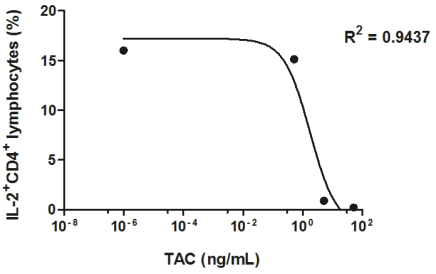
F % of $CD25^+$ in $CD4^+$ Lymphocytes



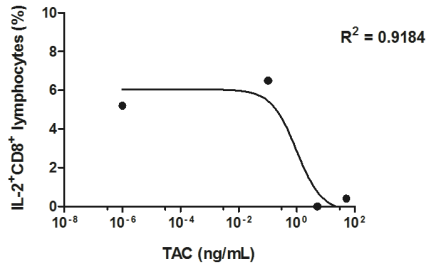
H % of $CD25^+$ in $CD8^+$ Lymphocytes

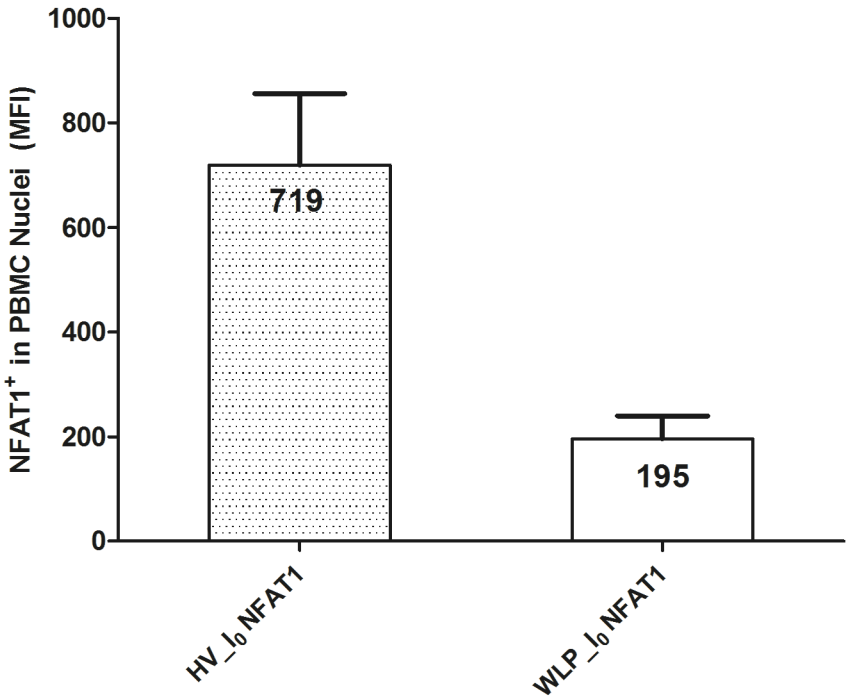


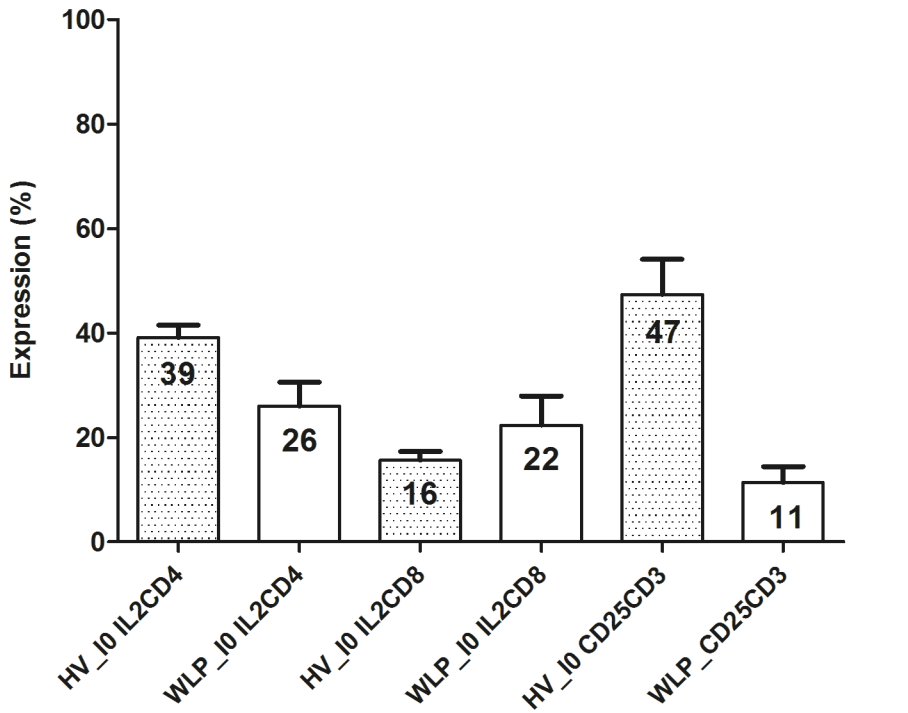
J % of $IL2^+$ in $CD4^+$ Lymphocytes



L % of $IL2^+$ in $CD8^+$ Lymphocytes




MFI NFAT1+ in PBMC Nuclei
NFAT1+ in PBMC Nuclei (MFI)
1000
800
600
400
200
0
719
195
HV_I0 NFAT1
WLP_I0 NFAT1


% of IL-2+ and CD25+ in CD4+ and CD8+ T Lymphocytes
Expression (%)
100
80
60
40
20
0
39
26
16
22
47
11
HV_I0 IL2CD4
WLP_I0 IL2CD4
HV_I0 IL2CD8
WLP_I0 IL2CD8
HV_I0 CD25CD3
WLP_CD25CD3

SUPPLEMENTAL MATERIAL AND METHODS

CHEMICALS AND REAGENTS

Lymphocyte density-gradient separation medium (Eurobio); RPMI 1640 1X medium, supplemented with 10% of heat-inactivated Fetal Bovine Serum (FBS), 10% of PenStrep 5000U/ml of penicillin, 5000 µg/ml of streptomycin and 10% of L-Glutamine 200 mM 1X; and phosphate buffered saline (PBS) 1X without calcium were purchased from Life Technologies; phorbol 12-myristate-13-acetate (PMA), calcium ionophore and Concanavalin A from Sigma Aldrich. Tacrolimus (TAC) pure substance was kindly provided by Astellas Pharma. PBMC lysis buffer (10 mM Pipes, $MgCl_2$ 2 mM, NaCl 0.1M and Triton 100X 0.1%) and 1% Bovine Serum Albumin (BSA) from Sigma Aldrich. IntraPrep kit and PE polyclonal IgG (Goat $F(ab')_2$ Fragment Anti-Mouse IgG(H+L) (IM0855) were purchased from Beckman Coulter. The anti-human T cell surface markers CD3 PerCP-Cy5.5 clone SK7 (332772), CD4 FITC clone SK3 (345768), CD4 PE clone (345769), CD8 PE-Cy7 clone SK1 (335822), CD25 APC clone MA251 (555434); the anti-NFAT1 antibodies (Purified Mouse Anti–NF-ATc2) clone 1 (610702) and Purified Mouse Anti–NF-AT1 clone 4G6-G5 (556601) and Golgi Plug protein transport inhibitor for IL-2 expression measurement were all from Becton Dickinson.

PHARMACODYNAMIC INVESTIGATIONS

PBMC separation

35ml of blood was drawn into 6ml sodium heparin vacutainer tubes (Becton Dickinson), under fasting conditions and before immunosuppressive drug intake on Monday mornings. Samples were processed within 1h of venipuncture and kept at room temperature in horizontal position. The fresh PBMC fraction was obtained by

lymphocyte density-gradient separation, using the MSL medium (Eurobio, France). PBMC washes were performed with RPMI 1640, without human serum addition. During PBMC incubation, we added RPMI 1640 medium with 10% of heat-inactivated Fetal Bovine Serum, 10% of PenStrep 5000U/ml of penicillin, 5000 µg/ml of streptomycin and 10% of L-Glutamine 200 mM 1X (Invitrogen, France). For incubation times longer than 24h, we also added beta-mercaptoethanol (Invitrogen, France).

White blood cells (WBC) yields measured using a calibrated cell counter were at least 10^6 in each well, sufficient for all our experiments. We did not optimize further our PBMC separation technique, as it fulfilled the MIQUE checklist requirements.

Ex-vivo Tacrolimus exposure

For each set of markers, aliquots of PBMC in supplemented RPMI were incubated ex-vivo for 30min at 37°C in a humidified atmosphere at 5% CO_2 (in 96-well sterile plates) with 0 to 50 ng/ml of TAC in RPMI 1640.

Cell activation strategy

For IL-2 and NFAT1 activation, we chose PMA/ionomycin because PMA exerts its action through PKC pathway activation and ionomycin increases the intracellular calcium level, acting on ion pump exchangers in the cell membrane. This combination bypasses the T-cell receptor (TCR) (Wang H et al. Transplantation Proceedings, 2013, 45:1822–1831) and exerts a vigorous stimulus on T cell cytokines synthesis, providing the best conditions for intracellular cytokine determination (Foster et al. Curr Protoc Immunol. 2007 Aug;Chapter 6:Unit 6.24).

For CD25 T cell surface marker activation quantification, we employed concanavalin A (ConA) because it induces T cell proliferation more effectively than PMA/I. ConA

activates phospholipase C via Gα protein leading to the IP3 pathway, and crosslinks TCR, guaranteeing activation via the TCR.

Using these two approaches best served our purposes because one of the secondary objectives of the clinical trial 3PIGREF is to discover biomarkers of acute cellular rejection, episodes where cytokine synthesis and surface marker activation are upregulated in T cells, leading to T cell proliferation [Wieland et al, Ther Drug Monit *2010* 32(5):560-572].

NFAT1 translocation to the nucleus of PBMC cells

To assess the degree of NFAT1 translocation into the nucleus, an indirect (secondary) antibody staining technique was employed to improve dye sensitivity to signal amplification. The procedure started with aliquots of 1 million cells in 1000 µl of supplemented RPMI, treated with 0 - 50 ng/ml TAC during 30 min at 37°C and 5% CO_2, followed by incubation with 50 ng/ml PMA and 2.5 µg/ml calcium ionophore for another 30 min at 37°C and 5% CO_2. After two washes with cold PBS 1X, cells were incubated 30 min on ice with PIPES lysis buffer (pH 7.4 in PBS 1X). Subsequently to two extra washes with BSA 1% in cold PBS 1X, a mix of 50 µL of anti-NFAT1 antibodies at 5 µg/ml in PBS 1X was added to the medium for another 30 min incubation on ice. Then a labeled PE polyclonal IgG (Goat $F(ab')_2$ Fragment Anti-Mouse IgG(H+L) at 0.33% was added. After 15 min incubation on ice in the dark, the resulting fluorescent signal of labeled PBMC nuclei was measured by flow cytometry, and the mean NFAT1 fluorescence intensity in the separated nuclei used for statistical analysis (8).

Intracellular IL-2 expression in $CD4^+$ and $CD8^+$ T cell subsets

After TAC exposure or directly after PBMC separation, aliquots of 1 million cells in 100 µl of supplemented RPMI were stimulated with 50 ng/ml PMA, 2.5 µg/ml calcium ionophore and 1 µg/ml Golgi Plug for 5h at 37°C in a humidified atmosphere with 5% CO_2. Cells were then i) stained with anti-human T lymphocyte surface markers (CD3 PerCP-Cy5.5 10uL, CD4 FITC 10 uL, CD8 PE-Cy7 3uL; ii) fixed; iii) permeabilized (IntraPrep); iv) stained with PE Rat Anti-Human IL-2 10uL; and v) analyzed by flow cytometry [Noceti et al. Clin Chem. 2014 Oct;60(10):1336-45.].

IL-2Rα expression at the surface of $CD4^+$ and $CD8^+$ T cells

For CD25 expression measurement, PBMC aliquots of 1 million cells in 100 µl of supplemented RPMI were incubated for at least 72h at 37°C and 5% CO_2 in the presence of 7.5 µg/ml ConcA in RPMI 1640 1X. After two additional washes in PBS 1X, direct staining was performed using: CD3 PerCP–Cy5.5 10uL, CD4 PE 10uL, CD8 PE-Cy7 3uL and CD25 APC 10uL. After 30 min incubation in darkness and several washes with BSA in PBS 1X / PBS 1X, flow cytometry analysis was performed.

IL-2 and CD25 expressions were reported as the percentage of fluorescent cells over the number of $CD4^+$, $CD8^+$ or $CD3^+$ cells, respectively.

FLOW CYTOMETRY STANDARDIZATION

An IVD certified Canto II flow cytometer equipped with 3 lasers (Becton Dickinson, BD) was employed for PD measures. The instruments settings were standardized and we employed the same protocols, reagents, antibodies and gating strategy as with the LSRII Fortessa flow cytometer equipped with 5 lasers (Becton Dickinson) used in France for our previous study on healthy volunteers.

Lymphocytes were chosen as an internal biological control population to place adequately the light scatter settings of the instrument. Inclusion of the FSC-H parameter allows discriminating doublets in a FSC-Area (FSC-A) versus FSC-Height (FSC-H) bivariate plot, to discard dead cells and duplets, contributing further to the accuracy of the results.

The instrument was standardized following the Euroflow Consortium guidelines (Kalina et al., Leukemia. 2012;26:1986–2010) and the Human ImmunoPhenotyping Project (Maecker et al., Nat Immunol. 2010;11(11):975–978) regarding the mean fluorescence intensity, variation coefficient, daily laser performance check and compensation of fluorochromes panel, to assure the reproducibility and performance of the instrument.

Instrument performance was verified on a daily basis (at each cold start) after laser stabilization for 30 min, using Cytometer Setup & Tracking Beads (CST, 642412) to check baseline detection efficiency, optical background and laser power and alignment; the bright bead % robust coefficient of variation (rCV) for the primary detector of each laser was set at 6.0.

MFI setup was performed using BD matrix template according to the values recommended for the Cytometer Set-up Tracking beads (CST) lot in use, under 'disabled compensation' conditions. The MFI of the brightest peak in each fluorescence channel was compared with the corresponding target MFI value. The following criteria had to be reached for the instrument to pass the check: i) MFI values within the target MFI±15%, and ii) CV of the brightest peak <4% for the blue and violet laser channels, while <6% for the red laser channels and the PECy7 channel. Whenever instrument performance failed, measures such as thorough cleaning, flow cell de-gassing and laser delay verification were undertaken.

Compensation was automatically calculated using the BD automatic fluorescence compensation tool available in DIVA software provided with the instrument, employing the Compbeads Plus kit negative (560497) and Anti Mouse Ig,k (555749) plus the whole panel of selected working fluorochromes.

Antibodies were titrated to optimize the best ratio of fluorescence and fluorescence-minus-one controls (FMO) were run during protocol validation. Our FMO protocol sensitivity gave LOD values of 0.18% for IL-$2^{+}CD4^{+}$, 0.25% for IL-$2^{+}CD8^{+}$ and 0.25% for NFAT1.

As antigen expression of $CD4^{+}$ T cells is stable across individuals and its stability is well validated, we decided to use $CD4^{+}$ subset as the stopping gate in our assays whenever possible, to ensure instrument performance. We systematically gated 50,000 events for intracellular IL-2 or CD25 activation marker in the $CD4^{+}$T cell subset, or 50,000 $NFAT1^{+}$ in PBMC nuclei.

ANALYTICAL AND INTRA-INDIVIDUAL VARIABILITY OF PHARMACODYNAMIC ASSAYS

We previously studied the analytical and intra-individual variability of our methods for all of our pharmacodynamic markers using PBMC from healthy volunteers enrolled in the 3-PIGREF study (Noceti et al. Clin Chem. 2014;60(10):1336–45). Flow cytometry measurements were performed by the same Operator (O.N.) on another Becton Dickinson flow cytometer (Fortessa LSRII, with 5 lasers), using the same fluorochromes and antibody clones, under standardized conditions of the equipment, filter settings and band pass filters, following the Euroflow guidelines regarding the mean fluorescence intensity, variation coefficient, daily laser performance check and compensation of fluorochromes panel, which assure the reproducibility and

performance of the experiments independent from the instrument employed. For our validation process, we adopted the FDA Clinical Laboratory Improvement Amendments (CLIA) validation criteria for Laboratory Developed Test (LDT) assays. Also, our methods complied with the International Council for Standardization of Haematology (ICSH) and the International Clinical Cytometry Society (ICCS) Guidelines for Cell-based Fluorescence Assays Validation [Davis et al. ,Cytometry Part B (Clinical Cytometry). 2013;84B:286–290; Davis et al., Cytometry Part B (Clinical Cytometry). 2013;84B:329–337; Wood et al., Cytometry Part B (Clinical Cytometry). 2013;84B:315–323]. However, what was not covered in the guidelines mentioned above is the way the flow cytometer is standardized (instrument setting and performance monitoring). For this, we followed Kalina et al.'s recommendations (Leukemia. 2012;26:1986–2010), including compensation and fluorochrome matrix with their MFI coefficient of variation for each channel, and this practice made the whole difference at the time to compare instruments performance between our two centers. For the evaluation of the analytical variability, three replicates of PBMC aliquots from 2 healthy volunteers (a male and a female) were employed. For intra-individual variability estimation, biomarker expression was measured on the same weekday over three consecutive weeks in PBMC aliquots from three other healthy subjects. The tacrolimus concentrations tested were 0, 0.5, 5 and 50 ng/mL. Flow cytometry measurements were performed in a single run for each subject. Analytical and intra-individual variability were obtained through analysis of variance (ANOVA) of log-transformed data to avoid the effect of inter-individual variability.

For the analytical variability, coefficients of variation at physiological levels (I_0) were always less than 9%, while those at 5 ng/ml TAC (i.e., approximately at TAC 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%. Indeed, the smaller the acquired values, as in the case of $IL\text{-}2^+CD8^+$ expression at 5 ng/ml TAC (<2% and often undetectable), the larger the imprecision of the readout. The intra-individual variability in 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%)

PHARMACOGENETIC STUDY

Genomic DNA was isolated from peripheral blood EDTA tubes using a commercial kit (the PureLink™ Genomic DNA, Invitrogen, USA in Montevideo and the QIAamp DNAMini Kit 51306 from Qiagen, France) according to the manufacturer's protocol. The DNA concentration was quantified using a NanoDrop 1000 spectrophotometer (Thermo Scientific, USA).

DNA amplifications were performed in a total volume of 20µL containing 50 ng of genomic DNA, 0.8 mM of each dNTP (Fermentas), 0.2 mM of each primer, 2mM $MgCl_2$, 5% DMSO (Bio Basic Inc), and 0.3U Taq DNA polymerase (Invitrogen).

The thermal cycler program was set for an initial denaturation at 95°C for 5min, followed by 30 cycles of 30s at 95°C, 30s at the annealing temperature (Tm), 30s at 72°C and a final extension for 5min at 72°C.

Tm was set at 54ºC for the amplification of PPP3CA and FKB1A fragments, and at 55ºC and 58°C when amplifying the PPP3R1 and PP1A promoter regions, respectively.

Promoters sequencing procedure

For DNA sequencing we used: the PCRx Enhancer system 11495-017 (10x PCRx Rxn Buffer, 10xPCR Enhancer Solution, 50nM Magnesium Sulphate, Platinum® Taq DNA Polymerase); 10 mM dNTP Mix 18427088 and BigDye® Terminator v3.1 Cycle Sequencing Kit 4337456 from Life Technologies, France; the QIAquick PCR Purification Kit 28106 and Dye-Ex 2.0 Spin Kit 63206 from Qiagen, Hilden, Germany. Customized primers were provided by Eurofins MWG Operon, Germany.

For each promoter region studied, the PCR reaction involved a first denaturation step at 94°C for 2min, followed by 35 cycles of denaturation at 95°C for 30sec, annealing at 55°C for 30sec, and extension at 68°C for 1min. The last step consisted of a final denaturation at 72°C for 7min. The reaction mix was composed of 5µL of PCRx

amplification buffer, 10mM of dNTP, 75mM of MgSO4 and 2.5UI of Platinum TAQ DNA polymerase, and 10µL of PCRx Enhancer Solution at a final concentration of 2X. Amplimers were purified with the QIAquick PCR Purification Kit. The sequencing reaction mix contained 4µl of BigDye® Terminator v3.1 Cycle Sequencing Kit, 4µL of amplimers, and 2µl of either the forward or the reverse primer. Confirmation using the opposite primer was performed whenever required. Sanger sequencing reactions consisted of a first denaturation step (94°C for 3 min), followed by 25 cycles of denaturation at 94°C for 10 sec, hybridization at 50°C for 5 sec and elongation at 60°C for 4 min. The final denaturation step lasted 1 min at 60°C. Finally, the sequencing products were purified using Dye-Ex 2.0 kit before separation by capillary electrophoresis on ABI 3130 XL Genetic Analyzer, Life Technologies. Sequences were then mapped to the reference sequence of the corresponding gene, and genetic variants identified, using SEQUENCHER 4.8 sequence analysis software, Gene Codes Corporation, Ann Arbor, MI USA.

The PCR products to be sequenced (*PPP3CA*, *PPP3R1* and *PP1A*) were purified using a 2% agarose gel (QIAquick Gel Extraction Kit, Qiagen, USA).

The sequencing reactions were performed by the Macrogen Service Center (Republic of Korea) or the Institute of Clinical Molecular Biology, Christian-Albrechts University Hospital Schleswig Holstein, Kiel (Germany).

Genotyping assays

The region containing the c.-215C>A *FKBP1A* polymorphism was digested with 0.3U of AvaI (Fermentas) for 3h at 37ºC and separated by electrophoresis through a 6% polyacrylamide gel; afterwards the product was exposed to silver nitrate 0.2%. All primer sequences are available on request.

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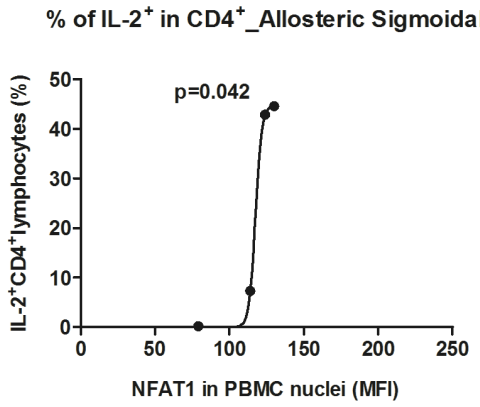
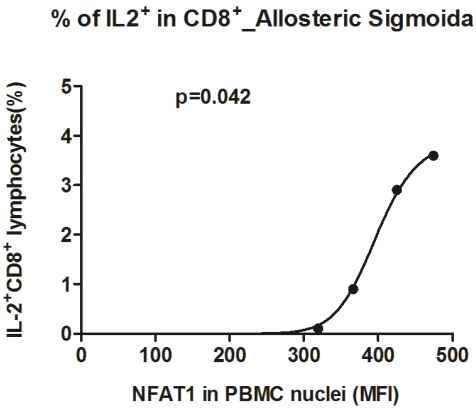
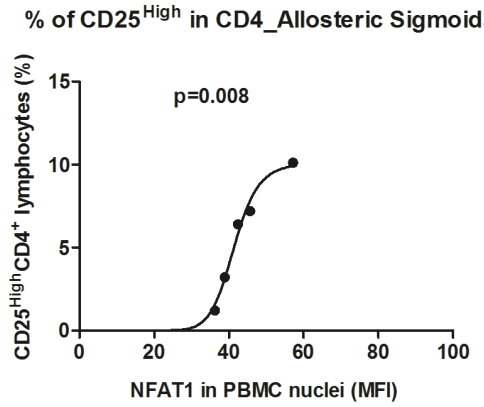
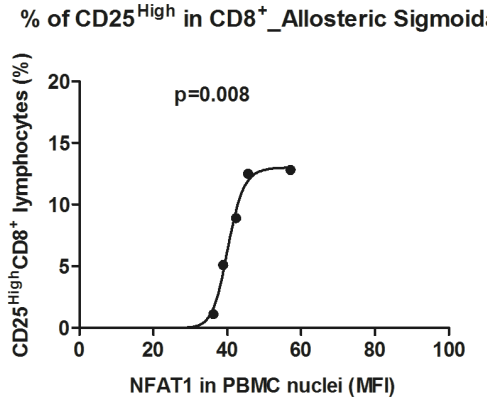
Best fits









Worst fits