The Effects of Tacrolimus on T-Cell Proliferation Are Short-Lived: A Pilot Analysis of Immune Function Testing

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**Background.** Optimal immunosuppression after organ transplant should balance the risks of rejection, infection, and malignancy while minimizing barriers to adherence including frequent or time-sensitive dosing. There is currently no reliable immune function assay to directly measure the degree of immunosuppression after transplantation. **Methods.** We developed an immune function assay to measure T-cell proliferation after exposure to immunosuppression in vivo. We tested the assay in mice, and then piloted the approach using single time point samples, 11 pediatric kidney transplant recipients prescribed tacrolimus, mycophenolate, and prednisone 6 months to 5 years posttransplant, with no history of rejection, opportunistic infection, or cancer. Twelve healthy adults were controls. **Results.** We demonstrated that our assay can quantify suppression of murine T-cell proliferation after tacrolimus treatment in vivo. In humans, we found a mean 25% reduction in CD4 and CD8 T-cell proliferation in pediatric renal transplant recipients on triple immunosuppression compared with adult healthy controls, but the pilot results were not statistically significant nor correlated with serum tacrolimus levels. We observed that cell processing and washing reduced the effects of tacrolimus on T-cell proliferation, as did discontinuation of tacrolimus treatment shortly before sampling. **Conclusions.** T-cell proliferation is currently not suitable to measure immunosuppression because sample processing diminishes observable effects. Future immune function testing should focus on fresh samples with minimal washing steps. Our results also emphasize the importance of adherence to immunosuppressive treatment, because T-cell proliferation recovered substantially after even brief discontinuation of tacrolimus.

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U.H.B., P.P.R. and B.L.L. conceived and designed the study, J.J. conducted most experiments with help from U.H.B. and T.A., B.L.L. and U.H.B. wrote the manuscript. U.H.B., B.L.L. and P.P.R. analyzed data. H.J.B., S.A., S.L.F., T.A., W.W.H. and M.H.L. provided access to patents and unique reagents. T.A. and W.W.H. advised experimental procedures.

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antibody assays, and the presence of opportunistic infections to estimate a patient’s degree of immunosuppression and adherence with medications. Prior attempts to directly measure a patient’s immune function have relied on nonspecific metabolic assays that have produced inconsistent results. 

We developed an assay quantifying the ability of T cells to proliferate in response to fixed, exogenous stimulation as a readout of immunosuppression after transplantation. We first tested the methodology in a murine model and then piloted the assay in children who had received a kidney transplant, using healthy adult blood donors as controls. This report describes the initial findings of our assay and their implications for assessment of medication adherence and future efforts to measure an individual patient’s overall degree of immunosuppression.

MATERIALS AND METHODS

Patients and Healthy Donors

We performed a cross-sectional study focusing on children and adolescents who had received a kidney transplant at the Children’s Hospital of Philadelphia. Patients were eligible for enrollment if they were 2 to 25 years of age; were on therapeutic immunosuppression, including prednisone, tacrolimus and mycophenolate; and were 6 months to 5 years after their first kidney transplant. Exclusion criteria were a history of renal allograft rejection, evidence of viral infection (Epstein-Barr virus, BK, or cytomegalovirus) within 3 months before sample collection, history of cancer or posttransplant lymphoproliferative disorder, or documented medication nonadherence. Comparison analyses were performed on healthy, deidentified adult donor peripheral blood mononuclear cell (PBMC) samples obtained through the Human Immunology Core at the University of Pennsylvania. All patients and guardians, as well as healthy adult blood donors, gave written informed consent as approved by the institutional review boards at Children’s Hospital of Philadelphia (IRB 14-010784) and the University of Pennsylvania (IRB 70906).

Murine Studies

We purchased C57BL/6 mice from Jackson Laboratories. Mice were housed under specific pathogen-free conditions using protocols approved by the Institutional Animal Care and Use Committees of the Children’s Hospital of Philadelphia and University of Pennsylvania (13-000561).

Sample Collection and Storage

For murine studies, we obtained spleens and processed them into single-cell suspension after red blood cell lysis. For human studies, PBMC and T cells were isolated, cryopreserved, and recovered as previously described. Briefly, each human subject provided a single whole blood sample (1-10 mL) drawn in an ethylenediaminetetraacetic acid tube during a scheduled outpatient visit at the time of routine, 12-hour trough level testing. Blood samples were processed in SepMate tubes (Stemcell Technologies, Vancouver, Canada) to isolate PBMC. The PBMC were counted, and either stimulated or suspended in CryoStor solution (Sigma-Aldrich), and stored in cryotubes in liquid nitrogen.

T-Cell Function Testing and Flow Cytometry

Our assay was designed to directly quantify the ability of T cells to proliferate. Specifically, in both murine and human studies, T cells were exogenously stimulated with a soluble, plate-bound, or bead-bound CD3 monoclonal antibodies (mAb) directed against the T-cell receptor in the presence of variable tacrolimus doses. Costimulatory signals were provided by antigen-presenting cells, or in case of purified CD4+ T cells, via CD3/CD28 mAb stimulation. Murine splenocytes, human PBMC, or isolated CD4+ T cells were labeled with carboxyfluorescein succinimidyl ester (CFSE), and proliferation was measured by flow cytometric analysis of CFSE dilution. Murine and human antibodies and key reagents for flow cytometry as well as stimulation are listed in Table 1. Costimulated samples were incubated in culture for 72 (mouse) or 96 (human) hours. For cell culture, we used Roswell Park Memorial Institute 1640 medium supplemented with 10% fetal bovine serum, penicillin (100 U/mL), streptomycin (100 mg/mL), and 55 nM β-mercaptoethanol. Cells were cultured at 37°C with 5% CO2. T-cell proliferation was determined by flow cytometric analysis of CFSE dilution and calculation of the area under the curve from multiple dilutions of stimulating antibodies in the same experiment, based on a previously published method. All flow cytometry data were captured using Cyan (Dako/Agilent Technologies, Santa Clara, CA) as well as Cytoflex (Beckman Coulter, Brea, CA) and analyzed using the FlowJo 10.1 software. Pooled histogram data are shown as percent of maximum (% of max), which is a normalization of overlaid data and represents the number of cells in each bin divided by the number of cells in the bin that contains the largest number of cells.

Data Analysis

The normally distributed data were displayed as means ± standard error of the mean. Measurements comparing 2 groups were performed with a Student t test. Likewise, comparisons involving groups of 3 or more subjects were analyzed by 1-way analysis of variance. For correlation, we used Pearson correlation for normally distributed data. A 2-sided P value less than 0.05 was considered statistically significant. Data were analyzed using GraphPad Prism software, version 6 (La Jolla, CA).

| TABLE 1. Antibodies and reagents |
|---------------------------------|
| Reagent or antibody | Source | Identifier |
|---------------------|--------|------------|
| Human CD3 antibody  | BD Pharmingen | Cat. 551020 |
| Dynabeads anti-human CD3 | Thermo Fisher | Cat. 11131D |
| APC mouse anti-human CD8 | BD Pharmingen | Cat. 555369 |
| MitoTracker Green | MitoProbe | Cat. 555349 |
| CellTrace CFSE cell proliferation kit | Thermo Fisher | Cat. 11131D |
| CFSE dilution | Thermo Fisher | Cat. 11131D |

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RESULTS

Murine Ex Vivo T-Cell Proliferation is Impaired by Tacrolimus Exposure Dose Dependently

We started the investigation of our assay by measuring T-cell proliferation in C57BL/6 mice subjected to intraperitoneal treatment of various concentrations of tacrolimus. After treating the mice with 0.5 to 10 mg·kg⁻¹·d⁻¹ tacrolimus or dimethyl sulfoxide (DMSO) vehicle control intraperitoneally for 4 days, we stimulated the isolated splenocytes with anti-CD3ε mAb and observed a dose-dependent reduction in CD4⁺ T-cell proliferation (Figures 1A and B). These findings suggested that it may be possible to quantify the immunosuppressive effects of tacrolimus ex vivo by directly measuring T-cell proliferation and supported further testing of the assay in human samples.

Human CD4⁺ T Cells From Different Healthy Donors Respond Like Costimulation

An optimal assay for use in human transplant recipients should quantify immunosuppression, allow for testing on frozen samples, and produce comparable results between different patients. In contrast to splenocytes from inbred C57BL/6 mice, human PBMC, especially after exposure to induction therapy after transplantation, can contain variable amounts of lymphocyte subsets, which can make patient-to-patient comparisons difficult. We therefore focused our initial assay development on cryopreserved and thawed CD4⁺ T cells from healthy adult blood donors, which were remarkably resilient to cryopreservation and showed excellent function after thawing. More importantly, the thawed CD4⁺ T cells also demonstrated very limited variability in response to proliferative stimuli, as anti-CD3ε/CD28 mAb-coated
beads induced a consistent greater than 80% proliferation down to a 1:4 bead per cell ratio in all samples tested from the healthy subjects (Figure 1C). In contrast, PBMC were much more variable in their response to the exogenous T-cell receptor stimulation (Figures 1D-G and Figure 2). The low variability in isolated CD4\(^+\) T-cell responses to standardized stimulation conditions allowed better comparisons between individual donors, which was limited in PBMC cells not undergoing CD4\(^+\) T-cell isolation (Figure 1). In addition, even a low concentration of tacrolimus at 3.125 ng/mL strongly suppressed T-cell proliferation in the cells isolated from healthy blood donors (Figure 3). We hypothesized that the combination of low variability of CD4\(^+\) T-cell proliferation responses between donors and strong suppression by even low tacrolimus levels would make it easier to spot evidence of immunosuppression by tracking T-cell proliferation in patients on tacrolimus treatment. Therefore, we aimed to use purified CD4\(^+\) T cells as the readout for our assay because of their resilience to cryopreservation, persistently strong response to T-cell proliferation across all donors, and evidence for suppression in the presence of tacrolimus.

**Isolated CD4\(^+\) T Cells From Immunosuppressed Patients Do Not Show Reduced Proliferation**

To evaluate our assay in transplant recipients, we performed a cross-sectional analysis of 11 children and adolescents who had received a kidney transplant at our center (Table 2). We selected patients who had stable, adequate immunosuppression, defined as (a) receiving triple immunosuppression therapy (tacrolimus, mycophenolate, prednisone), (b) no history of allograft rejection, (c) no history of opportunistic infection or cancer, and (d) long enough after induction (>6 months) so that T cell counts were fully recovered. However, we soon noticed that there was little difference in T-cell proliferation between immunosuppressed renal transplant recipients and healthy donor (HD) control purified CD4\(^+\) T cells, both in freshly isolated, as

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**TABLE 2. Patient characteristics**

|                           | N = 11 |
|---------------------------|--------|
| Age, y                    | 12.2 [8.9-18.2] |
| Non-Hispanic ethnicity (vs Hispanic) | 10/11 (90.9%) |
| White race (vs Black)     | 10/11 (90.9%) |
| Male sex (vs female)      | 6/11 (54.6%) |
| Time posttransplant (months) | 12.2 [11.0-20.1] |
| Living donor (vs deceased) | 8/11 (72.7%) |
| Human leukocyte antigen MM 1 | 1/11 (9.1%) |
| MM 2                      | 10/11 (90.9%) |
| MM 3                      | 4/11 (36.4%) |
| Induction therapy         |        |
| Thymoglobulin             | 8/11 (72.7%) |
| Basiliximab               | 2/11 (18.2%) |
| Alemtuzumab               | 1/11 (9.1%) |
| Creatinine-estimated GFR (mL·min\(^{-1}\)·1.73m\(^2\)) | 70.8 [63.9-89.1] |
| Tacrolimus trough level (ng·mL\(^{-1}\)) | 7.4 [3.9-8.6] |
| Donor-specific antibodies present | 1/11 (9.1%) |
| Prednisone dose (mg × d\(^{-1}\)) | 4.3 [2.0-5.0] |
| Mycophenolate dose (mg/m\(^2\) per dose) | 290.7 [151.5-314.5] |

Data shown as n (%) or median [interquartile range]. GFR, glomerular filtration rate; MM, mismatch.
well as frozen and thawed samples (Figure 1H). Because we had observed strong suppressive effects in murine T cells after injecting tacrolimus to C57BL/6 mice and in human T cells exposed to tacrolimus in vitro, we hypothesized that the difference between murine and human cell processing might be a potential cause for the absence of observable impairment of T-cell proliferation in transplant recipient CD4+ T cells. In contrast to murine splenocytes, human CD4+ T-cell isolation requires extensive washing and processing steps, in addition to freezing and thawing procedures. We hypothesized that these washing steps could have diluted out the immunosuppressive effects of tacrolimus. Therefore, we switched from isolated CD4+ T cells to using freshly isolated PBMC for our assay readout, PBMC allowed for more rapid processing and less washing procedures, potentially retaining the in vivo immunosuppressive effects of tacrolimus. However, this would be at the expense of higher variability between individual donors (Figures 1D-E and 2), compared with isolated CD4+ T cells (Figure 1C). Among the different modalities to stimulate freshly isolated PBMC, platebound CD3 mAb showed less interdonor variability relative to anti-CD3 mAb-coated beads (Figure 2). Accordingly, using freshly isolated PBMC from pediatric kidney transplant recipients, we observed a 24.3% and 25.3% reduction in their CD4+ and CD8+ T-cell proliferation, respectively, compared with adult healthy controls without immunosuppressive treatment (Figure 4). However, this suppression in proliferation was less than we had expected and was therefore unlikely to be clinically significant as an assay to quantify immunosuppression.

Washout of Tacrolimus Rapidly Diminishes Its Suppressive Effects

Because we could not detect any evidence of immunosuppression in kidney transplant recipients who were receiving immunosuppression using isolated CD4+ T cells, and observed only marginal suppression of T-cell proliferation using PBMC, we questioned if even the abbreviated process of isolating PBMC diluted the immunosuppressive effects of tacrolimus, limiting the overall utility of our assay. To specifically assess the effects of drug washout, we exposed HD PBMC to high-concentration tacrolimus at 200 ng·mL⁻¹ for 1 hour. Considering our previous observation that a low tacrolimus concentration of only 3.125 ng·mL⁻¹ reduced human T-cell proliferation by more than 50% (Figure 3), this 64-fold higher dose was, as expected, fully ablative of any T-cell proliferation (Figure 5A). However, after washing the cells just a few times with phosphate-buffered saline before costimulation, we observed a dose-dependent recovery of T-cell proliferation despite their recent previous exposure to high-dose tacrolimus (Figure 5A).

The Immunosuppressive Effects of Tacrolimus Are Short-Lived Once the Drug Is Removed

Our observations of declining suppression of T-cell proliferation after tacrolimus washout led us to 2 conclusions. First, the necessary cell processing and washing steps may limit immune function assays that rely on isolating T cells to quantify a patient’s overall degree of immunosuppression. Second, and perhaps more importantly, this finding also implies that falling in vivo serum tacrolimus levels, such as...
due to nonadherence, might also rapidly lower immunosuppressive coverage. We tested this theory by administering daily tacrolimus or vehicle control to C57BL/6 mice in 4 different conditions to mimic patients (1) with full adherence to tacrolimus treatment who would have therapeutic drug levels, (2) who generally take their medication but just forgot to take their last 2 doses before having a tacrolimus trough checked and would have low drug levels, (3) who never take their medication and would have undetectable drug levels, and (4) who take 1 dose just before their clinic visit so as to appear to have a therapeutic level (Figure 5B). We found that treatment on the last day was sufficient to achieve similar suppression compared with the fully adherent group, whereas missing the last 2 doses showed the same T-cell function as nonadherent animals receiving only vehicle control throughout (Figures 5C and D). Together, these data show that the effects of tacrolimus on T-cell proliferation are very short-lived and become undetectable soon after the drug is discontinued, or the cells are removed and washed during processing.

DISCUSSION

Optimizing immunosuppression after transplantation remains complex. Prescribing just the right amount of medication to weaken recipient immunity and prevent allograft rejection is particularly challenging in the absence of a reliable test that can inform clinicians when an individual patient is experiencing too little or too much immunosuppression. Proxy readouts, such as screening for viral infections or measuring therapeutic drug concentrations, are far from ideal, especially as individual patients can respond differently to the same treatment. Current biomarker research aims to identify early signs of impending allograft injury by examining endothelial damage, impaired glomerular filtration, or markers of general inflammation. However, these proposed biomarkers cannot yet guide clinicians to adjust immunosuppressive therapy proactively, because the reason for the allograft injury could arise from both too little (rejection), or too much immunosuppression (e.g., BK virus nephropathy).

A reliable immune function assay is an unmet need in the field of transplantation. Prior efforts to develop an assay, such as measuring adenosine triphosphate (ATP) within CD4+ T cells have not translated well into clinical practice, and cannot distinguish effectively between infection and rejection. In addition, this method can be confounded by the divergent modalities of ATP production among different T-cell subsets: Cytotoxic and effector T cells require a glycolytic metabolism to function, which is less efficient at ATP production than oxidative phosphorylation prominent in immunosuppressive, regulatory T cells.

In contrast to measuring ATP, assays that are directly related to the mechanisms of key effector T-cell functions may provide better alternatives. Our assay was designed to quantify T-cell proliferation in transplant recipients on immunosuppression. Unfortunately, the results were less robust in cells from children and adolescents after kidney transplant receiving stable immunosuppression. The processing steps required to isolate human T cells washed away the
immunosuppressive properties of tacrolimus, pointing to a transient effect of tacrolimus which we confirmed in murine in vivo studies. Tacrolimus’ mechanism of action works through impairing NFAT signaling, which impedes upon IL-2 production, and can have adverse effects on T-cell proliferation. It is therefore possible to speculate that T cells regain the ability to proliferate after tacrolimus is removed. In the context of adherence research and intermittently subtherapeutic tacrolimus, our findings support previous clinical studies showing the development of donor-specific antibodies is associated with medication nonadherence after kidney transplantation.

Our study has several limitations. One is the small sample size of 11 patients. Although it may have been possible to accumulate more patients, and reach statistical significance, we felt that the observed 25% reduction in T-cell proliferation using PBMC as the readout was not high enough to distinguish biologically meaningful outcomes (rejection, infection) in clinical practice. Furthermore, the test was unlikely to be practical for clinical care because we were unable to use frozen and thawed CD4+ T cells due to washout of the immunosuppressive effects of tacrolimus. The difficulty in acquiring control samples from HDs is another limitation of the test in its current form. Requiring a fresh, HD control sample, obtained the same day that a transplant recipient would undergo immune function testing, may not be a feasible approach going forward.

In conclusion, our proliferation-based assay is insufficient to measure therapeutic immunosuppression. However, in addition to this negative finding, the observation that the immunosuppressive effects of tacrolimus are rapidly lost by washing can aid in future assay development, which should consider minimal sample processing to prevent washing out the in vivo immunosuppressive properties of tacrolimus. Furthermore, our study illustrates how rapidly the effects of tacrolimus are lost once dosing is even briefly interrupted. We hope that this finding can help guide adherence education for transplant patients and motivate efforts to incorporate longer-acting immunosuppressive therapies into transplant regimens. Our results emphasize that patient adherence to currently available medications is crucial, because the immunosuppressive effect from tacrolimus treatment is rapidly lost.

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