Job Foundation Report
"The role of fibroblast specific STAT3 expression in pneumatocele development and gastrointestinal perforation in AD-HIES"

This project aims to study the role of the fibroblast, and specifically the role of STAT3 signaling in the fibroblast, and the relation of potential fibroblast dysfunction to the development of pneumatocele and gastrointestinal perforation in Autosomal Dominant Hyper IgE Syndrome (AD-HIES) due to dominant negative mutations in STAT3 (STAT3DN).

STAT3 has emerged in recent years as a focal mediator of fibrosis in a number of pathologic fibrosis states, and conversely, STAT3 pathway signaling inhibition by pharmaceutical treatments such as tocilizumab and sorafenib have appeared to impair wound healing and result in the formation of pneumatoceles or intestinal perforations in patients. In patients with AD-HIES, fibroblast dysfunction in would healing has been assessed by other investigators.

We sought to define the role of fibroblasts on the origin of pneumatoceles and gastrointestinal perforation in AD-HIES by attempting to create a model of pneumatocele and perforation in the STAT dominant negative mouse model. In addition, via direct cell culture models and interaction with *s. aureus*, a major source of early pathology and complications for patients with AD-HIES.

Thus far in our project, we have successfully developed a reproducible model wherein *s. aureus* infection in STAT3 DN mice results in large abscesses visible both on histology and computed tomography (CT), while wild type (WT: normal mice) are spared. Interestingly, both WT mice and STAT3 DN mice appear to have similar control of the bacterial load in the lung at 24 hours after infection suggesting that this abscess formation is not related to initial bacterial clearance. Alterations in expression of cytokines – chemical messengers that control aspects of inflammation – are different in certain specific cytokines between mice with the Job syndrome genetic change and WT mice. In cell cultures, we find that expression of a specific inflammatory cytokine appears to be altered in fibroblasts with the STAT3DN genetic change of both human cells and mice cells. Finally, we see that a different STAT gene appears to have increased phosphorylation in the

Our work is continuing. Our next steps are – 1) to create cell line specific knock-in of the STAT3DN gene in the mouse model, and determine whether the immune cells, fibroblasts, or epithelial cells (lining of the lung) are responsible for the abscess formation, considering the abscess as a potential analogue for pneumatocele in humans. 2) to further understand the meaning of the cytokine changes, gene expression changes and phosphorylation of STAT genes in STAT3DN fibroblasts, especially in the setting of *s. aureus* co-culture. 3) to then move towards the abdominal/intestinal perforation aspect of our grant.

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Detailed (Technical) Summary:

A.1 Brief overview

Patients with AD-HIES develop recurrent pyogenic pneumonia, especially due to *S. aureus,* and also other commonly encountered bacterial organisms, within the first few years of life. These infections often present without vigorous systemic inflammatory response, but with significant airway inflammation. Delay in diagnosis is common due to the impaired systemic response, and development of complications are frequent. These include empyema, pneumatocele and bronchiectasis, which are seen in about 70% of patients. Pneumatoceles become sites of persistent infection by fungal and gram negative bacteria, and a source of major morbidity.

Our goal in this grant was to determine whether fibroblasts, which are known to have impaired function in wound healing in patients with AD-HIES, play a role in the development of these pneumatoceles. Our second aim was to explore this in the intestinal perforation seen in some of these patients and whether this may be the same. We have not yet tackled this second aim but have made progress in the first.

Our initial aims were as follows:

AIM 1: Establish pneumatocele and intestinal perforation in transgenic STAT3mut mice to model the pathologic changes seen in AD-HIES and interrogate the fibroblast phenotype.

AIM 2: Establish role of hematopoetic system vs. fibroblasts in STAT3-dependent lung and gut pathology via bone marrow chimera rescue and cre-inducible STAT3 fibroblast model

AIM 3: Determine the effect of dominant-negative *STAT3* mutations on TGFβ, IL-4/IL-13 and STAT1 signaling in primary patient fibroblasts:

Our overall hypothesis was that in AD-HIES, abnormal fibroblast function would result in impaired extra cellular matrix creation, resulting in the pneumatocele/intestinal perforation phenotype. We proposed that this abnormal STAT3 signal would result in excess STAT1 phosphorylation, which in fibroblasts would result in abnormal and suppressed collagen/extracellular matrix formation.

We also were fortunate enough to acquire a STAT3 cre-mediated dominant negative mice which would allow us to investigate the role of individual cell lines without requiring the use of bone marrow chimera; instead, using cell line specific cre-activation to alter the genetic expression.

Our work focused in the past year on the lung aspect of this project. While we have yet to explore the gut, we do intend to continue further on this path, likely by leveraging our findings in the lung to acquire further funds and continue this work.

As a note, the post-doctoral fellow assigned to this project was able to start her work/hire date in July of 2021, and as with any new individual, time spent in orienting/bureaucracy also push back effective start – thus, we continue to work on this project (now with our own funds) with the intent of acquiring additional funding from the NIH for further exploration.

A.2 Overall milestones/achievements

Our project thus far has achieved the following – 1) creation of a reproducible and robust model of pulmonary abscess formation in the lung of the AD-HIES mouse after *s. aureus* infection. This model is novel and has not been previously reported in the literature. 2) Finding differential cytokine expression in the BAL fluid of WT vs AD-HIES mice after *s.aureus* infection. 3) Finding continued pulmonary *s. aureus* presence in both WT and AD-HIES mice after infection. 4) Differential IL-6 expression by fibroblasts with AD-HIES mutation vs. WT after

TGF-beta stimulation. 5) Differential IL-6 expression by human dermal fibroblasts from patients with AD-HIES vs wild type controls after exposure to *s. aureus*. 6) Altered STAT3 and STAT1 (the latter requires additional experiments) in AD-HIES fibroblasts. The alteration of STAT1 would be consistent with our initial hypothesis. 7) Altered function (in terms of extra cellular matrix secretion) of fibroblasts from patients with AD-HIES.

A.3 Establishment of lung model

A major, if not the major hurdle in this project was the establishment of a lung model of pneumatocele or an analogue thereof. At present, while we have not yet seen evidence of gross pneumatocele development in the mouse, what we do see is very impressive abscess formation in the AD-HIES mouse which generally does not occur in the wild type. Over the course of the year, one of the major difficulties was achieving a technique in which we could robustly reproduce this finding in every set of mice we used for experiment. Initially, response was variable, but with some change in technique and dosing, we now can reliably induce this abscess formation in the AD-HIES mouse with introduction of S. Aureus. To our knowledge, this has not been demonstrated in a mouse model of AD-HIES previously. Notably, despite these massive abscesses, the mice overall were well appearing and in no distress, which is consistent with the 'cold' abscess seen in human patients with AD-HIES.

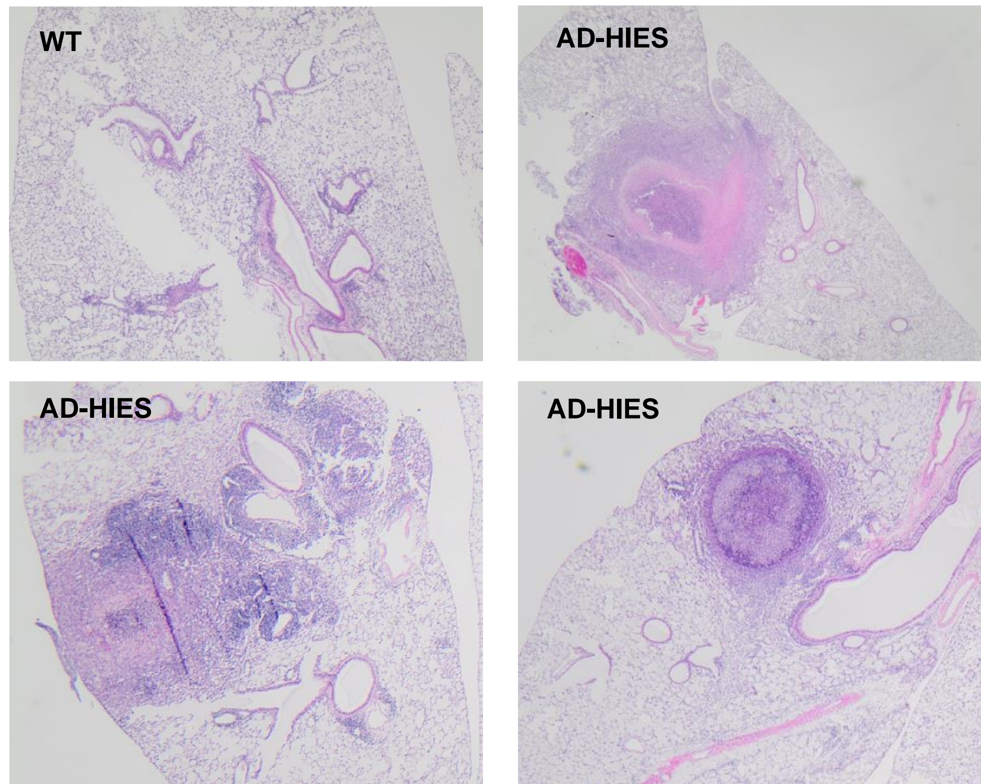

Figure 1: Representative samples of lung changes at 7 days after S. Aureus infection in WT mice and three separate AD-HIES mice.

In addition, we also used CT scanning to identify mice with abscess formation; we found that in severely affected mice, we could identify radiologic abnormalities, a finding that will allow us to identify mice with abscesses without dissection (and thus allow us to obtain tissue with known abscess formation for studies such as flow cytometry.

Overall, the frequency of abscess was far in excess of WT in the AD-HIES mice at both 7 and 14 days after infection:

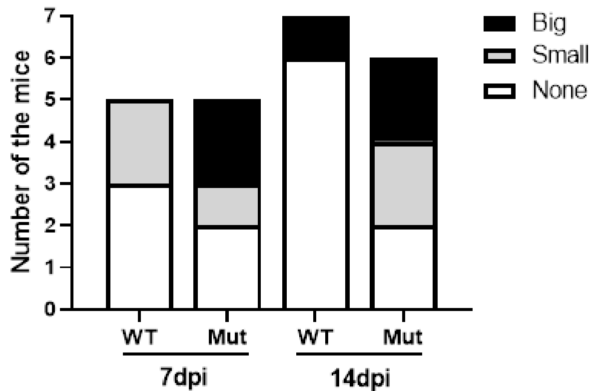

Figure 2: frequency of abscess formation in WT and AD-HIES (mut) mice.

Importantly, we also note that in both AD-HIES mice and WT mice, S. Aureus colonies from BAL fluid occur at the similar frequency in the AD-HIES mice and the WT mice, suggesting that initial bacterial clearance at 24 hours is not differentially impaired in the AD-HIES mice.

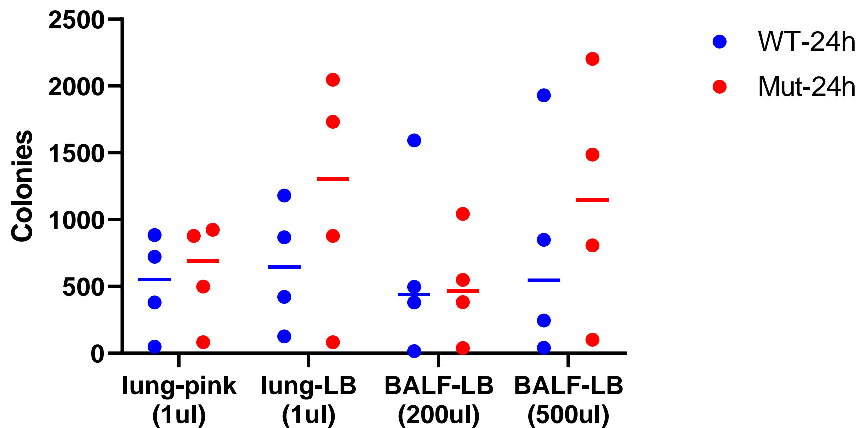

Figure 3: Colony number of S. Aureus from whole lung (lung) and BAL fluid (BALF). 'pink' refers to ChroMagar plate, LB is Luria broth plate.

At 14 days, culture of BAL fluid from mice with AD-HIES does result in very small numbers of bacterial colonies, whereas in WT mice, no colonies are demonstrated.

Following this, we attempted to do a preliminary analysis of multiple cytokines on a multiplex assay to gather clues as to whether specific cytokine and chemokine expression was altered in AD-HIES mice vs WT mice. These were performed at two weeks post infection and evaluated some 30+ cytokines and chemokines. At this point, WT mice have cleared infection.

Notably, cytokines between AD-HIES mice and WT mice were not tremendously different, despite the fact that AD-HIES harbored abscesses in the lung. This could be consistent with the 'cold abscess' wherein humans with AD-HIES have depressed inflammatory responses. At this timepoint, differences in IL-2, IL-5 and MIP2/CXCL2 were significantly elevated in the AD-HIES mice vs the WT mice:

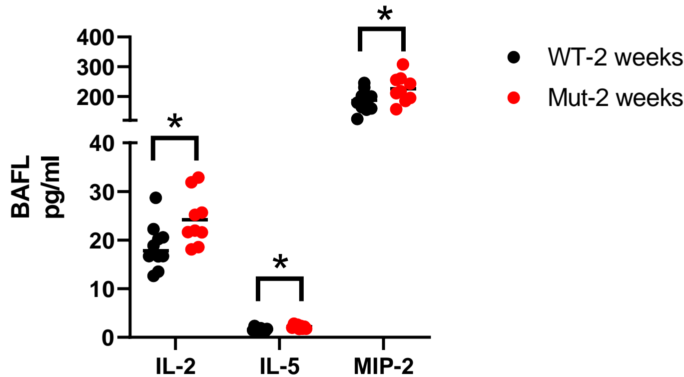

Figure 4: Cytokines in WT vs. AD-HIES (Mut) mice from lung BAL fluid after S. Aureus infection.

To further understand the interaction between *S. Aureus* and the fibroblast in AD-HIES, we co-cultured S. Aureus with fibroblasts from AD-HIES mice lung tissue and with human fibroblast tissue; these fibroblasts were obtained from single cell suspension of AD-HIES lung tissue using standard methods of fibroblast isolation. Here we found that mouse fibroblasts were able to withstand S. Aureus infection whereas human fibroblasts were killed by S. Aureus infection. (not shown)

We then wanted to identify whether AD-HIES fibroblasts produced differential amounts of inflammatory cytokines as opposed to WT fibroblasts. To do this experiment, we cultured human dermal fibroblasts from patients with AD-HIES and healthy donors with both S. Aureus and also with stimulation by TGF-beta. Here, we found that fibroblasts from patients with AD-HIES appear to produce lower levels of IL-6 than healthy donors (Figure 5):

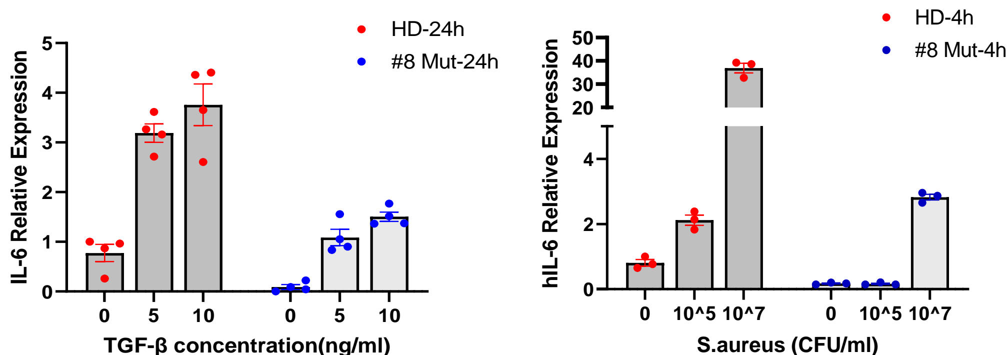

Figure 5: IL-6 expression after TGF-beta stimulation (LEFT) and after incubation of 30 minutes with S. Aureus (RIGHT) in healthy donor vs. AD-HIES (mut) donated fibroblasts. Both after TGF-beta stimulation and with S. Aureus co-culture, AD-HIES dermal fibroblasts produce diminished IL-6.

Following this experiment using a single healthy donor, we increased the numbers of patients to include 3 healthy donor lines and 3 AD-HIES patients, and this finding remained

consistent. To our knowledge the impaired IL-6 production in AD-HIES fibroblasts has not previously been reported. We also studied IL-6 secretion from fibroblasts in the setting of incubation with a STAT3 inhibitor (pharmacologic) and found that the addition of STAT3 inhibition at low doses (high doses are toxic) did not impair IL-6 secretion (not shown), suggesting that simple STAT3 inhibition is not likely the mechanism of this altered cytokine secretion.

Following this, we assessed the response of fibroblast STAT3 and STAT1 phosphorylation in fibroblasts in AD-HIES cells and WT cells. Here, we found that STAT3 was hyper-phosphorylated in the fibroblasts, and that overall expression of STAT3 at baseline was also increased. (Figure 6) Despite this, as would be expected, SOCS3 expression was decreased in AD-HIES. (Figure 7) SOCS3 is indicative of response to STAT3 signal via binding of phosphostat3 to nuclear DNA, which is impaired in AD-HIES.

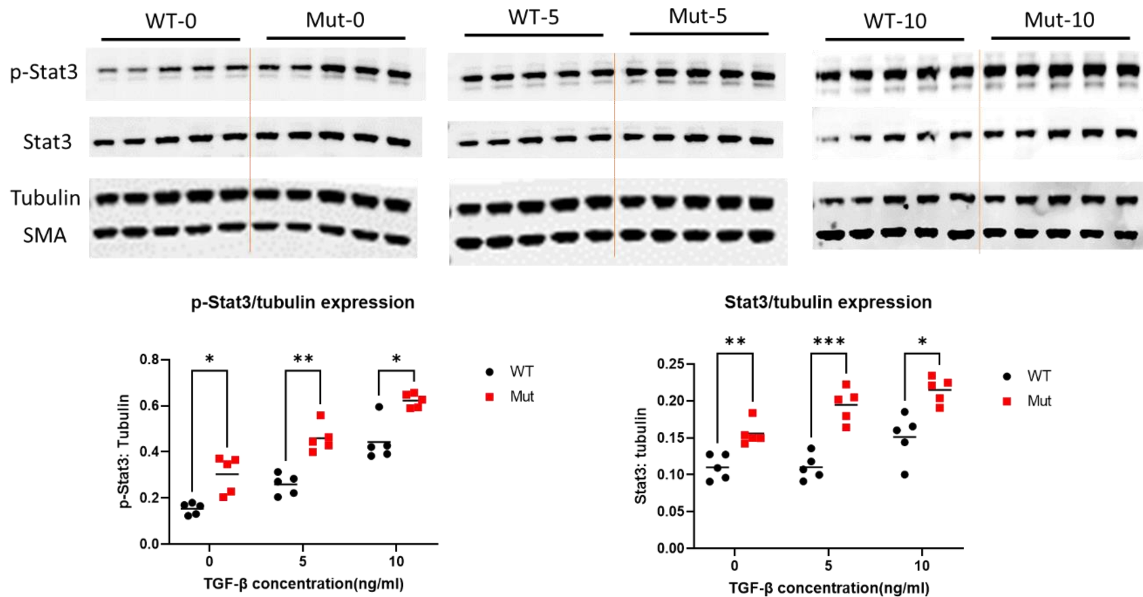

Figure 6: phosphorylated-STAT3 expression (LEFT graph) vs. Total STAT3 expression (RIGHT graph). AD-HIES fibroblasts demonstrate elevated STAT3 phosphorylation and elevated overall STAT3 expression vs. WT cells.

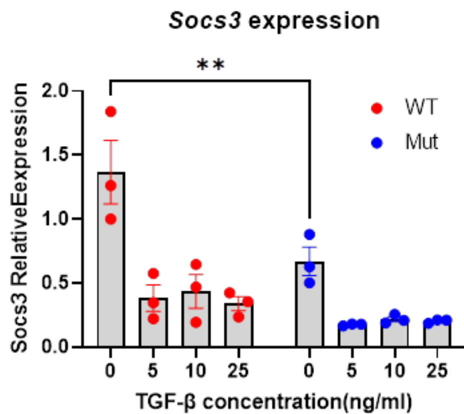

Figure 7: Socs3 expression is decreased in AD-HIES (mut) fibroblasts despite elevated STAT3 phosphorylation and STAT3 total expression.

We then assessed STAT1 phosphorylation in the dermal fibroblast, and found that STAT1 phosphorylation appeared increased in AD-HIES fibroblasts vs WT fibroblasts, even with TGF-beta stimulation (which does not cause increased STAT1 signal under normal conditions). This recent finding is being further studied. The overall signal of STAT1 phosphorylation was very low, which is consistent with low response to TGF-beta from STAT1. (Figure 8). Forthcoming experiments will investigate this finding using stimulation with interferon gamma, which is the cytokine known to induce STAT1 signal. This finding is central to the overall hypothesis, which posits that excess STAT1 signal in fibroblasts will result in impaired function of fibroblasts, resulting in pneumatocele and intestinal rupture due to impaired ECM formation.

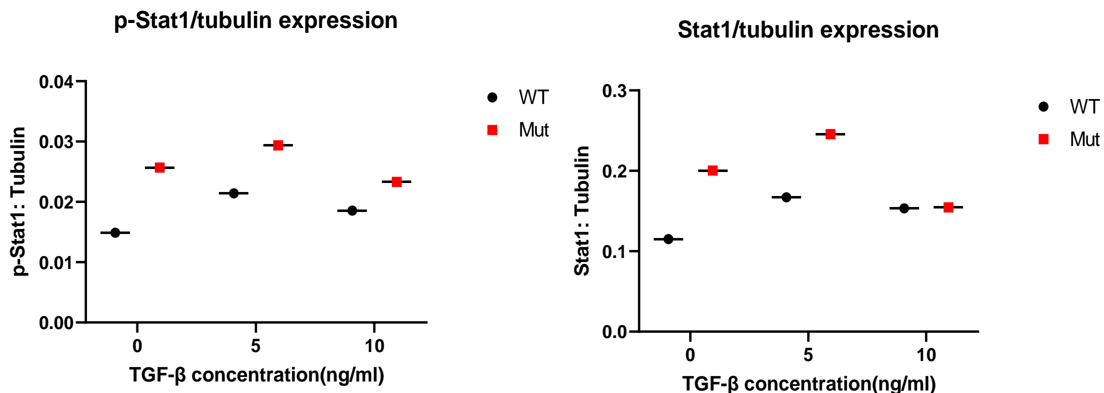

Figure 8: STAT1 is hyper-phosphorylated in dermal fibroblasts from patients with AD-HIES vs WT controls. (LEFT). STAT1 overall expression also appears increased in AD-HIES fibroblasts (RIGHT).

Returning to the mouse fibroblasts, we assessed IL-6 production from isolated fibroblast cells from the lung – here we were surprised to find that we had initially assayed two separate populations of fibroblast like cells – CD45+ and CD45 negative. CD45+ fibroblast like cells are termed 'fibrocytes' and represent bone marrow derived fibroblast like cells with some macrophage properties. Upon sorting CD45- fibroblasts from CD45+ fibroblast like cells, we found that while fibroblasts themselves have impaired IL-6 production, fibrocytes appear to have enhanced IL-6 production from patients with AD-HIES. Overall, this finding suggests that IL-6 expression in the lung may be complicated by cell of origin.

Finally, we sought to study the function of fibroblasts using mouse fibroblasts from AD-HIES mice and WT fibroblasts. Here we found that pulmonary fibroblasts from the mouse lung from AD-HIES mice demonstrated impaired migratory ability as compared to wild types, using a 'scratch assay' In this simple test, a pipet tip scrapes a line across a slide, and the rate of fibroblast migration across the slide is tested. Here, we found that AD-HIES fibroblasts migrated more slowly when unstimulated by TGF-beta, but that addition of TGF-beta stimulation seemed to result in a similar speed of migration.

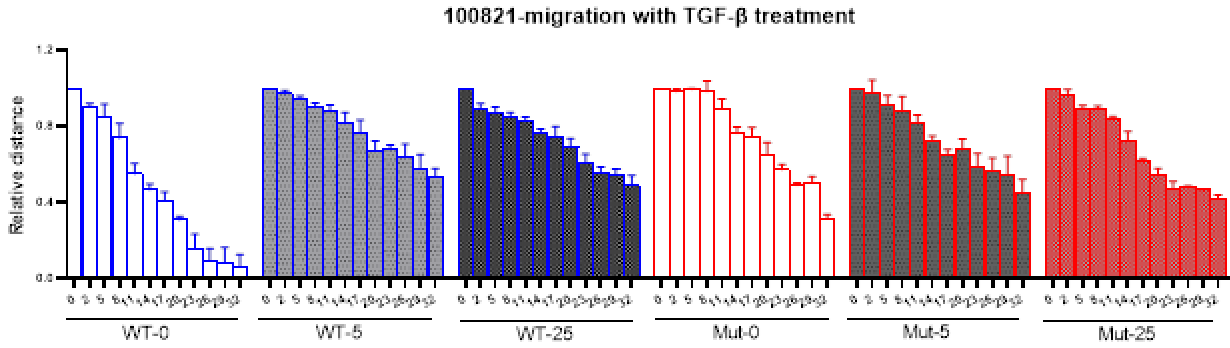

Figure 9: Migration of fibroblasts assay. Number of cells (y axis) is plotted against time on the x-axis (hours). At 0 TGF-beta incubation, by 32 hours, WT cells are approximately 0.2 units of relative distance, whereas Mut (AD-HIES) cells still are approximately 0.4 units apart, and the rate of migration has been much slower.

We then studied the functional differences in terms of collagen secretion of our AD-HIES fibroblasts and WT fibroblasts. Here, we found that AD-HIES mutant cells secrete overall less collagen than WT cells, and secrete less collagen type 1 and type 3 specifically; this was performed using two different assays. This experiment was complicated by the unexpected presence of CD45+ fibrocytes in the cells used, and we are now able to separate these cells by flow cytometry and work with the cell populations separately to understand the more complex nature of these cells function (ie, it may be that CD45+ fibrocytes function differently in terms of collagen secretion than CD45- fibroblasts and vary between themselves in the setting of STAT3 dominant negative mutation vs wild type).

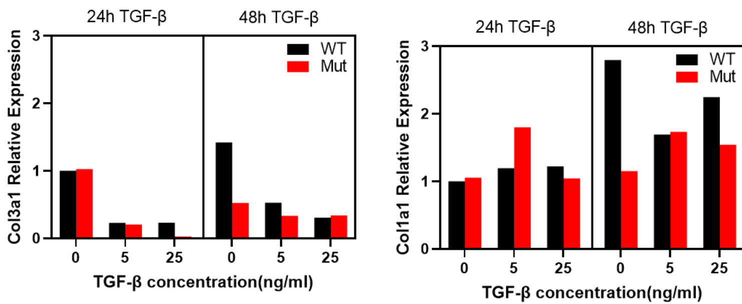

Figure 10: Collagen 3 (LEFT) and Collagen 1 (RIGHT) secretion shows that after 48 hours, Mut (AD-HIES) murine fibroblasts/fibrocytes have lower collagen specific mRNA message than WT cells.

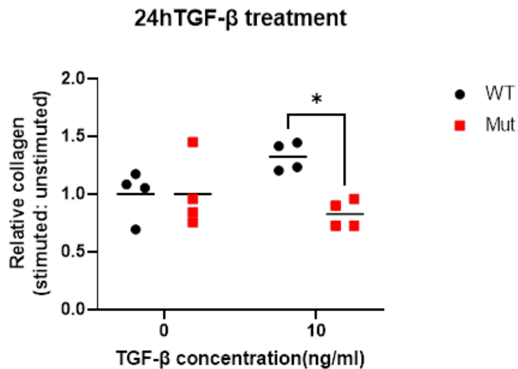

Figure 11: Mut (AD-HIES) fibroblasts/fibrocytes secrete less collagen protein when stimulated with TGF-beta than WT fibroblasts (murine).

Conclusions and next steps:

This project was overall challenging in that the development of a pneumatocele or abscess in a STAT3 mouse after S. Aureus incubation has not been previously achieved. We were fortunate in some ways that we saw a robust abscess formation, however as yet have not seen true pneumatocele formation. We have attempted to let mice remain with abscesses after several weeks to see if this resulted in pneumatocele, but so far this has not yet resulted in this finding; however, we have fine tuned our ability to derive abscesses (initially this was inconsistent) in the mice and now are able to retry these experiments to see if over the time period of many weeks (ie, 8-12 weeks) pneumatoceles occur after abscess formation. Another consideration is that antibiotic therapy itself could perhaps result in pneumatocele formation by clearance of abscess with damaged lung tissue, and this is another model we wish to pursue. Overall, the generation of abscesses gives us a pneumatocele analogue that we can now use to understand lung abscess formation in the AD-HIES mouse model and be able to extrapolate to the human.

We have now bred STAT3 dominant negative cre inducible mice that have both a bone marrow cre promoter which functions by deleting (when cre is activated) a stop codon and this then unlocks the translation of a mutant STAT3 protein which leads to the dominant negative phenotype:

STAT3_R328Q
Map of the Rosa locus

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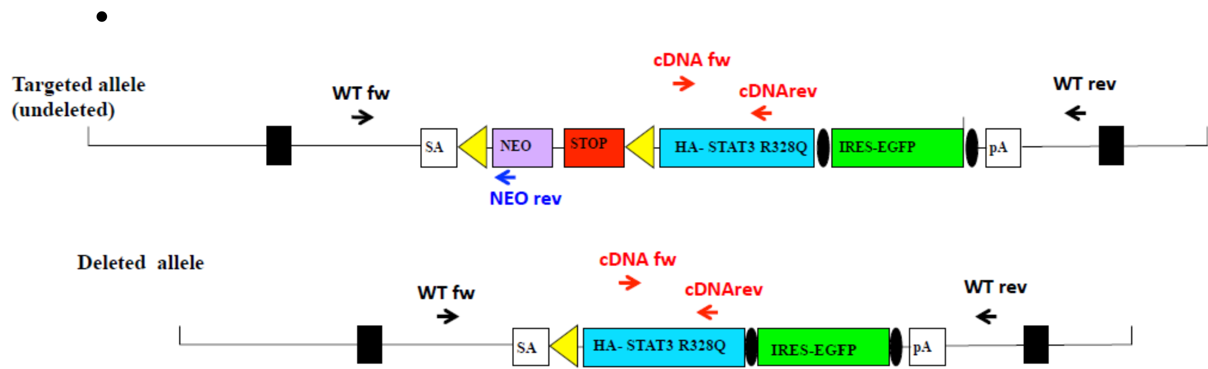

Using this cre mouse and our abscess model, our plan (these experiments are now underway) is to generate bone marrow STAT3 dominant negative vs fibroblast STAT3 dominant negative mutant mice and determine whether it is the leukocytes, fibroblasts or both that play a role in this abscess formation, by observing whether abscesses do not occur in the setting of either marrow or fibroblast STAT3DN mutation. If abscesses occur in both settings, we will then crossbreed an epithelial STAT3DN mouse and assess whether epithelial cells control for this abscess.

Our other experiments going forward (and underway) are to understand relative gene expression by fibroblasts in the setting of S. Aureus infection. We know from our previous experiments that IL-6 expression by AD-HIES fibroblasts is impaired after S. Aureus co-incubation. We are currently waiting on results of RNA seq from human STAT3 DN fibroblasts to determine what other genes are disordered in expression in this setting, and hope to use this data along with our current findings for a publication, likely in *Journal of Allergy and Clinical Immunology*.

These current experiments are taking place using our own funds (as other funds have been exhausted) and we hope to use this data to apply for further grants from the NIH.

We are very appreciative of the Job Foundation for their funding, which has made all of this work possible.