1. **Study Title:**
   Mapping graft-infiltrating T cells using TCR sequencing

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5. **Study Rationale, Background, and Preliminary Data:**

Cumulative results from five years of routine 6-month surveillance biopsies of renal allografts at UCSF show that 20% of patients have immune infiltrates in the graft without an associated rise in their serum creatinine, termed subclinical inflammation (SCI). These patients were then shown to have a faster decline in the graft function over time as compared to patients with no inflammation. (1) It has also been shown that regulatory T cell (Treg) therapy can prevent rejection in pre-clinical models. Seemingly paradoxically, however, in human studies FOXP3, which is highly expressed in Tregs, increases in peripheral blood and urine samples of some patients undergoing rejection. This subpopulation of patients, though, has better preservation of renal function than those who do not have increased expression of FOXP3. (2,3) These data all together suggest the presence of two broad categories of T cells involved in allograft inflammation – pathogenic and protective. The more gradual decline of graft function in patients with increased Tregs may be explained by a protective effect of the Tregs.
Graft rejection therefore would depend on the balance between graft-reactive, pathogenic T cells and graft-protective T cells. Current analysis of graft infiltrates does not distinguish between graft-reactive and other nonspecific T cells. The Tang Lab in collaboration with Laszik Lab propose using T cell receptor (TCR) sequencing to analyze intra-graft T cells in patients with both acute rejection and subclinical inflammation to determine the balance between graft-reactive pathogenic T cells and graft-protective regulatory T cells.

6. **Hypothesis and Study Objective(s):**

We hypothesize that there are two populations of T cells found in graft inflammation – graft-reactive pathogenic T cells and graft-protective regulatory T cells – and that in acute rejection,

graft-infiltrating T cells are enriched for graft-reactive T-cells with donor-reactive CD4 conventional T cells and CD8 T cells dominating over regulatory T cells. In subclinical inflammation, regulatory T cells will predominate the infiltrate.
We will test this hypothesis by combining TCR sequencing technology with in vitro stimulation of graft recipient cells using donor antigen presenting cells to generate TCR sequence "maps" of donor-alloantigen-reactive T cells which can then be compared with the TCR sequences found in graft biopsies.

Aim 1: Generate a TCRseq map of donor-reactive CD4 Tconv, CD8 and Tregs using peripheral blood samples collected at the same time as allograft biopsy.

Aim 2: Generate a catalog of TCRseq of intra-graft T cells from allograft biopsy.

Aim 3: Comparison of graft-derived TCR catalog to the map generated from peripheral blood donor-reactive CD4 Tconv, CD8 and Tregs to determine the relative frequencies of each subset in the graft.

7. Study Design and Methodology:

Aim 1: TCRseq map of donor-reactive T cells in peripheral blood. Donor stimulated B cells (sBc) and monocyte-derived dendritic cells (mDC) are potent stimulators of allo-reactive T cells. It is unknown, however, if these two antigen-presenting cells stimulate the same repertoire of T cells. Resting B cells are poor antigen presenting cells (APC) and only stimulate T cell proliferation after they are activated. Conversely, dendritic cells are potent APCs at baseline and are more likely to be the initiators of the allograft response. In our first series of experiments, using peripheral blood samples of patients obtained at the time of their allograft biopsies, we will FACS sort CD4 Tconv, CD8 and Treg. Aliquots of 200,000 cells of each subset will be used for direct TCR sequencing to determine an overall map of circulating T cells at the time of rejection. The remaining cells will be divided into two groups, one half of which will be stimulated with sBc and the other half of which will be stimulated with mDC. Both will be cultured for 7 days and the resulting donor-reactive cells will be collected, along with the previously described unstimulated cells. RNA will be extracted from these three cell populations and submitted to an outside company, iRepertoire, for TCRb sequencing. By imposing the donor-alloantigen-reactive maps onto the unstimulated polyclonal map we will be able to determine the frequency of donor-reactive CD4 Tconv, CD8 and Tregs in peripheral blood at the time of the graft biopsy. Additionally we will be able to compare the effector cell lineages stimulated by sBc versus mDC.

Aim 2: TCRseq map of intra-graft T cells. Paired graft samples accompanying the peripheral blood samples will be collected. RNA will be extracted from sections of formalin-fixed paraffin-embedded (FFPE) biopsy tissue and submitted to iRepertoire for TCRseq. Previous experiments in our lab using flow cytometric analysis have demonstrated a yield of 0.5-1 million cells from a 16G core needle biopsy with about 10,000-100,000 CD45+ cells. Of these cells, roughly 75% will be T cells, yielding about 5,000-70,000 per biopsy. Patients with subclinical inflammation will be on the lower end of the range of T cells collected as compares to those with acute rejection. RNA will be extracted and submitted to an outside company, iRepertoire, for TCRb sequencing, thereby generating a map of intra-graft T cells.

Aim 3: Relative frequencies of donor-reactive T cells in graft biopsies. The map of intra-graft T cells can then by compared to the sBc- or mDC-stimulated blood CD4 Tconv, CD8 and Tregs in order to calculate the relative-abundance of donor-reactive cells in the graft. As a separate comparison, we will analyze parallel FFPE biopsy samples using immunofluorescence. All patients undergoing biopsy at UCSF have two core biopsies taken. On will be used for TCRseq, the other we will use for immunofluorescence for CD3, CD4, CD8 and FOXP3. We will quantify the immunofluorescence results to calculate percentages of CD4+FOXP3- Tconv, CD8+ T cells and CD4+FOXP3+ Tregs among the total CD3+ T cells. These numbers will be compared with measurements derived from TCRseq analysis. Once this assay is in place, we plan to use it on samples of patients with no pathology, acute cellular rejection, subclinical inflammation and tubulointerstitial nephritis (TIN). We expect patients with SCI and ACR to have a dominance of donor-reactive T cells in the grafts. Conversely, in patients with no pathology and patients with TIN from infections, we expect that the infiltrating T cells will be mostly non-reactive to donor antigens. We also expect that patients with SCI to have higher ratio of Tregs to donor-reactive Tconv or CD8 cells when compared with patients with ACR. Anticipated results are summarized in Table 1 below.

Table 1.

Measurements	Normal	ACR	SCI	TIN
% donor-reactive T cells in blood	Baseline reference	Higher than normal baseline	Higher than normal but lower than ACR	Unchanged or lower than normal
Overlap between donor-reactive T cells in blood and graft	Low	High, most of the TCR seq in graft to map to donor-reactive pool	High, most of the TCR seq in graft to map to donor-reactive pool	Low
Donor-reactive Treg:donor-reactive Tconv or CD8	High, >1 to 2, if present	Low, less than 1:2	Higher than ACR	Likely not present

8. <u>Anticipated Challenges:</u>

In our initial experiments, we are planning to sequence peripheral blood sample T cell aliquots, each containing roughly 200,000 cells, at a depth of 1 million sequencing reads. It is possible that these cell populations are sufficiently diverse such that this will not yield an adequate map for comparison to the intra-graft T cell population. There are a few approaches to overcome this, should it be necessary. We can first try deeper sequencing using the same methods described above. An alternative approach uses iRepertoire's iPair technology for paired sequencing of TCRb and simultaneous profiling of T cell effector transcripts from a single cell. iRepertoire uniquely sequences TCR from RNA, which therefore allows the additional benefit of being able to identify RNA transcripts to report the lineage and functional state of the cell. We will use 16G core-needle biopsies which have been cryopreserved as a cell suspensions. They will be thawed and CD3+ cells will be FACS sorted into single T cell wells in a 96-well plate containing primers for TCR and various cell phenotyping transcripts (Table 2). The remaining cells will be bulk sequenced in order to rank clone types by copy number. Those lineages with high copy number or high expression of effector markers along with a representative sampling

of CD4 Tconv, CD8 and Tregs will be selected for donor-alloantigen reactivity screening. The TCR chains from these clones will be cloned into our lentiviral expression cassettes and expressed in hybridomas lacking TCR and subsequently stimulated with the donor sBc and mDC cells to assess for donor reactivity.

Table 2.

Marker category	Genes
Th1	IFNg, TNFa, Tbet, CD4
Th2	IL4, GATA3
Th17	IL17A, IL22, Rorgt
Tfh	IL21, BCL6, PD1
Treg	FOXP3, IL-10, Ebi3, p35, CD39, CTLA-4
CTL	CD8, Granzyme B, perforin, PD1, LAG3, HAVCR2

9. Expected Outcome and its Impact on Transplantation:

Previous assays measuring frequency of T cell populations in grafts do not provide any information on their donor-reactivity and in vitro assays which provide quantitative and qualitative information of graft-reactive T cells require a large number of purified cells which cannot easily be obtained from a graft biopsy. Using novel technologies to perform TCR sequencing is an obvious opportunity to gather information about graft-reactive cells when there are limited cells to analyze. Knowing the relative populations of graft-reactive cells in the biopsy of a patient with acute rejection will allow us to gradually become more specific in our prevention of or treatments for rejection. Additionally, by comparing the cell populations in the graft with those in the peripheral blood at the time of the biopsy, especially in patients with subclinical inflammation, we may be able to work toward a more readily available blood test to monitor for early signs of rejection before there is clinically apparent graft compromise (i.e. a rising creatinine).

10. Coursework Plan (100 hours per year):

As part of the FAVOR T32 training grant, I will participate in the FAVOR T32 Immersion Workshop (20 hours), the Training in Clinical Research (TICR) Summer Workshop (20 hours), the Scientific Writing Course (20 hours), Designing Clinical Research (EPI 202), Biostatistical Methods for Clinical Research (BIOSTAT 200), and Introduction to Statistical Computing in Clinical Research (BIOSTAT 212). Additional course work will include the Transplant Seminar Series, the Human Immunology Seminar Series, the Immunology Journal Club and additional pertinent BMS mini-coursework.

11. Tentative Budget for Project Completion:

Our approximate budget for reagents, supplies, patient samples and sequencing services totals $132,400. This project has been funded by a CTOT21 ancillary study grant (parental grant PI Vincenti; ancillary grant PI Tang), CTOTC mechanistic study grant (PI Feng and Tang) and a U24 grant (PI Laszik and Tang).

Our budget includes:

Reagents and supplies ($10,000):

- The costs include Media, serum, growth factors etc to produce sBc, mDC; FACS immunofluorescence antibodies; molecular biology reagents including RNA extraction kits and RNA preservation solutions; histology supplies; disposable lab supplies.

Other costs ($122,400):

TCRseq Amp2Seq service by iRepertoire: $117,000

- Experiments in C2: 4 patients x 10 samples/patient x $650/sample = $26,000
- Experiments in C3: 20 patients x 7 samples/patient x $650/sample = $91,000

Flow core recharge: $5,400

- 5 hr/patient x $45/hour x 24 patients = $5,400

References:

1. Park WD, Griffen MD, Cornell LD, Stegall MD. Fibrosis with inflammation at one year predicts transplant functional decline. J AM Soc Nephrol 2010;21(11):1987-1997.
2. Muthukumar T, Dadhania D, Ding R, Snopkowski C, Naqvi R, Lee JB et al. Messenger RNA for FOXP3 in the urine of renal-allograft recipients. N Engl J Med 2005;353(22):2342-2351.
3. Park WD, Griffin MD, Cornell LD, Cosio FG, Stegall MD. Fibrosis with inflammation at one year predicts transplant functional decline. J Am Soc Nephrol 2010;21(11):1987-1997.