1. **Study Title:**

Microbiome alterations in the etiopathogenesis of fibrosis in kidney and lung transplantation

2. **Proposing Trainee Investigator:**

[redacted]

PGY-2, UCSF Department of Surgery

[redacted]

3. **Clinical Mentor/Principal Investigator:**

Peter Stock, MD, PhD

UCSF Department of Transplant Surgery

[redacted]

4. **Scientific Mentor/Principal Investigator:**

Minnie Sarwal, MD, PhD

[redacted]

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

The most common cause of graft failure after the first year is an incompletely understood clinicopathological entity, variously called chronic rejection. This is further defined as chronic renal allograft nephropathy (**CAN)** in the context of kidney transplant (tx); subtyped into IFTA (interstitial fibrosis and tubular atrophy) and TG (transplant glomerulopathy)[1]. Chronic lung allograft dysfunction (**CLAD**) in lung tx is subtyped into BOS (bronchiolitis obliterans) and RAS (restrictive airway disease)[2]. Unfortunately, there are no real targeted therapies/ interventions that have proven successful to intervene to reverse or delay the progression of chronic rejection. Part of this difficulty may be from an incomplete understanding of the underlying etiologies that lead to such a process. The Sarwal Lab has done extensive transcriptional and proteomic studies that demonstrate up regulation of both alloimmune gene-sets, specific to T,B, monocyte, and dendritic cells, as well as innate immune gene-sets specific to granulocyte, NK, and mast cells in fibrosis[3] and chronic rejection. They are examining fibroblasts as heterogeneous populations of cells with diverse features and considerable functional variations during wound repair. Preliminary data show that certain scar forming, novel fibroblast lineages are enriched in human organ tx fibrosis. Murine En1 (Engrailed-1) positive fibroblasts (EPFs) have a propensity for fibrosis and scar formation[4] and highly coordinated gene expression (Fig.1A). Human:murine gene homology mapping for EPF enriched genes demonstrate enrichment for a likely novel human fibroblast lineage in transcriptional data generated from human CAN (Fig.1B) and CLAD samples (Fig.1C). The Sarwal Lab has expertise in laser capture and microCT guided tissue dissection of regions of tissue fibrosis, allowing for further interrogation of these novel fibroblast lineages.

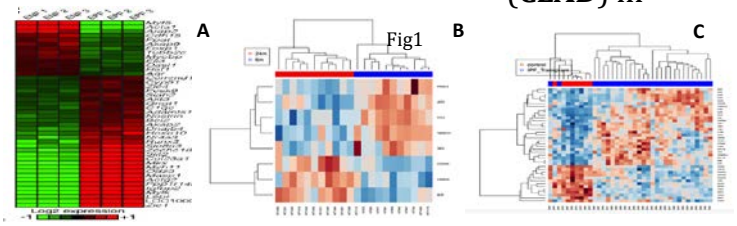

Fig1

One aspect of the pathogenesis of chronic rejection that has yet to be studied adequately is the role of the microbiome/virome. Data suggests that the microbiome/dysbiosis has a role in the pathogenesis of chronic kidney disease, by disruption of gut barrier function that allows translocation of endotoxin and

bacterial metabolites to the systemic circulation, which then contribute to uremic toxicity, inflammation, and progression of chronic kidney disease[5-7]. One could hypothesize that these same mechanisms that exist in non-transplant patients would also be present in transplanted patients, and contribute to the development of chronic fibrosis/rejection.

6. Hypothesis and Study Objective(s):

We hypothesize that kidney and lung allografts that develop CAN and CLAD, respectively, will show specific alterations in microbial biodiversity in regions of fibrosis, which can be both diagnostic and predictive. We plan to address our hypothesis through the following specific aims:

AIM1. Microdissection of regions of fibrosis in CAN and CLAD injuries in kidney and lung allografts prior to microbiome analysis. We will perform laser capture microdissection (LCM) (Carl Zeiss MicroImaging; Laboratory for Cell Analysis, UCSF) for regions of interstitial and glomerular fibrosis on archived kidney tx slides with CAN from UCSF pathology (Laszik/Sarwal), using established Banff histological criteria for diagnosis of CAN[8]. I will perform LCM on 10 CAN samples and use 10 normal kidney tx protocol biopsy samples for controls. The frozen tissue blocks will be cut into 10 µm sections (n=50), mounted on a thermoplastic (polyethylenenaphthalate) covered glass slide, fixed in ice cold 70% ethanol, rinsed in deionized water, then stained briefly (5-10 s) with hematoxylin, immediately rinsed, dehydrated in graded ethanol and air dried for immediate capture. For each biopsy we expect many fibroblasts (~7000 cells captured in prelim studies).

For CLAD samples (persistent decrease in FEV1 and total lung capacity decrease >10%, in combination with the presence of persistent infiltrates on microCT), established protocols have previously generated BOS and RAS micro-dissected alveolar regions (ongoing Sarwal Lab collaboration with the Leuven lung tx program) for me to use. Briefly, end-stage BOS and RAS lungs have been collected at time of re-tx (explant). Explanted lungs have been inflated using 30cm of water pressure and rapidly frozen in fumes of liquid N2 after deflation to 10cm of water pressure. After a high resolution CT, the lung is cut into 2cm thick slices from lung apex to base, scanned with a microCT scanner in frozen state, and serial cryosections have been prepared for microbiome analysis. We will use 10 explant lungs from patients suffering from BOS, 10 from patients with RAS, and compare our findings to 10 unused donor lungs (lungs rejected for tx for a reason not related to the lung itself, e.g. kidney tumor, recipient dying, logistical issues, etc). We expect 1000-3000 cells/alveolar duct. To avoid bacterial contamination, the middle part of the explant lung (the least chance of contamination) will be used for microbiome determination.

AIM 2. Determine microbiome variations in microdissected regions of fibrosis in CAN and CLAD injuries in kidney and lung tx. RNA will be extracted from LCM alveolar cells and fibroblasts. Nucleic acid extraction will be performed from the 3rd part of the core, which will be homogenized to enable detection of bacteria. Comprehensive molecular characterization of the kidney and airway microbiome will be performed using the lllumina MiSeq NGS platform targeting the 16S rRNA marker gene. Following initial quality filtering of sequence data, the generated amplicon libraries will be grouped into OTUs (Operational Taxonomic Units) on the basis of sequence similarity cutoffs, followed by a classification to a particular taxonomic level. This data will then be used to assign percentage relative abundance to each genera present in the sample. Preliminary microbiome analysis of microCT guided dissection of lung tissue from normal, COPD, cystic fibrosis, and from regions of fibrosis in lung tx BOS injury patients show high microbial diversity in the control core and no diversity in BOS. Additionally, the dysbiosis of the BOS microbiome deserves additional investigation as it was noted to be different to the other diseased conditions (Fig.2), and it is likely it would be different in RAS. Differentially expressed microbes will be identified using Significant Analysis of Microarrays (SAM) and the Limma Package at Bioconductor[9-11]. We will adjust for multiple testing[12], meta-analysis of multiple sources of data will be performed through the MetaOmics package[13] and network approaches will be applied including mirConnX (http://www.benoslab.pitt.edu/mirconnx/), RVnet and DREM[14]. Mixed linear

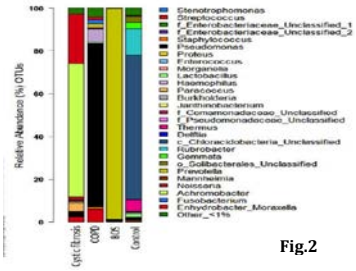

Fig.2

regression models will be generated to compare measures of structural alterations, inflammatory responses (generated by RNASeq data on the same samples being conducted by others in the Sarwal Lab), and microbes.

AIM 3. Non-invasive microbiome biomarkers to predict risk of development of CAN and CLAD. A similar approach will be conducted as outlined in Aim 2. We have biobanked serial urine and BAL samples from CAN and CLAD patients at 1,3,6,12 and 24 months post-tx. 4-5 serial urine and BAL samples will be examined for progressive changes in microbiome dysgenesis over time, and if there are differences in microbiome diversity during evolution of BOS and RAS sub-types of CLAD injuries. Our working hypothesis is that there will be a reduction in microbial biodiversity in samples of patients that subsequently go on to have chronic rejection, with comparison to normal samples. If confirmed, this could provide important biomarkers for non-invasive prediction of tx patients at risk of accelerated fibrosis.

7. Study Design and Methodology:

Please see details under each aim listed in section 6.

8. Anticipated Challenges:

The potential pitfalls in these proposed studies would be largely technical in nature. If the LCM in all kidney biopsies does not yield adequate RNA quality and quantity then we will perform the microbiome analysis on whole kidney sections instead. Our preliminary studies in microCT sections on CLAD samples in lung tx suggests that this will be feasible.

9. Expected Outcome and its Impact on Transplantation:

We are poised to make significant advances in the study of tx fibrosis through interrogation of the microbiome changes in novel fibroblast lineages that drive chronic rejection. In addition, our studies span two different solid organs, allowing us to compare and contrast the impact of microbiome diversity in different organs. This is especially important given the rich resident microbiome of the normal lung. We also stand to unravel specific triggers that may drive the determination of either RAS or BOS injuries in lung tx, each with different clinical course and prognosis. Understanding the role of the microbiome in the development of chronic rejection in organ tx may be invaluable in the development of future interventions or therapies to help prevent or reduce its occurrence.

10. Coursework Plan:

I have limited experience with LCM, microbiome sequencing, and statistical/bioinformatic data analysis. Though the Sarwal Lab has necessary expertise to guide me, I would like to undertake the following program in addition to the required base curriculum:

****Advanced Training In Clinical Research (ATCR) Certificate Program (one year)****

I plan to complete the base curriculum in the first year. The required base curriculum course "Biostatistical Methods for Clinical Research" is also one of the required courses of the ATCR program, so this will go towards my credits required for completing the certificate program in my first year.

References:

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