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Project Title: Investigating a non transcriptional role for IL-10-STAT3 in mitochondrial metabolism and AD-HIES

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Questions asked by the Job Research Foundation:

1. In the initial check-in, you mentioned that your lab had to be shut down and no laboratory work occurred from March through June 15th and that there were delays in patient sample collection but that data analysis continued from home. Please provide an update on your return to the lab and the status of the sample collection up to this point.

Since June 15, 2020, albeit with some setbacks that have occurred over the year, laboratory operations have been in person as needed. We received human AD-HIES samples from Dr. Alexandra Freeman at the NIH in July 2020.

2. Have there been any additional delays in your research due to factors outside of Covid?

There have not been any other additional delays.

3. At the previous check-in you provided the information below. Please update each of these for our records:

Specific Aim 1:

- Metabolomics on murine macrophages: 3-8 months Dec 2019 - Aug 2020.

We have completed these studies. I am including the data from our paper that we hope to be submitting in the next month.

Metabolic analysis reveals differences in arginine metabolism in WT and *Stat3*^{tgmuRtDBD} BMDM when compared to *Stat3*$^{\Delta mye}$ BMDM. Microbial (LPS) stimulation of macrophages results in extensive metabolic reprogramming which is suppressed by IL-10. To determine the impact of the STAT3 AD-HIES mutation on IL-10 mediated metabolic reprogramming, we stimulated WT, *Stat3*^{tgmutDBD} and *Stat3*$^{\Delta mye}$ BMDM with LPS in the presence or absence of IL-10 and performed metabolic profiling. Principal component analysis demonstrated that principal component 1 (PC1) was the primary driver of metabolic variation and correlated with host genotype (WT, *Stat3*^{tgmutDBD} and *Stat3*$^{\Delta mye}$) (**Supplemental Fig. 1**). Notably, *Stat3*$^{\Delta mye}$ BMDM metabolites localized to a distinct cluster separated from both WT and *Stat3*^{tgmutDBD} BMDM metabolites. No observable differences were discerned between WT and *Stat3*^{tgmutDBD} BMDM metabolites, suggesting that IL-10 mediated metabolic effects were largely unaffected by the STAT3 AD-HIES mutation (**Fig. 1A**).

We next subjected the metabolic profiles of *Stat3*^{tgmutDBD} and *Stat3*$^{\Delta mye}$ BMDMs to pathway analysis and revealed that arginine biosynthesis was the pathway most signficantly enriched

distinguishing the two genotypes (**Fig. 1B**). In macrophages, arginine is mainly metabolized to citrulline and nitric oxide (NO) under pro-inflammatory conditions (via *Nos2*) or to ornithine and proline under anti-inflammatory M2 conditions (via *Arg1*) (**Fig. 1C**). Citrulline can be recycled and combined with aspartate to produce arginine, or it can be routed into the TCA cycle (**Fig. 1C**). IL-10 suppressed the production of citrulline in WT but not $Stat3^{\Delta mye}$ cells (**Fig. 1D**). Consistent with the PCA analysis, IL-10 also suppressed citrulline in $Stat3^{tgmutDBD}$ BMDM (**Fig. 1D**). It has been reported that citrulline is exported during NO production. Consistent with the intracellular data, levels of citrulline in the media were reduced upon IL-10 stimulation in WT and $Stat3^{tgmutDBD}$ BMDM compared to $Stat3^{\Delta mye}$ BMDM (**Fig. 1E**). Together, these findings reveal that IL-10 mediated metabolic reprogramming is largely intact in $Stat3^{tgmutDBD}$ BMDM.

• Patient sample collection: 3-8 months Jan 2020 - Aug 2020

As noted above, we obtained patient samples from the NIH in July 2020.

• Testing STAT3 inhibitors on human macrophages: 3 months Aug 2020 - Nov 2020

Experimentation with TAT3 inhibitors were performed on human macrophages from healthy controls. Unfortunately, the data with specific STAT3 inhibitors (e.g. Stattic, C188-9) gave inconclusive results. Moreover, the number of viable monocytes in AD-HIES patients recovered was lower than expected (~50%), likely due to cell death during cryopreservation and thawing. As a result, there were insufficient patient monocytes to assess the effect of the inhibitors.

• Functional analysis on patient macrophages: 6 months Dec 2020 -May 2021

IL-10 mediated suppression in AD-HIES patients is gene-dependent. To determine whether macrophages derived from AD HIES patients exhibited defects in responding to the suppressive effects of IL10, we compared the ability of IL10 to suppress inflammatory gene expression in LPS-stimulated monocyte-derived macrophages. As expected, IL-10 inhibited the LPS-induced expression of both *TNF* and *IL1B* in macrophages derived from healthy controls (**Fig. 2A-B**). In AD-HIES patients, IL-10 was able to significantly suppress LPS induced TNF (**Fig. 2A**) but not IL1B (**Fig. 2B**). Together, in monocyte derived macrophages from AD-HIES patients, the ability of IL10 to suppress LPS-induced inflammatory genes remains intact for at least TNF.

• Metabolomics on patient monocyte-derived macrophages and plasma: 6 months Nov 2020 - Apr 2021

Due to issues with cell recovery as explained above, we did not have sufficient patient macrophages to perform metabolomic analyses. Metabolomics on plasma samples was considered. However, samples from healthy controls were not collected along with AD-HIES patients, as these samples had been collected prior to this project. The control samples are important in controlling for technical factors (e.g., type of collection tube, processing time) that can affect the metabolic profile. While experiments can be performed to address the impact of these factors, these would require additional time and were thus de-emphasized.

• Metabolomics on murine macrophages on an Il10-/- background if necessary: 3-6 Months Jan 2021 - Jun 2021
These experiments have not been necessary

4. As we head into the next six months of your funded research, please explain any additional changes to your aims or goals and specifically list and describe the changes and reasoning behind them.

Our efforts over the next 6 months, will be to finalize our murine and human data for publication. We are planning a submission of a manuscript entitled, “The STAT3 AD-HIES mutation differentially impacts IL-10 mediated induction and suppression”. This experimental work is encompassed in Aim 1 – which has occupied much of our efforts. The remaining patient samples have been set aside to address reviewer comments. Our work in Aim 2, “Elucidating the dependence of IL-10R signaling on mitochondrial STAT3”, has been de-emphasized as the experimentation for Aim 1 has occupied most of our time. Our data has pointed to a need to understand the mechanisms behind the ability of IL-10 to affect the functional and metabolic state of macrophages harboring the STAT3 AD-HIES mutations.

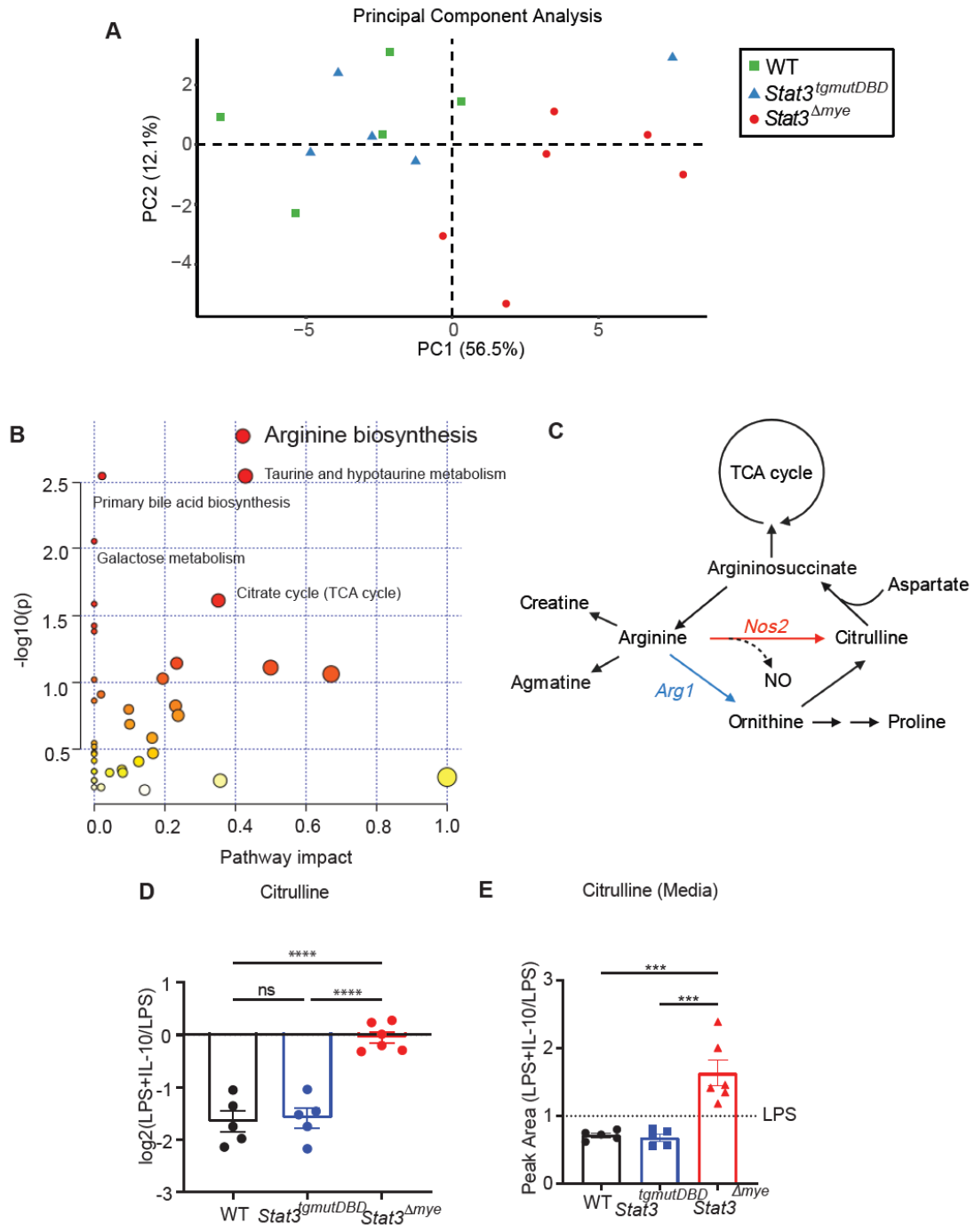


Figure 1. IL-10 mediated changes in arginine metabolism in the presence of the STAT3 AD-HIES mutation. WT, *Stat3*^{tgmutDBD} or *Stat3*$^{\Delta mye}$ BMDM were stimulated with LPS (100ng/ml) ± IL-10 (20ng/ml) for 24h and subjected to metabolic profiling. (**A-B, D-E**) Internal standard normalized intensities for the LPS+IL-10 condition were normalized to that of the LPS alone conditions and subjected to **(A)** Principal Component analysis or (**B**) pathway analysis (comparing *Stat3*^{tgmutDBD} vs *Stat3*$^{\Delta mye}$ BMDM). (**C**) Schematic of arginine metabolism in macrophages, with M1 macrophages utilizing *Nos2* (red) and M2 macrophages utilizing *Arg1* (blue). (**D**) Levels of citrulline in cells stimulated with LPS+IL-10. (**E**) Citrulline levels in the media for macrophages stimulated with LPS+IL-10, expressed as raw intensity normalized to that of the LPS alone condition. Graphs shown as mean ± S.E.M., with each individual point representing a biological replicate. N=5-6 biological replicates. *p<0.05, *** p<0.001, ****p<0.0001 by one-way ANOVA with Tukey's multiple comparisons test.

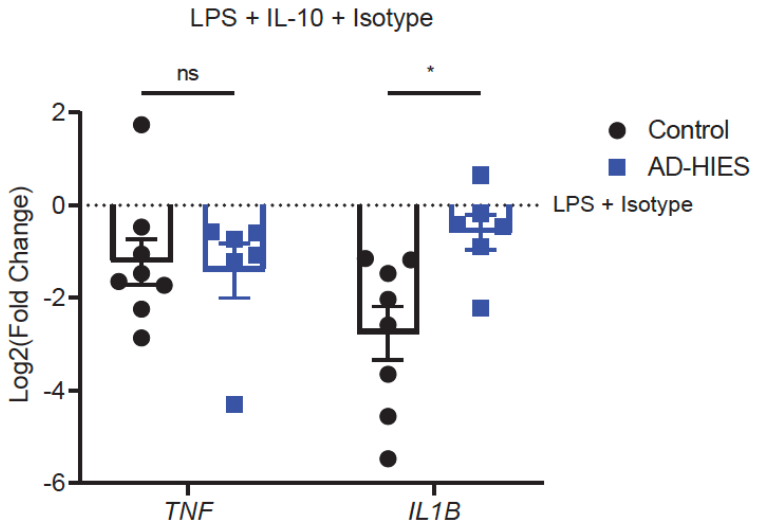
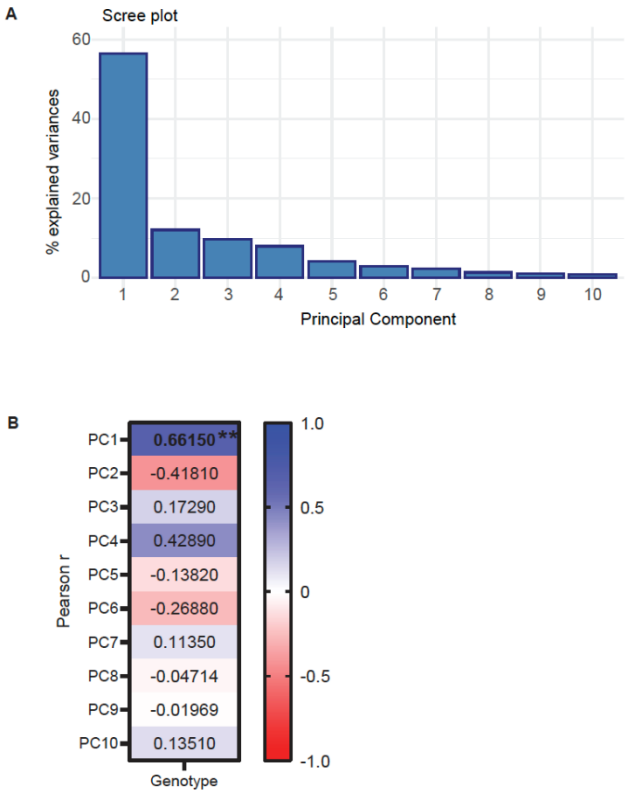


Figure 2. IL-10 mediated suppression in human STAT3 AD-HIES is gene-dependent. Monocyte derived macrophages from healthy controls or STAT3 AD-HIES patients were stimulated with LPS (100ng/ml) ± IL-10 (20ng/ml) and either isotype control or anti-human IL-10R blocking antibody (10µg/ml) for 24h. Expression of (A) *TNF* or (B) *IL1B* were assessed by qRT-PCR. Graph shows mean of $\log_2$(Fold Change relative to LPS + Isotype) ± S.E.M., with each point representing one subject. n = 7-8 controls or patients, done across 3 independent experiments. ns not significant, **p<0.01 by 2-way ANOVA with Šídák's multiple comparisons

Supplementary Figure 1



Supplementary Figure 1. PC1 captures most of the metabolic variation and is significantly correlated with Genotype. (A) Scree plot of PCA analysis used in Fig. 4A. **(B)** Pearson correlation analysis of the rotated values of each PC to the genotype, with pearson r shown. ** p<0.01