TANGELA: Tacrolimus Adjustment by NFAT-related Gene Expression in Lung Allograft Recipients.

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Principal Investigator: **John Greenland, MD, PhD**

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I. Study Objectives and Design

Study Design: This is a non-interventional cohort study to assess a novel assay to detect excessive or insufficient immunosuppression from the drug tacrolimus in lung transplant recipients. The assay measures mean residual expression (MRE) of genes downstream of nuclear factor of activated T cells (NFAT), a transcription factor regulated by tacrolimus. The investigators will assess whether MRE levels identify subjects at risk for rejection (insufficient immunosuppression) or infection (excessive immunosuppression).

Hypothesis: The degree of tacrolimus-induced immunosuppression, as assessed by the MRE of NFAT-controlled genes, can identify lung allograft recipients at increased risk for rejection or infection.

Study Objectives:

1. Determine tacrolimus dosing cutoffs that maximally classify lung allograft recipients as at risk for rejection or infection.
2. Assess the likelihood that tacrolimus dosing, informed by MRE level, will lead to a decreased frequency of rejection and/or infection.
3. Compare MRE levels and lung concentrations of tacrolimus for the prediction of lung transplantation-related outcomes.
4. Compare tacrolimus trough levels with MRE values in lung allograft recipients

II. Background:

Tacrolimus is a critical component of most post-lung transplant maintenance immunosuppression regimens.[1] Following initial observations of highly variable efficacy and side-effect profiles with fixed dosing regimens, dosing by trough has become the standard of care.[2] However, variation in clinical effects between individuals with similar trough levels has led to the concern that tacrolimus trough levels inaccurately reflect the degree of immunosuppression. Several clinical studies have investigated an assay based on the mean residual gene expression (MRE) at peak versus trough calcineurin inhibitor concentrations following a single dose. The 3 genes assayed are downstream of the nuclear factor of activated T cells (NFAT) family of transcription factors that are activated by calcineurin, so this gene expression ratio may assess the degree of immunosuppression attributable to a given dose of calcineurin inhibitor.[3,4] It is hoped that tacrolimus titration based on this assay could lead to decreased incidence of rejection, infection, and cancer in renal allograft recipients.[5,6]

Optimizing immunosuppression in lung allograft recipients remains challenging. In the first year after transplant, 35% of lung allograft recipients have at least 1 episode of acute rejection requiring treatment.[7] Acute cellular rejection is a risk factor for chronic lung allograft dysfunction (CLAD), which affects nearly 50% of patients at 5 years and is the most significant cause of death after the first year.[7,8] Acute cellular rejection and CLAD occur despite high doses of tacrolimus combined with mycophenolate mofetil and prednisone. At the same time, these immunosuppression regimens have been linked with increased rates of infection and malignancy.[9]

The difficulties tailoring immunosuppressive therapy for lung transplant recipients have fueled demand for a blood-based assay to monitor the immune system. The ImmuKnow assay measures $CD4^+$ T cell production of ATP in response to stimulation with phorbol 12-myristate 13-acetate (PMA). However, enthusiasm for this assay diminished after studies demonstrated poor test characteristics for diagnosing infection and rejection in lung transplant recipients.[10] The MRE assay is thought to be more specific to tacrolimus because the comparison of peak and trough gene expression can mitigate impacts of peripheral blood mononuclear cell (PBMC) lymphocyte count and of other immunosuppressive medications.[11, 12] The use of ionomycin to trigger calcium-dependent NFAT signaling and the measurement of the expression of genes under the NFAT promoter should further enhance the specificity for calcineurin inhibitors.

This MRE assay has shown early success in monitoring kidney, liver, and heart allograft recipients.[3, 13, 14]. However, the specificity of this assay for tacrolimus dosing and performance characteristics in identifying infection and rejection risk previously have not been evaluated in lung allograft recipients. We hypothesized that MRE levels would be associated with the level of tacrolimus-based immunosuppression and could identify lung transplant recipients at increased risk for infection or rejection.

III. Methods

Study Population:

This prospective observational cohort study was approved by the University of California, San Francisco (UCSF) institutional review board under protocol No. 14-13221. The study was performed in accordance with the Declaration of Helsinki and International Conference on Harmonization Good Clinical Practice guidelines. Assay optimization and post-hoc analyses were performed on anonymized whole blood samples from healthy, non-transplanted volunteers obtained under protocol No. 15-16072. Lung transplant recipients at UCSF were recruited 1 to 2 months after transplant and followed for 6 to 18 months after transplant. Subjects not treated with tacrolimus were excluded. All subjects provided written informed consent.

Immunosuppression practices followed institutional protocols. Induction regimens included basiliximab and methylprednisolone. In the absence of rejection, prednisone was administered at 20 mg daily for the first 3 months, tapered to 0.2 mg/kg daily over the next 3 months, and then gradually tapered to 0.1 mg/kg by 12 months. Targeted tacrolimus troughs were 10 to 14 ng/mL in the first 3 months, 10 to 12 ng/mL in the next 3 months, and 8 to 10 ng/ mL thereafter. Mycophenolate mofetil was started in the immediate postoperative period targeting 2 g daily in divided doses but could be reduced or stopped in the setting of leukopenia, skin cancer, or other side effects.

Procedures:

MRE Assay

Blood was collected in heparinized tubes before and 90-120 min after a scheduled dose of tacrolimus and within one day of a bronchoscopy scheduled for cause, such as suspected infection or rejection, or for surveillance (conducted 2, 3, 6, 12, or 18 months after transplantation per UCSF protocol). If a subject was scheduled for a bronchoscopy the same day, the trough tacrolimus level was drawn during placement of a peripheral venous catheter before bronchoscopy, and tacrolimus was taken following the bronchoscopy as soon as the treating nurse determined that swallowing was safe, again 90-120 minutes before the peak sample blood draw.

MRE assays were performed within 1 day of a bronchoscopy. In triplicate, 1 ml of blood was combined with 1 ml of Roswell Park Memorial Institute (RPMI, #61870127, Thermo Fisher Scientific, Waltham, MA) medium containing 10% Human AB serum (#HS-25, Omega Scientific, Tarzana, CA) and 1% penicillin/streptomycin (#54140, Thermo Fisher) for 3 h at 37°C. Stimulated wells included 100 ng/ml phorbol 12-myristate 13-acetate (PMA, #P1585, Sigma-Aldrich, St. Louis, MO) and 5 ug/ml ionomycin (#I9657, Sigma-Aldrich) with the RPMI-based medium during incubation. Red blood cells were lysed with RBC lysing buffer (#555899, BD Biosciences, Franklin Lakes, NJ) prior to mRNA isolation using the High Pure RNA Isolation Kit (#11 828 665 001, Roche, Indianapolis, IN). RNA quality was assessed using a NanoDrop Spectrophotometer (#ND-2000, Thermo Fisher). cDNA was synthesized using the SuperScript III First Strand Protocol (#18080-051, Thermo Fisher). Quantitative PCR was performed according to the RT2 qPCR Primer Assay Protocol (Qiagen, Germantown, MD) using a Bio-Rad CFX96 (Hercules, CA) or Applied Biosystem Viia7 (Thermo Fisher) device. We used Qiagen primers for β-actin, IFN-γ, IL-2 and GM-CSF (#PPH00073E-200, PPH00380B-200, PPH00172B-200, and PPH00576B-200).

The MRE was calculated as:

$$\frac{100\%}{3} \cdot \sum_{x \in \{\text{Il-2},\ \text{GM-CSF},\ \text{IFN}\gamma\}} 2^{\left[(Ct_x - Ct_{actin})_{pre-dose\ trough} - (Ct_x - Ct_{actin})_{post-dose}\right]}$$

Where Ct denotes cycle threshold for stimulated whole blood samples assayed with the given primer, x. MRE values > 140% were excluded, as these were associated with low RNA quality and quantity.

MRE and Steroid Interaction Assay

Non-transplant control samples were incubated in RPMI media containing serum, 0 or 10 ng/ml, and 0, 20, 100, or 200 ng/ml of prednisone at 37°C and 5% CO2 for 90 min prior to PMA/ionomycin stimulation as described above.

Outcome Measures:

Acute cellular rejection and bronchoscopically detected infection were the primary outcomes. Acute cellular rejection was determined based on clinical interpretation of transbronchial biopsy specimens graded for rejection according to International Society for Heart and Lung Transplantation (ISHLT) guidelines, predominantly by a single thoracic transplant pathologist, who was not aware of MRE values.[15] Infection was defined based on the presence of potentially pathogenic organisms in bronchoalveolar lavage (BAL) fluid and 1 or more of the following: symptoms consistent with acute infection, computed tomography findings suggesting active infection, or moderate or greater organism burden on semiquantitative culture. This study definition was based on the 2010 ISHLT definitions of pneumonia and tracheobronchitis. Because moderate or greater burdens of pathogenic organisms are typically treated with antibiotics per UCSF protocols, we added high organism burden as a criterion.[16] Daily mycophenolate mofetil, prednisone, and tacrolimus doses were abstracted from clinical charts. Tacrolimus troughs were assayed on whole blood by the clinical laboratory using the Architect Immunoassay (Abbott Park, IL). The tacrolimus trough value closest in time to the study visit was used.

Sample Size and Statistical Analysis:

The target enrollment was 50 subjects, which was estimated to provide 97% power to identify subjects at risk for acute cellular rejection and 89% power to identify subjects at risk for infections, assuming a 5% incidence of ≥A2 grade rejection and an 18% incidence of infection. These power calculations were performed based on 2-sample t tests[17] with effective sample sizes adjusted for intracluster correlation.[18] Reproducibility of MRE measurements was determined by Pearson product moment correlation. Associations between MRE, weeks after transplant, and tacrolimus trough level were visualized by using generalized additive modeling. Associations with infection and rejection status, time post-transpant, and immunosuppressive agent doses and trough levels were tested by using separate linear models adjusted with generalized estimating equations (GEEs) to account for repeated measures on individual subjects. The local hazard ratios for infection versus MRE and tacrolimus trough level were determined by using a logistic generalized additive mixed model, with subject identifier as a random effect and MRE and tacrolimus trough level as fixed effects. Statistical analyses were performed in R (version 3.3.2, R Foundation for Statistical Computing, Vienna, Austria) using the “pwr,” “gee,” “mgcv,” and “nplr” packages.[17, 19, 20]

IV. References

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