AWARD NUMBER: W81XWH-20-1-0147

TITLE: Modulation of T-Cell Activation During Mycobacterium tuberculosis Infection

PRINCIPAL INVESTIGATOR: Andrew Olive

CONTRACTING ORGANIZATION: Michigan State University, East Lansing, MI

REPORT DATE: June 2023

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Development Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
June 2023	Final	01Mar2020-28Feb2023

4. TITLE AND SUBTITLE

Modulation of T-Cell Activation During Mycobacterium tuberculosis Infection

5a. CONTRACT NUMBER
W81XWH-20-1-0147

5b. GRANT NUMBER
PR192504

5c. PROGRAM ELEMENT NUMBER
W81XWH-19-PRMRP-DA

6. AUTHOR(S)
Andrew Olive

E-Mail: oliveand@msu.edu

5d. PROJECT NUMBER
0011420096

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

MICHIGAN STATE UNIVERSITY
426 AUDITORIUM RD RM 2
EAST LANSING MI 48824-2600

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Development Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)
USAMRMC

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES
None

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.

15. SUBJECT TERMS
None listed.

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRDC
Unclassified	Unclassified	Unclassified	Unclassified	8	19b. TELEPHONE NUMBER *(include area code)*

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 Mtb modulates infected macrophages to prevent effective detection and control in the respiratory tract. Furthermore, long-term antibiotic treatment may directly blunt productive T cell responses. To address these possibilities, we are characterizing host pathways that regulate key molecules in macrophages that interact with/modulate T cell function and control using forward genetic screens. We reason that identifying host pathways that can reprogram infected cells to more effectively signal to T cells and control antibiotics 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 and control during treatment 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, antibiotics, proteasome.

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 award funding period for this project we made significant progress on the overall goals proposed even with the Covid-19 pandemic that will be described in detail below.

1. *Summary of progress in previous funding periods*: In the first two years of our funding period, we successfully screened for regulators of T cell modulatory markers MHCII, PD-L1 and CD40 on macrophages using our loss-of-function approach. We found an unexpected role of mitochondrial respiration in regulating the induction of these genes during activation/infection.

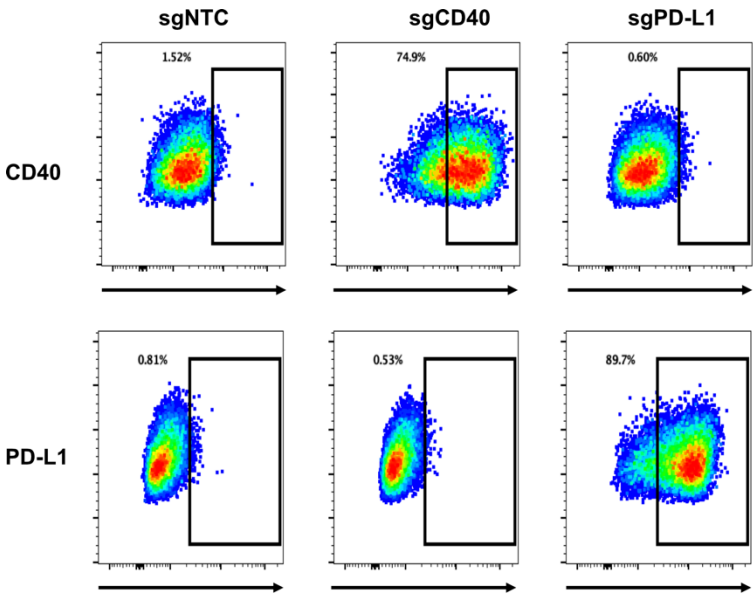

Figure 1. Using SAM to overexpress key co-stimulatory molecules in macrophages. SAM macrophages with the indicated sgRNAs were analyzed by flow cytometry for surface expression of CD40 and PD-L1. The percent positive cells are indicated.

Through this study we optimized knockout editing and co-culture assays with T cells that now will enable mechanistic understanding of these key interactions during infection. ***We were excited to publish these findings in eLife, a high impact journal in 2021 while receiving very positive reviews that were published with the manuscript.*** We have attached this manuscript again for our final report on this award.

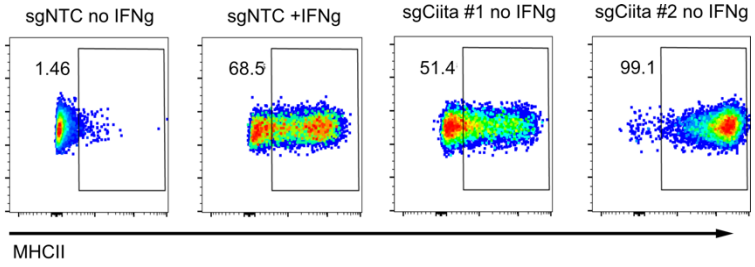

Figure 2. Targeting Ciita with sgRNAs results in robust MHCII expression independently of IFNg activation. Control SAM macrophages were left untreated or treated with IFNg while SAM macrophages targeting Ciita were left untreated. Cell were then stained for surface MHCII and analyzed by Flow Cytometry

2. *Validation of the MHCII high screen.* Last year we noted that the follow-up studies from our gain-of-function screen from cells expressing high levels of MHCII were unsuccessful. Over the last year we have made a key focus of our no-cost extension to better understand the shortcomings of this approach and re-evaluate candidates. We have made incredible progress on this over the last year. A talented DO/PhD student Mahima Thapa, took over this project and not only re-analyzed the screen, but she re-optimized our gain-of-function approach to be successful. As a first step she wanted to engineered macrophages that express high levels of the T cell modulatory molecules PD-L1 and CD40. We reasoned that the transcriptional regulation of these molecules is less complex and would enable us to optimize our approach. We now have several macrophage lines that each express high levels of these co-stimulatory molecules that will enable us to better dissect T cell macrophage interactions during infection (**Figure 1**). As a next step she examined whether she could make Ciita overexpression macrophages drive high levels of MHCII expression without activation. Indeed, her new sgRNAs resulted in the robust activation of MHCII without IFN activation (**Figure 2**). One interesting analysis that remains ongoing is completing global transcriptomics on Ciita over-expression macrophage lines to better understand MHCII independent genes controlled by Ciita. While there is heterogeneity in this approach that we continue to investigate, it raised the possibility that our screen did have bona fide MHCII regulators. We have now generated new macrophage lines targeting 10 new genes from our sequencing results. From these studies we have now identified at least 4 regulators beyond Ciita that directly modulate MHCII expression in resting cells including, Mfn2, Ptch2, Bhlhb9 and SLC25a37 (**Figure 3**). Interestingly several of these genes modulate mitochondrial function including oxidative phosphorylation. Final studies are examining how these genes regulate MHCII, but these data appear to link our findings related to mitochondrial respiration we previously published with our gain-of-function approach. **This is an exciting discovery that we anticipate will be ready for publication soon.**

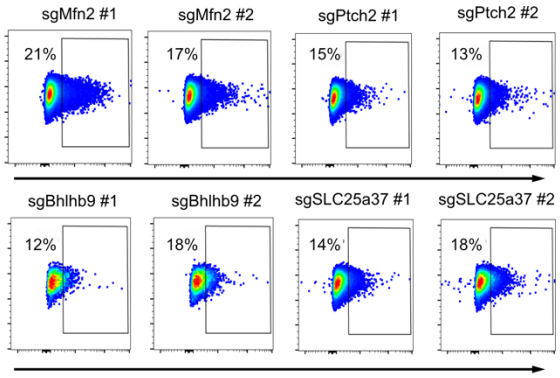

Figure 3. Validated candidates show increased MHCII expression in resting macrophages. Resting SAM cells expressing the indicated sgRNAs were examined by flow cytometry for MHCII surface expression. Shown is the percent MHCII positive for each cell line.

3. *Determine the intersection between oxidative phosphorylation and antibiotic control of Mycobacterial infections*. In the last funding period, we found that the key Anti-mycobacterial drug linezolid, directly modulates mitochondrial respiration, and can inhibit macrophage activation. Given our findings that OxPhos are key for MHCII expression, and Mtb directly targets mitochondrial function, this suggested a potential mechanism by which Mtb inhibits MHCII expression. This type of connection between pathogenesis and the host response was the ultimate goal for this award. These data also suggest that antibiotics like Linezolid may have direct effects on macrophages independent of directly targeting Mtb that modulate their efficacy at controlling intracellular Mycobacterium. Over the no-cost extension period of this award we have now used these preliminary data to complete a genome-wide screen to understand how intracellular Mycobacterial control by Linezolid is dependent on host pathways. To do this we infected the genome-wide knockout library with GFP-Mycobacteria then treated these cells with Linezolid. Using cell sorting we isolated cells with high and low levels of GFP that correlate with total intracellular bacterial load. We are initially interested in genes that when lost result in increased bacterial survival. These are

genes required for Linezolid to work effectively at controlling intracellular Mycobacteria, and thus modulate the host response. We are now validating the pathways identified including OxPhos and the host proteasome. As an example, we now have confirmed that inhibition of the host proteasome chemically with Lactacystine results in decreased Mycobacterial killing by Linezolid (**Figure 4**). From these validated pathways we are now examining how they modulate MHCII, PD-L1 and CD40 during infection. We will further use T cell co-culture assays to dissect how these pathways alter Mtb mediated inhibition of T cell modulatory markers. These highly innovative studies lay the groundwork for impactful future studies and show our success during the award period. ***We are finalizing experiments for a subset of this work that we anticipate submitting for publication in the coming months.***

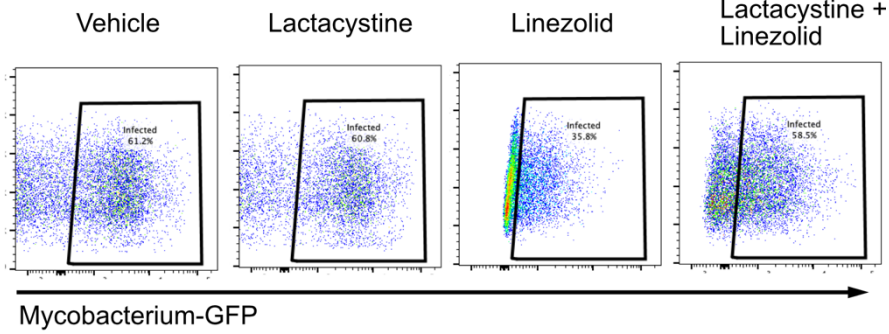

Figure 4. Blocking host proteasome function inhibits Mycobacterial killing by Linezolid. Macrophages were infected with GFP-Mycobacteria and treated with the indicated compounds. Four days later flow cytometry was used to quantify intracellular mycobacterial levels.

Summary: Even though our gain-of-function approach has some 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 we anticipate at least two more publications directly related to our data from this award. Thus, this project is already a huge success. A key thread through our data is the centrality of host mitochondrial function for macrophages to appropriately respond to infection and interact with T cells. Through all three of our screening approaches we have now shown that OxPhos is needed for induction of T cell modulatory markers and intracellular control of Mycobacteria by antibiotics.

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. Nothing to Report

4. Impact:

1. 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. Through this award we discovered a new immunometabolism network that modulates T cell responses and inflammatory responses that play a key role in TB disease. These results are very impactful and suggest new potential mechanisms by which Mtb evades the immune response. Our findings of new genes that can turn on MHCII expression is also impactful as it suggests a new mechanism to overcome Mtb inhibition of antigen presentation and T cell responses. Based on these paradigm shifting results we are further exploring the interconnections between Mycobacteria, host, and antibiotics. An important reason for this is the fact that patients must be on multiple antibiotics for

over 6 months to control TB, thus understanding how Mtb evades rapid killing by drugs and T cells and how the drugs themselves alter the host response is essential. A key impact of this award is linking the antibiotic Linezolid directly to mitochondrial respiration pathways that modulate Mycobacterial virulence and the expression of antigen presentation machinery. Our work is impactful because it now has identified host pathways that may regulate the efficacy of Mycobacterial drugs and identify new host-directed therapy targets that drive more rapid bacterial control. Taken together, our work supported by this Discovery Award, has resulted in a range of key and impactful findings directly related to the host response of Mycobacterial disease.

2. While our work mostly focused on host-pathogen interactions, these results are also impactful for a broad range of disease states including cancer and autoimmunity. Co-stimulatory and co-inhibitory molecules such as CD40 and PD-L1 are some of the best immunotherapies against cancers. Understanding how macrophages modulate these key molecules is foundationally impactful to define mechanisms of immunotherapy resistance by aggressive cancers. With regard to autoimmune responses, many of these such as Systemic Lupus Erythematosus (SLE) are mediated by self-activation of the adaptive immune response. Understanding how antigen presentation machinery is regulated as we have done here, identified new potential targets to prevent autoreactive immune cells during active flares. Thus, by defining key mechanisms controlling host-pathogen interactions during Mycobacterial infections, we have made a large impact on a range of key fields that involve the host immune response.

3. Nothing to Report

4. Nothing to Report

5. Changes/Problems:

A. Nothing to Report

B. *In vivo* genetic manipulations using gain-of-function approaches proved inefficient and not reproducible (ie editing in bone marrow cells). Rather than focus on the *in vivo* portion of this award in the no-cost extension we instead chose to focus on understanding limitations of the gain-of-function approach in macrophages. This was highly successful and will enable us to publish 1-2 additional reports based on data from this award. In ongoing work we are developing new editing approaches using the electroporation of mRNAs that we anticipate being more successful to unlock in vivo studies with these important approaches.

C. Nothing to Report

D. Nothing to Report

6. Products

7. 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

D. Poster Presentation. Conference Presentation. Using a CRISPR-mediated gain-of-function approach in macrophages to dissect interactions with CD4+ T cells. AAP/ASCI/APSA Joint Meeting. April 2023.

No other products to Report.

7. Participants and other Collaborating Institutions

A. Individuals who worked on the Project during no-cost extention

Name: Dr. Andrew Olive
Project Role: PI
Research Identifier: https://orcid.org/0000-0003-3441-3113
Nearest Person Months Worked: 2
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: Mahima Thapa
Project Role: Graduate Student (DO/PhD Program)
Research Identifier: https://orcid.org/0009-0006-8953-1677

Nearest Person Months Worked: 6
Contribution to Project: Conducted sequencing and validation of repeated MHCII high screen. She created new overexpression lines of MHCII, PD-L1 and CD40 and is examining the underlying mechanisms of function. She has validated a subset of hits required high MHCII expression that we are preparing for submission.
Funding Support: MSU DO/PhD funds

B. Nothing to Report

C. Nothing to Report

8. Special Reporting Requirements:

Nothing to Report