Award Number: W81XWH-14-1-0225

TITLE: Environmental Mycobiome Modifiers of Inflammation and Fibrosis in Systemic Sclerosis

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REPORT DATE: September 2017

TYPE OF REPORT: Annual

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Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
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This project is focused on Systemic Sclerosis (SSc), a progressive fibrotic disease characterized by skin fibrosis and damage to internal organs. While a wide range of environmental and biological triggers have been proposed, no definitive etiologic agents have yet been identified. Metagenomic analysis of non-human sequences in SSc RNA-seq data was used to detect microbial sequences in human tissues in an unbiased, quantitative manner. Our studies suggest that disease pathogenesis includes a common environmental fungal trigger, *Rhodotorula glutinis*, which we hypothesize elicits immune activation in a permissive host genetic background.

Skin biopsies have been collected from SSc patients and analyzed by high-throughput sequencing, providing substantial gene expression data as well as detailed information regarding the host microbiome. Data have been compared against that of healthy control samples.

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1. INTRODUCTION

Systemic sclerosis (SSc) is a heterogeneous disease of fibrosis and inflammation, concomitant with significant autoimmunity. SSc often presents with skin manifestations and Raynaud’s phenomenon; the extent and location of fibrotic lesions in people with SSc contributes to the diagnoses of disease subtypes and prognosis. My laboratory has pioneered the use of gene expression subsets in SSc [2-6]. Most recently we have demonstrated enrichment of a mycobiome component (Rhodotorula glutinis) in SSc patient skin [7].

We describe our studies from the first year of the grant below. This work was accomplished by researchers at Geisel School of Medicine at Dartmouth, Boston University Medical Center and University of California, San Francisco under the partnering PI option.

2. KEYWORDS:
IMSA, systemic sclerosis, scleroderma, SSc, mycobiome, microbiome, fibrosis, gene, genetics, RNA-seq, Next Generation Sequencing, skin, R. glutinis, Rhodotorula, Metagenomics

3. ACCOMPLISHMENTS

Milestones were assigned to this proposal, with tasks to be accomplished by each investigator. The overall summary of our progress relative to these tasks is given below, followed by a complete discussion of our work this past year.

Milestone 1 Determine the identity and distribution of microbiome components across SSc skin.

Task 1 (Months 1-36) Whitfield Laboratory to perform RNA-seq analysis of SSc skin biopsies.

Including technical replicates, RNA-seq has been run on 22 SSc patient skin biopsies and 6 healthy controls to date; an additional 8 SSc patient biopsies are currently being processed. Recruitment of additional SSc patients and healthy controls is ongoing.

Task 2 (Months 6-36) Whitfield Laboratory to perform RNA-seq analysis for differentially expressed mRNAs and non-coding RNAs.

Raw sequence reads have been analyzed using publicly available software packages that have been optimized and validated by us.

Task 3 (Months 6-36) Arron group to perform IMSA and determine the identity of microbiome components.

Dr. Arron’s group is currently performing metagenomic analysis on 28 new and existing skin biopsy samples (22 SSc and 6 healthy controls) from the Whitfield Laboratory.

Task 4 (Months 1-24) Arron group to create scaffolds from aligned reads for each microbiome component and develop nested PCR followed by targeted multiplexed sequencing assays for cost-effective screening.

We developed a fast and efficient nested PCR reaction targeting microbiome components specific for fungal species identification. We then evaluated the identity and number of fungal reads by next generation sequencing (see Task 5). We have found the data from this targeted mycobiome method to be lower quality than our metagenomic RNA-seq analyses and we are exploring targeted microbiome methods.

Our preliminary findings suggest significant bacterial dysbiosis in affected skin, with only modest changes in fungal abundance. Validation of these findings will therefore be run using targeted 16S sequencing to identify changes in bacterial composition. DNA has been collected for 116 skin samples (SSc and healthy controls) to date, with an additional 178 samples awaiting DNA purification. Targeted sequencing of the V2-4-8 and V3-6,7-9 hypervariable regions of 16S rRNA from these samples will be performed using the Ion 16S Metagenomics Kit (ThermoFisher), and sequenced on the Ion Torrent.

The original goal of this task was validation. Therefore, validation will also be performed by analyzing RNA-seq data generated as part of other studies (n > 100). This approach enables direct assessment of all microbiome components associated with affected skin, including archaea, bacteria, viruses, fungi, and
parasites, as well as comparisons between microbiome composition and gene expression of associated tissues using the same computational methods.

Task 5 (Months 1-12) Whitfield Laboratory to examine a larger population of archived skin biopsy RNA to determine the prevalence of microbiome components across the SSc population.

See above.

Task 6 (Months 1-24) Culture microbiome components from the skin of SSc patients. Use of skin biopsies as a method for fungal culture was not successful.

Disappointingly, swabbing of affected skin of SSc patients has yet to result in recovery of clinically relevant fungi or other organisms. Swabbing and other culture-based methods of microbial detection will be revisited following completion of our RNA-seq analyses, enabling targeted isolation of organisms associated with SSc lesional skin.

**Milestone 2** Identify the inflammatory infiltrates in SSc skin and their response to microbiome components

Task 1 (Months 1-6) Whitfield Laboratory to perform computational analysis/prediction of inflammatory cell infiltrates from whole genome expression data.

We used single sample Gene Set Enrichment Analysis (ssGSEA) to identify the cellular subsets in SSc skin at different stages of disease.

Task 2 (Months 6-24) Perform immunohistochemistry to validate the computational predictions of task 1.

We have optimized markers for different cell types in SSc skin. We have used CD163 for macrophages, CD1c for myeloid dendritic cells (mDCs), and CD3 for T cells in a separate study. Current work focuses on the identification of T cell subsets (CD4 and CD8), and B cells (CD19 and CD20). We can now use these markers to look at innate and adaptive immune cells in the patients of this study.

Task 3 (Months 1-18) Whitfield Laboratory to develop protocols for the isolation and characterization of immune cells from skin using the sclerodermatous Graft-Versus Host Disease (sclGVHD) mouse including detailed characterization of cell types.

We established the sclGVHD model in the laboratory. We have performed initial cell isolations and phenotyping of these samples.

Task 4 (Months 6-18) Identify the secreted mediators of fibrosis / inflammation being produced (Whitfield / Pioli). Once cells are isolated, we will screen for secreted pro-fibrotic mediators.

We have analyzed profibrotic mediators in these cells, work is ongoing.

Task 5 (Months 12-36) Apply protocols to characterize the inflammatory infiltrate in the skin of SSc patients (Whitfield / Pioli). After cell isolation procedures have been optimized in the sclGVHD mouse, we will examine the infiltrate and profibrotic mediators in SSc skin biopsies.

Protocols for isolating these cells from mouse skin have been optimized. Work is underway for human skin.

**Milestone 3** Determine if SSc patients have a specific immune response against *R. glutinis* that is different from healthy controls and if this response can drive fibrosis.

Task 1 (Months 1-24) Test patient sera for cross-reactivity against *R. glutinis* antigens (Whitfield/Lafyatis).

We have performed western blots using whole cell lysates and probed with sera collected from both healthy controls and SSc patients.

Task 2 (Months 1-24) Identify the cross-reacting proteins by mass spectrometry (Whitfield).

Serum-immunoprecipitation of *R. glutinis* and human HeLa cell whole cell lysates followed by mass spectrometry was performed to identify immunoreactive proteins associated with *R. glutinis*. We have written a manuscript on the human cross-reactivity. We are having difficulty with the annotation state of the *R. glutinis* genome for annotating those spectra.

Task 3 (Months 12-36) Use isolated PBMCs and isolated monocytes to examine the cytokines secreted and changes in gene expression when cells are exposed to *R. glutinis* or other putative micro / mycobiome triggers (Whitfield/Pioli).
Work underway.

Task 4 (Months 12-24) Determine if chronic exposure to *R. glutinis* or other micro / mycobiome components stimulate a fibrotic response in a mouse model of SSc. (Whitfield).

Work underway.

**PRELIMINARY RESULTS BY MILESTONE**

**Milestone 1:** Determine the identity and distribution of microbiome components across SSc skin

**Task 1: RNA-Seq analysis of SSc skin.** Including technical replicates, RNA-seq has been run on 18 SSc patient skin biopsies to date. Recruitment of additional SSc patients and healthy controls is ongoing. mRNA from 22 SSc patients and 6 healthy controls have been sequenced, yielding 30-237 million paired-end reads per sample. Reads were then aligned to the human genome (hg19 assembly). Approximately 70%-80% of reads were uniquely mapped, which is in line with expectations (Table 1).

Table 1. Statistics of alignment
Task 2 RNA-seq analysis for differentially expressed mRNAs. In order to identify differentially expressed mRNA, we used RSEM software to estimate the abundance of each mRNA transcript. Each sample was normalized using quantile normalization. Batch biases generated by the inclusion of previously sequenced samples from a separate study (N_Base samples) was performed with ComBat (Figure 1).
As an initial analysis of these data, we chose to examine the consensus genes from Mahoney et al. [4]. These are genes that were consistently and reproducibly associated with individual SSc intrinsic gene expression subsets across three independent patient cohorts. Expression of these genes in our RNA-seq data reveals increased expression in the inflammatory and fibroproliferative subsets of patients (Figure 2). Expression of these genes is shown both before and after batch correction. Intermixing of samples is clearly evident after ComBat correction, indicating that batch correction was successful.

**Figure 2. Heatmap of Mahoney_267modules.** (Left) no ComBat correction; (Right) with ComBat correction. The attached heatmap only shows the samples from year 1 as the current set of samples are undergoing analysis.

**Task 3: IMSA analysis to identify microbiome components.** In order to map the microbiome components present in these skin biopsies, IMSA has been performed on RNA-seq data from 28 skin biopsies (22 SSc and 6 healthy controls). We performed quality filtering and human sequence filtering using human genome (hg19). Over 99% of the total readset was derived from human or nonhuman primates in both SSc and control samples. IMSA was used to map reads to the NCBI non-redundant nucleotide (nt) database and generate taxonomy reports.

In preliminary data, we found that only inflammatory samples have high *Rhodotorula glutinis* target read counts (Figure 3), consistent with the preliminary data we presented in our initial grant proposal. Therefore, this preliminary analysis validates those original data.
Figure 3. IMSA analysis of RNA-seq data from SSc skin biopsies. SSc skin biopsies were divided by intrinsic gene expression subset, as previously described [3, 8]. Each biopsy was analyzed for *R. glutinis* sequences and IMSA score plotted for each subset.

We used a process called rarefaction to account for the variable read depth of different samples. We used Quantitative Insights Into Microbial Ecology (QIIME) to perform rarefaction of outputs, a process by which taxa are randomly sampled without replacement; this process is necessary to ensure even sampling depth across patients. Alpha and beta diversity measures are calculated from these data.

Comparisons of SSc and controls revealed statistically significant differences in abundance between five of the top seven genera, with observed increases in SSc skin in the mRNA of major bacterial commensals such as *Propionibacterium*, *Staphylococcus*, and *Corynebacterium*; decreases were observed in *Citrobacter* and *Vibrio*, relative to controls (p = 0.019, p = 0.004, and p = 0.002, respectively; Figure 4A). *Rhodotorula* levels overall were not significantly different between SSc and controls in this cohort, which we believe is likely the result of differences in patient populations, with this second patient cohort consisting primarily of later disease duration. Biopsies from additional 8 untreated, early-stage SSc patients are being processed further validate the presence of *R. glutinis*. 
Dysbiosis changes with disease duration. Alpha diversity provides a measure of inter-sample diversity. Analysis of our SSc samples reveals significant changes in microbiome composition associated with disease duration ($p = 0.011$ by Chao1). Comparisons were performed using 4 disease duration groups, defined as < 2 years, 2-5 years, 5-10 years, and > 10 years, with or without healthy controls. Early stage patients (disease duration < 2 years after first non-Raynaud’s symptoms) exhibit less microbiome diversity, followed by increasing diversity thereafter (Figure 4B). The most significant differences were observed between patients <6yr disease duration compared with those with >6yr ($p = 0.001$).

Linking microbiome dysbiosis to host gene expression changes. We examined our data for gene expression changes that were linked to differences in microbiome composition in each patient sample. Gene expression for each of the 28 skin biopsies was calculated from the RNA-seq data for the intrinsic genes from Johnson, et al. [1] (Figure 5). Hierarchical clustering of these data showed clear inflammatory (purple branches) and non-inflammatory (green branches) patients (Figure 5A). The inflammatory group shows high expression of genes associated with immune activation (Figure 5C). In these data, this split is significantly associated with disease duration, with early stage patients (red blocks; disease duration <5 years) clustering within the inflammatory group (Figure 5B). With one exception, all late stage patients (blue blocks; disease duration >5 years) and healthy controls (green blocks) clustered on the non-inflammatory branch (Figure 5B). The strong fatty acid and lipid metabolism signature evident in healthy controls, and a small number of inflammatory patients (Figure 5D), suggest multiple intrinsic subsets. Consistent with our data above, patients in the inflammatory group have low microbiome diversity (i.e. significant dysbiosis) while samples in the non-inflammatory group have increased microbiome diversity. Therefore, there is a correlation between presence of the inflammatory gene expression signature and decreased microbiome diversity.

Based on these data, we find substantial differences between microbial populations of SSc skin with increasing disease duration. Within SSc patients, alpha diversity was significantly lower in the inflammatory group relative to non-inflammatory patients ($p = 0.004$ for Chao1), generally characterized by
a small number of dominant genera, such as *Escherichia* and *Propionibacterium*. These data suggest that changes in the cutaneous microbiome of SSc patients may directly contribute to immune activation in these patients.

**Tasks 4 and 5. Develop a nested PCR-based assay followed by targeted multiplexed sequencing as a cost-effective method for screening archived skin biopsy RNA to determine the prevalence of microbiome components across the SSc population.** Improvements in our sample-processing pipeline now allow for simultaneous extraction of DNA, RNA, and miRNA from all patient biopsies. DNA is being used as a template for targeted sequencing of the intergenic transcribed spacer regions (ITS), a region widely regarded as the gold standard for fungal species identification. To date, targeted ITS sequencing libraries have been analyzed from 48 archived samples (39 SSc and 9 controls), which includes both paired lesional and non-lesional skin as well as multiple time points from a single patient (Figure 5). Sequencing outputs are being analyzed by IMSA to identify differences in microbial diversity and species abundance between patients and controls, between lesional and non-lesion skin, as well as how these populations change over time.

Our preliminary findings from our RNA-seq data suggest significant bacterial dysbiosis in affected skin, with only modest changes in fungal abundance. Validation of these findings will therefore be run using targeted 16S sequencing to identify changes in bacterial composition. DNA has been collected for 116 skin samples (SSc and healthy controls) to date, with an additional 178 samples awaiting DNA purification. Targeted sequencing of the V2-4-8 and V3-6-7-9 hypervariable regions of 16S rRNA from these samples will be performed using the Ion 16S Metagenomics Kit (ThermoFisher), and sequenced on the Ion Torrent.

**Figure 6. Targeted ITS sequencing of normal and SSc skin biopsies.** Below is a preliminary analysis of targeted fungal ITS sequencing which shows a subset of patients have increased *R. glutinis* sequences (red). The most prominent fungal species detected on skin were *Malassezia* spp. (blue), the most common genus of skin commensal fungi.

**Proportional Distribution of Fungi On Normal and SSc Skin**

**Task 6: Culture microbiome components from the skin of SSc patients.** As mentioned above, swabbing of affected skin of SSc patients has unfortunately yet to result in recovery of clinically relevant fungi or other organisms. Swabbing and other culture-based methods of microbial detection will be revisited following completion of our RNA-seq analyses, enabling targeted isolation of organisms associated with SSc lesional skin. This may be due to the use of antiseptics prior to biopsy collection as a means of preventing infection of the biopsy site.

**Milestone 2:** Identify the inflammatory infiltrates in SSc skin and their response to microbiome components

**Task 1: Computational prediction of inflammatory cell infiltrates from genomic expression data.** We have used single sample Gene Set Enrichment Analysis (ssGSEA) to identify the cellular subsets in SSc skin at different stages of disease. We first benchmarked the ssGSEA method in my laboratory using publicly available gene expression data from pools of cell lines that had a known composition (data not shown). These data demonstrated that ssGSEA accurately predicted cell type enrichment. We then analyzed a set of patients for whom we had whole genome expression data and that had strong expression of the inflammatory signature. We
find the inflammatory signature is most strongly correlated with gene expression signatures from activated Dendritic Cells (DCs) and macrophages (MØs) (Figure 6). These methods are being applied in conjunction with the samples being analyzed in milestone 1.

**Figure 7. Correlation of cell type signatures with a patient’s inflammatory signature normalized enrichment score (NES).** The inflammatory signature in SSc skin is most highly correlated with activated DCs and MØs.

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**Task 2: Perform immunohistochemistry to validate the computational predictions of task 1 above.** We have optimized markers for different cell types in SSc skin.

We have optimized markers for different cell types in SSc skin. We have used CD163 for macrophages, CD1c for myeloid dendritic cells (mDCs) and CD3 for T cells in a separate study. We can now use these markers to look at innate and adaptive immune cells in the patients of this study. We have optimized these stains in a set of SSc samples and will now be performing these stains in samples for this study. T cell subsets will be identified using antibodies against CD4 and CD8, and CD19 and CD20 will be used to determine B cell localization.

**Task 3: Develop protocols for the isolation and characterization of immune cells from skin using the sclerodermatous Graft-Versus Host Disease (sclGVHD) mouse including detailed characterization of cell types.**

We established the sclGVHD model in the laboratory and can recapitulate both skin thickening (Figure 8) and the aberrant gene expression profiles observed in our prior studies (Figure 9). We have performed initial cell isolations and phenotyping of these samples (Figure 10).

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**Figure 8. Immunohistochemistry was performed to show increased fibrosis at 2 weeks after disease initiation. As expected we observe skin thickening in the sclGVHD mouse that is not observed in controls.**

Gene expression analyses were performed on skin biopsies from the sclGVHD mouse and compared to our prior study of this model [9]. We find gene expression changes were produced and consistent with those observed when the model was generated at Harvard (Figure 9). Therefore, we can clearly reproduce this model faithfully, including the molecular SSc phenotype.
Figure 9: Gene expression analysis of the sclGVHD mouse. We performed gene expression microarray analyses of the skin of the sclGVHD mouse generated in Dr. Whitfield’s lab for this study with data from the sclGVHD taken from Greenblatt et al. 2012 [9]. We find that samples from mice generated in this project (highlighted boxes) faithfully recapitulate the aberrant gene expression observed in our original study. These gene expression data are consistent with what we observe in the inflammatory subset of SSc.

Skin tissue was minced, digested with collagenase D and DNase I, and filtered through 70- and 40-micron mesh to facilitate cell dispersion. Single cell suspensions were stained with antibodies directed against the pan-leukocyte marker CD45, myeloid cell markers CD11b and CD11c, and CD115, CD206, and murine MHC-Class II (IA/IE) for flow cytometric analysis (Figure 10). Gating of positively stained cells was determined using fluorescence-minus-one (FMO) controls. CD45 positive live cells were gated and surface expression of CD11c, which is a murine dendritic cell marker, and CD11b, which is highly expressed on mouse macrophages, was analyzed on the CD45+ cell population (Figures 10A and 10B). Consistent with previous reports [9, 10], there is an increase in skin macrophages (CD11b+CD11c) in sclGVHD mice compared with syngeneic transplant controls. As demonstrated in Figure 10C, the macrophage cell population is characterized by significantly increased expression of the CSF-1R CD115 and murine MHC Class II (IA/IE). Surface levels of the mannose receptor CD206 were also elevated, although they did not reach statistical significance. These findings are consistent with results obtained by Greenblatt et al., indicating that we have established this model for our future analysis. Furthermore, these results suggest that the activation profile of SSc macrophages is unlikely to conform to a uniformly pro- or anti-inflammatory polarization state, as CD115 and CD206 are typically expressed by alternatively activated macrophages and enhanced IA/IE surface levels are characteristic of pro-inflammatory macrophages.

Figure 10. Characterization of the Myeloid Cell Population in skin of the 2 week old sclGVHD mouse. Flow cytometric analysis of CD45+ cells derived from back skin of BALB/c Rag2-/- hosts that received either syngeneic BALB/c or allogeneic B10.D2 splenocytes two weeks prior to cell harvest (n=3 per group): A. Gating strategy for selection of CD11b vs. CD11c positive cells. B. Percentage of CD11b/CD11c cell populations in syngeneic (control) vs. allogeneic (experimental) recipients measured in panel A. and C. Percentages and mean fluorescence intensity (MFI) of three characteristic macrophage markers (CD115, CD206, and IA/IE) on gated macrophage population (CD11b+/CD11c+) in syngeneic (black bars) vs. allogeneic (red bars) recipients.

Task 4: Identify the secreted mediators of fibrosis / inflammation being produced (Whitfield / Pioli). Once cells are isolated, we will screen for secreted pro-fibrotic mediators. We have analyzed profibrotic mediators in these cells, work is ongoing.

Task 5: Apply protocols to characterize the inflammatory infiltrate in the skin of SSc patients. Protocols for isolating these cells from mouse skin have been optimized. Work is underway for human skin.
Milestone 3: Determine if SSc patients have a specific immune response against *R. glutinis* that is different from healthy controls and if this response can drive fibrosis.

**Task 1: Test patient sera for cross-reactivity against *R. glutinis* antigens.** In these experiments, we set out to test the hypothesis that autoantibody reactivity observed in SSc could recognize the same proteins in fungi, indicating that autoantibodies may have originated in response to fungal infection. Western blots were performed using *R. glutinis*, Malassezia furfur, Saccharomyces cerevisiae, and HeLa whole cell lysates (to test cross-reactivity with humans), and probed with sera collected from both healthy controls and SSc patients representing the three major autoantibody groups (Controls, CENP, TOP1, and RNAP3). Clear differences in cross-reactivity were evident between patient subsets. SSc patients showed a pattern of cross reactivity against *R. glutinis* lysates that was distinct from that observed in healthy controls. Among clinical autoantibody groups, a band consistent with the presence of TOP1 was seen in 3 of 4 TOP1 patients against *R. glutinis* and HeLa cells (Figure 7) this band was not observed in either M. furfur or S. cerevisiae, suggesting the possibility of cross-reactivity between *R. glutinis* and human TOP1 (Figure 7). Specific cross reactivity was also observed in CENP and RNAP3 patients; the identity of these proteins is being investigated.

![Figure 11. Western blots using SSc and control Sera](image)

**Task 2. Identify the cross-reacting proteins by mass spectrometry.** Serum-immunoprecipitation of *R. glutinis* and HeLa cell whole cell lysates followed by mass spectrometry was performed to identify immunoreactive proteins associated with *R. glutinis*. As part of this investigation, we performed a high-throughput analysis of all autoantibodies present in SSc sera, revealing novel targets and associated processes associated with SSc, which is now in press (Johnson, et al. Arthritis Research and Therapy, 2016).

Serum-immunoprecipitation of *R. glutinis* whole cell lysates followed by mass spectrometry revealed considerable reactivity in both SSc patients and healthy controls; however, identification of target peptides was not possible due to the absence of a sufficiently well-annotated *R. glutinis* proteome. Cross reactivity of autoantibodies against human and fungal proteins has been confirmed in *S. cerevisiae*, with autoantibodies against human TOP1 strongly cross-reactive to the equivalent protein in *S. cerevisiae*.

**Task 3: Use isolated PBMCs and isolated monocytes to examine the cytokines secreted and changes in gene expression when cells are exposed to *R. glutinis* or other putative micro / mycobiole triggers.**

*Work is underway. We have shown that PBMCs and macrophages from SSc patients (monocytes isolated from peripheral blood of SSc patients that are differentiated in autologous sera) are activated under basal conditions, as these cells secrete pro-and anti-inflammatory mediators in the absence of exogenous activation. Therefore, additional stimulation with LPS does not appear to further activate the cells. These activated cells produce a wide range of pro-fibrotic molecules that have been implicated in SSc, including IL-6 and TGFbeta. We believe these are the major drivers of fibrosis in SSc.*
Task 4: Determine if chronic exposure to *R. glutinis* or other micro/mycobiome components stimulate a fibrotic response in a mouse model of SSc.

*Work underway.* The sclGVHD mouse has been established and this task should be completed in the coming year.

**CONCLUSION:**

We have made significant progress on all milestones for the first two years of our grant. We have sequenced samples, and are actively analyzing gene expression changes and performing metagenomic analyses for micro- and mycobiome composition. Analyses of the data have revealed widespread dysbiosis in SSc skin, with additional sequencing ongoing to both confirm and expand on these results. Computational analyses have identified activated DCs and MØs as the key cell types driving the inflammatory signature, a phenotype consistent with the presence of a mycobiome trigger; these results are now being confirmed experimentally. We have generated the sclGVHD mouse model, optimized our cell isolation procedures and methods for isolating macrophages from these mice. Immuno-phenotyping and response to microbiome components is in process. We have analyzed the cross-reactivity of autoantibodies with human and fungal components. A paper reporting the autoantibody cross-reactivity to human proteins in HeLa cells has been published [11].

**KEY RESEARCH ACCOMPLISHMENTS Summary**

- RNA-seq followed by metagenomic analyses for microbiome components reveals dysbiosis as a common feature of SSc skin, which increases with disease duration.
- Our work has shown that the innate immune system (macrophages and DCs) are likely drivers of SSc and that these cells respond to microbiome components.
- We have successfully established the sclGVHD model in the laboratory and are examining the immune drivers and their response to microbiome components.
- Cross reactivity with microbiome component is observed and there are data to suggest basal activation of immune cells in SSc.

**The next reporting period:**

September 2016-September 2017

**4. IMPACT**

What was the impact on the development of the principal discipline(s) of the project?

The major impact of this project is that we are demonstrating a novel paradigm for the initiation of SSc. This has the potential to dramatically change the way we think about SSc and the role of the innate immune system in driving disease.

What was the impact on other disciplines?

This study impacts areas of genomics, metagenomics, microbiology, innate immunity, and autoimmunity. The methods we demonstrate and develop here will affect all of these fields. In particular, this study begins to develop methods for both systems biology and metagenomic sequencing analyses that can be used in other rare diseases.

What was the impact on technology transfer?

Technical demands associated with this project necessitated the development of a novel method to isolate DNA, RNA, and miRNAs from a single skin biopsy. This method has been submitted as a disclosure to our technology transfer office (TTO).

What was the impact on society beyond science and technology?

Scleroderma is an incurable disease that often has a very poor prognosis. If our metagenomic results are confirmed, this will provide not only a better understanding of the molecular processes driving disease pathogenesis, but also identify alternative strategies, such as anti-fungal treatment, as a possible treatment for SSc.
5. CHANGES/PROBLEMS
None to report.

6. PRODUCTS:
None at this time.

Oral Presentations: (Chronological Order)
Presentations for Michael L. Whitfield, PhD

6/16  “Systems biology and bioinformatics approaches to evaluating disease mechanisms and therapeutic trials in autoimmunity and fibrosis” University of North Carolina at Chapel Hill, Chapel Hill, NC

6/16  “Burroughs Wellcome Training Program: Big Data in the Life Sciences” Burroughs Wellcome Fund, Durham NC

5/16  “Systems biology and bioinformatics approaches to evaluating disease mechanisms and therapeutic trials in autoimmunity and fibrosis” University of Michigan, Rheumatology Grand Rounds, Ann Arbor MI

4/16  “The genome and scleroderma’s social network” Scleroderma Foundation New England, Patient Education Seminar, Boston MA

3/16  “Multi-organ systems biology reveals a common immune-fibrotic axis in systemic sclerosis, pulmonary fibrosis and pulmonary arterial hypertension” Scleroderma Research Foundation Annual Workshop, San Francisco, CA

2/16  “Systems biology and bioinformatics approaches to understand complex autoimmune diseases” University College London, London UK

2/16  “Integrative, multi-organ systems biology of systemic sclerosis reveals a macrophage signature associated with disease severity in multiple end-target tissues” Scleroderma World Congress, Lisbon Portugal

2/16  “Systems biology and bioinformatics approaches to understand complex autoimmune diseases” Pittsburgh School of Medicine, Pittsburgh, PA

2/16  “Advances in understanding pathogenesis and treatment in systemic sclerosis” Rheumatology Grand Rounds, Dartmouth-Hitchcock Medical Center.

12/15  “Big Data in the Life Sciences” North Carolina Central University, Durham, NC

12/15  “Big Data in the Life Sciences” North Carolina State University, Raleigh, NC

12/15  “Big Data in the Life Sciences” University of North Carolina, Chapel Hill, NC

11/15  “Multi-tissue genomic networks and systems biology in systemic sclerosis”. Scleroderma Foundation Workshop, ACR Annual meeting, San Francisco CA

8/15  “Systems Biology in Systemic Sclerosis.” Session Chair and topic introduction. Scleroderma Basic Science Workshop, Cambridge UK.

6/15  “Defining overlapping pathology between SSc patients and commonly used mouse models of disease” Actelion, Basel Switzerland. (cancelled due to illness)
6/15 “Genomic and Proteomic Quantification of the Heterogeneity of SSc: Implications for Pathogenesis and Treatment”. EULAR. Rome, Italy. (cancelled due to illness)

6/15 “Genomics, Bioinformatics and Systems Biology for Precision Medicine in Systemic Sclerosis”. NIH CORT (P50) Advisory Committee meeting. Boston University Medical Center, Boston MA

4/15 “Genomics, Bioinformatics and Systems Biology for Precision Medicine in Systemic Sclerosis”. SScores (NIH P30) Advisory Committee meeting. Boston University Medical Center, Boston MA.

3/15 “A macrophage-associated inflammatory signature is found in all SSc tissues and associated with more severe disease” Scleroderma Research Foundation Workshop on Scleroderma, San Francisco, CA

3/15 “Molecular stratification and drug response for SSc clinical trials” Pfizer, Cambridge, MA.

2/15 “Enabling Precision Medicine in SSc Clinical Trials” Discussion leader and presenter, NIAMS roundtable discussion on Scleroderma: Advancing Potential Drugs to Patient Care


1/14 “Mechanisms of Systemic Sclerosis (Scleroderma) pathogenesis by systems level genomic analyses” Genomic Medicine Grand Rounds, Geisel School of Medicine.

12/14 “Untangling molecular changes in SSc clinical trials: Gene expression subsets, response signatures and pathway changes” ASSET Investigator Meeting. University of Michigan, MI

11/14 “Identification of the Microbiome As a Potential Trigger of Systemic Sclerosis By Metagenomic RNA-Sequencing of Skin Biopsies” ACR Basic Research Conference Boston, MA.

Dr. Sarah Arron reports no presentations on this topic in the past year.

Abstracts and Presentations: (Chronological Order)


Manuscripts:

The following manuscripts from Dr. Whitfield’s lab have relevance to this proposal. Publication 5 directly derives from work performed to accomplish the aims of this proposal.


8. Zhenghui Li, Guoshuai Cai, Michael S. Ball, Kun Qu, Patricia A. Pioli, Howard Chang, Sarah Arron, Robert Lafyatis, and Michael L. Whitfield. Functional Characterization of Systemic Sclerosis Transcriptome Identifies a Coding Region Polymorphism more Prevalent in Africans that affects IL6 Production. *In preparation*

The following additional papers were published by Drs. Whitfield, Lafyatis and Arron during the funding period.


6. Lisa M. Rice, Jessica Ziemack, Eric Stratton, Sarah Mc Laughlin, Cristina Padilla, Allison Mathes, Romy Christmann, Giuseppina Stifano, Jeff Browning, Michael L. Whitfield, Robert Spiera, Jessica Gordon,


**Degrees obtained that are supported by this award**

Dr. Zhenghui Li, who worked on the microbiome project, will complete his Ph.D during year 2 of funding. He has received direct support from this grant.

**Development of cell lines, tissue or serum repositories**

None

**7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS**

None

**8. SPECIAL REPORTING REQUIREMENTS**

**COLLABORATIVE AWARDS:** For collaborative awards, independent reports are required from BOTH the Initiating PI and the Collaborating/Partnering PI. A duplicative report is acceptable; however, tasks shall be clearly marked with the responsible PI and research site. A report shall be submitted to https://ers.amedd.army.mil for each unique award.

An identical final progress report will be sent from Dr. Arron

**9. REFERENCES**


10. APPENDIX