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TITLE: Modulation of T Cell Activation During Mycobacterium Tuberculosis Infection

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CONTRACTING ORGANIZATION: Michigan State University, East Lansing, MI

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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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## 1. Introduction:

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.

**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 gain-of-function platform using CRISPR-Cas9 approaches to modulate the expression of key molecules in macrophages that alter T cell function. We created 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 result in the increased expression of key molecules on macrophages that modulate T cell responses.

2) To determine how modulating these molecules directly alters T cell effectors function and the control of Mtb infection.

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

1. We identified genes that result in increased MHCII expression on macrophages during Mtb infection in the absence of IFNγ activation and have begun validation and mechanistic studies.

2. We expanded our gain-of-function screen to identify host genes that modulate the surface expression of MHCII and the co-stimulatory/co-inhibitory markers CD40/PD-L1 during IFNγ activation similar to what occurs in vivo during Mtb infection.

3. We developed and optimized an ex-vivo T cell activation assay with TCR-transgenic CD4+ T cells that we will now use to understand how candidate genes directly impact T cell activation and effector function.

**1.**

**I.** A first milestone in the first year of funding was to identify host genes in the MHCII high library that we enriched for elevated MHCII expression in the absence of cytokine stimulation. Last year we sequenced two replicate libraries from the MHCII high enrichment using illumina sequencing. Prior to sequencing we examined if MHCII expression was altered following Mtb infection and we found no significant changes. Thus, we sequenced the library from uninfected

conditions for simplicity. Even though the gain-of-function library used contains over 70,000 unique sgRNAs, found roughly 600 unique sgRNAs were present in the MHCII high enriched library. This suggests a very strong selection and represents less than 1% of the sgRNAs in the library. Even more, only a subset set of genes had more than one sgRNA represented in these libraries, thus our selection for high MHCII expression in the absence of cytokines resulted a severe bottleneck. The advantage of this bottle neck is the ability to deeply validate a high percentage of these candidates to understand their mechanisms of action. As of today we have cloned roughly 200 sgRNAs targeting almost 50 unique genes identified in this screen with 4 unique sgRNAs. Importantly, one of the top candidates was the gene Ciita which is known to directly modulate MHCII expression in macrophages. We identified other high priority candidates that were plausibly link to the MHCII expression including several transcription factors such as FosB and Cnot6, chromatin remodeling genes like H2al2a and Pygo2. Thus our enriched MHCII library identified a range of candidates that could modulate MHCII expression.

**II.** As a second milestone, we are currently validating top genes from the screen identified in A. We have begun identifying genes that directly modulate Ciita and MHCII RNA expression directly in the absence of cytokine treatment. Within the screen we identified the master MHCII regulator Ciita. Given Ciita is normally induced by IFNγ, this highlights the effectiveness of our initial approach. We cloned 4 sgRNAs targeting distinct regions of Ciita and examined the expression of Ciita and the MHCII gene H2-Aa (**Figure 1**). We found 3 of the 4 sgRNAs induced Ciita expression by at least 10 fold compared to non-targeting controls. Even more importantly we found that the same 3 sgRNAs significantly induced the expression of MHCII by 10-20 fold. Thus, our screen effectively identified a key regulator of MHCII through the enrichment scheme we developed.

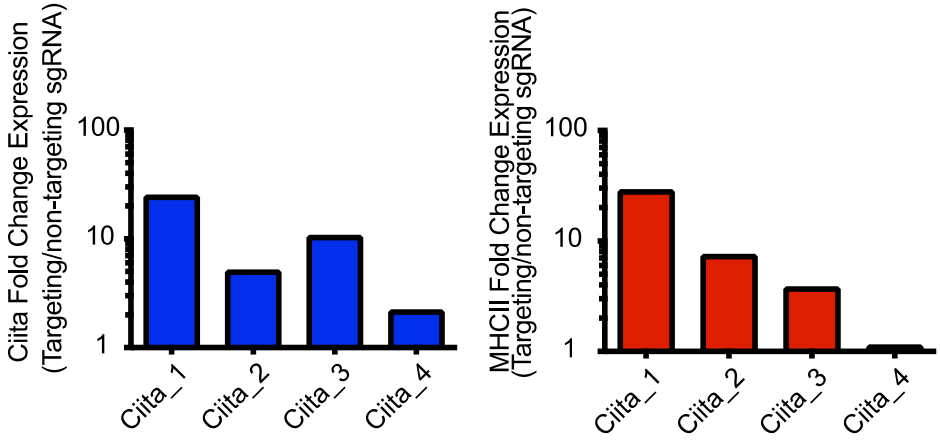


**Figure 1. Distinct sgRNAs targeting Ciita differentially induce MHCII.** Four independent sgRNAs targeting Ciita were cloned into the dCas9 macrophage line. Following selection RNA was isolated and the relative expression of Ciita and MHCII (H2-Aa) were quantified by qRT-PCR

In addition to Ciita we have generated >50 cell lines targeting a range of genes identified in the screen. We are in the beginning phase of validating these genes and classifying their impact on MHCII expression. Already we have identified 3 genes with sgRNAs that induce Ciita or MHCII almost two fold (**Figure 2**). Given the tight regulation of MHCII expression it is expected that most genes will not drive MHCII expression as highly as Ciita but any expression is consistent with our screens results. These genes, Snx29, Neu2 and Ssbp4, likely function through distinct mechanisms which will be the next focus of our studies in understanding the mechanisms by which each gene controls MHCII expression.

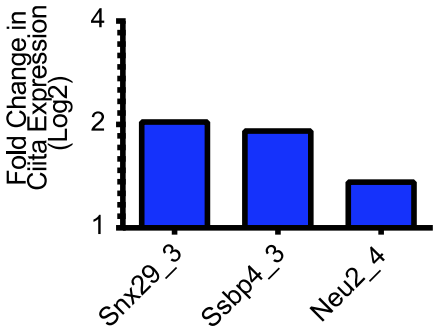


**Figure 2. Distinct sgRNAs targeting Candidate genes induce Ciita.** Candidate sgRNAs for the indicated genes were transduced into dCas9 macrophages. Following selection RNA was isolated and the expression of Ciita was quantified and fold change was determined compared to control cells.

Overall our studies characterizing the MHCII high library are generally on the timelines proposed, even with significant delays due to Covid-19. We have streamlined our validation and characterization strategy such that we are combining a subset of these studies into single experiments. Thus, we expect these experiments to

completed in the next several months. With the remaining time on the award (~8-9 months) we will focus on Aim 1.2 described in the proposal which is characterizing the mechanisms of

2. Given the surprising results that infection of the MHCII high library with M. tuberculosis did not result in changes in MHCII expression in resting macrophages, we further expanded our gain-of-function approach to examine previously described immune evasion functions of Mtb, namely inhibiting IFNγ-mediated MHCII and CD40 expression. Several previous studies showed Mtb infection blunts the effective induction of these molecules. In addition, recent work suggests that the expression of PD-L1 expression on macrophages directly modulates Mtb-specific T cell activation. We reasoned that using our gain-of-function and loss-of-function libraries to identify genes that increase or suppress IFNγ-mediated expression of these markers would broaden our ability to overcome Mtb virulence tactics and more finely tune subsequent T cell responses (**Figure 3**) . By screening for parallel surface markers, we also will be positioned to identify broad and specific regulators of surface protein expression in macrophages, similar to an approach we have taken in separate studies using a loss-of-function library we developed. Over the last year we completed replicate screens using the gain-of-function library to identify genes that when expressed modulate the IFNγ-mediated induction of MHCII, CD40, and PD-L1. Using an identical validation scheme to that described in section 1 and in the proposal, we are validating a specific hits and broad regulators. While the loss-of-function studies are further along, we anticipate full validation of the loss- and gain-of-function in the next 3-4 months. The advantage of expanding our screening approach is that it will increase the likelihood that we will identify new genes or pathways that are capable of subverting M. tuberculosis immune evasion. This will increase our likelihood of overall success in this proposal, and presents a range of new datasets for us to explore into the future to broadly reach our overall goals within this proposal. Already, we have identified a new pathway of immunometabolism that is required for effective IFNγ responses and requires mitochondrial complex I of the electron transport chain. Deleting Complex I directly alters Mtb replication and the response to IFNγ which we will continue to examine in the future beyond the end of this award (**Figure 4**) . Taken together, our functional genetic approach has proved incredibly fruitful to identify new mechanisms that regulate macrophage responses and modulate host-pathogen interactions during Mtb infection.

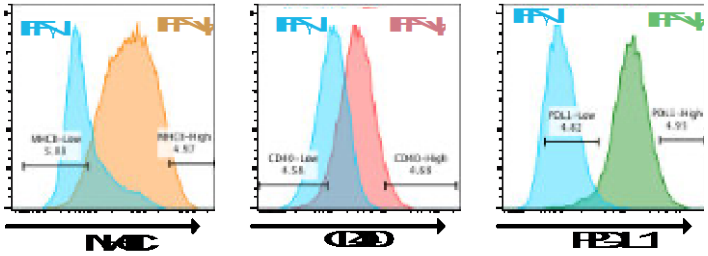


**Figure 3. Sorting of IFN-gamma induction of MHCII, CD40 and PD-L1.** Shown are representative histograms of the induction of key markers that modulate T cell function after IFN-gamma activation. Both the gain-of-function and loss-of-function CRISPR Cas9 libraries were stimulated and the top and bottom 5% of cells for the indicated markers were sorted and sequenced to find regulators that may bypass Mtb mediated T cell inhibition.

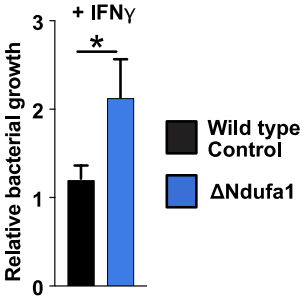


**Figure 4. Cell autonomous responses are altered in ΔComplex I cells.** Cells were infected with Mtb-Lux then treated with IFNγ 4 hours later. Shown is the relative growth over 3 days compared 4hrs as determined by luciferase production. **p<.01 *p<.05 by two-tailed t-test.

3. One of the major goals for our first year of funding was optimizing ex vivo T cell assays that could be used to dissect how distinct macrophage pathways modulate the T cell responses during Mtb infection. Using T cells derived from TCR-transgenic mice, we developed an assay that effectively allows us to examine how changes in macrophage antigen presentation machinery directly affect the effector function of CD4+ T cells (**Figure 5**). This assay is highly specific and requires macrophages to express

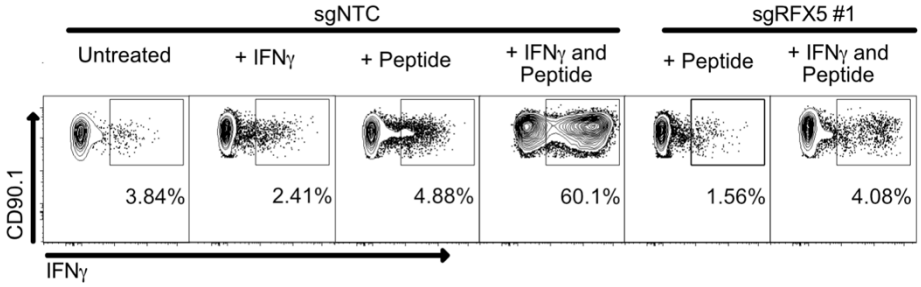


**Figure 5. Optimization of Macrophage/T cell activation *ex vivo* assay.** TCR transgenic T cells were isolated and incubated with control macrophages or macrophages that are unable to induce MHCII (sgRFX5) from the indicated conditions (+/- peptide +/- IFN-gamma). Intracellular cytokine staining was then used to examine the activation of the TCR transgenic T cells by quantifying IFN-gamma production.

high levels of MHCII and be pulsed with peptide. The optimization of this assay has allowed us to lay the groundwork to understand how distinct genes identified in our functional genetic approaches directly alter the host response to Mtb infection and modulate TB disease and pathogenesis. We have begun examining how distinct pathways such as the Complex I immunometabolism pathway directly alters intracellular control of Mtb during infection this has led us to the finding that lacking complex I and Oxphos blunts CD4+ T cell mediated control of Mtb. This finding is significant, and we are currently investigating the role of this pathway in vivo in mice lacking complex I or are edited for distinct complex I subunits.

**Summary:** Even with some slight delays due to covid, we are on track with our project and we expect to meet all milestones laid out in our statement of work by the completion of the award. We have expanded our functional genetic screens and have a high number of confirmed candidates that alter MHCII expression or co-stimulatory/co-inhbitory receptors, one of these projects has already resulted in a pre-print (see Appendix) that is currently in revision at eLife and will likely be published soon. Over the next year of funding we will employ the optimized ex vivo T cell assays in combination with in vivo experiments to better understand how candidate genes that modulate the interface between APCs and T cells changes TB disease.

**C.** The award has allowed many training opportunities for my graduate student Laurisa Ankley to present her research in several forums including internal department seminars, international meetings including the international cytokine society, the American Association for Immunologists annual meeting and a Keystone meeting on innate immunity. Furthermore, a recently hired post-doc is following up on aspects of the project which will help me train him and my graduate student in many professional activities including obtaining more funding, drafting and submitting manuscripts as well as career development and networking opportunities. I continue to meet weekly with all lab members to provide mentorship in experimental design and professional development. Annually I hold a state of the lab meeting to educate all lab members on the inner workings of running a lab. This includes describing budgeting and strategic planning that many students do not formally receive in their training but is essential for their success in science.

**D**. Nothing to report.

**E**. In the final year of funding for this project we have two major milestones left to accomplish all the goals described in the original proposal.

I. As we identify high priority hits from all the functional genetic screens identified above we will examine their mechanisms of action and dissect their importance to host-pathogen interactions during Mtb infection. We already have several high priority candidates including the Complex I pathway, FosB, and Pygo2 which will validate and followup in detail. These follow up studies will allow us to identify how these genes control antigen presentation machinery, identify if driving their expression with our gain-of-function approaches improves T cell effector function during Mtb infection and gain a broader understanding of the regulatory pathways that control antigen presentation during infection.

II. The last milestone in our project is understanding how candidate genes modulate the in vivo T cell response to TB and disease progression. Studies are already underway to understand complex I in TB disease as described above. We are finalizing in vivo gene editing to broaden our candidates for follow up in vivo and we anticipate these experiments will be optimized by the fall allowing 6 months to complete the in vivo TB experiments as described in the proposal. By using a combination of already constructed mutant mice, and leveraging bone marrow editing we are uniquely positioned to understand how any high priority candidate functions in vivo during Mtb disease. Because of the unique TCR-transgenic resources, we will be able to rapidly test how candidate genes directly alter Mtb specific T cell activation and effector function during Mtb infection and identify new pathways that can be targeted to overcome immune evasion.

4. **Impact:**

   **A.** Using our functional genetic approaches with both loss-of-function and gain-of-function libraries has broadly impacted our understanding of regulatory networks that control antigen presentation in macrophages. Our initial success with these libraries led us to further expand these approaches to understand how key cytokines seen during Mtb infection further modulate the expression of important molecules beyond just MHCII, such as CD40 and PD-L1. By expanding our focus on a range of important pathways we are now uniquely positioned to broadly understand the complicated regulatory networks that modulate T cell functions during Mtb infection. The long-lasting impact of these findings will allow us to identify new host-direct targets that can be modulated during infection and result in better disease outcomes and improved protective immunity. Given the fact that Mtb continues to kill over 1.5 million people each year, these findings have the potential to have a major impact on controlling TB disease in the future. Furthermore, optimizing the range of assays that dissect macrophage interactions with T cells will broadly impact other important diseases that T cells play a critical role in controlling such as autoimmunity and cancer progression. Thus the impact of our findings to date already will impact a range of immunological fields.

   B. Nothing to Report

   C. Nothing to Report

   D. Nothing to Report

5. Changes/Problems:

   A. The only change to our proposal is expanding our scope to include cytokine activated conditions and other important molecules that modulate T cell function during Mtb infection. These changes will increase the impact of our findings, and given our skill in conducting these screens only took 2 months to complete. Thus by expanding the scope we are even better positioned to modulate the APC/T cell interface and overcome Mtb-mediated immune evasion even if driving MHCII alone does not significantly alter TB disease.

   B. The lab was shutdown for 8 weeks due to Covid-19 which did put us slightly behind our original timelines/milestones. However, with a new post-doc hire who is skilled in BSL3 work, we are already making up lost ground and we do not anticipate any further delays on the project in the coming year.

   C. Nothing to Report

   D. Nothing to Report

6. **Products**

**Publications, Conference Papers and Presentations**

   A. Preprint Posted at BioRxiv. Kiritsy MC, Mott D, Behar SM, Sassetti CM, **Olive AJ.** (Updated 11/24/20). Mitochondrial respiration contributes to the interferon gamma response in antigen presenting cells. Pre-print BioRxiv. DOI:https://doi.org/10.1101/2020.11.22.393538**. In Revison at eLife.**

   B. Oral Presentation. Dissecting the contributions of IFNγ in the outcome of *Mycobacterium tuberculosis* infection. Invited Seminar. Senda Biosciences. April 2020.

   C. Oral Presentation. Mechanisms of interferon gamma-mediated protection during *Mycobacterium tuberculosis* infection. **Boston TB Meeting. January 2021**

No other products to Report.

## 7. 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:** 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:** Justin Trombley
**Project Role:** Technician
**Research Identifier:** https://orcid.org/0000-0002-3856-3138
**Nearest Person Months Worked:** 5
**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:** 3
**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

## 8. Special Reporting Requirements:

Nothing to Report