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PRINCIPAL INVESTIGATOR: Andrew Olive

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Andrew Olive

E-Mail: oliveand@msu.edu

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MICHIGAN STATE UNIVERSITY
426 AUDITORIUM RD RM 2
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14. ABSTRACT
Infections with Mycobacterium tuberculosis (*Mtb*) cause the severe pulmonary disease Tuberculosis (TB and are responsible for over 1.5 million deaths each year. Additionally, over 1/3 of the world's population has been exposed to infected individuals. One of the major reasons *Mtb* infections are so lethal is the normal host response is unable to eradicate the pathogen, leading to chronic infections in some individuals that can develop into active disease. We continue to lack effective tools to eradicate *Mtb* infections, including a protective vaccine, fast acting anti-infective drugs, and biomarkers that predict who will or will not progress to active TB. In most *Mtb* infected individuals the expansion and activation of *Mtb*-specific T cells is robust. With effective initial activation, why are these cells not sufficient to sterilize *Mtb* infections? One possibility is that although these T cells are functional, they are unable to detect *Mtb* within infected cells limiting their ability to control infection. Several studies suggest that *Mtb* can modulate T cell effector functions and evade T cells ability to detect infected cells. We reason that identifying host pathways that can reprogram infected cells to more effectively signal to T cells would be an important addition to the host-directed therapy toolbox. The overall goal of our Discovery Award is to identify targets that can be augmented in infected cells to improve adaptive immune responses against Mtb infection. Here we will focus on modulating key molecules that directly signal to T cells on infected macrophages and test whether altering their expression directly impacts T cell function and control during Mtb infection.

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TABLE OF CONTENTS

1. Introduction:

Infections with Mycobacterium tuberculosis (*Mtb*) result in the lethal pulmonary disease Tuberculosis which is responsible for over 1.5 million deaths each year. One of the major reasons *Mtb* infections cause disease is that the normal host response is unable to eradicate the pathogen, leading to chronic infections in some individuals that can develop into active disease. We continue to lack effective tools to eradicate *Mtb* infections, including a protective vaccine, fast acting anti-infective drugs, and biomarkers that predict who will or will not progress to active TB. In most *Mtb* infected individuals the expansion and activation of *Mtb*-specific T cells is robust but delayed. With effective activation in the draining lymph nodes, why can these immune cells not sterilize *Mtb* infections? One possibility is that although these T cells are functional, Mtb modulates infected macrophages to prevent effective detection and control in the respiratory tract. To address this possibility, we are characterizing host pathways that regulate key molecules in macrophages that interact with/modulate T cell function. We reason that identifying host pathways that can reprogram infected cells to more effectively signal to T cells would be an important addition to the host-directed therapy toolbox. The overall goal of our Discovery Award is to identify targets that can be augmented in infected cells to improve adaptive immune responses against Mtb infection. Here we will focus on modulating key molecules that directly signal to T cells on infected macrophages and test whether altering their expression directly impacts T cell function and control during Mtb infection.

2. Keywords: Mycobacterium tuberculosis, Host-pathogen interactions, Immune evasion, MHCII, antigen presentation, CD40, PD-L1, co-stimulation, Co-inhibition, Interferon gamma, Protective immunity, CRISPR-Cas9, functional genetics, Macrophages, CD4+ T cells.

3. Accomplishments:

A. The major goals of this project are to leverage our innovative loss-of-function and gain-of-function platforms using CRISPR-Cas9 approaches to understand how the expression of key molecules in macrophages are regulated and modulate T cell function. We created a genome-wide knockout macrophage library and a genome-wide synergistic activator mediator (SAM) library in murine macrophages, where every cell drives expression of a unique sgRNA and subsequently a gene via a catalytically inactive dCas9 coupled to a transcriptional activator. Using this approach, our overall goals are:

1) To determine the underlying mechanisms that regulate the expression of key molecules on macrophages that modulate T cell responses.

2) To determine how these regulatory networks in macrophages directly alter T cell effectors function and the control of Mtb infection ex vivo and in vivo.

B. Over our second year of funding for this project we made significant progress on the goals proposed that will be described in detail below.

1. We created a series (>50) of gain-of-function macrophage lines based on candidates from an MHCII overexpression library in resting macrophages. However, while we saw increased target gene expression, almost none of these candidates impacted MHCII expression in resting cells. This included Ciita, a known regulator MHCII expression. *This suggests that in the absence of activation, such as following IFNg stimulation, single genes are not sufficient to drive MHCII surface expression in macrophages.* This also suggests noise in our initial screening approach that we are currently investigating. We additionally have completed Gain-of-Function screens for parallel markers including CD40 and PD-L1. We are in the process of validating these screens and infecting candidate cell lines with Mtb to determine the outcomes of interactions with Mtb-specific T cells using our optimized *ex vivo* T cell assay described further below. We completed the analysis of our parallel loss of function screens in activated macrophages for genes regulating MHCII, CD40 and PD-L1 which have been very successful as described more below. Importantly, as mentioned briefly in the last reporting period, we continue to be unable to reproduce previous studies that show that Mtb directly blocks MHCII expression in resting macrophages. This suggests the hypothesis that inhibition of T cell responses during Mtb may occur through modulating other molecules including

co-stimulatory molecules or require macrophage activation. We are actively testing this hypothesis through our no-cost extension phase of this award.

2. To address the findings in #1 above we expanded our screening to include activated macrophages by adding IFNg and identified host genes that modulate the surface expression of MHCII and the co-stimulatory/co-inhibitory markers CD40/PD-L1 similar to what occurs in vivo during Mtb infection in the lungs. These screens were VERY successful. We developed a novel pooled analysis pipeline and discovered a novel role of oxidative phosphorylation (OxPhos) in regulating MHCII, CD40 and PD-L1 expression in activated macrophages. ***OxPhos pathways were significantly enriched in our bioinformatic analysis and suggested a unique role for Complex I of the electron transport chain (ETC) but not other ETC complexes (Figure 1).*** We validated these results using

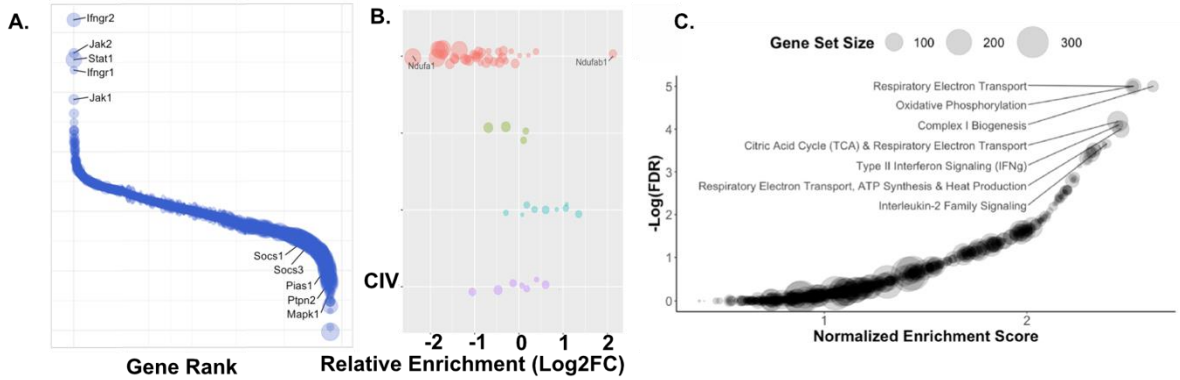

Figure 1. CRISPR screens Identify Complex I as a key regulator of T cell modulatory molecules. (A) Genome-wide screens for MHCII/CD40/PD-L1 identify shared regulation in activated macrophages. Shown is a rank plot of the combined analysis for all screens for surface marker induction. (B) Relative enrichment of electron transport genes grouped by complex. (C) Pathway analysis identifies OxPhos as a key regulator of T cell modulatory marker expression

both genetic and chemical approaches and showed Complex I prevents effective MHCII expression upstream of transcriptional activation of MHCII genes. Further mechanistic studies found that Complex I function is required to activate the Jak/Stat phosphorylation cascade that initiates during macrophage activation (Figure 2).

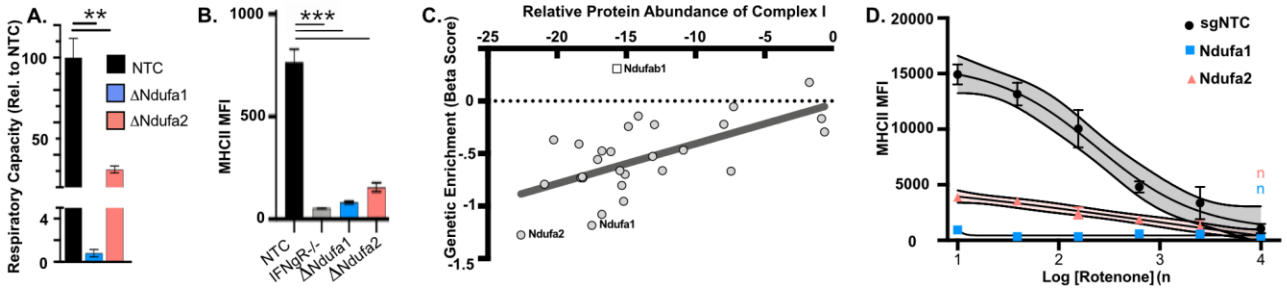

Figure 2. Complex I mutants are deficient in IFNγ-signaling. (A) Indicated cells were grown in pyruvate as the sole carbon source then treated with IFNγ. The relative ATP levels compared to NTC controls were determined 24 hours later. (B) Indicated Møs were stimulated with IFNγ and stained for MHCII and the mean fluorescence intensity (MFI) was quantified. Shown is the mean of 4 biological replicates for >3 independent experiments. (C) Correlation between the relative effect of each complex I subunit on the structural integrity of Complex I (x-axis) with the relative requirement of each complex I subunit for the IFNγ-response (y-axis; Beta score). The Pearson correlation coefficient (r) was calculated to be 0.6452 p-value = 0.0002. *Ndufab1* (empty square) is an essential gene. (D) MHCII MFI of macrophages stimulated with IFNγ and treated with rotenone at the indicated concentrations. Mean +/- the standard deviation. Representative of four experiments all experiments gated on live cells ***p<.001 **p<.01using one-way anova with Tukey test.

While Complex I inhibition blocked T cell markers, the cells were entirely capable of inducing cytokines following the activation of pattern recognition receptors. These exciting results go against current dogma in the Mtb/macrophage field that suggest macrophage activation and Mtb infection drives aerobic glycolysis with a limited role for mitochondrial respiration. We now hypothesize that Mtb drives cells towards glycolysis as an immune evasion mechanism to blunt the IFNg response and prevent effective T cell responses towards infected macrophages. We are directly testing these exciting hypotheses in the last phase of the no-cost extension and better understanding the direct links between Complex I and IFNg activation. Our goal, in line with this DOD award is to define ways we can target this pathway and overcome Mtb immune evasion tactics. We are in the process of deriving mice lacking the S4 complex I subunit to examine the function of this complex in vivo. We continue to be optimizing our in vivo gene editing approach. Unfortunately, the percent of cells effectively edited remains below what is usable in the assays, recent advancements in Cas9 loaded with RNAs, or RNPs, will be our next approach in the no-cost extension period. ***We were excited to***

publish these findings in eLife, a high impact journal while receiving very positive reviews that were published with the manuscript. I anticipate the findings funded by the DOD with drive 2-3 more manuscripts based on these initial findings alone.

3. While investigating our novel discovery of OxPhos regulation of macrophage T cell interacting markers, we made an important finding with regards to TB treatment. Anti-TB therapy requires over 6 months of continued antibiotic therapy with multiple antibiotics including linezolid. Because linezolid has been suggested to modulate host mitochondrial function in addition to targeting Mtb, we tested how this antibiotic impacts OxPhos in macrophages and expression of macrophage T cell interaction markers. We tested how prolonged exposure to Linezolid modulated mitochondrial function in macrophages and how this impacts the expression of important T cell stimulatory markers. We observed persistent exposure of macrophages to linezolid reduced mitochondrial number, lowered OxPhos-dependent ATP production, and prevented robust expression of MHCII and other macrophage markers (Figure 3). ***This suggests a new paradigm in TB biology where there is a key need to better understand how antibiotic therapy may have off-target effects that directly impact immune clearance.*** These data are being used to better understand how antibiotic therapy may inhibit Mtb responses and may have large clinical implications worldwide. We are currently designing new projects beyond the scope of this award to better understand how antibiotic therapy may cause immunosuppression and determining how this impacts susceptibility to infections.

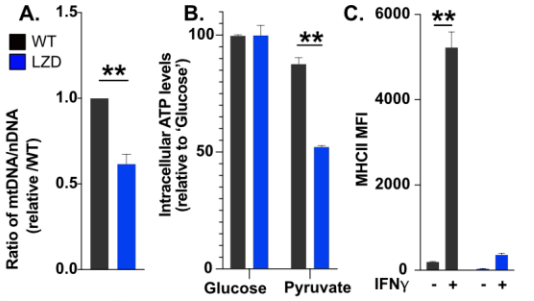

Figure 3. LZD-conditioned macrophages phenocopy Complex I loss. Macrophages were cultured in vehicle (WT) or 50ug/ml linezolid (LZD) for 4 weeks. (A) qPCR was used to quantify Mt genomes in relation to diploid genomes. (B) ATP abundance was determined in glucose or pyruvate only media. (C) MHCII expression was determined in WT and LZD cells by flow cytometry after 24 hours in the presence or absence of IFNγ. Data are representative of two independent experiments. **p<.01 by t-test (A and B) and One-way ANOVA (C).

4. Last reporting period we optimized an ex-vivo T cell activation assay with TCR-transgenic CD4+ T cells. Based on our findings related to OxPhos described above we have now used this ex vivo model to examine how OxPhos in macrophages directly modulates the function of Mtb-specific CD4+ T cells, a key goal of this DOD award (Figure 4). We found that both macrophages and dendritic cells with blocked complex I function were unable to activate Mtb-specific CD4+ T cells and Complex I was required to control Mtb growth. We also validated our OxPhos phenotype in human cells suggesting that we have found an important new immunometabolism pathway that controls T cell activation during Mtb infection. These findings were included in our eLife publication. ***This exciting result further shows the importance of Complex I in the immune response during Mtb infection***.

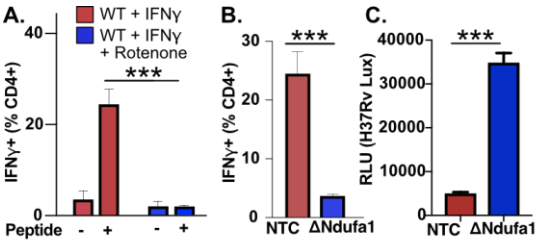

Fig 4. Complex I is required for Macrophages to activate CD4+ T cells and control *Mtb*. (A) BMDMs were IFNγ stimulated with and without peptide or rotenone or (B) NTC or ΔNdufa1 Møs from HoxB8 cells were stimulated with IFNγ and peptide. (C) or were were infected with Mtb-Lux. For all panels resting C7 cells were then co-cultured. For (A) and (B) the % of IFNγ+ C7 Cells was determined by ICS 6 hours later. For (C) the total luciferase levels were determined 3 days later luciferase signal. All data are representative of 2 independent experiments. For ***p<.001 (A) one-way ANOVA with tukey test or (B) and (C) two-tailed t-test.

Summary: Even though our initial gain-of-function approach has limitations, expanding the scope of our screens was incredibly successful. We already published one high impact manuscript in eLife with the support of this DOD award and have been invited to present on these results at department seminars across the country and national/international meetings. These studies thus far have identified a novel immunometabolism pathway that modulates T cell modulatory markers, identified a possible negative effect of long-term antibiotic use on TB immune responses, and developed a strong genetic platform to unravel host-pathogen interactions during Mtb infection. Thus, this project is already a huge success. With the remaining support in the no-cost extension phase, we will further validate our results, understand the underlying mechanisms of T cell modulatory pathways in macrophages, and examine how these pathways converge with host-pathogen interactions during Mtb infection. Over the next year of funding, we will examine the gain-of-function screens

to validate screens from activated macrophages and further explore the role of Complex I in driving protective immunity during Mtb infection.

C. The award has allowed many training opportunities to present our research in several forums including internal department seminars, international meetings including the international cytokine society, the American Association for Immunologists annual meeting and the Midwest Microbial Pathogenesis Conference. My postdoctoral fellow Dr. White contributed greatly, generating cell lines to validate screens and to expand our troubleshooting of the gain-of-function approach. Dr. White and my student Laurisa Ankley are working to better understand the immunometabolism pathways that we have identified and determining how these pathways directly impact Mtb pathogenesis and TB disease progression. I currently meet weekly with all lab members to provide mentorship and help them with all experimental design as well as professional development. I held a state of the lab meeting in January of 2022 to educate all lab members on the inner workings of running a lab, including how to apply for funding, budgeting and planning for the expansion of my research group in the near future. This award generated significant data that has played a large role in receiving new funds from the NIH that will be awarded soon to better understand the mechanisms of metabolism and Mtb infection.

D. Nothing to report.

E. In the no-cost extension for this project there are two key milestones that we would like to accomplish.

I. Over the no-cost extension phase of this award we are committed to understanding the limitations of our original MHCII screen to better understand MHCII regulation in even more detail. We are currently developing co-expression approaches to combine our gain-of-function approach genetic tools to determine if this helps us better understand MHCII regulation, trafficking and expression in more detail. Furthermore, we have completed parallel gain-of-function screens for key T cell modulatory markers in macrophages that we are starting to validate. We expect to use these results to A) better understand fundamental regulation of key immune pathways in macrophages B) determine how these pathways directly impact Mtb growth and Mtb-specific T cell functions and C) examine if these pathways can be modulated to overcome Mtb-mediated immune evasion both ex vivo and in vivo. We are working to improve in vivo editing over the next year to further the impact of these studies.

II. Over the no-cost extension phase of this award we will continue to examine the mechanisms controlling our novel immunometabolic pathway controlled by Complex I. Studies are underway to better understand the direct links between Complex I and Jak/Stat signaling. This includes RNAseq analysis, metabolomic studies, and directed genetic/chemical approaches. We are also designing new experiments to better understand how Mtb targets mitochondrial function and determine if modulating OxPhos alters the host response to Mtb ex vivo and in vivo. This will include in vivo editing and examination of Mtb disease in a mouse lacking a complex I subunit.

1. Impact:

A. Mtb infections continue to result in over 1.5 million death each year and is endemic in particular geographic locations around the globe putting our military at risk of infection and disease. Our discovery of a new immunometabolism network that modulates both Mtb inflammatory responses as well as the T cell response against Mtb is very impactful. First it shows a new immune evasion mechanism used by Mtb to sustain infection and avoid killing by T cells. This was a foundational goal of this DOD award and this discovery will enable new targets for therapy to tested with the hope of overcoming Mtb virulence tactics and driving more rapid bacterial control. Second these results are impactful because they show an unexpected requirement for distinct metabolic pathways for the host to effectively respond to infections. Current literature focuses solely on glycolysis pathways but our discovery from this DOD award expanded the scope of host metabolic networks that contribute to immunity to include OxPhos. This paradigm shifting discovery would not have been possible without this award. Finally, our observation that long-term antibiotic treatment blocks this new immunometabolism network and suppresses host responses is a critical discovery that

requires further exploration. The possibility that antibiotic treatment, while killing the pathogen, has side-effects that prevent effective immune responses is compelling and must be further examined. It is possible civilians and military personnel on long-term antibiotic treatment may become more susceptible to other infections which would have large implications in disease monitoring of these individuals. Thus, this DOD award has already had a large impact on TB disease, immunometabolism and antibiotic therapy. We are motivated to further these discoveries in the no-cost extension phase of this award.

B. Nothing to Report

C. Nothing to Report

D. Nothing to Report

2. Changes/Problems:

A. Over the last year we have expanding our scope to understand the regulation of key T cell stimulatory genes in macrophages. These have been incredibly successful as described above as we have begun to better understand immune evasion by Mtb and identify new therapeutic targets. Thus, expanding the scope of these studies has been incredibly fruitful and provide robust datasets that we continue to explore. This is a key goal of the no-cost extension phase of this award.

B. Examining MHCII expression in resting macrophages proved to be more complex than our original screen suggested. We anticipate leveraging this complication to better understand MHCII regulation and further identify immune evasion tactics employed by Mtb. We are validating parallel screens and combining our genetic approaches in the no-cost extension phase to understand the mechanisms regulating T cell stimulatory markers on macrophages in mechanistic detail.

C. Nothing to Report

D. Nothing to Report

3. Products

Publications, Conference Papers and Presentations

A. Kiritsy MC, Mott D, Behar SM, Sassetti CM, **Olive AJ**. (2021) Mitochondrial respiration contributes to the interferon gamma response in antigen-presenting cells. *eLife* 10:e65109.

B. Oral Presentation. Conference Presentation. Mitochondrial respiration contributes to the interferon gamma response in antigen presenting cells. Keystone Meeting on Innate Immunology. April 2021

C. Oral Presentation. Conference Presentation. Mitochondrial respiration contributes to the interferon gamma response in antigen presenting cells. AAI. June 2021

No other products to Report.

4. Participants and other Collaborating Institutions

A. Individuals who worked on the Project

Name: Dr. Andrew Olive
Project Role: PI

Research Identifier: https://orcid.org/0000-0003-3441-3113
Nearest Person Months Worked: 3
Contribution to Project: Supervised work done on the project. Conducted experiments related to Aim 1 and Aim 2.
Funding Support: MSU Startup Funds, National Institute of Health

Name: Justin Trombley
Project Role: Technician
Research Identifier: https://orcid.org/0000-0002-3856-3138
Nearest Person Months Worked: 6
Contribution to Project: Maintained animals, conducted T cell experiments in Aim 2 to understand how Mtb T cell activation is modulated by loss or gain of macrophage genes.
Funding Support: National Institutes of Health

Name: Dr. Dylan White
Project Role: Postdoctoral Fellow
Research Identifier: https://orcid.org/0000-0001-6712-9448
Nearest Person Months Worked: 6
Contribution to Project: Conducted sequencing IFN-gamma activated SAM library, is conducting validation studies of MHCII-Hi library
Funding Support: MSU Startup Funding

Name: Laurisa Ankley
Project Role: Graduate Student
Research Identifier: https://orcid.org/0000-0002-0635-9001
Nearest Person Months Worked: 2
Contribution to Project: Conducted sequencing and validation of loss-of-function studies examining MHCII, PD-L1 and CD40 and is examining the underlying mechanisms of function.
Funding Support: National Institutes of Health

B. Nothing to Report

C. Nothing to Report

5. Special Reporting Requirements:

Nothing to Report