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Dissecting *Mycobacterium tuberculosis* immune evasion of dendritic cell-T cell crosstalk

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Dissecting *Mycobacterium tuberculosis* immune evasion of dendritic cell-T cell crosstalk

By

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Bachelor of Science, University of Florida, 2016

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Abstract

Dissecting *Mycobacterium tuberculosis* immune evasion of dendritic cell-T cell crosstalk

By Ana Beatriz Enriquez

Mycobacterium tuberculosis (Mtb), is the causative agent of tuberculosis (TB), an infectious disease that continues to cause a significant burden to public health worldwide. While the only licensed vaccine, BCG, is able to prevent disseminated disease in children, it is ineffective at preventing pulmonary TB. Current efforts to develop efficacious vaccines and adjuvants are hindered by a lack of understanding of the mechanisms that contribute to protective immune responses against TB. Moreover, we need to understand how to overcome Mtb evasion strategies in order to induce protective immunity through vaccination. Therefore, insights that address these critical gaps in knowledge are necessary to advance the development of efficacious TB vaccines and adjuvants.

Recent studies have identified a critical role for $CD4^+$ T helper 17 (Th_{17}) cells in protective immunity to TB. However, Th_{17} responses are limited during Mtb infection and the molecular signals that contribute to restricting these responses are poorly understood. Mtb limits Th_{17} responses during infection by impairing the functions of dendritic cells (DCs), which are important for presenting pathogen-derived antigens and for initiating T cell responses. Our laboratory has previously demonstrated that Mtb restricts Th_{17} polarization by impeding CD40 signaling on DCs, and that exogenous engagement of the CD40-CD40L pathway enhances Th_{17} polarization during Mtb infection. However, the molecular basis for CD40-dependent Th_{17} polarization remained unknown. We found that the canonical Notch ligand, DLL4, is upregulated downstream of CD40 signaling on DCs and is necessary for Th_{17} polarization. Exogenous CD40 engagement increased *Dll4* mRNA and surface expression of DLL4 on Mtb-infected DCs. Blocking DLL4 impaired the ability of DCs to polarize Th_{17} responses *in vitro* and *in vivo* in mouse models of TB. Interestingly, DLL4 blockade also led to a significant reduction in the polarization of multifunctional Th_{17} responses. We then elucidated the mechanism by which Mtb impedes these responses and found that the Mtb Hip1 serine protease limits DLL4 expression by impairing CD40 signaling on DCs.

Having demonstrated that engaging CD40 augments protective immune responses during infection, we then used RNA sequencing to elucidate additional transcriptional mechanisms downstream of CD40 on DCs that contribute to enhanced T cell functions. We found that engaging CD40 on Mtb-infected DCs led to reprogramming of the DC transcriptome, including significant increases in the ability of DCs to secrete Th_1- and Th_{17}-polarizing cytokines and DC migration-inducing chemokines, and upregulation of co-stimulatory molecules. Moreover, we found that exogenously engaging CD40 on DCs led to a significant increase in the capacity of T cells to proliferate, and that blocking key co-stimulatory molecules led to a significant decrease in proliferation.

Collectively, these insights address critical gaps in knowledge by elucidating the molecular mechanisms that dictate polarization of protective T cell responses during Mtb infection. Moreover, we have demonstrated how Mtb is able to evade host immunity by impairing DC-T cell crosstalk and have shown that engaging CD40 is able to overcome pathogen evasion to induce critical DC functions. The insights generated here will contribute to the development of more efficacious TB vaccines and adjuvants for TB.

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Introduction

Mycobacterium tuberculosis (Mtb) is the causative agent of tuberculosis (TB), an infectious disease that remains a threat to public health worldwide. In 2020, 9.9 million individuals fell ill with TB[1]. In the same year, 1.5 million individuals, including those co-infected with HIV, died as a result of TB[1]. Although TB deaths have been steadily declining, those recorded in 2020 were particularly concerning as they represent an increase compared to the previous year. In fact, TB deaths in 2020 were up 5.6% compared to 2019 records, the first increase observed in over two decades since 2005[1]. While the rise in cases can be attributed to disruptions in care due to the current outbreak of SARS-CoV-2 and COVID-19 pandemic[2], it highlights the necessity of prioritizing TB research. Importantly, the failure to meet public health goals and the rise in deaths underscore the need to develop effective TB vaccines and adjuvants as a strategy to limit disease burden worldwide.

This chapter will cover different topics related to TB, from diagnosis and treatment to pathogenesis and disease. It will also cover TB immunology and summarize the function of innate and adaptive cells that have been identified to play critical roles in protective immunity. Importantly, it will discuss how Mtb has evolved different strategies to limit the function of innate immune cells in order to dampen protective immune responses. Finally, it will end with a summary on the progress in developing the next generation of TB vaccines.

Part 1 – Tuberculosis Disease, Treatments, and Diagnostics

Part 1.1 – *Mycobacterium tuberculosis*

In 1882, Dr. Robert Koch presented a ground-breaking discovery: TB is caused by the bacterial pathogen *M. tuberculosis*[3]. This discovery set the stage for future studies that characterized the causative agent of an important infectious disease. Mtb is a slow-growing, rod-shaped bacterium with a peculiar lipid-rich outer membrane[4]. While other bacteria are classified according to their ability to test negative or positive on Gram staining, the outer membrane of Mtb precludes the use of these tests and is therefore considered "acid-fast[5]." Diagnostic testing therefore requires acid-fast stains such as Ziehl-Neelsen or fluorochrome staining (fluorescent dye) in order to definitively identify the presence of acid-fast mycobacteria[5]. Progress in characterizing Mtb has been challenging in comparison to other bacterial pathogens, in part due to its longer doubling time (i.e., ~21 hours in liquid culture, 3 weeks for a colony to appear on a plate), and the need for Biosafety Level-3 facilities (BSL3) for handling virulent mycobacteria and performing experiments. TB research has benefited greatly from key developments such as genome sequencing[6] and the application of newer technologies such as CRISPR[7] which allows researchers to genetically manipulate Mtb genetics to better understand microbial functions. Genome sequencing highlighted some important characteristics of Mtb. When all 4,043 genes of Mtb were annotated, 17% of these genes encoded proteins that functioned in cell wall processes[6,8]. Moreover, 28% of the genes were found to encode proteins related to metabolism (intermediary and lipid) and respiration[6,8]. While these provide clues about important processes for Mtb lifestyle, 52% of annotated genes have yet to be assigned a function[6,8]. Further research into the functions of these genes will undoubtedly yield critical insights that will allow us to better understand bacterial functions of Mtb.

Part 1.2 – Transmission and Disease

Koch's landmark discovery in the late 19th century that TB is caused by the bacterium Mtb[3] challenged a long-held notion that TB transmission is genetic. However, how exactly Mtb was spread had yet to be empirically demonstrated. Cornerstone studies in the 1960's using guinea pigs exposed to air from TB sanatoriums were crucial to establishing that TB is transmitted via the aerosol route[9,10]. It is now well established that TB is spread through aerosolized particles containing the bacterium that are expelled by individuals experiencing TB disease, through coughing or other actions such as talking and singing[11]. The efficiency of transmission, however, is dependent on a variety of genetic and environmental factors and not all individuals are uniformly infected after being exposed[12]. When infection is successful, aerosol particles containing Mtb bacteria enter the airways. Mtb is recognized and phagocytosed by alveolar macrophages, which transport the bacteria into the lungs[13]. Once in the lungs, additional innate immune cells are recruited to the site of infection, such as dendritic cells (DCs). Interactions between innate, epithelial cells and Mtb forms the basis of granulomas, the characteristic hallmark structures of TB disease. Granulomas serve dual purposes: they aid the pathogen by providing a protected niche in the lung and they also aid the host by effectively controlling further spread of the pathogen[14]. Granulomas typically consist of a "core" that is composed of infected innate immune cells (such as macrophages and neutrophils), that is surrounded by additional innate immune and epithelial cells which are surrounded by a lymphocytic "cuff" that consists of T and B cells[15]. These structures are highly heterogenous, both between different individuals and within the same individual[16]. In most cases, following exposure, the immune system is able to mount a response that successfully controls (albeit does not clear) infection. This results in a disease state known as latent tuberculosis infection (LTBI), in which the individual is not infectious and does not develop symptoms but continues to harbor low levels of

persistent bacteria within macrophages. However, in 4% - 14% of individuals exposed to an index case of TB develop primary active tuberculosis (ATB)[17] disease. ATB is characterized by the inability of the immune system to mount a response that controls infection, thereby leading to excessive bacterial replication and tissue damage in the lungs. Individuals with LTBI can also re-activate to ATB due to conditions such as co-infection with HIV and taking immunosuppressant drugs. While TB is primarily a pulmonary disease, uncontrolled bacterial infections can disseminate to other parts of body allowing Mtb to infect other organs in what is known as extrapulmonary TB, or EPTB. Individuals with ATB are infectious and develop mild to severe symptoms including gradual weight loss, productive cough and breathlessness[18]. Although disease is often presented as a dichotomy between either latent or active for simplicity, TB is complex and disease is more accurately represented as a continuum of infection states ranging from incipient or subclinical TB to full-blown symptomatic ATB[18]. Incipient TB represents individuals that are likely to progress to ATB but have not yet developed symptoms or test positive on diagnostic assays[19] while subclinical TB represents those who are asymptomatic but test positive for TB on diagnostic assays[19]. Although this continuum of disease states presumes that infection has occurred, some individuals appear remain uninfected despite prolonged or repeated exposure to Mtb[20]. While individuals with this phenotype have been termed “resisters”, it is currently difficult to definitively prove the absence of infection. Nevertheless, studies on these cohorts have provided insights into immune mechanisms that may contribute to immune control and protection[20].

Part 1.2 – Tuberculosis Diagnosis

Current TB diagnostics aim to diagnose the following infection states: LTBI, drug-susceptible ATB or single/multidrug resistant TB[18]. Patients suspected of having ATB typically exhibit characteristic disease symptoms, particularly 2 weeks of coughing, fever, and night sweats. A sputum sample is collected for determining the presence of Mtb and radiological assessments such as chest X-rays or more advanced computed tomography (CT) scans will be used to screen for the presence of granulomas or lung damage[18,21]. The presence of Mtb in sputum can be assessed by smear microscopy, which is the least sensitive and consists of visualizing acid fast-stained sputum under a microscope or the more sensitive PCR-based Gene-Xpert tests which amplify Mtb nucleic acid[22]. Smear microscopy is unable to detect extrapulmonary TB, is not effective in patients who are co-infected with HIV, and poorly detects pediatric TB[18,23]. Given these drawbacks, diagnosis now favor the using PCR-based tests that detect Mtb-specific genes known as nucleic acid amplification tests (NAATs)[18,22]. This is also the technology used in tests to diagnose single-drug resistance. Xpert MTB/RIF is used not only to detect Mtb quickly but is used to detect rifampicin resistance[22]. However, because treatment regimens consist of multiple antibiotics and Mtb is able to develop resistance to all of these concurrently, additional methods to test antibiotic resistance include culturing sputum on different solid growing medium with different antibiotics to determine resistance, known as drug-susceptibility testing[22,24]. The gold standard for diagnosis of TB is currently culture-based microbiological assessments such as BACTEC or MGIT assays which can take 4-6 weeks. In these tests, sputum is collected, processed, and cultured in order to check for replicating bacteria[22,25,26]. These tests are considered the gold-standard as they not only test for the presence of bacteria but also for viability.

Although no specific test can definitively diagnose true LTBI, tests for LTBI primarily focus on detecting prior exposure to Mtb antigens by eliciting memory responses. The Mantoux Tuberculin Skin Test (TST) consists of injecting a combination of Mtb proteins, known as purified protein derivative (PPD), intradermally and assessing the size of induration after a few days[18]. This test, however, is affected by confounding variables such as vaccination with the licensed TB vaccine bacillus Calmette-Guérin (BCG) or previous infection with nontuberculous mycobacteria (NTMs), which can elicit similar responses to Mtb infection and could result in a false positive[18]. Prior Mtb exposure is best measured using Interferon Gamma Release Assays, or IGRAs. These tests require collection of whole blood or PBMCs and subsequent stimulation with ESAT-6/CFP-10 antigens, which are specific to Mtb and absent in BCG[18]. The release of IFN-γ or the number of cells expressing IFN-γ is then measured and positivity is determined[18]. Additional tests that serve in a limited function include testing for the presence of LAM in patient urine samples, which is an Mtb-specific cell wall molecule[22]. Although IGRAs are more specific than TSTs, these assays cannot distinguish between LTBI and ATB and are not able to provide important information such as risk of progression to disease[22].

Critical to the control of TB is the ability to accurately diagnose disease state. While some diagnostics are available, each of these assays carry important limitations and are unable to address crucial TB questions. These tests are unable to definitively discriminate between LTBI, ATB, incipient, or subclinical TB. Importantly, while sputum-based assays are widely used, these are unable to be used to diagnose LTBI, EPTB, or TB in certain populations such as children[22]. A more effective means of diagnosis is through the use of non-sputum-based biomarkers. Biomarkers are measurable markers (either host or bacterial) that can serve as

indicators of specific conditions. Research on biomarkers for TB diagnosis have yielded promising results[27]. Various studies have highlighted how specific markers on T cells can serve to distinguish between LTBI and ATB[28-31] and monitor treatment response. A recent tour-de-force study on RNA sequencing on whole blood was able to generate markers that distinguish between incipient/clinical/subclinical TB and even response to treatment[32]. To better be able to develop diagnostic biomarkers, more work needs to be done to characterize host and pathogen immune responses during different disease states.

Part 1.3 – Tuberculosis Treatment

The mid-20th century discovery that the antibiotic streptomycin was able to effectively kill Mtb ushered a new era of treatment for TB[33]. However, scientists quickly observed resistance to antibiotics administered singly[34]. This observation and a subsequent trial demonstrating the effectiveness of combinatorial antibiotic chemotherapy[35-37] set the precedent for the multi-drug treatment regimens recommended today. The current standard treatment regimen for drug-susceptible TB, in place since the 1990's, consists of 4 antibiotics: rifampicin, isoniazid, pyrazinamide and ethambutol[38]. Patients take all four antibiotics in what is considered the "initiation" phase of treatment for 4 months while the "continuation" phase of treatment requires only rifampicin and isoniazid to be taken for 2 months[39]. While the TB treatment regimen is significantly longer compared to treatment regimens for most other bacterial infections (6 months compared to ~1 week[40]), it is highly effective with a 76% - 85% rate of effectiveness according to a meta-analysis[41] when administered as Directly Observed Therapy (DOT). TB treatment is longer due to the slow-growing nature of Mtb, which requires administering antibiotics for longer in order to clear bacteria[42]. Successful completion of treatment is also

necessary to prevent emergence of resistant Mtb strains. Although antibiotic resistance in Mtb has been documented since the 1940s[34] recent decades have seen the emergence of multi- and extensively drug-resistant strains of Mtb (MDR- and XDR-TB)[43]. Treatment regimens for MDR/XDR-B need to be tailored to the antibiotic-susceptibility profile of Mtb isolated from each patient. In the case of rifampicin mono-resistance, for instance, patients need to take isoniazid (or another first-line antibiotic) for 18 months[21]. In cases of MDR-TB, second-line TB drugs are used, which are generally regarded as weak in effectivity but highly toxic, which requires some to be taken for up to 24 moths[43]. Apart from being a more challenging treatment regimen, the effectiveness of treatment lowers with resistance. The most recent statistics demonstrate MDR-TB treatment is 59% effective while XDR-TB treatment is 20% effective[1,44]. While these statistics are alarming, advances have been made to develop new, effective drugs for drug-resistant TB. The drug Bedaquiline was recently approved by the US Food and Drug Administration to be used in conjunction with other prescribed drugs for drug-resistant TB[43].

Although patients who are experiencing LTBI are not symptomatic or infectious, there is a 10% lifetime chance of re-activation to ATB[18]. Current treatment regimens for LTBI include similar drugs to those used for ATB but are often administered for a shorter timeframe[45]. There are currently two different treatment courses for LTBI: 3HP and 4R[45]. 3HP requires taking isoniazid rifapentine weekly for 3 months, while 4R requires a daily dose of rifampicin for 4 months[45]. Although there is also a treatment regimen that consists of daily doses of isoniazid for 6-9 months, this treatment is often least recommended due to issues such as length and risk of liver damage[45]. In fact, the original trial for 3HP treatment was found to be more effective in preventing re-activation while the 4R treatment trial found this regimen was comparable to the 9-

month isoniazid treatment for LTBI[46,47]. Future treatment regimens for LTBI consist of developing antibiotic courses that are even shorter in length. For instance, a recent study outlined the 1HP treatment regimen, which requires a daily dose each of isoniazid and rifapentine for 1 month[45]. While this trial found the treatment regimen to be comparable to 9-month isoniazid[48], it is yet to be known how effective this regimen is outside of the study.

Part 2 – The Immune Response to Tuberculosis

Part 2.1 – Role of Innate Immune Cells in *Mycobacterium tuberculosis* Infection

Innate immunity plays a critical role in Mtb infection by contributing to pathogen control and dictating adaptive immunity. This section covers the role of select innate immune cells and how these interactions determine TB disease. Specifically, it covers the role of innate immune cells who have well-established roles in TB immunity and whose function shapes the outcome of infection and T cell responses.

Macrophages

Macrophages play a pivotal role in the immune response to Mtb by dictating the course of infection and provide a niche for intracellular replication of Mtb. Among the first cells that Mtb encounters in the airway are alveolar macrophages (AMs), a subset of pulmonary macrophages that are often described as the first line of defense in respiratory infections[49]. A recent study in mice using a fluorescently-labeled Mtb strain found that AMs are a major target of Mtb early in infection and their movement to the lung precedes infection of other innate immune cells with Mtb, thereby demonstrating the central role of AMs in disseminating Mtb[13]. While AMs promote

initial Mtb replication, once in the lungs Mtb also infects interstitial macrophages (IMs). In contrast to AMs, IMs have a less permissive environment which restricts Mtb replication, as demonstrated by a study which found depletion of IMs resulted in increased bacterial burden[50,51]. While the primary function of macrophages is to phagocytose and clear microbes through antimicrobial activities, macrophage responses can also shape adaptive immune responses. Through use of surface recognition receptors, which include toll-like receptors (TLRs) and C-type lectin receptors (CLRs), macrophages recognize and engulf Mtb[52] into a phagosomal compartment. Mtb inhibits phagosome-lysosome fusion via multiple virulence factors, thus subverting macrophage microbicidal functions. [53]. In addition, recognition of Mtb through surface receptors also promotes pro-inflammatory cytokine secretion including TNF-α, IL-6, and IL-12[54,55]. TNF-α secretion during infection is important for stimulating production of other cytokines such as IL-1 and IL-6, inducing chemokine secretion (to recruit additional immune cells), and granuloma integrity[55-57]. IL-6 and IL-12 in addition to being important for inducing pro-inflammatory pathways also function in skewing T-helper (Th) cell responses[55,58,59]. Other methods that macrophages use to eliminate bacteria include the production of reactive oxygen species and reactive nitrogen intermediates which also have antimicrobial functions[54].

Dendritic Cells

Dendritic cells (DCs) are a subset of monocytic immune cells termed "professional antigen-presenting cells" due to their enhanced capability of initiating T cell responses through the following key functions: processing and presenting antigen, upregulating co-stimulatory molecules and producing cytokines and chemokines. These signals function to activate naïve CD4 T cells and promote their differentiation and polarization to specific T-helper (Th) subsets.

DCs are among the first immune cells that comes in contact with Mtb following translocation of bacteria to the lungs[13,60,61]. Like macrophages, DCs are able to recognize Mtb through binding of bacterial antigens to different surface recognition receptors with DC-SIGN serving as the primary receptor[62]. While Mtb is able to infect DCs *in vitro* and *in vivo*[58,60,62-67], Mtb replicates at low levels within these cells compared to macrophages[67] and are less adept at restricting the growth of bacteria[66,68]. Following infection, DCs are then able to upregulate functions important for activation of T cell responses, including co-stimulatory molecules and cytokine production[58,60,63,66]. These cytokines include TNF-α and IL-12, which we introduced earlier as important for shaping immune responses[66] while IL-12 also functions in DCs to promote migration to the lymph node[69]. The critical role of DCs in initiating T cell responses during Mtb infection is well-established[70]. In fact, the earliest studies on DCs in the context of Mtb infection found that these cells were superior to macrophages at inducing T cell proliferation *in vitro*[58,66,71]. An early *in vivo* study in which DCs were depleted using a diptheria toxin deletion of CD11c (a pan-DC marker) demonstrated that deleting these cells led to a delay in T cell responses[72]. Subsequent studies have found that the migration of DCs to the lymph nodes (LNs) precedes the detection of T cell resposnes[60,73]. Overall, while DCs do not contribute to bacterial control, they are necessary for initiating T cell responses and carrying out effector functions that shape TB protective immunity.

Neutrophils

A study examining pulmonary TB patients found that infected neutrophils are the predominant subset of cells found in sputum[74], thereby demonstrating that these cells are readily found in the lung during infection. Despite their abundance, neutrophils are a challenging population of cells

to study due to their short-lived lifespan. Current literature on neutrophils in the field has revealed that these subsets are more pathogenic than protective during TB[75,76]. A study on the peripheral white blood cells in household contacts found an inverse correlation between neutrophil count and developing pulmonary TB[77]. While the formation of neutrophil extracellular traps (NETs) can be beneficial in other bacterial infections to promote clearance[78], in the case of TB these NETs were more likely to be found in the plasma of ATB patients compared to LTBI or healthy controls[79]. A study found that neutrophils, through secretion of S100A8/A9 proteins, mediate inflammation and disease in ATB, in both NHP and mouse models of TB[80]. Mtb readily infects neutrophils[74], thereby further recruitment of neutrophils provides a pool of cells that can maintain infection and promote tissue pathology. In addition to the pathogenic role of neutrophils, a few studies have highlighted some beneficial functions of neutrophils in TB[76,81]. Moreover, a study found that depletion of neutrophils in mice *in vivo* led to reduced trafficking of DCs to the lymph nodes and demonstrated that neutrophil-dependent antigen (by ingesting infected neutrophils) facilitated activation of antigen-specific T cells[82]. Interestingly, separate studies have revealed that apoptotic neutrophils enhance immune responses by increasing antimicrobial infections of macrophages[83] and by releasing bacteria that can be engulfed by DCs and speed up T cell priming[84]. It appears that while infecting neutrophils provides a cellular pool that maintains infection, the infection and death of these cells also promotes immunity. Overall, studies suggest that fine-tuning neutrophil responses is an important component of a protective immune response.

Additional Innate Immune Subsets

While the role of macrophages, DCs and neutrophils is relatively well characterized, the recent literature, particularly studies using single cell technologies, has allowed us to discover additional innate immune cells that contribute to the immune response to TB. Myeloid-derived suppressor cells (MDSCs) are a heterogenous population of cells that possess suppressive functions[85]. Although these populations, which consist of monocytic and granulocytic subsets, were originally characterized in different cancer models, their role in infectious diseases has recently come to be appreciated[86]. While the presence of suppressive myeloid cells have been observed in the context of TB for decades[87], a publication in 2013 was the first to formally characterize suppressive cells as MDSCs[88]. In the study, authors found increased percentages of MDSCs in active TB patient samples that when co-cultured with T cells resulted in reduced activation[88]. Subsequent studies have found that MDSCs correlate with suboptimal immune responses[87]. While more remains to be known about the role of MDSCs in TB, these subsets are likely to play a role in shaping protective versus pathogenic responses.

Part 2.2 – Role of Adaptive Immune Cells in *Mycobacterium tuberculosis* Infection

Adaptive immunity is critical to controlling Mtb infection. This section covers the most critical components of protective immunity in the context of TB.

CD4 T cells

The role of CD4 T cell in protective immunity to TB is undisputed. Depletion of CD4 T cells in mice exacerbates Mtb infection[89-92] and has been shown to be detrimental for disease in NHP models[93]. The role of CD4 T cells in humans is also highlighted in Mtb-HIV co-infection, where CD4 T cell depletion leads to a higher risk of re-activation from LTBI as well as a higher risk of

developing TB and increased mortality[18,94,95]. Moreover, CD4 T cells provide "help" for CD8 T cells[96-98] and specific T helper (Th) subsets contribute to protective immunity. Following activation, naïve T cells differentiate into distinct Th cell subsets. Studies in mice demonstrated that infection with Mtb primarily results in an IFN-γ-producing Th_1 response which is critical for antimicrobial immunity[99]. Infection of IFN-γ-knockout mice results in significantly higher CFU in the lungs, enhanced lung pathology and reduced survival compared to WT mice[91,100,101]. IFN-γ is also able induce nitric oxide synthase 2 (NOS2)[102] and autophagy[103] in infected macrophages to promote bacterial clearance. Moreover, individuals with genetic deficiency in IFN-γ, particularly receptors IFNγR1 and IFNγR2, have increased susceptibility to mycobacterial diseases and are considered to have Mendelian susceptibility to mycobacterial diseases (MSMD)[104-107]. Th_1 subsets are also able to produce TNF- α, which is important for containing infection. Mice deficient for TNF-α have increased susceptibility to infection,[108] and patients treated with anti-TNF-α antibodies exhibited reactivation TB[109], highlighting the importance of this cytokine in pathogen control and granuloma integrity. Although Th_1 responses are necessary for important aspects of TB immunity, these responses are insufficient for generating sterilizing immunity. This is exemplified by the MVA85A vaccine trial; despite eliciting strong IFN-γ responses, the vaccine failed to provide increase protection over placebo[110].

In addition to Th_1, research has found that Th_{17} responses are critical for beneficial immunity. The Th_{17} subset, originally discovered for its role in autoimmunity[111-113], produces cytokines IL-17A, IL-17F, IL-21, and IL-22[55]. Th_{17} cells and the cytokines they produce have been shown to be important for protective immunity during Mtb infection in mice[114-118], NHPs[119], and in human patients with TB[120-123].Several vaccine and adjuvant strategies that have been found to be

protective against Mtb infection are driven by induction of Th_{17} responses[124-126]. Th_{17} responses can also function in protection by synergizing with Th_1 responses[127]. In humans, Th_{17} cells also produce additional cytokines, such as IL-26, that have antimicrobial functions themselves or increase functions in cells that promote bacterial clearance[128,129]. In a model of *C. acnes*, Th_{17} cells were found to encode antimicrobial function genes and release extracellular traps, a method that could also function in Mtb but has yet to be demonstrated[130]. Moreover, individuals who have impaired IL-17 responses through genetic deficiencies have increased susceptibility to mycobacterial infections[131,132]. While early Th_{17} responses in the lung appear to be necessary for protective immunity, excessive IL-17 can also drive pathogenic responses during Mtb or BCG infection[133-135]. While the basis for pathogenic versus protective Th_{17} responses in Mtb infection remains poorly understood, literature from other fields suggest timing, location, and capacity to secrete other cytokines could play important roles[136].

Other Th subsets related to Th_{17} cells, including Th_{22} cells have been recently implicated in the immune response to Mtb. Th_{22} cells, originally discovered in inflammatory skin conditions, primarily secrete IL-22 (in the absence of other Th_{17}-producing cytokines)[137-140]. Although IL-22-deficiency did not exacerbate pathology or Mtb burden in mice in an initial study[141], a subsequent study found that IL-22 provides protection at chronic stages of infection as KO mice had significantly increased bacterial burden in the lungs compared to WT mice at 100 days post-infection[142]. IL-22 can promote protection by increasing the antimicrobial capacity of macrophages[143-145], promoting epithelial cell function, and recruiting macrophages and monocytes at later stages of infection[142]. A recent study found Th_{22} subsets were detected in PBMCs of LTBI patients and co-infection with HIV led to a reduction in these subsets[146]. We

have also come to appreciate the role of multifunctional T cells, or T cells that have the capacity to produce multiple cytokines. A human study found that Th_1* cells, or those that co-express IFN-γ and IL-17A, were increased in patients with LTBI compared to healthy controls[122] and $CXCR3^+CCR6^+$ Th_1-Th_{17} cells correlated with protection in an NHP model of LTBI[119]. While we have gained an appreciation of the contributions that these CD4 T cell responses make to protective immunity, a critical gap in knowledge exists on how exactly these immune responses can be augmented during infection. This information is necessary as Mtb actively dampens the polarization of these responses. Future research should aim to elucidate the mechanisms that lead to these responses so they may instruct the design of more efficacious vaccines and adjuvants.

CD8 T cells

While CD8 T cell responses were previously thought to play limited roles in protection, we have come to appreciate their contributions to TB immunity[147]. Mice that are deficient in β_2 macroglobulin, a component of MHC-I, succumb sooner following infection compared to WT mice[148]. Similarly, mice deficient in TAP-1 genes, which function in MHC-I antigen processing, have higher bacterial loads and reduced survival compared to WT mice[149,150]. A later NHP study also definitively showed that CD8 T cells contribute to protection by demonstrating that depleting CD8 populations decreased BCG immunity in a vaccination model and anti-tuberculous immunity in a re-infection model[151]. CD8 T cells contribute to protective immunity in a variety of ways. Human CD8 T cells express the cytolytic molecule granulysin, which was found to directly kill intracellular Mtb[152]. Another molecule, granzyme B, was found to be expressed by CD8 T cells in LTBI but not ATB patients[153]. Moreover, a study on human resected lung tissue found reduced CD8, granulysin and perforin in granuloma regions compared to

healthy tissue[153], suggesting CD8 T cells and their effector molecules are important for protection. CD8 T cells are able to express similar cytokines to CD4 T cells, such as IFN-γ, that contribute to protection[97]. Recent literature has also highlighted the ability of Mtb to subvert CD8 responses by modulating antigen. During infection, high frequencies of antigen-specific CD8 T cell responses are specific to the $TB10.4_{20-28}$ antigen of Mtb[154,155]. Despite constituting a significant portion of the CD8 pool, a subsequent study found that these cells are unable to induce antimicrobial functions in macrophages[156]. This demonstrates that Mtb modulates expression of antigens by secreting "decoy" antigens that stimulated non-protective antigen-specific CD8 T cells, thereby limiting the ability of CD8 T cell to provide protective immune responses.

B Cells and Antibodies

In the late 19th century, half a century before the advent of antibiotics, attempts at TB treatments took the form of serum therapy, in which serum from vaccinated animals was administered to patients in order to passively transfer antibodies. Many studies demonstrated sera were unable to successfully treat TB[157]. These early experimental failures, and subsequent notion that intracellular infections preclude the need for B cells and antibodies precluded the need to study these responses during infection[158]. Although most vaccination strategies against TB aim to elicit strong cell-mediated as opposed to humoral immunity[159], we have come to appreciate that B cells and antibodies can also contribute to protection. B cells are a multi-faceted group of cells that in addition to producing antibodies are able to present antigens and produce cytokines[158]. B cells can be found in granulomas, particularly in the lymphocytic cuff, demonstrating that they can influence responses at the site of infection[160-162]. A study in mice found that B cell deficiency

leads to increased neutrophil influx and excess IL-17 inflammation responses following infection, which suggests that B cell responses can fine-tune responses to prevent pathology[163]. Some of the strongest evidence for the role of B cells in protection are studies on inducible bronchus-associated lymphoid tissue (iBALT). iBALT are tertiary lymphoid structures that contain B cell follicles and have been suggested to be important for protection in other respiratory infections[164]. In primate models, iBALT structures were more likely to be found in LTBI vs ATB animals[165]. Moreover, formation iBALT structures are also a potential mechanism of protection by mucosal vaccines[166,167]. Similarly, recent studies have highlighted unappreciated roles of antibodies in protection. Health care workers that did not develop disease following Mtb exposure were able to produce antibodies that when transferred passively into mice led to moderate protection against challenge[168]. Antibodies from LTBI patients compared to ATB patients enhanced intracellular killing of Mtb by macrophages[169]. A subsequent study to try to elucidate the mechanism of protection following i.v. BCG found this vaccination strategy induced strong IgM loads in the lungs and that these correlated with reduced Mtb burden[170]. Another study found that the "resistors", or those patients who do not "convert" following exposure, have increased IgM and IgG antibodies compared to those who progress to disease[171]. Most recently, a study found that changes in antibody changes, specifically Fc glycosylation and IgG subclass, can be observed following TB antibiotic treatment[172]. Taken together, these data demonstrate that B cells and antibodies have novel roles in TB immunity.

Additional adaptive immune responses cell subsets

DURTs, or donor-unrestricted T cells, are cells that comprise three different lymphocytic populations that do not recognize "traditional" antigens which are restricted by MHC genetics[173].

DURTs include the following populations of cells: CD1-restricted T cells, mucosal-associated invariant T cells (MAITs), gamma-delta (γδ) T cells, and HLA-E restricted T cells[174]. γδ T cells are $CD3^+$ cells like conventional αβ (CD4/CD8) T cells but comprise a significantly smaller population of the T cell pool[175]. These cells are highly enriched in mucosal sites, including the lungs[175]. Although Th_{17} cells contribute to IL-17 production during infection, a critical early source of IL-17 is produced by γδ T cells in the lungs[176]. The role MAIT cells is less defined and studies have found contradictory results. While MAIT cells are able to produce IL-17 similar to γδ T cells[177], and some studies have found these cells to be reduced in TB patients[174], other studies in humans and mice found a dispensable role for these cells in protection[174,178-180]. Some of the better-defined ligands that bind CD1-restricted T cells are lipids belonging to Mtb[181]. The role of CD1-restricted T cells in protection against TB is not completely understood, and while a human study found these cells correlated with exposure but not necessarily disease state[182], isolated CD1 T cells from humans were able to induce intracellular killing of Mtb in macrophages[183]. While the role of HLA-E-restricted T cells in TB is even less known than other DURT members, HLA-E can activate CD8 T cells and these populations of cells can become activated and expand in tissues during infection[174].

Part 3 – *Mycobacterium tuberculosis* Immune Evasion Mechanisms

Upon entering the lung, Mtb encounters a hostile immune environment. Through thousands of years of co-evolution with humans, Mtb has effectively developed immune evasion strategies to subvert protective immune responses in order to promote infection. While only 2% of the annotated genes of the Mtb genome encode proteins that are only known to function in virulence, various proteins that serve other functions also serve to dampen immune cell function[6,8]. The

following section will cover how Mtb is able to impair the ability of key innate immune cells to perform vital functions during infection and will highlight some virulence factors which mediate these effects.

Part 3.1 – Modulation of macrophage responses

Although macrophages perform important functions for the host such as eliminating bacteria, carrying out these functions also paradoxically benefit Mtb. This is evident early in infection, as phagocytosis of Mtb by alveolar macrophages serves as a means of dissemination from the airways into the lungs[13]. Once inside, Mtb manipulates macrophage responses and subverts important functions to generate a protective niche[184]. Not only does making macrophages a protective home protect Mtb from immune response such as completement and antibodies, it also serves to protect it from antibiotics, an observation which has been documented since the 1950s[185]. Mtb enters macrophages through phagosomes, which eventually bind to lysosomes in order to degrade proteins and kill bacteria. Early macrophage studies in the 1970's revealed that Mtb remains in immature phagosomes in these cells and is able to replicate in these structures[186,187]. A recent study even found that Mtb is able to secrete a molecule known as 1-TbAd that coats bacteria and neutralizes acid in phagolysosomes[188]. Mtb is also able to secrete proteins that allow it to escape this compartment. The ESX-1 secretion system secrets important proteins, among them ESAT-6/CFP-10 heterodimer which aids in bacterial escape from the phagosome[184]. Literature also demonstrates that Mtb is able to manipulate macrophage response even before it is phagocytosed. Mtb recognition through the mannose receptor results in impaired ability of phagosomes to mature in macrophages[189]. A study found that Mtb uses lipids on its cell surface, such as PDIM, to recruit and infect macrophages that have less inflammatory and

dampened antimicrobial functions[190], thereby demonstrating that Mtb preferentially infects more permissive macrophages that promote disease. If inside a macrophage that is better able to perform anti-microbial functions, Mtb is able to impair the ability of these cells to upregulate co-stimulatory markers and pro-inflammatory cytokines[191]. Overall, Mtb subverts macrophages at both the recognition and intracellular level in order to generate a favorable niche that promotes infection.

Part 3.2 – Modulation of dendritic cell responses

DCs are attractive targets for immune modulation because of their positioning at the innate/adaptive interface. The importance of subverting DC responses as a mechanism of limiting protective immune responses during infection is demonstrated by studies which show that transfer of antigen-loaded or activated DCs (as a mucosal vaccine) augments T cell responses[192-195]. Moreover, some studies have even demonstrated that transfer of BCG-infected DCs is able to augment protective immune responses[196,197]. While DCs are able to upregulate activation markers following infection[58,63,66], studies with Mtb mutant strains have revealed that these responses are limited during infection[195,198], whereby Mtb impairs DC functions that are necessary for robust activation of naïve T cells. Mtb prevents optimal upregulation of co-stimulatory molecules on DCs, such as CD40 and CD86, and limits the secretion of Th_{17}-polarizing cytokines such as IL-6[195,198]. Mtb also prevents the ability of DCs to become activated by modulating recognition through surface receptors. Studies found that ligation of DC-SIGN on DCs, the major receptor through which Mtb gains entry into DCs, leads to upregulation of IL-10[199,200], an anti-inflammatory cytokine, and reduced maturation following LPS stimulation[199]. Similarly, Mtb uses immune evasion proteins to limit detection through TLR2 on DCs in order to

dampen activation[198]. DC activation is a precursor to migration to the lymph nodes (LN), which is necessary for activation of T cells[60,201]. In the context of viral respiratory infections, DCs quickly travel to the LN in hours[202], but in the context of Mtb infection, this process is significantly delayed[60] with the earliest antigen-specific T cell responses being detected at 12 days post-infection[203]. While a study found that Mtb can reduce expression of integrins and thereby impair the physical capacity of DCs to migrate[204], delayed travel to the LN can also be due to the suboptimal DC activation. IL-12p40, for instance, is a cytokine that is secreted by activated DCs and was shown to be necessary for DC migration and initiation of T cell responses[69], but its expression is limited during infection[195,198]. Interestingly, DC populations that are able to travel to the LN are not always the most protective. Like macrophages[190], Mtb might be able to preferentially infect DCs subsets that function poorly in inducing T cell responses. Myeloid DC populations in the lungs that are highly infected poorly activate naïve T cells[193]. Interestingly, this DC population is able to secrete unprocessed antigens such as Ag85B, which can then be taken and processed by resident lymph node DCs in order to initiate T cell responses[193]. While this may appear to be beneficial, a subsequent study, however, found that blocking exported antigen in DCs resulted in increased proliferation of CD4 T cells[205], thereby demonstrating that a strategy used to initiate T cells in the absence of proper DC function actually results in suboptimal T cell responses. Even cells that are able to express MHC class II molecules and present antigen, but are highly infected, are unable to initiate protective T cell responses[60]. These results suggest that Mtb is able to manipulate the immune cell landscape by selecting for DC populations during infection that are poorly immunogenic. An early study found that immature DCs are better able to be infected by Mtb than mature DCs[68]. Recently, a study found that $CD11c^+$ DCs (a subset of DCs in the lungs) which were shown to be important for

initiating Th_1 responses in the LN during Mtb infection are impaired by $CD103^+$ DCs, which produce IL-10[73]. Choosing to infect specific DC subsets may therefore dictate the outcome of immune responses. Collectively, these studies demonstrate that Mtb infection leads to suboptimal DC functions which in turn limits the initiation of protective T cell responses during infection. While it is clear that Mtb directly targets DCs, we need a more in-depth understanding of how to overcome pathogen evasion. Addressing this knowledge gap will therefore yield important insights that can then be applied towards augmenting protective immune responses against TB.

Part 3.3 – Bacterial immune evasion factors

Although many immune evasion factors have been identified in the Mtb genome, the functions of only a few have been well characterized[6]. While the studies discussed above highlight the ability of Mtb to dampen the ability of key innate immune cells to perform critical functions, some studies have been able to identify specific Mtb proteins that limit protective immune responses and are termed immune evasion proteins. Insights on the mechanisms underlying immune evasion proteins is important tool for identifying targets for augmenting protective immunity. In fact, many of the insights we have about vital immune cell functions during Mtb infection come from studying immune evasion proteins. For example, we know that apoptosis is important for the immune response to TB because of proteins such as SigH and NuoG. Mtb encodes sigma (*sig*) factors, which are the subunits of RNA polymerases that dictate specificity and initiation of transcription[206]. *sigH* is induced in conditions of stress (heat shock, oxidative stresses)[207,208] and following macrophage phagocytosis[209]. Infection with a Δ*sigH* mutant led to upregulation of chemokine and apoptotic genes in macrophages, and showed that Mtb interferes

with host apoptosis which is important for activation of host immunity[210]. During growth, Mtb is able to use respiration as an energy source but this requires transfer of electrons into the respiratory chain to fuel this process. To do this, Mtb encodes a proton-pumping type I NADH dehydrogenase, of which NuoG is one of the subunits[211]. Infection of mice with a Δ*nuoG* mutant led to an increase in apoptosis and was phagocytosed faster by myeloid cells *in vivo*[212]. Importantly, Δ*nuoG* infection led to earlier activation of T cells compared to wild type Mtb, demonstrating apoptosis is important in generating beneficial T cell functions[212]. We know antigen presentation is important because of proteins such as EsxH (TB10.4) and LprG. To transport molecules across the cell wall, Mtb encodes five type-VII secretion systems (T7SS) known as the Esx systems, of which EsxH is one[213]. EsxH, in conjunction with EsxG (the other protein encoded by the Esx-3 system) was found to disrupt ESCRT function and arrest phagosome maturation[214]. Follow-up studies found that ESCRT is important for antigen presentation in macrophages and dendritic cells, and EsxGH impairs priming of naïve T cells as transfer of MtbΔ*esxH*-infected DCs enhances T cell priming and proliferation *in vivo*[215]. LprG is a lipoprotein, which among it's different functions is to regulate the surface expression of LAM[216,217], a glycoprotein that plays a role in dampening immune responses[218]. An early study found that purified LprG inhibited MHC-II antigen presentation in macrophages by prolonged activation of TLR-2[219]. Deleting LprG in the BCG vaccine strain led to an increase in polyfunctional T cells in WT mice and upregulated pro-inflammatory cytokines and lowered CFU in a susceptible mouse strain[220].

A limited number of immune evasion proteins have been demonstrated to function by restricting polarization of protective Th cell subsets. One of these that has implications for activation of

DCs and polarization of protective immune responses is Hydrolase important for pathogenesis 1 (Hip1). Proteins that are expressed on the surface of Mtb are the first to interact with innate immune cells and therefore play a pivotal role on shaping immune responses during infection. Hip1 is a serine protease that has been extensively studies in our laboratory and is expressed on the surface of Mtb and cleaves GroEL2, a chaperonin[221-223]. Hip1 proteolytic activity converts GroEL2 from a multimer to a monomer and contributes to dampening immune responses during infection. The cleaved monomeric form of GroEL2 which predominates in wild type Mtb dampens the pro-inflammatory function of macrophages and DCs; however, the full-length multimeric form of GroEL2 promotes robust innate responses [222,224]. Hip1 was originally identified in a screen used to identify bacterial proteins necessary for growth in macrophages[225]. A follow-up study confirmed the requirement of Hip1 for growth in macrophages and demonstrated that despite modest reductions in CFU, infection with a *hip1* mutant led to improved survival and pathology[221]. Hip1 has been shown to restrict innate immune cell activation during infection by dampening TLR2 signaling[191] and limits the ability of macrophages and dendritic cells to produce proinflammatory cytokines[191,198]. Additionally, Hip1 limits the upregulation of co-stimulatory molecules and the ability of DCs to present antigen and activate T cells during infection[198]. DCs infected with *hip1* mutant are better able to polarize T cells in the lungs of mice following intratracheal transfer[195]. One of the co-stimulatory molecules that *hip1* dampens on DCs is CD40[198], which our lab has shown is critical for polarizing Th_{17} responses during infection[195]. Therefore, in addition to limiting innate immune cell activation, Hip1 also limits protective T cell responses during infection. Utilizing the *hip1* mutant to dissect how Mtb impairs CD40 dependent DC-T cell crosstalk will therefore yield important molecular insights into the mechanisms that contribute to protective immunity during infection.

Part 4 – Tuberculosis Vaccines

In 1921, physicians Albert Calmette and Camille Guérin developed a live-attenuated strain of *Mycobacterium bovis*, known as Bacillus Calmette-Guérin (BCG) for use as a vaccine against TB[226]. Despite being more than a century old, BCG is the only licensed vaccine against TB and the most widely used vaccine world-wide[227]. BCG is administered at birth and is able to provide protection against disseminated forms of TB in children[228,229]. However, studies have also demonstrated that BCG immunity wanes in adolescence and is poorly effective at preventing pulmonary TB, which is the transmissible form of TB, in either children or adults[228,230]. Although we do not yet have a new licensed vaccine that is able to replace BCG, several new vaccine candidates are currently in the TB vaccine development pipeline.

Most of the vaccines that have reached clinical trials in humans include adjuvants. The most promising of these candidates is the M72/AS01$_E$ subunit vaccine. This vaccine consists of an Mtb32A/39A recombinant vaccine with AS01$_E$ adjuvant, recently observed a 49.7% protection rate in the phase 2B clinical trial result[231]. The cationic peptide adjuvant IC31 is used in conjunction with two vaccine candidates, H4:IC31 and H56:IC31[232,233]. While H4:IC31 did not demonstrate significant protection (30.5% efficacy)[233], H56:IC31 was found to induce antigen-specific IgG responses and Th_1 cytokine expressing CD4 T cells[232]. In humans, ID93 + GLA-SE vaccination, which consists of the ID93 antigen (which is composed of 3 antigens Rv2608, Rv3619, and Rv3620) and adjuvant GLA-SE led to multi-cytokine producing T cells (TNF, IFN-γ and IL-2) and IgG1 and IgG3 antibody production[234,235]. Other vaccines that have not yet reached vaccine trials show promise in animal models. A protein-Lipokel vaccine conjugate was

able to reduce Mtb CFU in the lungs of mice and increase the frequency of DCs in lymph nodes following vaccination[236]. CysVac2/AdvaxCpG, have shown promise in pre-clinical models[237]. CAF01 has demonstrated efficacy as an Mtb vaccine adjuvant in pre-clinical mouse models and induces a strong Th_{17}-polarized immune response in mice[238].

In addition to providing valuable insight into protective immunity, deleting or mutating IEMs in BCG or Mtb also serves as a viable vaccination approach. Mucosal vaccination of NHPs with Mtb∆*sigH* lowers pathology and increases survival following challenge but increases T central and effector cells in the lung[239]. Further deleting *nuoG* in the BCG ∆*ureC*::*hly* strain led to a significant reduction in CFU following challenge and an increase in CD4 T cells in the draining lymph nodes of mice compared to parent vaccine[240]. Even so, deleting *nuoG* from BCG itself also led to a significant reduction in CFU following challenge compared to WT[240]. A recent study found that vaccination with a BCG strain in which the lprG-rv1410c operon was deleted (BCG∆*lprG*) Of particular note, the vaccine strain led to an increase in Th_{17} responses in the lungs of mice, which have been shown to play a role in protection against TB[241]. AERAS-402, an adenovirus-vectored vaccine containing sequences of Ag85A,B, and TB10.4 induces polyfunctional CD4 and CD8 T cells in humans following vaccination[242]. Deletion of Hip1 has also been explored in the context of BCG immunogenicity. A *hip1* knockout of BCG induces more pro-inflammatory cytokine production and increases the ability of DCs to polarize T cells compared to BCG[26]. In a DC mucosal vaccine model, DCs infected with BCG∆*hip1* are better able to active T cells (and polarize polyfunctional T cells) and induce immune responses that significantly reduce CFU following challenge[26].

Although most of these studies have administered vaccines as intra-muscular or intra-dermal (like BCG), changing the route of vaccinations has also been employed as a strategy to increase effectiveness. An important study in 2020 demonstrated that near-complete bacterial clearance can be achieved with intravenous (i.v.) vaccination of BCG in NHP models[243]. Most vaccines are injected into muscle although BCG is given as an intradermal injection. However, targeting mucosal immune subsets that can quickly transport antigen to stimulate an immune response is likely to be important for mucosal Mtb vaccine strategies[244,245]. Some TB vaccines, such as CysVac2/Advax candidate, have been observed to be more protective after intranasal or intrapulmonary administration, a quality attributed to local IL-17 production and the establishment of T_{RM}, which are resident-memory CD4 T cells that are found at the site of infection and can quickly respond upon activation[246,247].

Apart from serving to directly test vaccine constructs, clinical trials and vaccine research can also provide important insights beyond whether a new vaccine is efficacious. BCG re-vaccination control group in the H4:IC31 trial was found to be more effective at preventing TB than the new vaccine construct that was tested[233], suggesting that BCG re-vaccination may be a viable strategy to induce protective immunity. The MVA85A trial, despite demonstrating no efficacy, helped provide further evidence that strong Th_1 responses are not sufficient alone for protection[110]. Although the actual use of i.v. BCG in humans is controversial, the primate study can provide insights that can be used for vaccines that are more likely to be used[243]. Vaccine trials have also revealed that targeting important innate immune cells like DCs, through adjuvants, is important for protective immunity. This is demonstrated by the fact that most of the vaccines that are currently in development in TB use adjuvants[248].

The failure to find an effective replacement for BCG in the 100+ years of its creation highlights an important point: we lack fundamental insight into the mechanisms necessary for inducing protective immunity during TB. To develop better vaccines, we need to understand what constitutes protective immunity, and critically, we need to understand how to augment these immune responses during infection. Studies on which pathways facilitate protective immune responses and how we can overcome Mtb modulation in order to augment these responses will therefore be valuable in instructing the design of vaccines and adjuvants which can in turn serve to limit TB burden worldwide.

Dissertation Overview

While we have come to appreciate the contribution of Th_{17} subsets to TB immunity, the molecular mechanisms that contribute to restricting these responses during infection are poorly understood. Moreover, we lack comprehensive knowledge on how Mtb evades DC responses and how we can overcome immune evasion mechanisms to augment protective immunity. Addressing these questions and further characterizing DC-T cell crosstalk during Mtb infection is necessary to generate insights that can contribute to the development of effective TB vaccines and adjuvants.

In this dissertation, we address these fundamental questions by dissecting the pathways that contribute to Th_{17} polarization during infection, the mechanisms by which Mtb impedes these responses and identify molecular targets for developing novel adjuvants and vaccine approaches. We demonstrate that DLL4, a canonical ligand of the Notch signaling pathway, is necessary for

CD40-dependent Th_{17} polarization *in vitro* and *in vivo* and show that these responses are impeded by the Hip1 serine protease of Mtb (Chapter II). We then present findings from transcriptomics studies which demonstrate that exogenously engaging CD40 on Mtb-infected DCs reprograms DCs to increase their pro-inflammatory functions, Th_{17} polarization and proliferation capacity (Chapter III). Finally, we end by summarizing the major findings and discuss how insights generated in this dissertation advance fundamental knowledge on immunity to TB and contribute to the development of efficacious TB vaccines and adjuvants.

Chapter II

Mycobacterium tuberculosis impedes CD40-dependent Notch signaling to restrict Th_{17} polarization during infection

Chapter adapted from:

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Abstract

Early Th_{17} responses are necessary to provide protection against *Mycobacterium tuberculosis* (Mtb). However, we previously demonstrated that Mtb restricts Th_{17} polarization and that engaging the CD40 costimulatory pathway on dendritic cells (DCs) enhances Th_{17} responses and augments Mtb control. Defining the molecular mechanisms that limit Th_{17} polarization during Mtb infection will facilitate the design of TB vaccination strategies that induce protective Th_{17} immunity. Here, we identify the Notch ligand, delta-like canonical Notch ligand 4 (DLL4), as necessary for Th_{17} polarization and demonstrate that Mtb limits DLL4 signaling on DCs to prevent optimal Th_{17} responses. While Mtb infection induced only low levels of *Dll4* mRNA in DCs, exogenously engaging CD40 on Mtb-infected DCs increased *Dll4* expression and led to high frequencies of $DLL4^+$ DCs. Antibody blockade of DLL4 on DCs reduced Th_{17} polarization but did not affect Th_1 polarization. DLL4 was also required for inducing multifunctional $CXCR3^+$ $CCR6^+$ CD4 T cells co-expressing IL-17, IL-22 and IFN-γ in the lungs of mice, and DLL4-dependent Th_{17} responses inversely correlated with Mtb lung burdens. Additionally, we show that the Mtb Hip1 protease attenuates DLL4 expression on lung DCs by impeding CD40 signaling. Overall, our results demonstrate that Mtb impedes CD40-dependent Notch ligand signaling to restrict Th_{17} responses and identify the CD40-DLL4 pathways as targets for developing new Th_{17}-inducing vaccines and adjuvants for TB.

Introduction

Mycobacterium tuberculosis (Mtb) is the causative agent of tuberculosis (TB), a serious global health problem that led to the death of 1.5 million individuals worldwide in 2020[1] alone. The currently licensed vaccine against TB, *Mycobacterium bovis* Bacillus Calmette-Guérin (BCG), has poor efficacy against pulmonary TB in adults and children[228,230]. Significant barriers that hinder developing more efficacious TB vaccines include our limited understanding of protective immunity against infection and disease, and the Mtb immune evasion mechanisms that impede protective host immune responses. CD4 effector T cell responses are critical for immune control of Mtb. IFN-γ-producing CD4 T helper 1 (Th_1) cells are necessary for inducing antimicrobial functions in macrophages[100,101,249-251], but are insufficient for providing protection against TB disease. Studies in animal models and humans from several groups, including our own, have identified important roles for IL-17 and Th_{17} responses in protective immunity against Mtb[121,123,125,131,195,252-254] and suggest that vaccines that induce early Th_{17} responses will provide enhanced protection against TB. However, we have previously shown that Mtb actively limits the early generation of lung Th_{17} responses through the immunomodulatory functions of the Mtb serine protease Hip1[195,198]. Therefore, delineating the molecular basis for Th_{17} polarization following infection and the mechanisms employed by Mtb to limit Th_{17} generation will allow us to design efficacious TB vaccines that induce protective immunity.

Dendritic cells (DCs) are critical for initiating the activation, proliferation and polarization of naïve CD4 T cells by presenting pathogen-derived antigens, upregulating co-stimulatory molecules, and producing cytokines that contribute to polarization into Th_1, Th_{17} and other Th subsets[192,201,255]. However, Mtb is able to impede DC functions and impair DC-T cell crosstalk,

leading to sub-optimal effector Th cell responses that fail to eliminate infection[195,198,255]. We recently demonstrated that the CD40-CD40L costimulatory pathway is necessary for generating Th_{17} responses and that Mtb limits Th_{17} responses by impairing interactions between CD40 on DCs and CD40L on CD4 T cells through Hip1 protease[195,198]. Importantly, we showed that exogenously engaging CD40 on Mtb-infected DCs led to significant enhancement of mucosal Mtb-specific Th_{17} responses and improved control of Mtb lung burdens in mouse models[195]. These studies demonstrate that targeting the CD40-CD40L pathway is an attractive approach for developing new Th_{17}-inducing vaccine adjuvants and therapeutics.

In order to better inform development of vaccine and therapeutic strategies that would augment DC responses and Th_{17} polarization, we sought to elucidate the molecular pathways downstream of CD40 signaling in DCs that preferentially induce Th_{17} polarization. Here we identify the Notch ligand delta-like canonical Notch ligand 4 (DLL4) as necessary for Th_{17} polarization and demonstrate that Mtb limits DLL4 signaling on DCs to prevent optimal Th_{17} responses. Notch signaling is initiated by ligand-receptor binding and requires binding of any of the five ligands: DLL1, DLL3, DLL4, Jagged1, or Jagged2 to any of the four Notch receptors, NOTCH1-4, on neighboring cells[256]. We found that while Mtb infection induced only low levels of *Dll4* mRNA in DCs, exogenous CD40 engagement of Mtb-infected DCs substantially increased *Dll4* expression and led to high frequencies of $DLL4^+$ DCs. We hypothesized that CD40-dependent Th_{17} polarization is mediated by Notch ligands and tested this hypothesis by blocking Notch ligand signaling. Antibody blockade experiments showed that DLL4 is required for Th_{17} polarization during Mtb infection, both *in vitro* and *in vivo*, providing new evidence that links CD40, DLL4 and Th_{17} polarization. Moreover, CD40-engagement enabled DCs to induce

multifunctional CXCR3^{+} CCR6^{+} Th_{17} subsets in the lung, including double-positive (DP) IL-17^{+}IFN-γ^{+} and IL-17^{+}IL-22^{+} subsets and triple-positive (TP) IL-17^{+}IFN-γ^{+}IL-22^{+} subsets that have been associated with protective immunity against TB[120,253,257]. IL-17 and IL-22 expression positively correlated with NOTCH2 receptor expression on Th subsets and these responses were abrogated upon blocking DLL4 on DCs. Moreover, DLL4-dependent IL-17 responses inversely correlated with Mtb lung burdens, suggesting that DLL4 signaling promotes Th_{17} responses and augments Mtb control. Additionally, we found that the Hip1 protease impedes DLL4 expression on lung DCs through a CD40-dependent mechanism. To our knowledge, our studies are the first to show that Notch signaling downstream of CD40-CD40L interactions is necessary for generating Th_{17} responses during Mtb infection and that overcoming Mtb limitation of CD40 and Notch ligand signaling pathways can promote protective immunity. Our studies thus identify CD40 and DLL4 as targets for adjuvant-mediated immunomodulation during vaccination.

Results

Engaging CD40 on Mtb-infected dendritic cells augments pro-inflammatory cytokines and enhances expression of Notch ligand *Dll4*.

To identify the CD40-dependent mechanisms that enable DCs to polarize Th cells towards Th_{17} subsets, we employed our previously-described model in which we exogenously engaged CD40 using the multimeric CD40L reagent (CD40LT), which crosslinks CD40 and effectively simulates the membrane-assisted aggregation of CD40L[195]. We infected bone marrow-derived dendritic cells (BMDCs) from C57BL/6 (B6) mice in the presence or absence of CD40LT for up to 72 hours (Figure 1). At designated time points post-infection, we used quantitative polymerase chain reaction (qPCR) to assess gene expression and harvested supernatants to measure IL-6 and

IL-12p40 cytokine production by enzyme-linked immunosorbent assay (ELISA). IL-6 and IL-12p40 are two pro-inflammatory cytokines that are produced by activated DCs and contribute to Th_{17} and Th_1 polarization, respectively. We confirmed that Mtb-infected BMDCs stimulated with CD40LT produced significantly higher levels of pro-inflammatory cytokines IL-6 and IL-12p40, as previously demonstrated[195] (Figure 1A), along with significant increase in *Il6* and *Il12b* mRNA levels (Figure 1B). We next measured the expression of Notch ligands *Dll4* and *Jag1*. The addition of CD40LT to Mtb-infected DCs increased mRNA corresponding to *Dll4* by ~100 fold compared to Mtb alone and led to a significant increase at 72H (Figure 1C). In contrast, *Jag1* mRNA levels did not increase with the addition of CD40LT and were comparable to Mtb infection alone (Figure 1C). While stimulating BMDCs with CD40LT alone induced *Dll4* mRNA compared to uninfected (UI), addition of CD40LT in the context of Mtb infection induced substantially more expression of *Dll4* than CD40LT or Mtb alone (Supplemental Figure 1). We were unable to detect the expression of additional Notch ligands *Dll1, Dll3* or *Jag2* (A.B.E. and J.R., unpublished data). To compare our Mtb-DC infections to the responses induced by purified ligands that bind pattern recognition receptors (PRRs), we stimulated DCs with LPS (TLR4 agonist) or zymosan (TLR2/Dectin-1 agonist) (Supplemental Figure 2). LPS and zymosan each led to significant induction of IL-6 and IL-12p40 protein and corresponding mRNA (Supplemental Figure 2A-C). Importantly, both LPS and zymosan induced robust expression of *Dll4* mRNA at levels that were comparable to Mtb + CD40LT conditions (Supplemental Figure 2D). Consistent with Mtb infection results, neither LPS or zymosan induced robust expression of *Jag1* (Supplemental Figure 2D). We also found that addition of CD40LT significantly augmented *Dll4* mRNA following stimulation with LPS, zymosan or the Th_{17}-skewing fungal pathogen *Candidia albicans* [258] (*C. albicans;* Supplemental Figure 3). Taken

together, our results suggest that the expression of *Dll4* in DCs is limited during Mtb infection but can be augmented by engaging CD40 on DCs. Moreover, consistent with previous observations, *Jag1* is constitutively expressed in DCs and is not further induced by stimulation with either Mtb or PRR ligands[259]. These data show that engagement of CD40 on DCs augments *Dll4* mRNA in the context of Mtb infection as well other PRR ligands and Th_{17}- skewing pathogens.

We next sought to test whether the induction of *Dll4* observed following addition of CD40LT is dependent on Mtb viability. We stimulated BMDCs with heat-killed Mtb (HK Mtb) with or without CD40LT for up to 48 hours and collected supernatant and RNA for ELISA and qPCR assays, respectively, as described in Figure 1. As with live Mtb infection, we observed a significant increase in IL-6 and IL-12p40 protein following the addition of CD40LT (Supplemental Figure 4A) and increase in *Il6* and *Il12b* mRNA levels (Supplemental Figure 4B). Moreover, addition of CD40LT led to a significant increase in *Dll4* compared to HK Mtb alone while *Jag1* levels remained unaltered (Supplemental Figure 4C). These results show that the lack of *Dll4* upregulation following Mtb infection as well as the enhanced *Dll4* expression upon CD40 engagement is not dependent on the presence of live bacteria.

Engaging CD40 on Mtb-stimulated DCs enhances surface expression of DLL4 and Jagged1.

Notch ligands need to be present on the surface of cells in order to interact with Notch receptors on neighboring cells[256]. Therefore, having observed an increase in *Dll4* mRNA following the addition of CD40LT, we next sought to test whether CD40 engagement also induces Notch

ligand expression on the surface of Mtb-infected DCs. We stimulated BMDCs with HK Mtb and collected cells at different time points over a 24-hour time course and assessed surface expression of DLL4 and Jagged1 using flow cytometry. The addition of CD40LT led to a significant increase in the populations of DCs expressing DLL4 or Jagged1 compared to Mtb alone as assessed by frequencies (Figure 2A-B) and MFI (Figure 2C). Notably, the majority of CD40-enagaged DCs expressed both DLL4 and Jagged1 simultaneously (Figure 2A-B). These data demonstrate that in addition to increasing *Dll4* mRNA, CD40 engagement augments surface expression of DLL4 and that all DLL4-expressing DCs were also positive for Jagged1.

DLL4 is required for Th_{17} polarization but is dispensable for Th_1 polarization.

To investigate the role of DLL4 and Jagged1 in CD40-mediated Th_{17} polarization during Mtb infection, we set up DC-T cell co-culture assays as previously described[195]. Since deletion of either DLL4 or Jagged1 in mice is embryonically lethal[260-263], we used blocking antibodies corresponding to each of the two Notch ligands. DCs exposed to Mtb in the presence or absence of CD40LT for 24 hours were co-cultured with OT-II transgenic T cells and cognate OVA peptide ($OVA_{323–339}$). At the time of co-culture, blocking antibodies to either DLL4, Jagged1, or a combination of DLL4 and Jagged1 antibodies were added to each well at increasing concentrations. Following 72 hours of co-culture, cell-free supernatants were harvested and IFN-γ and IL-17 production were measured by ELISA to represent Th_1 and Th_{17} cytokines, respectively (Figure 3A-B). As previously demonstrated[195], addition of CD40LT enhanced IL-17 levels in co-culture but did not augment IFN-γ levels (Figure 3A-B). CD40LT stimulation alone did not lead to an increase in Th_{17} polarization and was comparable to the uninfected condition (Supplemental Figure 5). Antibody blockade of Jagged1 during co-culture led to a significant

reduction in both IFN-γ and IL-17 compared to controls, while blocking DLL4 alone preferentially decreased IL-17 levels without significantly altering IFN-γ production (Figure 3A-B). We also measured IL-2 production (Figure 3C) and found that blockade of Jagged1 resulted in a significant reduction in IL-2. In contrast, addition of anti-DLL4 did not alter IL-2 production, suggesting that blockade of DLL4 did not impact the expansion of CD4 T cells. Isotype controls for each of the Notch ligand antibodies demonstrated the specificity of these results (Supplemental Figure 6). Further, providing Notch ligand blockade also led to a reduction in Th_{17} polarization following heat-killed *C. albicans* + CD40LT DC stimulation (Supplemental Figure 7). These data indicate that CD40 engagement promotes Th_{17} polarization *via* Notch ligands. Importantly, DLL4 is specifically required for Th_{17} polarization but is not required for Th_1 polarization.

To further investigate the role of DLL4, either singly or in combination with Jagged1, in Th_{17} polarization *in vivo*, we used an intratracheal (IT) transfer model to transfer BMDCs directly into the lungs of mice[195]. This model allows us to specifically dissect how CD40 and Notch ligands on DCs impact T cell polarization *in vivo*. Groups of BMDCs exposed to different experimental conditions (depicted in Figure 4A) were intratracheally transferred into mouse lungs in the presence or absence of blocking antibodies to DLL4 alone, or both DLL4 and Jagged1. One day before IT transfer of DCs, purified naïve CD4 T cells from $ESAT_{1-20}$-Tg mice were adoptively transferred into mice *via* the intravenous (i.v.) route. Six days after intratracheal DC transfer, mice were euthanized and single-cell lung suspensions were stimulated with ESAT-6_{1-20} peptide (to stimulate antigen-specific T cells), and responses were measured using flow cytometry. We observed a significant increase in the frequencies of IL-17^+ CD4 T cells upon transfer of CD40-

engaged Mtb-DCs compared to controls, while IL-2$^+$ and IFN-γ$^+$ CD4 T cells were unaffected by CD40LT addition (Figure 4B-C). Providing DLL4 blocking antibodies led to significantly reduced frequencies of antigen-specific IL-17$^+$ CD4 T cells but did not affect IFN-γ$^+$ or IL-2$^+$ CD4 T cell frequencies (Figure 4B-C). This supports our *in* vitro polarization assay data (Figure 3) and demonstrates that DLL4 blockade specifically affects Th_{17} polarization but does not impact Th_1 polarization or overall T cell expansion. Blocking both DLL4 and Jagged1 significantly reduced IL-17$^+$ and IL-2$^+$ CD4 T cell frequencies but also resulted in lower IFN-γ$^+$ CD4 T cells (Figure 4B-C). These data support a critical role for DLL4 alone or in combination with Jagged1 in Th_{17} polarization during Mtb stimulation *in vivo*.

CD40-mediated Th_{17} polarization during Mtb infection is dependent on DLL4 in the lungs of mice.

Our data demonstrates that Mtb limits DLL4 during infection and that CD40 engagement of Mtb-infected DCs leads to significant induction of DLL4 which is required for Th_{17} polarization (Figure 3-4). To further examine the role of DLL4 and CD40 in polarizing endogenous Th cells towards Th_{17} and other subsets *in vivo*, we transferred Mtb-infected DCs directly into the lungs of naïve B6 mice and carried out detailed phenotyping of the Th subsets in the presence or absence of DLL4 blocking antibodies (Figure 5A). BMDCs were infected *in vitro* for 48 hours with Mtb in the presence or absence of CD40LT, with or without DLL4 blocking antibody. After the infection period, BMDCs were harvested and transferred into the lungs of mice as previously described[195] (Figure 5). At four weeks post-transfer, we euthanized mice and generated single cell lung suspensions to assess the *ex vivo* CD4 T cell cytokine responses induced by transferred DCs using flow cytometry. As with our previous data (Figure 4), CD40LT did not augment IL-2$^+$

and IFN-γ^+ CD4 T cell frequencies and DLL4 blockade did not impact these responses (Figure 5B). However, the transfer of CD40LT-treated DCs led to higher frequencies of IL-17^+ CD4 T cells and providing DLL4 blocking antibody in this context significantly reduced Th_{17} responses (Figure 5B). In addition to IL-17, the cytokine IL-22 has also been reported to be produced by Th_{17} cells in mucosal settings[264]. Notably, we found that CD40 engagement also led to significantly higher IL-22^+ CD4 T cell frequencies and blocking DLL4 reversed this effect (Figure 5B). We extended our analyses to examine multiple-cytokine producing CD4 T cells, which have been implicated in protection against TB[120,125,253,257]. Interestingly, the Boolean analysis showed that CD40LT also augmented the frequencies of double-positive (DP) IL-17^+IL-22^+ and triple-positive (TP) IL-17^+ IFN-γ^+ IL-22^+ CD4 T cells (Figure 5C). Moreover, the chemokine receptors CXCR3 and CCR6, which have been associated with protective Th_{17} and Th_1/Th_{17} subsets[253,257], were co-expressed on single-positive (SP) IL-17^+ and DP IL-17^+IL-22^+ populations induced by CD40LT but these were abrogated upon DLL4 blockade (Figure 5D). We obtained similar results upon assaying Mtb antigen-specific responses following *ex vivo* stimulation of lung cells with Mtb whole cell lysate (WCL) (Supplemental Figure 8). Isotype controls for either antibody demonstrated the specificity of these results (Supplemental Figure 9). Overall, these results demonstrate an essential role for DLL4 in generating both bulk and antigen-specific multifunctional Th_{17} responses during Mtb infection.

The dependence on DLL4 for Th_{17} polarization demonstrates a critical role for Notch signaling during Mtb infection. The Notch signaling pathway is activated once a Notch ligand binds to a Notch receptor to initiate downstream effector functions[256]. Therefore, we next sought to test Notch receptor expression on T cells. Since murine CD4 T cells express NOTCH1 and

NOTCH2[265], we tested the presence of these two receptors in the same groups of mice. Interestingly, we found that transfer of Mtb-infected DCs plus CD40LT significantly increased the frequencies of $NOTCH2^+$ and $NOTCH1^+NOTCH2^+$ CD4 T cells (Figure 6A). The median of values of $NOTCH1^+$ appears to be greater in the Mtb + CD40LT condition, but this result is not significant (Figure 6A). DLL4 blockade led to a reduction in $NOTCH2^+$ and $NOTCH1^+NOTCH2^+$ DP CD4 T cells (Figure 6A) and in $IL\text{-}17^+$ cells expressing NOTCH1 or 2 (Figure 6B). Taken together, these data support the role of Notch ligand-Notch receptor signaling downstream of CD40 in generating Th_{17} responses during Mtb infection.

DLL4-mediated Th_{17} responses correlate with NOTCH2 expression and lower lung Mtb burden.

To gain insights into the relationships between DC and T cell markers in the lung we next performed correlation analyses from our multiparameter flow cytometry data (Figure 7). We found that IL-17-producing CD4 T cells not only correlated with CD40L expression on CD4 T cells, consistent with our previous study[195], but also showed positive correlations with NOTCH1 and NOTCH2 expression (Figure 7A-B). IL-22 expression was positively correlated with CD40L and NOTCH2 but not with NOTCH1 (Figure 7B), while IFN-γ expression did not correlate with either of the two NOTCH receptors (Supplemental Figure 10). We were curious to study the relationship between T cell markers in our panels and Mtb load (measured by plating for CFU) in the lungs of mice. We found that transfer of infected DCs treated with CD40LT led to significantly lower Mtb burdens in the lungs compared to infection alone, while Mtb burdens in mice that received DLL4 blocking antibody had significantly higher Mtb CFU, comparable to the CD40LT-untreated group (Figure 7C). Correlogram analyses showed that only two markers,

IL-17 and NOTCH2, had significant negative correlations with CFU (Figure 7C). Taken together, these results suggest that CD40 engagement on DCs augments Mtb control through increased IL-17 responses and Notch signaling pathways.

Mtb restriction of CD40 and DLL4 signaling in lung DCs is mediated by the Hip1 serine protease.

We previously showed that Mtb prevents CD40 expression on infected DCs through the immunomodulatory serine protease Hip1[195,198,224]. A *hip1* mutant induced robust CD40 expression and higher Th_{17} responses compared to wild type Mtb[195,198,224]. We also showed that Mtb Hip1 restricts optimal Th_{17} polarization by dampening the CD40 costimulatory pathway and that engaging CD40 on DCs enhances Th_{17} responses to levels comparable to that induced by the *hip1* mutant[195,224]. Thus, the *hip1* mutant provides us with a unique tool to further probe the relationship between CD40 and DLL4 pathways during *in vivo* Mtb infection. We infected C57BL/6 mice with either wild type Mtb or the *hip1* mutant *via* the aerosol route and euthanized mice two weeks post-infection to assess early immune responses (Figure 8A). We harvested the lungs and stained single cell suspensions for cell surface and intracellular markers to analyze by flow cytometry. We observed that the *hip1* mutant induced robust CD40 expression on DCs in the lung compared to wild type Mtb (Figure 8B). We next examined the expression of DLL4 and Jagged1 in two populations of lung DCs: $CD11b^+$ and $CD103^+$ DCs. These are the two main populations of classical DCs in the lungs and have been implicated in Th polarization[73,266,267]. Infection with the *hip1* mutant, but not wild type Mtb, led to significantly higher frequencies of $DLL4^+$ and $DLL4^+Jagged1^+$ DCs of both types in the lungs of infected mice (Figure 8C-D). Frequencies of $Jagged1^+$ DCs in the lung were higher in both infected groups compared to

uninfected mice, but indistinguishable between wild type and *hip1* mutant groups (Figure 8C-D). We next assessed *ex vivo* T cell responses in these same groups of mice at this early time-point. Consistent with previous data, infection with *hip1* mutant resulted in significantly higher frequencies of IL-17$^+$ CD4 T cells[195,198,224] as well as significantly higher frequencies of IL-2$^+$, IFN-γ$^+$, and IL-22$^+$ CD4 T cells in the lung compared to wild type Mtb infection (Figure 8E). Interestingly, the *hip1* mutant also induced higher frequencies of Notch receptor-expressing T cells compared to Mtb infection (Figure 8E). Further, correlogram analyses showed that DLL4 expression on CD103$^+$ DCs positively correlated with IL-17 and NOTCH2 expression on T cells (Figure 8F) but did not show any association with IFN-γ, IL-22, or NOTCH1 (Supplemental Figure 11). These data suggest that DLL4-NOTCH2 interactions likely mediate Th_{17} polarization during Mtb infection. Thus, the absence of Hip1 augments CD40 and DLL4 signaling and subsequent Th_{17} responses whereas the presence of Hip1 impairs CD40-DLL4 signaling to limit Th_{17} responses.

The similarity between the responses elicited by exogenous engagement of CD40 in the context of wild type Mtb infection and infection with the *hip1* mutant led us to hypothesize that the *hip1* mutant increases DLL4 expression on lung DCs through CD40 engagement. To test the requirement for CD40 in Notch ligand expression, we infected mice lacking CD40 (CD40$^{-/-}$) or C57BL/6 mice with intact CD40 (CD40$^{+/+}$) with either wild type Mtb or the *hip1* mutant *via* the aerosol route. We assessed DC and *ex vivo* T cell profiles two weeks post-infection using flow cytometry (Figure 9). Figure 9A shows that DLL4$^+$ and DLL4$^+$Jagged1$^+$ lung DC populations induced by the *hip1* mutant are dependent on CD40. We observed a significant reduction in the frequencies of IL-2$^+$, IFN-γ$^+$ and IL-22$^+$ CD4 T cells and a reduction in IL-17$^+$ CD4 T cell

frequencies in the $CD40^{-/-}$ group relative to the $CD40^{+/+}$ group (Figure 9B). Additionally, we observed reduced levels of $NOTCH2^{+}$ and $NOTCH1^{+}NOTCH2^{+}$ co-expressing cells and significantly reduced levels of $NOTCH1^{+}$ (Figure 9C). Taken together, these data show that *in vivo* induction of DLL4 signaling is dependent on CD40 expression on lung DCs and suggests that DLL4-NOTCH2 interactions in turn promote optimal polarization of Th_{17} responses. Importantly, these data implicate the Hip1 serine protease in impeding CD40-dependent Notch ligand signaling and highlight limitation of CD40 and DLL4 signaling as an immune evasion mechanism that dampens Th_{17} polarization during Mtb infection.

Discussion

In this study, we identify Notch ligand signaling on DCs as a critical mechanism for Th_{17} polarization during Mtb infection. We show that induction of the Notch ligand DLL4 downstream of CD40 signaling augments Th_{17} responses, which correlated with lower Mtb lung burdens. DLL4 is required for inducing multifunctional $CXCR3^{+}CCR6^{+}$-expressing DP IL-17^{+}IFN-g^{+}, IL-17^{+}IL-22^{+} and TP IL-17^{+}IFN-g^{+}IL-22^{+} subsets in the lung. Additionally, we provide evidence that Mtb limits CD40-dependent Notch ligand signaling and dampens Th_{17} responses through the immunomodulatory Hip1 serine protease. Overall, our studies provide new molecular insights into Th_{17} responses during Mtb infection and reveal key innate pathways that can be targeted to enhance protective CD4 T cell responses and improve pulmonary control of Mtb.

DCs are critical for shaping adaptive immunity and are necessary for initiating T cell responses in the lung following Mtb infection. However, it is now well established that Mtb impedes DC

functions to subvert early protective T cell responses[195,198,224,255] and restrict Th_{17} polarization[195,198,224]. Our group reported that Mtb prevents optimal crosstalk between DCs and CD4 T cells by impairing the CD40 costimulatory pathway[195]. Specifically, we showed that interactions between CD40 on DCs and CD40L on T cells are necessary for Th_{17} polarization during Mtb infection, even when Th_{17}-polarizing cytokines such as IL-6, IL-1b and IL-23 are present, and that exogenously triggering CD40 signaling on DCs enhances Th_{17} responses and improves control of pathogen burdens in the lungs[195]. However, the mechanism by which CD40 orchestrates Th_{17} polarization in response to Mtb or other stimuli was not well understood. We now demonstrate that engaging CD40 on DCs during Mtb infection leads to upregulation of the Notch ligand *Dll4* (Figure 1, Supplemental Figure 4) and increases cell surface expression of DLL4 and Jagged1 on DCs (Figure 2). In contrast, *Jag1* is constitutively expressed and is not further induced by infection (Figure 1, Supplemental Figure 4). Notably all $DLL4^+$ DCs were also positive for Jagged1, leading to high frequencies of double-positive $DLL4^+Jagged1^+$ DC populations (Figure 2). We also show that blocking DLL4 abrogates CD40-dependent Th_{17} polarization *in vitro* (Figure 3) and *in vivo* (Figure 4-5) but does not significantly impact Th_1 polarization, highlighting a critical role for DLL4 in Th_{17} polarization. These findings reveal novel insights into how DC responses mediate Th_{17} polarization during Mtb infection. Additionally, our results on *C. albicans* and other PRR ligands (Supplemental Figure 3 and Supplemental Figure 7) suggests that these insights are likely to also be important for understanding Th_{17} polarization beyond Mtb infection.

While collaboration between CD40 and DLL4 in Th_{17} polarization has not been previously reported, expression of the Notch ligands *Dll4* and *Jag1* has largely been studied in the context

of Th_1/Th_2 differentiation in response to TLR stimulation and anti-CD40 antibody[268]. Some reports have linked DLL4 with Th_{17} polarization in response to stimulating with TLR ligands *in vitro* or following BCG infection, but these studies did not demonstrate a clear requirement for DLL4 or CD40-DLL4 collaboration in generating Th_{17} responses[269-271]. Thus, to our knowledge, this is the first study to link CD40 and DLL4 signaling on DCs to Th_{17} polarization during Mtb infection and provides key insights that can be applied to other experimental models where immunomodulation of Th_{17} responses is of interest. In addition to their roles in Th_{17} polarization, our study also identifies a role for CD40 and Notch ligands in promoting multifunctional lung Th responses *in vivo* (Figure 5), including induction of DP IL-17^+IL-22^+ and TP IL-17^+ IFN-γ^+ IL-22^+ CD4 T cells. Studies in humans and non-human primate models of TB have suggested that CD4 T cells that simultaneously expressing multiple cytokines are protective against disease[120,125,253,257]. IL-22, an IL-10 family member, is often co-expressed by Th_{17} cells[264] and has been implicated in promoting protective immunity to Mtb[120,142]. Additional Th subsets that are thought to promote protective functions in TB include $CXCR3^+CCR6^+$ T cells that co-express IFN-γ and IL-17, (sometimes referred to as Th_1* subsets), which were identified in the peripheral blood of latently infected individuals[257]. Interestingly, our group recently showed that $CXCR3^+CCR6^+$ dual Th_1/Th_{17} cells are also present in lung compartments of asymptomatic Mtb-infected rhesus macaques, where they were associated with pulmonary control of Mtb infection[253]. We now show that $CXCR3^+CCR6^+$ CD4 T cells that express both IL-17 and IFN-γ are induced *via* CD40 and DLL4 signaling, highlighting an essential role for crosstalk between DC costimulatory and Notch ligand pathways for generating these responses (Figure 6). Moreover, our data showing that triggering CD40-DLL4 signaling can overcome Mtb restriction

of IL-17, IL-22 and IFN-g producing multifunctional responses in the lung, provides new mechanistic insights that can be leveraged for inducing protective Th_{17} subsets *via* vaccination.

One of the ways by which Mtb evades host immunity is through expression of immunomodulatory proteins that interfere with DC-T cell crosstalk. Our lab has previously shown that Mtb prevents CD40 expression on DCs and restricts Th_{17} polarization through the Hip1 serine protease[195,198,224]. A *hip1* mutant strain of Mtb induces robust CD40 expression and higher Th_{17} responses relative to wild type Mtb[195,198]. We now show that in contrast to wild type Mtb, which did not induce DLL4 on the cell surface of lung DCs, mice infected with a *hip1* mutant significantly increased $DLL4^+$ and $DLL4^+Jagged1^+$ DC populations in the lung (Figure 8). This was accompanied by higher frequencies of IL-17- and IL-22-producing Th subsets along with increased expression of NOTCH1 and NOTCH2 receptors on T cells early in infection relative to wild type Mtb (Figure 8). Moreover, levels of DLL4 on DCs positively correlated with IL-17 and NOTCH2 expression on Th subsets suggesting that DLL4-NOTCH2 interactions likely mediate Th_{17} polarization (Figure 8). The induction of $DLL4^+Jagged1^+$ DC and $NOTCH1^+NOTCH2^+$ CD4 T cells following CD40 engagement or *hip1* mutant infection suggests that combinatorial Notch-ligand-Notch receptor interactions may promote balanced Th_1/Th_{17} responses. Infection of $CD40^{-/-}$ mice demonstrated that induction of $DLL4^+$ and $DLL4^+Jagged1^+$ DCs by the *hip1* mutant was abrogated in the absence of CD40 (Figure 9), demonstrating that Notch ligand expression is dependent on CD40 signaling. These results provide evidence that Mtb limits DLL4-Notch receptor interactions during infection *via* a mechanism that involves Hip1. We have previously shown that Hip1 prevents optimal CD40 expression through proteolysis of its substrate GroEL2[222,224]. We showed that full-length

recombinant GroEL2 protein induces robust CD40 expression on DCs through a TLR2-dependent mechanism[198]. However, the cleaved form of GroEL2, which is the form that predominates in wild type Mtb, and which is present in both live and killed cultures, is unable to induce CD40[224]. Therefore, we posit that the ability of the *hip1* mutant to induce Notch ligands on lung DCs is mediated by the presence of full length GroEL2, as this protein remains uncleaved in the absence of the Hip1 protease[221,222,224]. We posit that full length GroEL2 specifically limits CD40 signaling on DCs (and not general DC activation or other co-stimulatory markers such as CD80/CD86) as we have demonstrated that Hip1 limits Notch ligand expression through a CD40-dependent mechanism (Figure 9). Thus, by impeding the CD40 costimulatory pathway, wild type Mtb limits DLL4-NOTCH receptor signaling, leading to delayed and sub-optimal Th_{17} responses. Additionally, our data on *C. albicans* (Supplemental Figure 3 and Supplemental Figure 7) support the idea that modulating Notch signaling may be a strategy employed by Th_{17}-polarizing bacteria to promote Th_{17} responses as well as by other pathogens that manipulate DC-T cell crosstalk and promote disease, thereby extending our insights beyond TB.

Our results also suggest that Notch signaling plays a role in generating protective immune responses that help control mycobacterial burdens. Correlation analysis revealed that IL-17 and NOTCH2 inversely correlate with Mtb CFU (Figure 7). In our DC IT transfer experiment, we observed induction of $NOTCH2^+$ lung CD4 T cells following CD40-engagement was reduced after DLL4 blockade (Figure 6). Additionally, infection with the *hip1* mutant, which naturally engages the CD40-CD40L pathway, led to induction of DLL4 on lung DCs and NOTCH2 on T cells (Figure 8). These results suggest that wild type Mtb actively dampens Notch ligand and

Notch receptor expression to impede DC-T cell crosstalk in order to promote disease and maintain bacterial burdens in the lung during infection. Previous work from our lab showed that infection with the *hip1* mutant significantly prolonged survival of mice and resulted in dramatically lower lung immunopathology[221]. Our data suggest that early induction of CD40 and DLL4 in the absence of Hip1 leads to higher IL-17^{+}NOTCH2^{+} Th subsets and more protective immune responses. Our results that suggest Notch signaling is important for protection aligns with recent studies in other experimental models[269,272,273]. Of interest, a recent study on SARS-CoV-2 infection found that Notch signaling is upregulated in juvenile compared to older macaques and suggests that lack of Notch signaling could be a risk factor for the increased susceptibility of older individuals to COVID-19[274]. Our results on Notch signaling in Mtb-infected mice highlight the need to better understand this pathway in human TB, where there is limited data. A recent study measured the expression of Notch ligands and receptors in human PBMC samples and found that individuals with active TB exhibited higher expression of DLL4 on monocytes and NOTCH1 on T cells compared to healthy individuals[275]. Another study found that DLL4 expression on monocytes in individuals with TB was reduced following anti-TB treatment[276]. However, these studies focus on chronic stages of TB disease and do not study Notch ligand expression during early events following Mtb infection. Therefore, additional studies in humans and nonhuman primate models of latent and active TB are needed to dissect the role of Notch ligand signaling in initiating Th_{17} responses in lung compartments following Mtb infection as well as within granulomas in chronic stages of TB disease. The timing and location of DLL4 and Jagged1 signaling during Mtb infection will likely dictate Th_1/Th_{17} balance and protective versus pathogenic outcomes, along with other suppressive pathways that have been identified to function during Mtb infection[277]. Moreover, since we know that aberrant

IL-17 or DLL4 expression is not beneficial for the host[133,134,278-281], inducing balanced Th_1/Th_{17} immunity along with their temporal and spatial context will be important considerations in designing vaccines and host-directed therapies for TB. DLL4 has also been shown to be important for reducing inflammation in non-TB contexts[282,283], where it has been implicated in activating T cells and other responses that mediate graft-versus-host disease and autoimmunity[279-281]. Moreover, excessive IL-17 is well established as a mechanism of autoimmune-driven pathology and can also be detrimental in TB disease depending on the timing and location[133,134,278]. Further studies on immunomodulation of DLL4, either by targeting CD40 signaling or *via* small molecules that directly target Notch ligands on DCs, will be necessary for determining the utility of modulating the CD40-DLL4 axis for host directed therapies that prevent excessive TB pathology.

In addition to providing new insights into Mtb immune evasion strategies, identification of the CD40-DLL4 axis in Th_{17} polarization has implications for improving vaccine and adjuvant design. Studies on Mtb immunomodulatory proteins that subvert host protective immunity, such as those presented here, are vital for elucidating pathways that should be overcome in the context of live-attenuated vaccines. Several studies have demonstrated that deletion of immune evasion proteins in either Mtb or the BCG vaccine strain is a viable strategy for developing more efficacious vaccines against TB[220,239,284,285]. BCG has previously been shown to affect DC expression of Notch ligands[269,276] and TLR9 was reported to regulate granulomas induced by BCG *via* DLL4[269]. Our lab has developed a knockout of *hip1* in BCG (BCGΔ*hip1*) and found it to induce higher levels of IL-17 than wild type BCG[26]. Thus, it would be interesting to test whether DLL4 signaling is operant in the context of BCGΔ*hip1* vaccination and whether

engaging the CD40-DLL4 axis has an adjuvant effect on BCGΔ*hip1* vaccination. Further, designing adjuvants that crosslink CD40 and/or DLL4 on DCs during subunit vaccination can induce beneficial early Th_{17} polarization and a more balanced Th_1/Th_{17} response to vaccination.

In summary, our study demonstrates that engaging CD40 during Mtb infection is critical for inducing the Notch ligand DLL4, which is necessary for Th_{17} polarization during infection. By delineating the DLL4-CD40-Th_{17} axis in TB, our work provides clear targets that can be harnessed for new adjuvant and vaccination approaches not only in TB, but also in other pathogenic infections and autoimmune disease states where therapeutically manipulating Th_{17} response is desirable.

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Materials and Methods

Mice

C57BL/6 wild type (WT) and $CD40^{-/-}$ (B6.129P2-$Cd40^{tm1Kik}$) mice were purchased from The Jackson Laboratory. C57BL/6 OT-II $OVA_{323–339}$ Thy1.1+ mice (originally developed by Dr. F. Carbone, University of Melbourne) were kindly provided by Dr. Bali Pulendran (Stanford

University, formerly Emory University) and bred in the Yerkes vivarium. C57BL/6 ESAT-6_{1-20}/I-A^b transgenic mice were kindly provided by Dr. Andrea Cooper (Trudeau Institute) and bred in the Yerkes vivarium. All WT mice used for experiments were eight-to-twelve weeks of age and all transgenic mice used were eight-to-eighteen weeks of age. Mice were housed in either the Yerkes National Primate Center animal BSL-3 or BSL-1 vivarium under sterile conditions with food and water provided ad libitum. All animals were handled according to the regulations formulated by the Emory University Institutional Animal Care and Use Committee (IACUC).

Generation of bone marrow-derived dendritic cells

Bone marrow-derived dendritic cells (BMDCs) were generated from C57BL/6 WT mice purchased from The Jackson Laboratory as previously described[195]. Briefly, the femur and tibia of mice were extracted and flushed using cold RPMI-1640 (Lonza). Following red blood cell (RBC) lysis using RBC Lysis Buffer (Sigma), progenitor cells were spun down and plated at a concentration of 1E6 cells/ml in R10 media (RPMI-1640 [Lonza] with 2mM L-glutamine [Lonza], 0.1 mM NEAA [Gibco], 10 mM HEPES [Corning], 1mM Sodium Pyruvate [Lonza], 10% heat-inactivated FBS [Gemini]) supplemented with 1:100 Penicillin/Streptomycin (Lonza), 1:1000 2-mercaptoethanol (BME; Gibco) and 20 ng/ml murine rGM-CSF (R&D Systems). Cells were grown at 37ºC with 5% CO_2. On Day 3 and 6 following plating, cells were fed using R10 media supplemented with 2-mercaptoethanol and rGM-CSF. On day 8 following plating, cells were harvested and purified using mouse $CD11c^+$ beads (MiltenyiBiotec) according to manufacturer recommendations. Purity of BMDCs was confirmed using flow cytometry. For BMDC stimulations and infections, purified BMDCs were plated in 24-well tissue culture plates at a concentration of 6E5 cells/ml in R10 media supplemented with 1:1000 BME. For

intratracheal assay BMDC preparations, cells were plated in tissue culture plates at a concentration of 1E6 cells/ml. Cells were allowed to adhere to the plate (4 hours post-plating to overnight) before use. All cells throughout the study were counted using trypan blue stain (Thermo-Fisher Scientific) on a Countess Automated Cell Counter (Invitrogen).

Bacterial strains

Mycobacterium tuberculosis (Mtb) strains H37Rv and H37Rv *hip1* mutant[221] were used. As previously described[195,198,222], Mtb strains were grown in liquid media Middlebrook 7H9 (BD Difco) supplemented with 0.5% glycerol (Sigma), 10% oleic acid-albumin-dextrose-catalase (OADC) (BD) and 0.05% Tween 80 (VWR). Additionally, 20 µg/ml kanamycin (Sigma) was included for growing the *hip1* mutant. Stocks were prepared by growing cultures to an OD_{600} of 0.4 - 0.6, then filtered and resuspended in 7H9 media with 25% glycerol (Sigma) and stored at -80ºC. Before use, stocks were titered to determine CFU. Heat-killed stocks were prepared as previously described[195].

Mtb in vitro infection and stimulation of BMDCs

For *in vitro* infections with live Mtb, purified BMDCs were infected with H37Rv at an MOI of 1.0. Briefly, bacterial cultures were centrifuged and resuspended in R10 media supplemented with 1:1000 BME (and 1 µg/ml CD40LT (Adipogen) for relevant conditions). Plates were then placed in the 37ºC incubator (with 5% CO_2) for 6 hours. Following infection, a 200 µg/ml Amikacin (Sigma) solution (in R10 supplemented with 1:1000 BME) was added to cells for 30 minutes to kill extracellular bacteria. Afterwards, wells were washed 3X PBS (Sigma) and resuspended in R10 media supplemented with 2-mercaptoethanol (and 1 µg/ml CD40LT for

relevant conditions) and the plates were placed in the 37ºC incubator until designated time point. For certain wells, cells were lysed using PBS + 0.5% TritonX (Fisher Scientific) and plated to determine intracellular CFU on Middlebrook 7H10 agar plates (supplemented with 0.5% glycerol [Sigma], 10% OADC [BD] and 0.1 mg/ml cycloheximide solution [Sigma]). For BMDC stimulations, cells were allowed to adhere until stimulation, and existing supernatant was removed and replaced with R10 media with 1:1000 BME containing stimuli. Cells were then placed in the incubator until designated time points. Heat-killed bacteria were used at an MOI of 30. Stimulations with CD40LT alone used 1 µg/ml CD40LT. For PRR/TLR stimulations, 0.1 µg/ml LPS (Invivogen), 10 µg/ml Zymosan (Invivogen), or heat-killed *C. albicans* (Invivogen) at an MOI of 17 were used. Following stimulation, cell-free supernatants were removed from each well and stored for protein quantification (for live infection, supernatants were filtered using a 0.23 µm filter and removed from the BSL-3). To collect samples for RNA purification, wells were washed X1 using PBS (Sigma) and 300 µl of RNA Lysis Buffer (Zymo) was added to each well. Samples were then flash-frozen using 70% ethanol and dry ice and stored in the -80ºC until RNA purification.

RNA extraction, cDNA generation, and qPCR

RNA samples were purified using the Quick-RNA Miniprep Kit (Zymo) according to manufacturer's instructions. For BSL-3 samples, RNA was purified in the BSL-3 and removed at the elution step. Following purification, RNA was quantified using a spectrophotometer (NanoDrop ND-1000 or NanoDrop One [Fisher Scientific]). cDNA was made from each sample using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) using 100 ng of RNA and carried out in a C1000 Thermal Cycler (BioRad) according to manufacturer's

recommendation. Quantitative Polymerase Chain Reactions (qPCR) were carried out in a 384-well plate format on a QuantStudio 5 Smart Start (Thermo-Fisher Scientific) machine for "SYBR" reactions. SYBR Green PCR Master Mix (Applied Biosystems) was combined with molecular-biology grade water (Quality Biological), cDNA, and primers (10 μM) and pipetted into each well. Murine KiCqStart SYBR Primers used for this study (mouse m_*Il6*_1, m_*Il12b*_1, m_*Dll4*_3, m_*Jag1*_1) were purchased from Sigma. GAPDH primer was purchased from Eurofins (sequence: F: TGGCCTTCCGTGTTCCTAC R: GAGTTGCTGTTGAAGTCGCA). Prior to use, primer efficiency curves were generated for each primer. Each sample for qPCR was run in triplicate. All qPCR Data were analyzed using the ΔΔCt method and expression of genes was standardized to GAPDH. All qPCR Data are presented as $2^{-\Delta\Delta Ct}$.

Enzyme-Linked Immunosorbent Assay

Cell-free supernatants were used to enumerate cytokine protein levels using Enzyme-Linked Immunosorbent Assay (ELISA) assay. All the ELISAs were run according to manufacturer instructions: murine IL-6 (BD), murine IL-12p40 (BD), murine IFN-γ (Mabtech), murine IL-2 (BD), murine IL-17A (Invitrogen), and murine IL-22 (R&D Systems). Plates were washed using a Biotek ELx405 machine and measured using a BioTek ELx808 reader.

DC-T cell co-culture assays

Purified BMDCs were stimulated with Mtb (as described above) with different conditions for 24 hours. Following this time period, supernatant was removed and wells were washed 1X with PBS (Sigma). A mixture of R10 (supplemented with 1:1000 BME) with 10 μg/ml cognate

peptide ($OVA_{323-339}$, Invivogen) was then added and the BMDCs were "pulsed" for 1 hour. Afterwards, OT-II $OVA_{323–339}$-specific $Thy1.1^+$ CD4 T cells were added at a ratio of 4:1 T cell: DCs to each well. For conditions that required antibody blockade, the following antibodies (15 µg/ml - 60 µg/ml) were added at the co-culture step: murine DLL4 blocking antibody (Clone: HMD4; BioXCell), InVivoMAb polyclonal Armenian hamster IgG [DLL4 isotype] (BioXCell), Jagged1 blocking antibody (Clone: HMJ1-29; Biolegend), purified Armenian hamster IgG Isotype [Jagged1 isotype] (Biolegend). The cell culture plate was placed in a 37ºC incubator (with 5% CO_2) for 72 hours. Supernatants were harvested and then briefly spun to ensure a cell-free mixture. C57BL/6 OT-II $OVA_{323–339}$ $Thy1.1^+$ naïve CD4 T cells were purified using the mouse naïve $CD4^+$ T cell isolation kit (StemCell) according to manufacturers' instructions [isolated cells were >95% viable].

Intratracheal instillation of DCs and mouse tissue harvest

Purified BMDCs were stimulated or infected (as outlined above) in tissue culture plates for intratracheal (IT) transfer. BMDCs were harvested either at 24 hours (stimulated BMDCs) or at 48 hours (infected BMDCs). For conditions using CD40LT and antibody blockade, relevant blocking antibodies were provided during infection/stimulation. Afterwards, cells were harvested from the plates, washed 1X using PBS (Sigma), and then spun down followed by counting. Cells were then resuspended at 20E6/ml in PBS. For experiments in which Mtb-infected BMDC were transferred IT in the presence of blocking antibodies (anti-DLL4 or isotype controls), cells were resuspended in PBS and antibodies were added at 60 µg/ml. For experiments in which Mtb-stimulated BMDCs were transferred IT in the presence of blocking antibodies (anti-DLL4, anti-Jagged1, or isotype controls), cells were resuspended in PBS and antibodies were added at 30

μg/ml. For IT transfer, mice were anesthetized using isoflurane in a closed isoflurane chamber (Med-Vet) and 1E6 infected BMDCs (in 50 μl volume) were instilled into the trachea of mice as previously described[195]. For Mtb-stimulated BMDC IT experiments, C57BL/6 ESAT-6_{1-20}/I-A^b transgenic naïve CD4 T cells were transferred into mice one day before IT transfer. Briefly, spleens from ESAT-6 transgenic mice were harvested and made into a single-cell suspension. CD4 T cells were purified using mouse CD4 (L3T4) MicroBeads (MiltenyiBiotec) according to manufacturer's instructions. The resulting purified CD4 T cells were resuspended at a concentration of 1E7 cells/ml and 1E6 cells were transferred to mice *via* the intravenous (i.v.) route one day before IT transfer. Mice were euthanized at either six days or four weeks post-IT, depending on experiment, using isoflurane overdose. Lungs were placed into lung C-Tubes (MiltenyiBiotec) containing HBSS (Corning) supplemented with 2% heat-inactivated FBS (Gemini) and 10mM HEPES (Corning). A mixture of 0.1% collagenase, type IV (Worthington) and 0.01% DNAse I (Worthington) was added into each tube. Lung tissue was homogenized using an automated gentleMACS Dissociator (MiltenyiBiotec) using the manufacturer's murine lung processing program. After addition of collagenase/DNAse mixture, lungs were dissociated and placed in a 37ºC (with 5% CO_2) incubator for 30 minutes. Following this time, lungs were dissociated again using a murine lung processing program. The tubes were then spun down and red blood cells were lysed from the mixture using RBC lysis buffer (Sigma). Lungs were resuspended at 10E6/ml in R10 supplemented with 1:1000 BME and 1E6 cells were plated per well for *ex vivo* or ESAT-6_{1-20} stimulation in 96-well U-bottom propylene plates and placed in the 37ºC incubator (with 5% CO_2). For enumeration of bacteria, a portion of the lungs was harvested in sterile 2 ml tubes (Sarstedt) containing stainless steel beads (Next Advance) and PBS + 0.02% Tween80. The lungs were then homogenized in a Bullet Blender (Next Advance).

Serial dilutions were plated onto Middlebrook 7H10 plates (with or without 20 µg/ml Kanamycin for *hip1* mutant) to determine the CFU.

Aerogenic infection of mice with Mtb

Mtb cultures for aerosol infection were prepared as previously described [195]. Briefly, mice were infected *via* the aerosol route (~ 100 CFU) using a nose only exposure chamber (In-Tox Products). A day following aerosol infection, mice were euthanized to determine bacterial burdens as described above.

Flow cytometry

For lung suspensions, cells were either left unstimulated (*ex vivo*) or stimulated with 10 µg/ml ESAT-6_{1-20} peptide (Genemed Synthesis, Inc) or 10 µg/ml whole cell lysate (WCL) [BEI]. Plates were then placed in the 37ºC incubator (with 5% CO_2) and after 2 hours, a mixture of 5 µg/ml Brefeldin A (Sigma) and 1:1500 GolgiStop (BD) in R10 supplemented with 1:1000 BME was added. The plate was then returned to the incubator for 4H (6H total) for ESAT-6 stimulations or overnight for WCL stimulations and the cells were stained the next day. For staining BMDCs, cells were harvested from plates at designated time points and stained directly. To distinguish between live and dead cells, all cells were stained with Fixable Aqua Dead Cell Stain Kit (Molecular Probes) or Fixable Near-IR Dead Cell Stain Kit (Invitrogen). Additionally, mouse Fc block (BD) was used before staining. To stain BMDCs, the following antibodies were used (all surface): FITC anti-mouse I-A/I-E (clone: M5/114.15.2, Biolegend), PE anti-mouse DLL4 (clone: HMD4-1, Biolegend), PE-Cy5 anti-CD40 (clone: 1C10, eBioscience), PE-Cy7 anti-mouse CD11c (clone: N418, eBioscience), APC anti-mouse CD339 (Jagged 1) (clone: HMJ1-29,

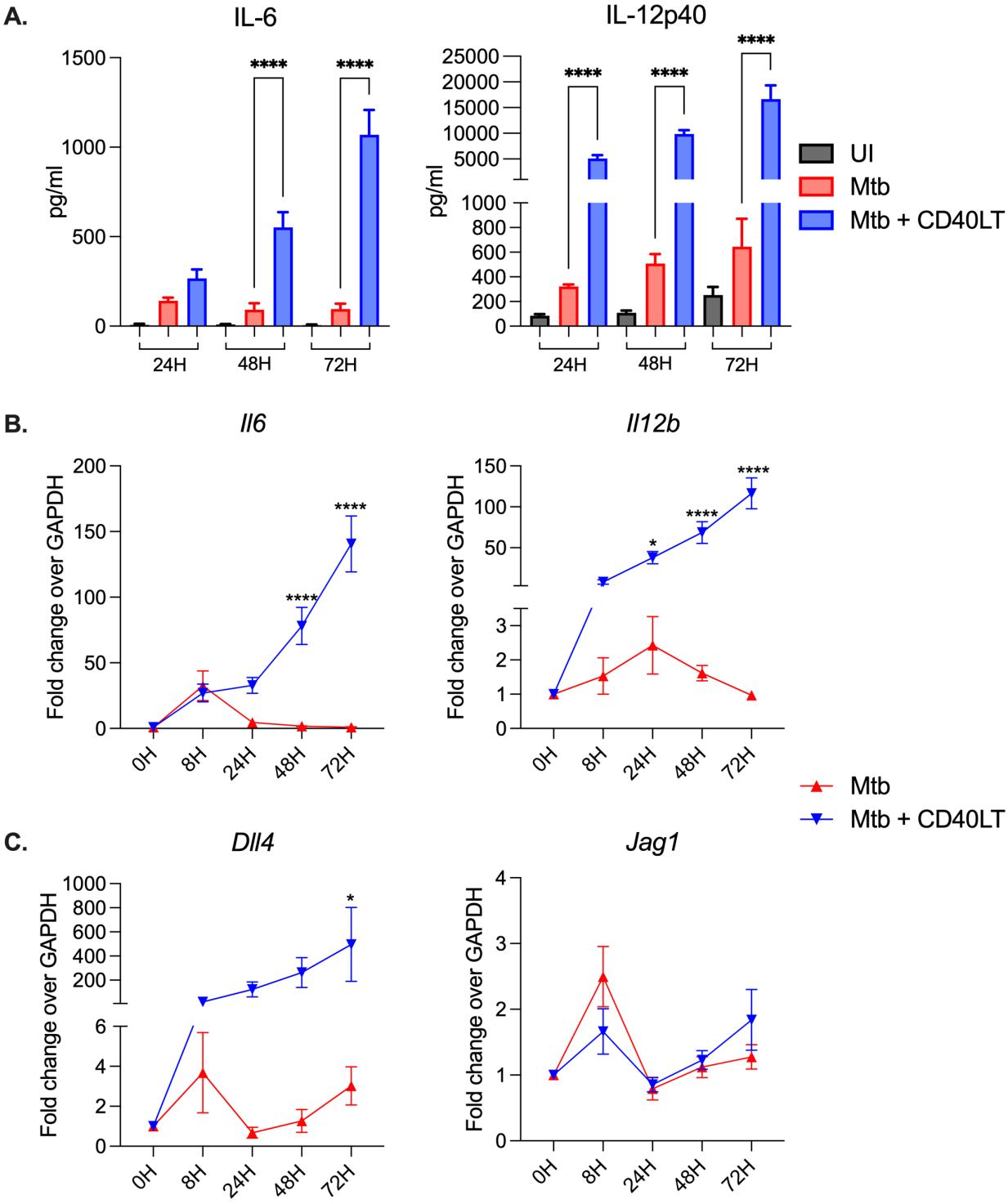
Biolegend), Alexa700 anti-mouse CD86 (clone: GL-1, Biolegend), APC-Cy7 anti-mouse CD11b (clone: M1/70, Biolegend). To stain lung T cells, the following surface stain antibodies were used: FITC anti-mouse CD14 (clone: Sa14-2, Biolegend), FITC anti-mouse NK-1.1 (clone: PK136, Biolegend), FITC anti-mouse TER-119/Erythroid (clone: TER-119, Biolegend), FITC anti-mouse CD19 (clone: 1D3/CD19, Biolegend), FITC Rat anti-mouse IL-2 (clone: JES6-5H4, BD), PE Rat anti-mouse Vβ6 T-Cell (clone: RR4-7, BD), PerCP anti-mouse CD45 (clone: 30-F11, Biolegend), Alexa 700, Hamster anti-mouse CD3ε (clone: 500A2, BD), APC-Cy7 anti-mouse CD8α (clone: 53-6.7, Biolegend), BV650 anti-mouse/human CD44 (clone: IM7, Biolegend), BV650 anti-mouse CD183 (CXCR3) (clone: CXCR3-173, Biolegend), BV711 anti-mouse CD8α (clone: 53-6.7, Biolegend), BV785 anti-mouse CD196 (CCR6) (clone: 29-2L17, Biolegend), APC-Cy7 Rat anti-mouse CD44 (clone: IM7, BD). To stain lung innate cells, the following surface stain antibodies were used: BUV395 Rat Anti-Mouse CD84 (clone: 1D3/CD84, BD), BUV496 Rat Anti-Mouse I-A/I-E (clone: 2G9, BD), BUV563 Hamster Anti-Mouse CD80 (clone: 16-10A1, BD), BUV661 Rat Anti-Mouse CD115 (clone: T38-320, BD), BUV737 Rat Anti-Mouse DLL4 (clone: 9A1.5, BD), BUV805 Rat Anti-Mouse F4/80 (clone: T45-2342, BD), BV421 Rat Anti-Mouse CD172a (clone: P84, BD), BV421 anti-mouse CD169 (clone: 3D6.112, Biolegend), BV480 Hamster Anti-Mouse CD103 (clone: 2E7, BD), BV570 anti-mouse CD3 (clone: 17A2, Biolegend), BV570 anti-mouse CD19 (clone: 6D5, Biolegend), BV650 anti-mouse/rat XCR1 (clone: ZET, Biolegend), BV711 anti-mouse CD11c (clone: N418, Biolegend), BV750 anti-mouse CD45 (clone: 30-F11, Biolegend), BV786 Mouse Anti-Mouse CD64 a/b (clone: X54-5/7.1, BD), FITC anti-mouse Ly-6G (clone: 1A8, Biolegend), BB700 Rat Anti-Mouse CD124 (clone: mIL4R-M1, BD), PE anti-mouse Jagged1 (clone: HMJ1-29, Biolegend), PE/Cy5 anti-mouse CD3ε (clone: 145-2C11, Biolegend), PE-Cy7 anti-mouse/human

CD11b (clone: M1/70, Biolegend), PE/Dazzle 594 anti-mouse Ly-6C (clone: HK1.4, Biolegend), PE-Cy5 anti-mouse CD24 (clone: M1/69, Biolegend), PE-Cy7 anti-mouse JAML (clone: 4/E10, Novus Biologicals), Alexa 647 Rat Anti-Mouse S100A9 (clone: 2B10, BD), and Alexa 700 anti-mouse/human CD11b (clone: M1/70, Biolegend). To stain lung T cells, the following intracellular stain antibodies were used: PE-CF594 Rat anti-mouse IL-17A (clone: TC11-18H10, BD), PE-Cy7 Rat anti-mouse TNF (clone: MP6-XT22, BD), BV786 Rat anti-mouse CD4 (clone: RM4-5, BD), APC anti-mouse IFN-γ (clone: XMG1.2, eBioscience), BV421 Rat anti-mouse IL-2 (clone: JES6-5H4, BD), PE Rat anti-mouse NOTCH1 (clone: 22E5.5, BD), PerCP-Cy5.5 anti-mouse IL-22 (clone: Poly5164, Biolegend), PE-Cy7 anti-mouse CD40L (clone: SA047C3, Biolegend), V450 Hamster anti-mouse CD3ε (clone: 500A2, BD), BV605 Rat anti-mouse NOTCH2 (clone: 16F11, BD), Alexa700 Rat anti-mouse CD4 (clone: RM4-5, BD). Prior to use, all antibodies were titrated for optimal concentration. The live/dead stain (1:500) and Fc block (1:50) mixture was diluted in PBS (Sigma). The surface stain antibodies were diluted in FACS buffer (PBS [Sigma], 2% heat-inactivated FBS [Gemini], and 2 mM EDTA [Corning]). For intracellular staining, the BD Cytofix/Cytoperm kit and buffers were used according to manufacturer's instructions. Following staining, cells were fixed in 1:1 FACS buffer to 4% paraformaldehyde (PFA) (Electron Microscopy Sciences) for BSL-3 samples, or 2% PFA for non-BSL-3 samples and placed in the 4ºC until acquisition (up to 24 hours post-staining). For compensation, Anti-Rat and Anti-Hamster Ig κ /Negative Control Compensation Particles (BD), UltraComp eBeads compensation beads (Invitrogen), or ArC Amine Reactive Compensation Bead Kit (Invitrogen) were used. All samples were acquired using an LSR-II machine (BD) or an A5 Symphony (BD) using FACSDiva (BD) software. All Data were analyzed using FlowJo software (FlowJo LLC). For certain experiments, FlowAI plugin was used to select for optimal

events[286]. For DC flow cytometry data, cells were pre-gated on singlets, live cells, and $CD11c^+$ MHC-II High populations (for *in vitro* experiments) or pre-gated on singlets, $CD45^+$, live cells, $CD3^-CD19^-$, MHC-II^+, $CD11c^+$ and either $CD11b^+CD103^-$ ($CD11b^+$ DC) or $CD11c^-CD103^+$ ($CD103^+$ DC) [for *in vivo* experiments]. For T cell flow cytometry data, cells have been pre-gated on singlets, live cells, and $CD3^+CD4^+$ populations.

Statistical analyses

Statistical analyses of data and graphs were mostly conducted using Prism (GraphPad). For correlations, R statistical software was used, in particular the corrplot[287,288] and ggscatter packages. The experimental schema figures were generated using BioRender.com. All data presented are representative of 2 to 4 independent experiments and are presented as mean ± S.D or mean ± S.E.M (indicated in figure legend). Statistical significance p-value key is the following: ns = no significance, * = ≤ 0.05, ** = ≤ 0.01, *** = ≤ 0.001, **** = ≤ 0.0001. Statistical tests performed for each figure are noted in individual figure key. All correlations presented are Pearson's correlations.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IL-12p40
pg/ml
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1000
500
0
25000
20000
15000
10000
5000
1000
800
600
400
200
****
24H
48H
72H
UI
Mtb
Mtb + CD40LT
B.
Il6
Il12b
Fold change over GAPDH
200
150
100
50
0
3
2
1
*
0H
8H
24H
48H
72H
Mtb
Mtb + CD40LT
C.
Dll4
Jag1
6
4

Figure 1: Engaging CD40 on Mtb-infected DCs augments pro-inflammatory cytokines and enhances expression of Notch ligand *Dll4*. BMDCs were infected with either media (UI) or Mtb H37Rv strain at an MOI of 1 in the presence or absence of 1 μg/ml of multimeric CD40 L reagent (CD40LT). At designated time points, cell free supernatants and RNA were collected to assay for cytokines and mRNA. A) ELISA measurements of IL-6 and IL-12p40 in supernatants. B) and C) qPCR analysis of genes was standardized to housekeeping gene GAPDH, analyzed using the $\Delta\Delta$Ct method, and presented as $2^{-\Delta\Delta Ct}$. Data are presented as mean ± SD (a) or mean ± SEM (B-C). Data are representative of 3 independent experiments. Data were analyzed in A) using a one-way ANOVA with a correction for multiple comparisons and B-C) using a two-way ANOVA with a correction for multiple comparisons. Statistical significance p-value key is the following: * = ≤ 0.05, **** = ≤ 0.0001.

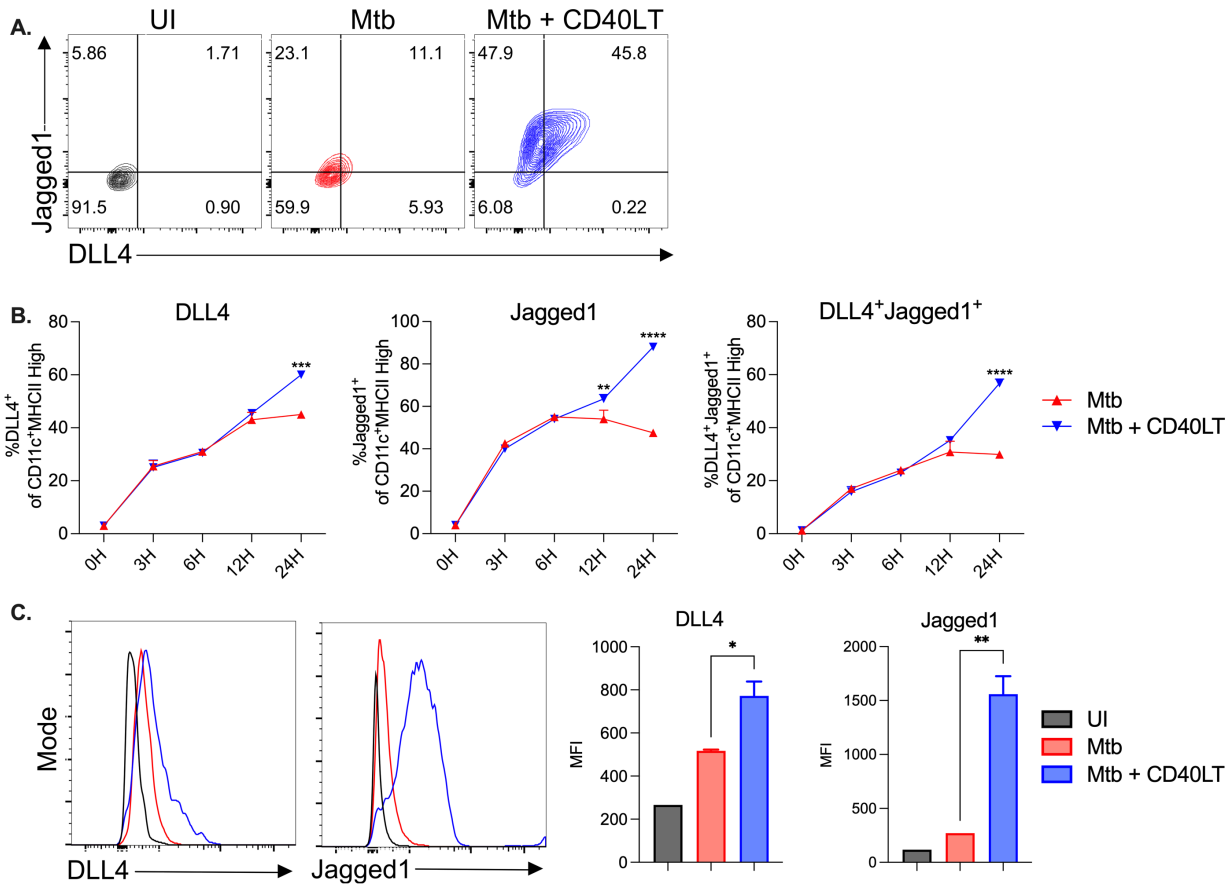


Figure 2: Engaging CD40 on Mtb-stimulated DCs enhances surface expression of DLL4 and Jagged1. BMDCs were stimulated with either media (UI) or HK Mtb (MOI 30) with or without the addition of 1 μg/ml of CD40LT. At designated time points, cells were collected and stained for surface markers. A) Representative flow plot of DLL4$^+$ and Jagged1$^+$ frequencies. B) Frequency of DLL4$^+$ and Jagged1$^+$ and DP populations. C) MFI of DLL4 and Jagged1 expression and representative graphs. Populations shown were pre-gated on singlets, live cells, and CD11c$^+$ MHC-II High populations. Data are presented as mean ± SD. Data are representative of 4 independent experiments. Data were analyzed in B) using a two-way ANOVA with a correction for multiple comparisons and C) using an unpaired student's t-test. Statistical significance p-value key is the following: * = ≤ 0.05, ** = ≤ 0.01, *** = ≤ 0.001, **** = ≤ 0.0001.

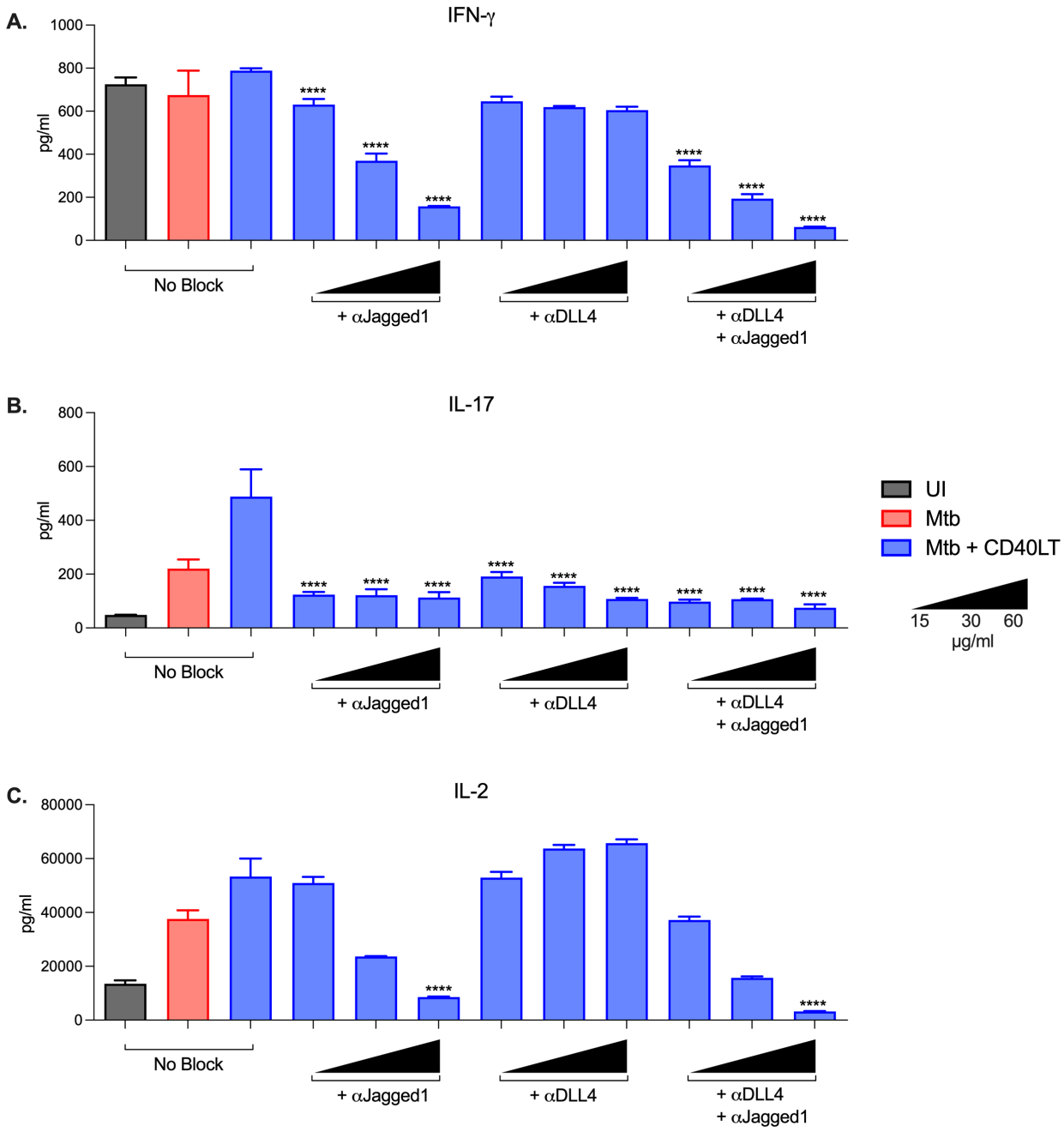

A.
IFN-γ
pg/ml
1000
800
600
400
200
0
****
No Block
+ αJagged1
+ αDLL4
+ αDLL4
+ αJagged1
B.
IL-17
pg/ml
800
600
400
200
0
UI
Mtb
Mtb + CD40LT
15
30
60
μg/ml
C.
IL-2
pg/ml
80000
60000
40000
20000
0

Figure 3: DLL4 is required for Th_{17} polarization but is dispensable for Th_1 polarization. BMDCs were stimulated with either media alone (UI), or HK Mtb (MOI 30) with or without 1 μg/ml of CD40LT. Following 24H of stimulation, cells were pulsed with 10 μg/ml of cognate peptide ($OVA_{323-339}$) for one hour and then co-cultured with purified naïve CD4 OT-II Tg Thy1.1 T cells at a ratio of 4:1. For blockade conditions, blocking antibodies to either DLL4, Jagged1, or both DLL4 and Jagged1 combined, were added at the time of co-culture at the following concentrations: 15, 30, or 60 μg/ml. After 72H of co-culture, cell-free supernatants were harvested and assayed for cytokines by ELISA. A) IFN-γ (Th_1). B) IL-17 (Th_{17}). C) IL-2. Data are presented as mean ± SD. Data are representative of 3 independent experiments. Data were analyzed using a one-way ANOVA with a correction for multiple comparisons (all data points were compared to Mtb + CD40LT). Statistical significance p-value key is the following: *** = ≤ 0.001, **** = ≤ 0.0001.

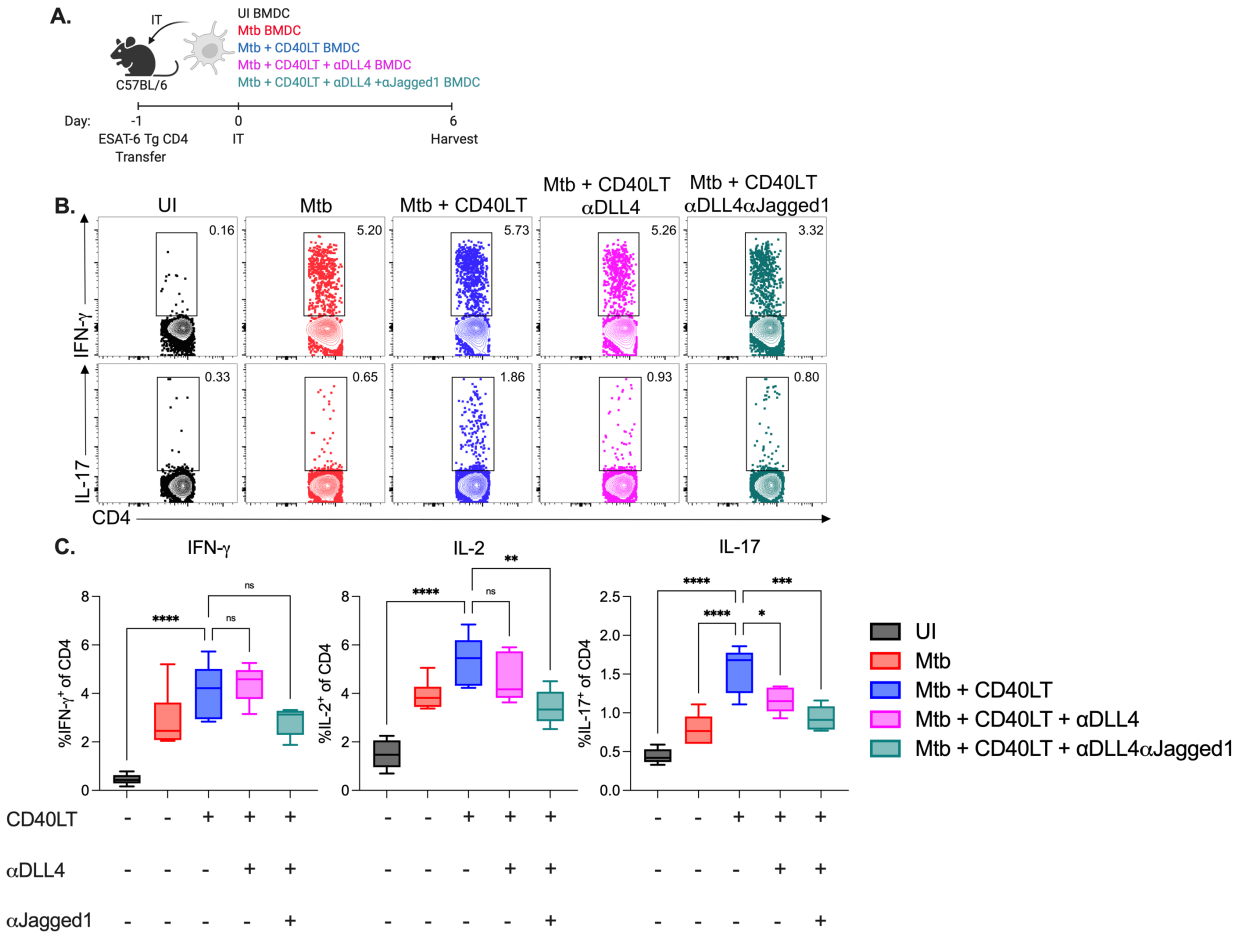

A.
IT
C57BL/6
UI BMDC
Mtb BMDC
Mtb + CD40LT BMDC
Mtb + CD40LT + αDLL4 BMDC
Mtb + CD40LT + αDLL4 +αJagged1 BMDC
Day:
-1
ESAT-6 Tg CD4 Transfer
0
IT
6
Harvest
B.
UI
Mtb
Mtb + CD40LT
Mtb + CD40LT αDLL4
Mtb + CD40LT αDLL4αJagged1
0.16
5.20
5.73
5.26
3.32
0.33
0.65
1.86
0.93
0.80
IFN-γ
IL-17
CD4
C.
IFN-γ
IL-2
IL-17
%IFN-γ+ of CD4
%IL-2+ of CD4
%IL-17+ of CD4
****
ns
**
*
***
CD40LT
αDLL4
αJagged1
UI
Mtb
Mtb + CD40LT
Mtb + CD40LT + αDLL4
Mtb + CD40LT + αDLL4αJagged1

Figure 4: Blocking DLL4 singly or in combination with Jagged1 on DCs reduces antigen-specific IL-17$^+$ CD4 T cell frequencies *in vivo*. A) Experimental schema. BMDCs we stimulated with either media alone (UI), or HK Mtb (MOI 30) with or without 1 μg/ml of CD40LT for 24H. At this time in the CD40LT condition, either DLL4 or DLL4 and Jagged1 blocking antibodies (30 μg/ml) were added. One day before transfer, 1E6 ESAT-6 transgenic T cells were transferred into mice *via* the i.v. route. On the day of intratracheal (IT) transfer, 1E6 DCs were transferred. At 6 days after transfer, mice were euthanized and lung single cell suspensions were stimulated with 10 μg/ml ESAT-$6_{1\text{-}20}$ peptide to assess antigen-specific responses. Cells were then stained for flow cytometry. B) Representative flow plots of IFN-γ and IL-17-positive populations. C) Cytokine-positive frequency of CD4 T cells. Populations shown were pre-gated on singlets, live cells, and CD3$^+$ cells with data plotted from CD4 T cells. Experimental schema was made with BioRender.com. Data are presented as mean ± SD. Data are representative of 2 independent experiments. Data were analyzed using a one-way ANOVA with a correction for multiple comparisons. Statistical significance p-value key is the following: ns = no significance, * = ≤ 0.05, ** = ≤ 0.01, *** = ≤ 0.001, **** = ≤ 0.0001.

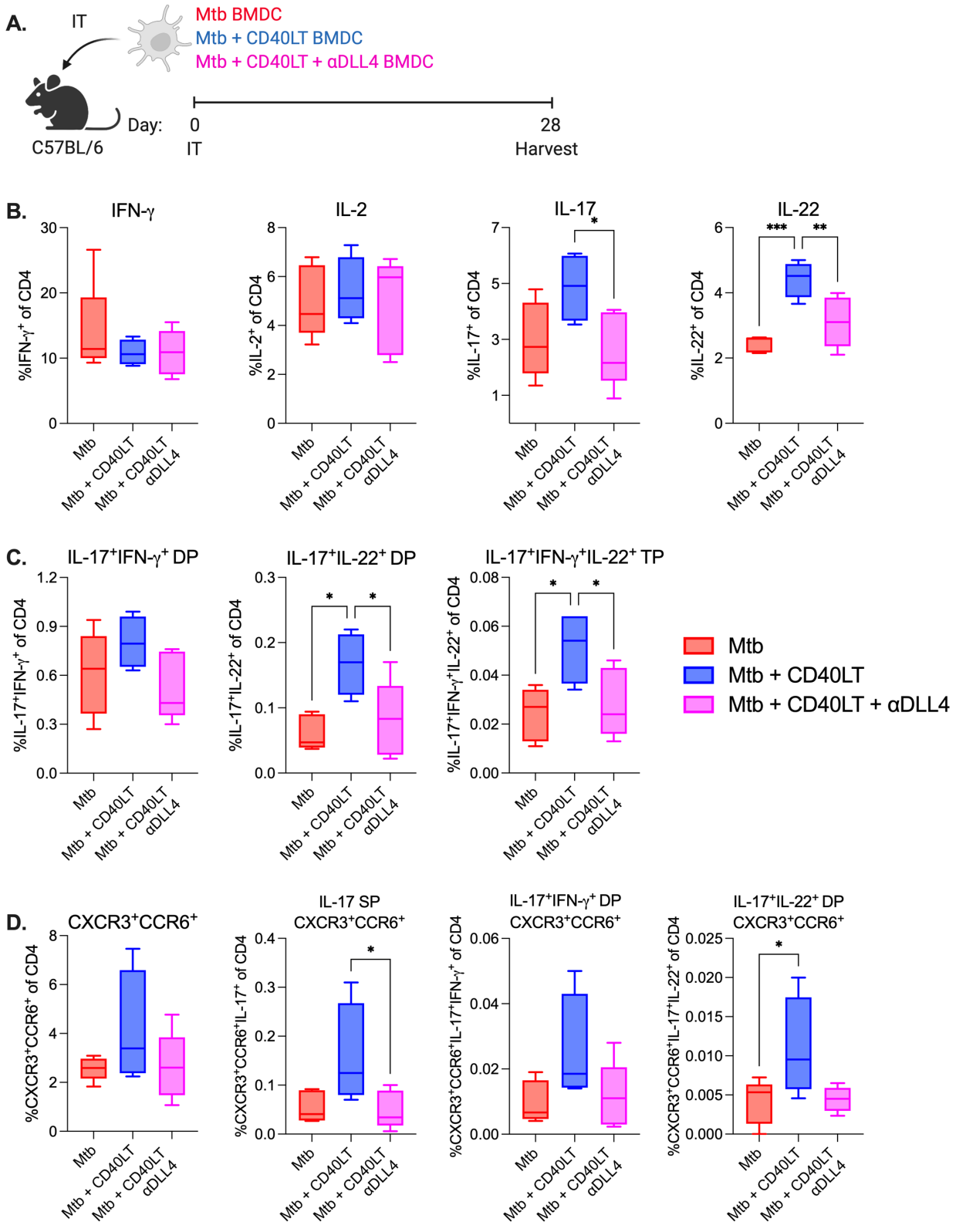

A.
IT
Mtb BMDC
Mtb + CD40LT BMDC
Mtb + CD40LT + αDLL4 BMDC
Day:
0
28
C57BL/6
IT
Harvest
B.
IFN-γ
IL-2
IL-17
IL-22
%IFN-γ+ of CD4
%IL-2+ of CD4
%IL-17+ of CD4
%IL-22+ of CD4
Mtb
Mtb + CD40LT
Mtb + CD40LT αDLL4
C.
IL-17+IFN-γ+ DP
IL-17+IL-22+ DP
IL-17+IFN-γ+IL-22+ TP
%IL-17+IFN-γ+ of CD4
%IL-17+IL-22+ of CD4
%IL-17+IFN-γ+IL-22+ of CD4
Mtb
Mtb + CD40LT
Mtb + CD40LT + αDLL4
D.
CXCR3+CCR6+
IL-17 SP CXCR3+CCR6+
IL-17+IFN-γ+ DP CXCR3+CCR6+
IL-17+IL-22+ DP CXCR3+CCR6+
%CXCR3+CCR6+ of CD4
%CXCR3+CCR6+IL-17+ of CD4
%CXCR3+CCR6+IL-17+IFN-γ+ of CD4
%CXCR3+CCR6+IL-17+IL-22+ of CD4
Mtb
Mtb + CD40LT
Mtb + CD40LT αDLL4

Figure 5: Blocking DLL4 on Mtb-infected DCs reduces Th_{17} and multifunctional CD4 T cell responses *in vivo*. A) Experimental schema. BMDCs were infected with Mtb at an MOI of 1, with or without CD40LT, for 48H. For antibody blockade studies, 60 μg/ml anti-DLL4 antibody was added at this time. DCs were then harvested and 1E6 were intratracheally (IT) transferred into the lungs of mice along with additional blocking antibody. At 4 weeks post transfer, mice were euthanized and lung suspensions were unstimulated to asses *ex vivo* responses using flow cytometry. B) Cytokine-positive frequency of CD4 T cells. C) Frequency of multiple-cytokine positive CD4 T cells as determined by Boolean analysis. D) Frequency of $CXCR3^{+}CCR6^{+}$ population and Boolean analysis of multiply marker/cytokine populations. Populations shown were pre-gated on singlets, live cells, and $CD3^{+}$ cells with data plotted from CD4 T cells. Experimental schema was made with BioRender.com. Data are presented as mean ± SD. Data are representative of 2 independent experiments. Data were analyzed using a one-way ANOVA with a correction for multiple comparisons. Statistical significance p-value key is the following: * = ≤ 0.05, ** = ≤ 0.01, *** = ≤ 0.001. SP = single-positive, DP = double-positive, TP = triple-positive.

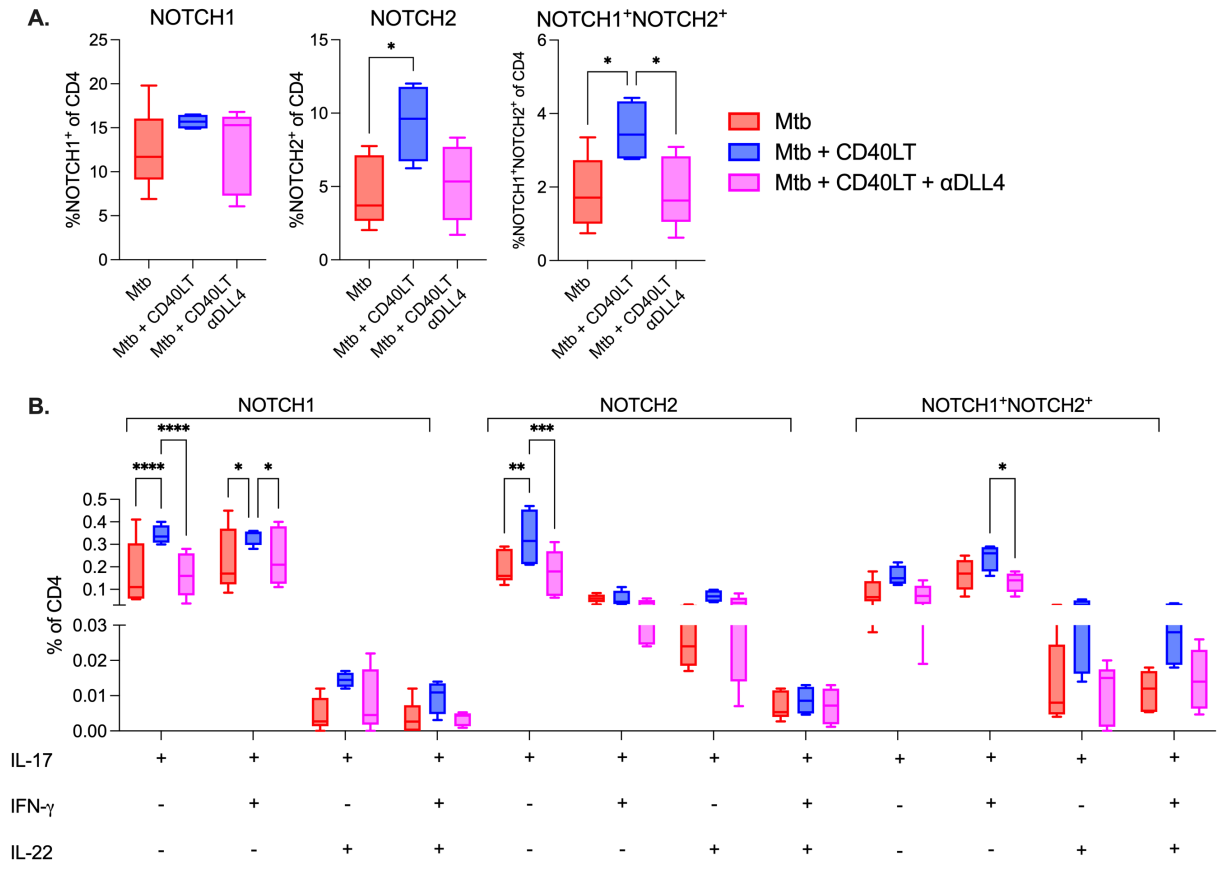

A.
NOTCH1
NOTCH2
NOTCH1+NOTCH2+
%NOTCH1+ of CD4
%NOTCH2+ of CD4
%NOTCH1+NOTCH2+ of CD4
Mtb
Mtb + CD40LT
Mtb + CD40LT + αDLL4
B.
NOTCH1
NOTCH2
NOTCH1+NOTCH2+
% of CD4
IL-17
IFN-γ
IL-22

Figure 6: Engaging CD40 on Mtb-infected DCs increases NOTCH2 expression on CD4 T cells in the lung. BMDCs were infected with Mtb at an MOI of 1, with or without CD40LT, for 48H. For blockade, 60 μg/ml anti-DLL4 antibody was added at this time. DCs were then harvested and 1E6 were intratracheally (IT) transferred into the lungs of mice along with additional blocking antibody. At 4 weeks post transfer, mice were euthanized and lung suspensions were unstimulated to asses *ex vivo* responses using flow cytometry. A) Frequency of T cells expressing NOTCH receptors. B) Frequency of NOTCH receptor positive and multiple-cytokine positive CD4 T cells as determined by Boolean analysis. Populations shown were pre-gated on singlets, live cells, and $CD3^+$ cells with data plotted from CD4 T cells. Data are presented as mean ± SD. Data are representative of 2 independent experiments. Data were analyzed using a one-way ANOVA with a correction for multiple comparisons. Statistical significance p-value key is the following: * = ≤ 0.05, ** = ≤ 0.01, *** = ≤ 0.001, **** = ≤ 0.0001.

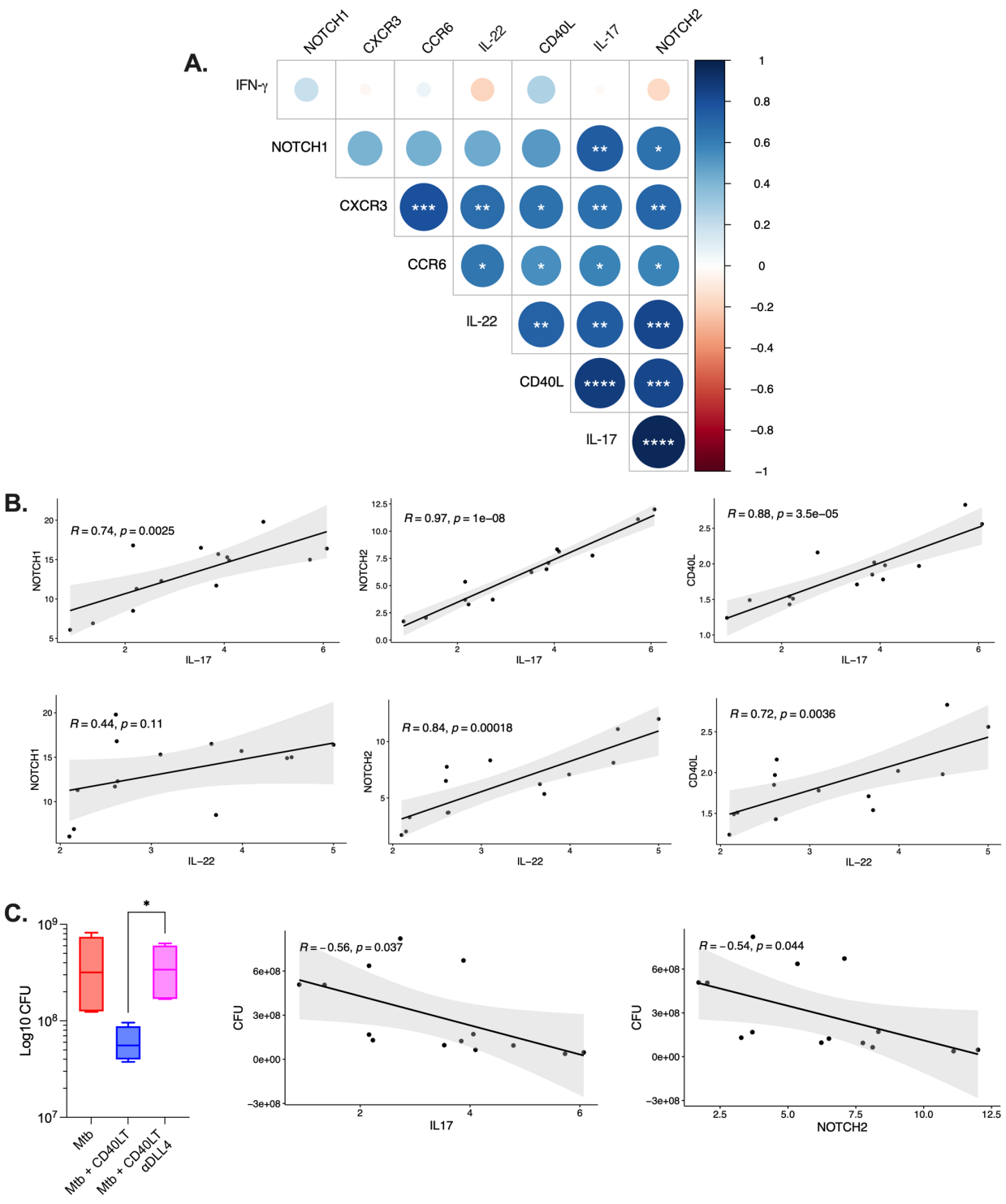

A.
NOTCH1
CXCR3
CCR6
IL-22
CD40L
IL-17
NOTCH2
IFN-γ
1
0.8
0.6
0.4
0.2
0
−0.2
−0.4
−0.6
−0.8
−1
B.
R = 0.74, p = 0.0025
R = 0.97, p = 1e−08
R = 0.88, p = 3.5e−05
R = 0.44, p = 0.11
R = 0.84, p = 0.00018
R = 0.72, p = 0.0036
IL−17
IL−22
C.
Log10 CFU
Mtb
Mtb + CD40LT
Mtb + CD40LT αDLL4
R = −0.56, p = 0.037
R = −0.54, p = 0.044
CFU
IL17
NOTCH2

Figure 7: Th_{17} responses correlate with NOTCH2 expression and lower lung CFU. BMDCs were infected with Mtb at an MOI of 1, with or without CD40LT, for 48H. For blockade, 60 μg/ml DLL4 blocking antibody was added at this time. DCs were then harvested and 1E6 were intratracheally (IT) transferred into the lungs of mice with additional blocking antibody. At 4 weeks post transfer, mice were euthanized and lung suspensions were unstimulated to asses *ex vivo* responses using flow cytometry. A) Correlogram using R package "corrplot" showing frequencies of cytokine-positive and marker-expressing CD4 T cells. B) Correlations in R using "ggscatter" package. C) Lung homogenates were plated and bacterial CFU was measured 4 weeks post infection, correlations between CFU and frequencies of markers on CD4 cells using R "ggscatter" package". All correlations presented are Pearson's correlations. Data in C are presented as mean ± SD and analyzed using an unpaired Student's t-test. Data are representative of 2 independent experiments. Statistical significance p-value key is the following: * = ≤ 0.05, ** = ≤ 0.01, *** = ≤ 0.001, **** = ≤ 0.0001.

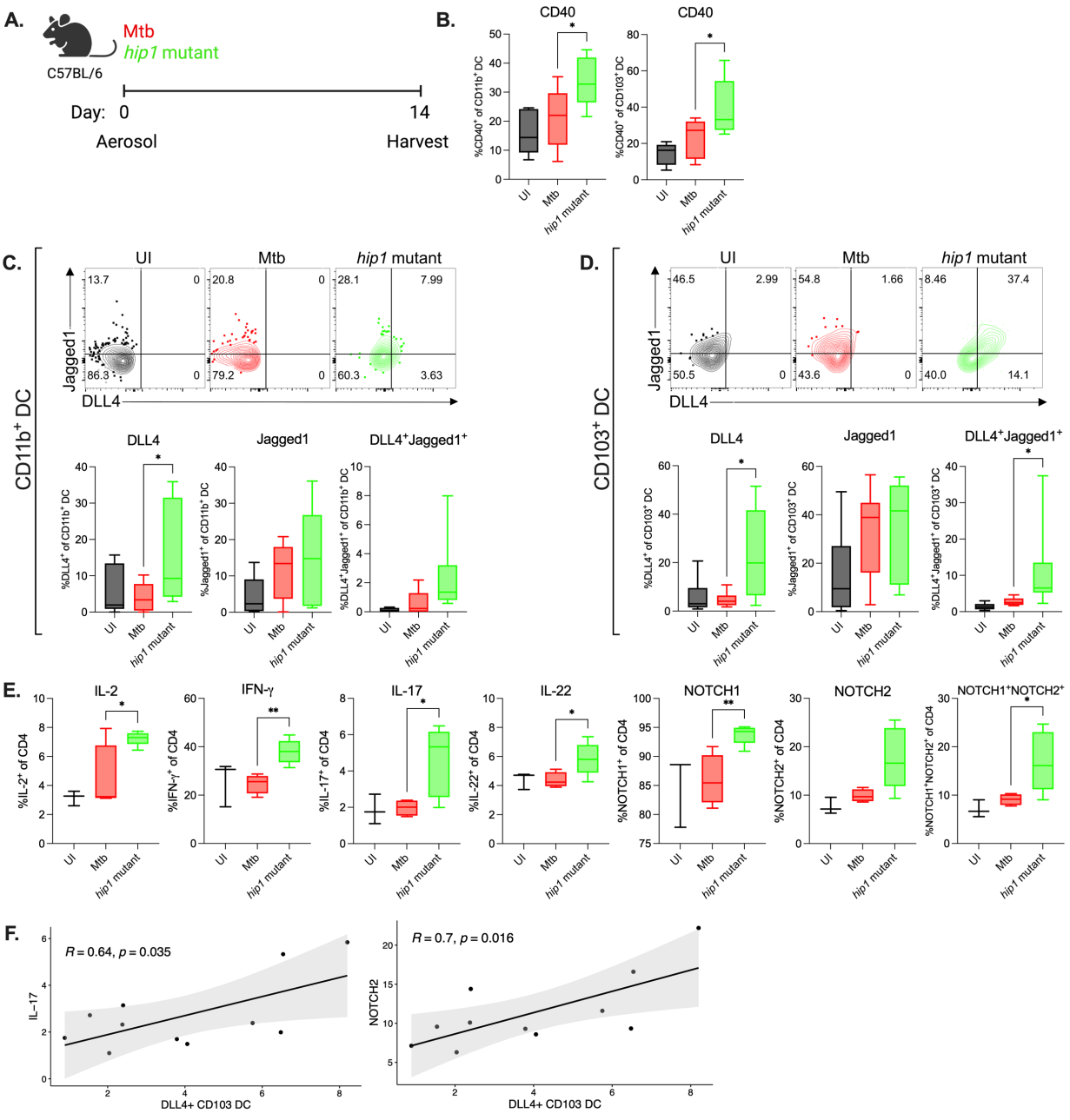

A.
Mtb
hip1 mutant
C57BL/6
Day: 0
14
Aerosol
Harvest
B.
CD40
CD40
%CD40+ of CD11b+ DC
%CD40+ of CD103+ DC
UI
Mtb
hip1 mutant
C.
CD11b+ DC
UI
Mtb
hip1 mutant
Jagged1
DLL4
13.7
0
86.3
0
20.8
0
79.2
0
28.1
7.99
60.3
3.63
DLL4
Jagged1
DLL4+Jagged1+
%DLL4+ of CD11b+ DC
%Jagged1+ of CD11b+ DC
%DLL4+Jagged1+ of CD11b+ DC
D.
CD103+ DC
46.5
2.99
50.5
0
54.8
1.66
43.6
0
8.46
37.4
40.0
14.1
%DLL4+ of CD103+ DC
%Jagged1+ of CD103+ DC
%DLL4+Jagged1+ of CD103+ DC
E.
IL-2
IFN-γ
IL-17
IL-22
NOTCH1
NOTCH2
NOTCH1+NOTCH2+
%IL-2+ of CD4
%IFN-γ+ of CD4
%IL-17+ of CD4
%IL-22+ of CD4
%NOTCH1+ of CD4
%NOTCH2+ of CD4
%NOTCH1+NOTCH2+ of CD4
F.
R = 0.64, p = 0.035
IL-17
DLL4+ CD103 DC
R = 0.7, p = 0.016
NOTCH2
DLL4+ CD103 DC

Figure 8: Mtb restricts DLL4 expression and early CD4 T cells responses in the lung through the Hip1 serine protease. A) Experimental schema. B6 mice were infected *via* the aerosol route with a low-dose of Mtb or *hip1* mutant. Following 2 weeks post-infection, mice were euthanized and lung *ex vivo* responses were measured using flow cytometry. B) Frequency of CD40-expressing $CD11b^+$ DCs and CD40-expressing $CD103^+$ DCs. Representative flow plots and frequency of $DLL4^+$, $Jagged1^+$, and $DLL4^+Jagged1^+$ for C) $CD11b^+$ DCs and D) $CD103^+$ DCs. E) Cytokine and NOTCH receptor positive CD4 T cell population frequencies. F) Correlations between different CD4 T cell and innate immune population markers using the R "ggscatter" package. DC data were pre-gated on singlets, $CD45^+$, live cells, $CD3^-CD19^-$, $MHC\text{-}II^+$, $CD11c^+$ and either $CD11b^+CD103^-$ ($CD11b^+$ DC) or $CD11c^-CD103^+$ ($CD103^+$ DC). T cell data were pre-gated on singlets, live cells, and $CD3^+$ cells with data plotted from CD4 T cells. All correlations presented are Pearson's correlations. Experimental schema was made with BioRender.com. Data in B-E are presented as mean ± SD. Data were analyzed in B-E using unpaired Student's t-tests. Data are representative of 2 independent experiments. Statistical significance p-value key is the following: * = ≤ 0.05, ** = ≤ 0.01.

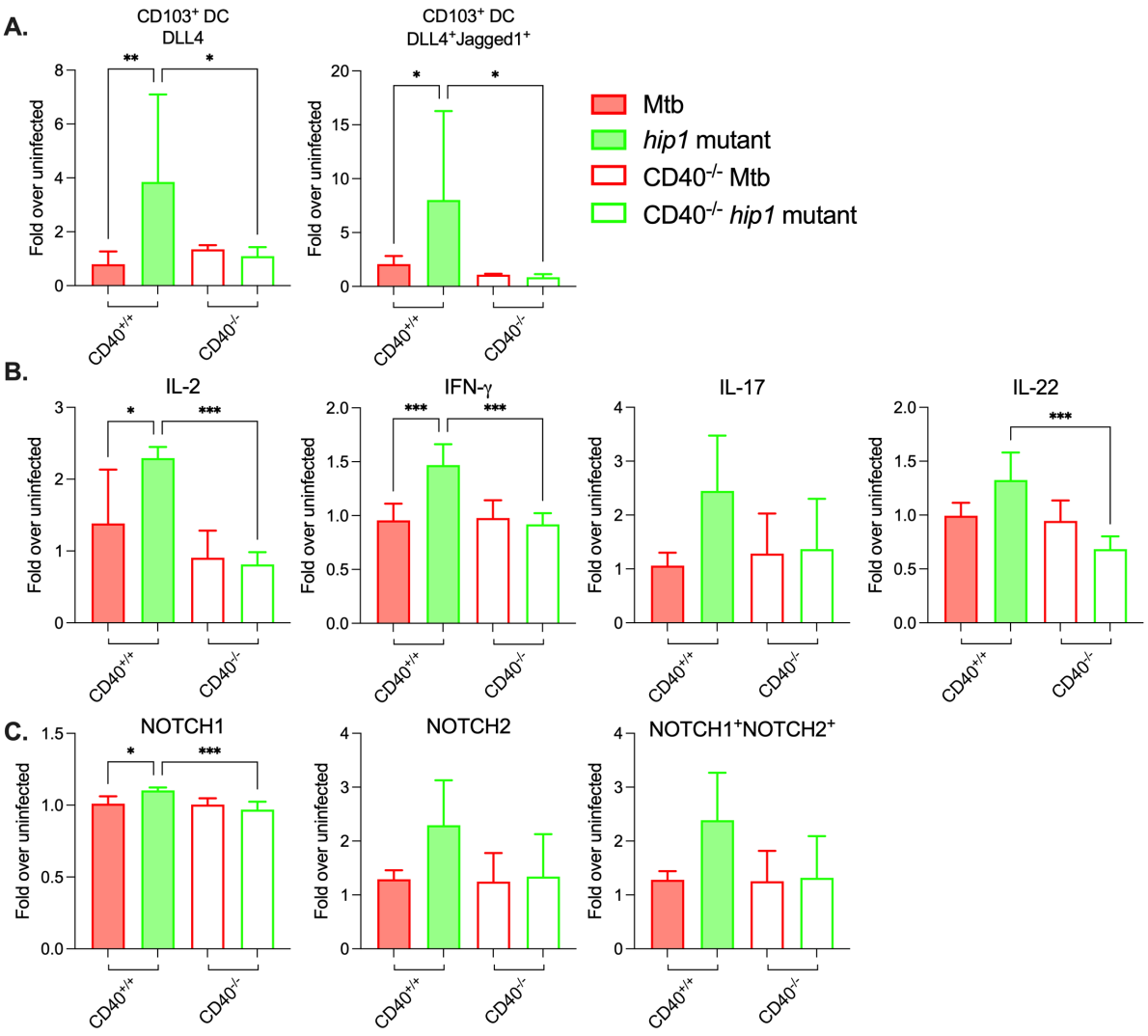
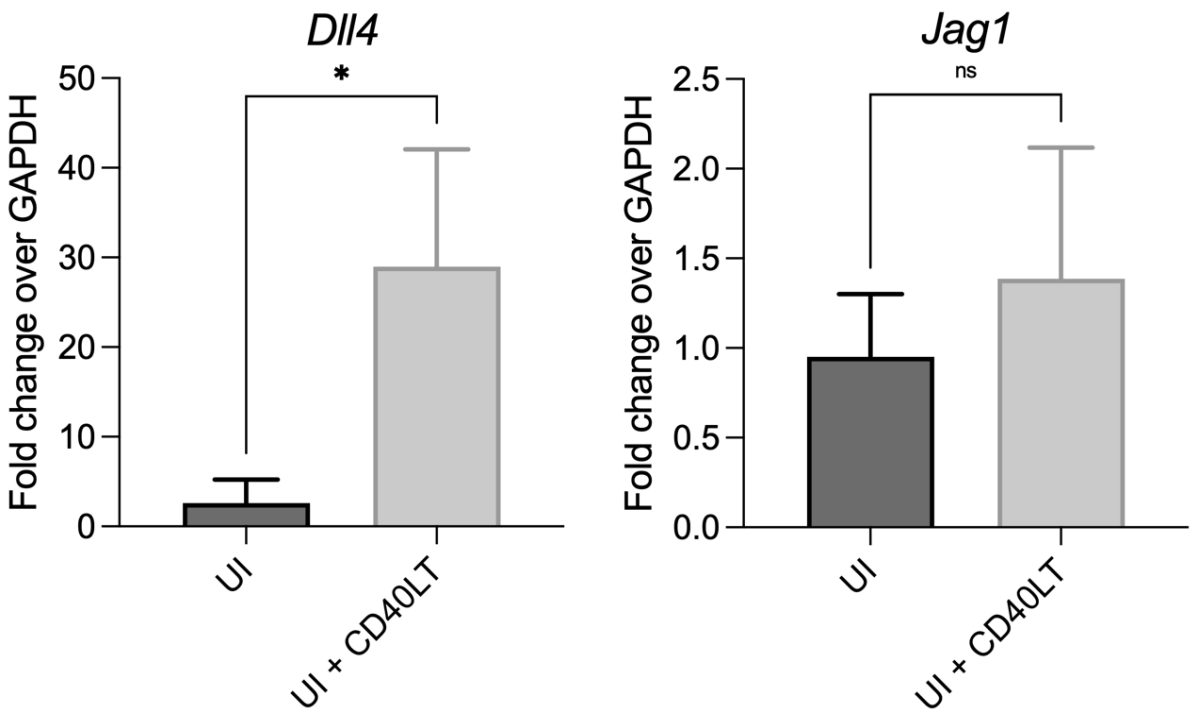
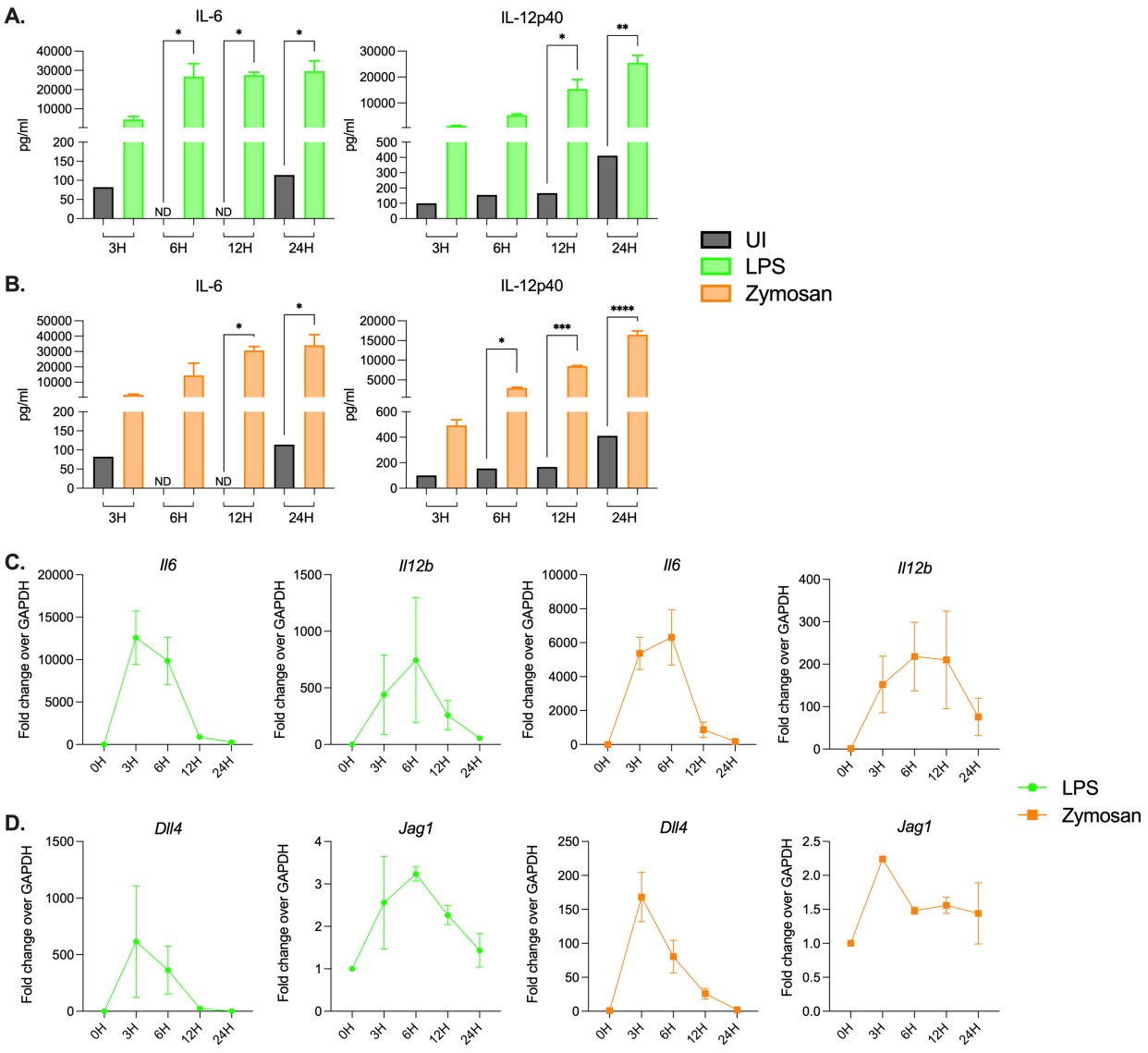

A.
CD103+ DC
DLL4
CD103+ DC
DLL4+Jagged1+
Fold over uninfected
Mtb
hip1 mutant
CD40-/- Mtb
CD40-/- hip1 mutant
CD40+/+
CD40-/-
B.
IL-2
IFN-γ
IL-17
IL-22
C.
NOTCH1
NOTCH2
NOTCH1+NOTCH2+

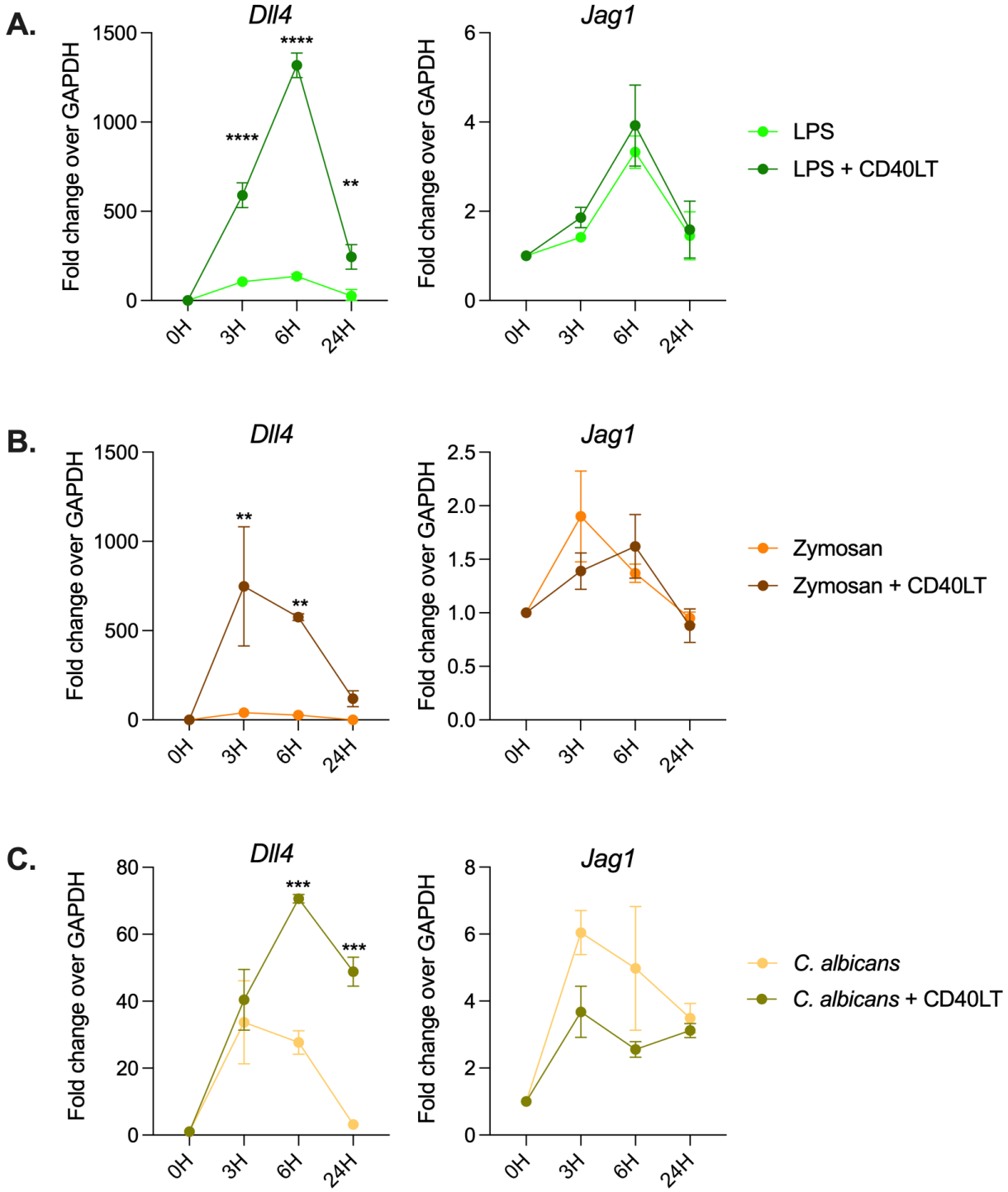
Figure 9: Hip1 impedes DLL4 expression on lung DCs *via* a CD40-dependent mechanism. C57BL/6 ($CD40^{+/+}$) or $CD40^{-/-}$ mice were infected *via* the aerosol route with a low-dose of Mtb or *hip1* mutant. Following 2 weeks post-infection, mice were euthanized and lung *ex vivo* responses were measured using flow cytometry. A) Fold of infected mice over uninfected mice (from the same mouse strain) for $DLL4^{+}$ and $DLL4^{+}Jagged1^{+}$ in the $CD103^{+}$ DC population. B) Fold over uninfected for cytokine-positive CD4 T cells. C) Fold over uninfected for NOTCH-receptor expressing CD4 T cells. DC data were pre-gated on singlets, $CD45^{+}$, live cells, $CD3^{-}$ $CD19^{-}$, MHC-II^{+}, $CD11c^{+}$ and either $CD11b^{+}CD103^{-}$ ($CD11b^{+}$ DC) or $CD11c^{-}CD103^{+}$ ($CD103^{+}$ DC). T cell data were pre-gated on singlets, live cells, and $CD3^{+}$ cells with data plotted from CD4 T cells. Data were analyzed using a one-way ANOVA with a correction for multiple comparisons. Data are presented as fold over uninfected mean ± SD. Data are representative of 2 independent experiments. Statistical significance p-value key is the following: * = ≤ 0.05, ** = ≤ 0.01, *** = ≤ 0.001.



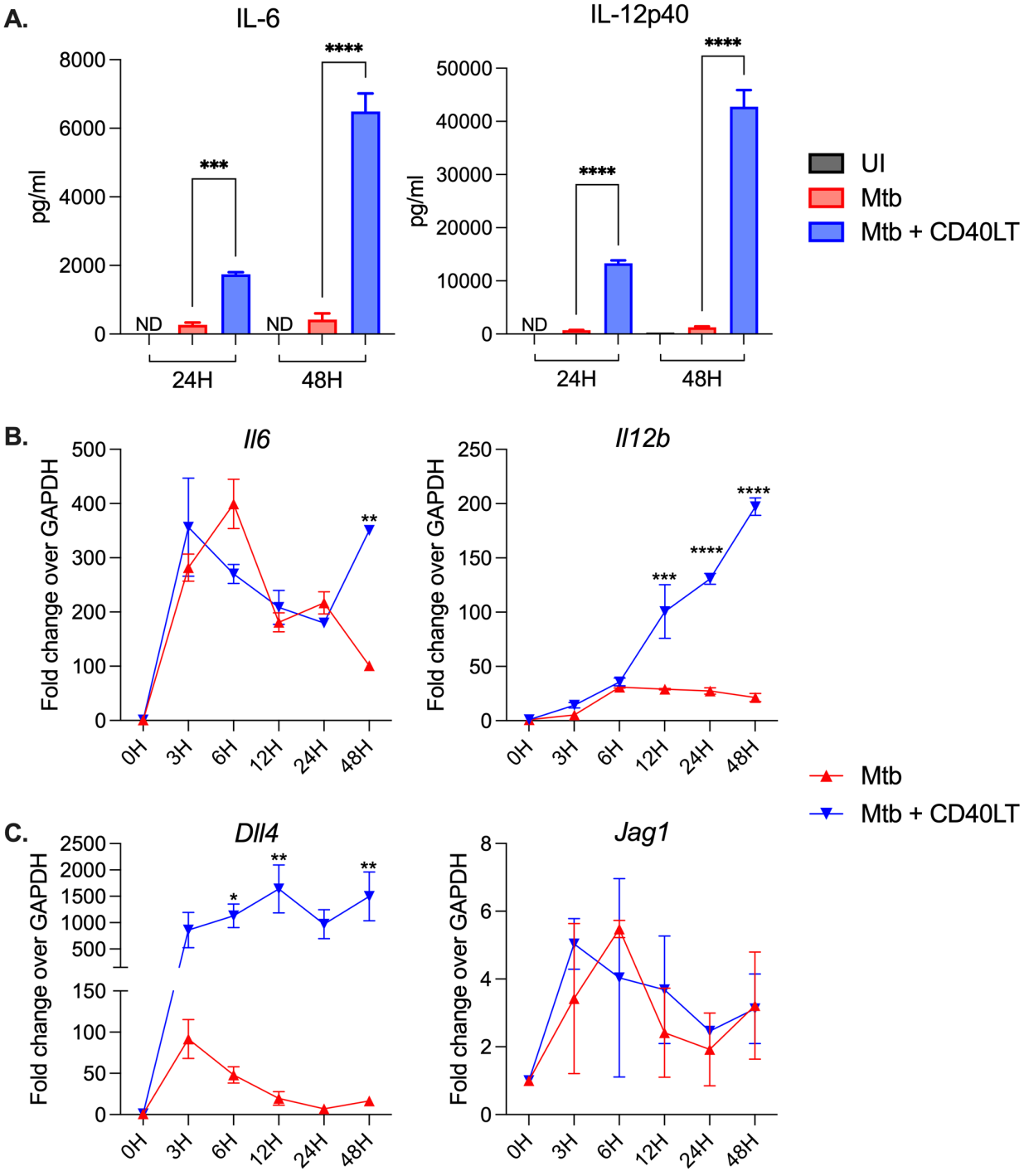
Supplemental Figure 1: Stimulation of CD40LT alone increases *Dll4* mRNA. BMDCs were stimulated with either media (UI) or 1 μg/ml of CD40LT for 8H. Following stimulation, RNA was collected to assay for *Dll4* and *Jag1* mRNA. qPCR analysis of genes was standardized to housekeeping gene GAPDH, analyzed using the ΔΔCt method, and presented as $2^{-\Delta\Delta Ct}$. Data are presented as mean ± SD. Data are representative of 3 independent experiments. Data were analyzed using an unpaired Student's t-test. Statistical significance p-value key is the following: ns = no significance, * = ≤ 0.05.


A.
IL-6
IL-12p40
pg/ml
ND
3H
6H
12H
24H
UI
LPS
Zymosan
B.
C.
Il6
Il12b
Fold change over GAPDH
0H
D.
Dll4
Jag1

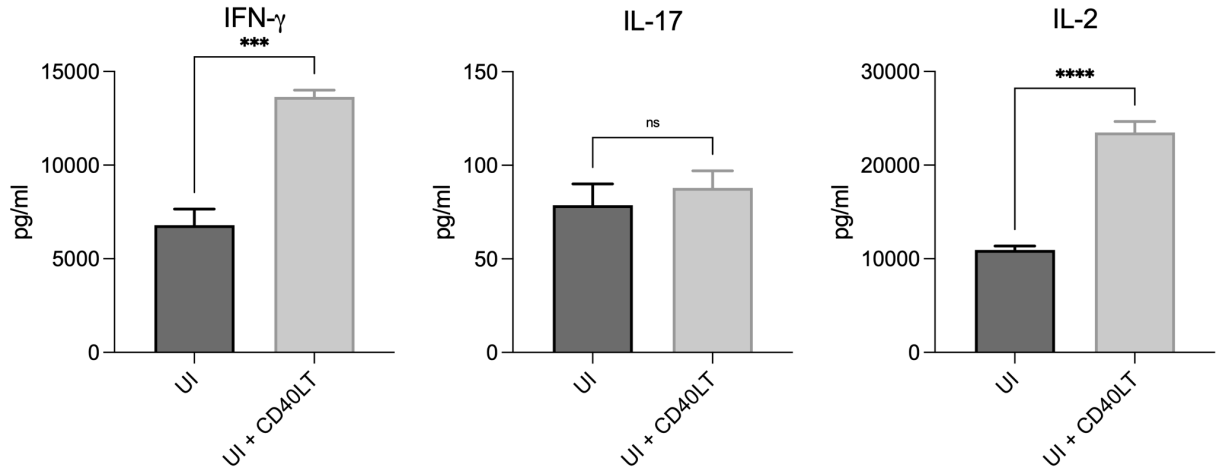
Supplemental Figure 2: LPS and zymosan stimulation of DCs enhances pro-inflammatory cytokines and upregulation of *Dll4*. BMDCs were stimulated with either media (UI), 0.1 μg/ml LPS or 10 μg/ml Zymosan. At designated time points, cell free supernatants and RNA were collected to assay for protein and mRNA. A)-B) ELISA measurement of IL-6 and IL-12p40 in supernatants. C)-D) qPCR analysis of genes was standardized to housekeeping gene GAPDH, analyzed using the ΔΔCt method, and presented as $2^{-\Delta\Delta Ct}$. Data are presented as mean ± SD (A-B) or mean ± SEM (C-D). Data are representative of 3 independent experiments. Data were analyzed using a one-way ANOVA with a correction for multiple comparisons. Statistical significance p-value key is the following: * = ≤ 0.05, ** = ≤ 0.01, *** = ≤ 0.001, **** = ≤ 0.0001. ND = not determined.


A.
Dll4
Jag1
Fold change over GAPDH
1500
1000
500
0
6
4
2
0H
3H
6H
24H
****
****
**
LPS
LPS + CD40LT
B.
Dll4
Jag1
**
**
2.5
2.0
1.5
1.0
0.5
0.0
Zymosan
Zymosan + CD40LT
C.
Dll4
Jag1
80
60
40
20
8
***
***
C. albicans
C. albicans + CD40LT

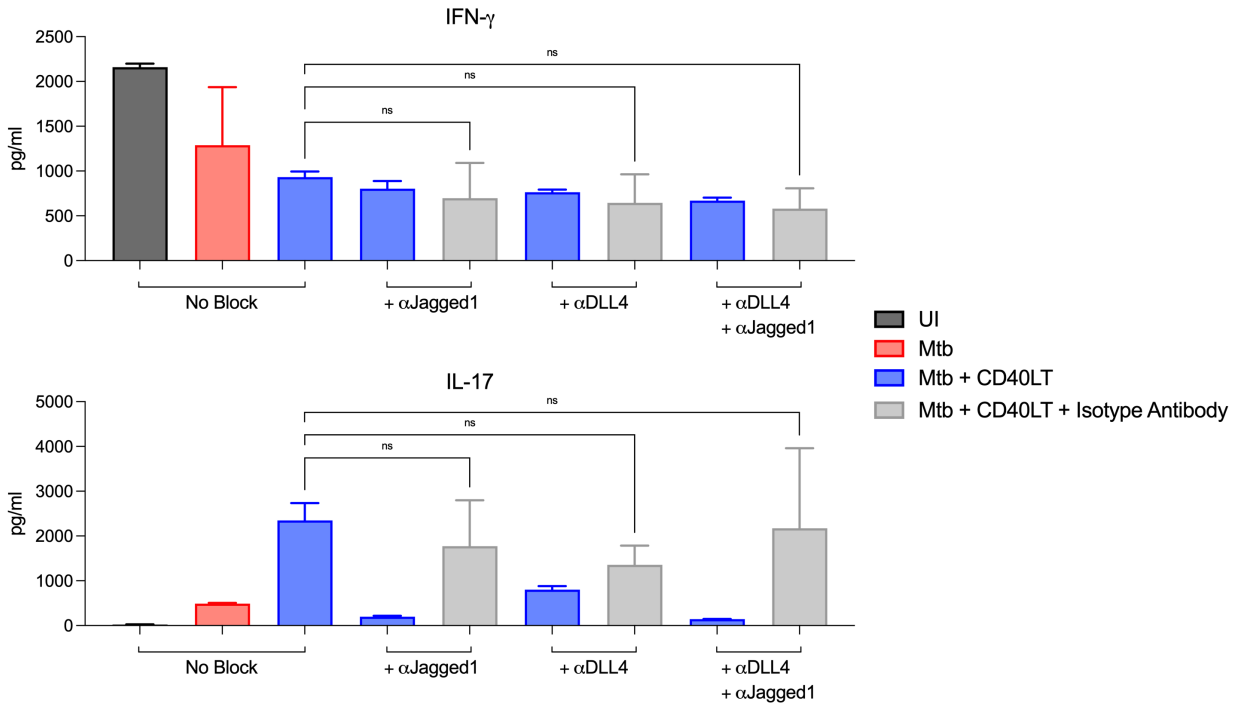
Supplemental Figure 3: Addition of CD40LT significantly augments *Dll4* mRNA transcript in PRR/TLR and *C. albicans*-stimulated DCs. BMDCs were stimulated with either 0.1 μg/ml LPS, 10 μg/ml Zymosan, or heat-killed *C. albicans* at an MOI of 17 with or without 1 μg/ml of CD40LT. At designated time points, RNA was collected to assay for mRNA. qPCR analysis of genes was standardized to housekeeping gene GAPDH, analyzed using the $\Delta\Delta$Ct method, and presented as $2^{-\Delta\Delta Ct}$. Data for LPS A), Zymosan B), and *C. albicans* C). Data are presented as mean ± SD. Data are representative of 3 independent experiments. Data were analyzed using a two-way ANOVA with a correction for multiple comparisons. Statistical significance p-value key is the following: ** = ≤ 0.01, *** = ≤ 0.001, **** = ≤ 0.0001.


A.
IL-6
IL-12p40
pg/ml
8000
6000
4000
2000
0
50000
40000
30000
20000
10000
***
****
ND
24H
48H
UI
Mtb
Mtb + CD40LT
B.
Il6
Il12b
Fold change over GAPDH
500
400
300
200
100
250
150
50
0H
3H
6H
12H
24H
48H
C.
Dll4
Jag1
2500
2000
1500
1000
8
6
4
2
*
**

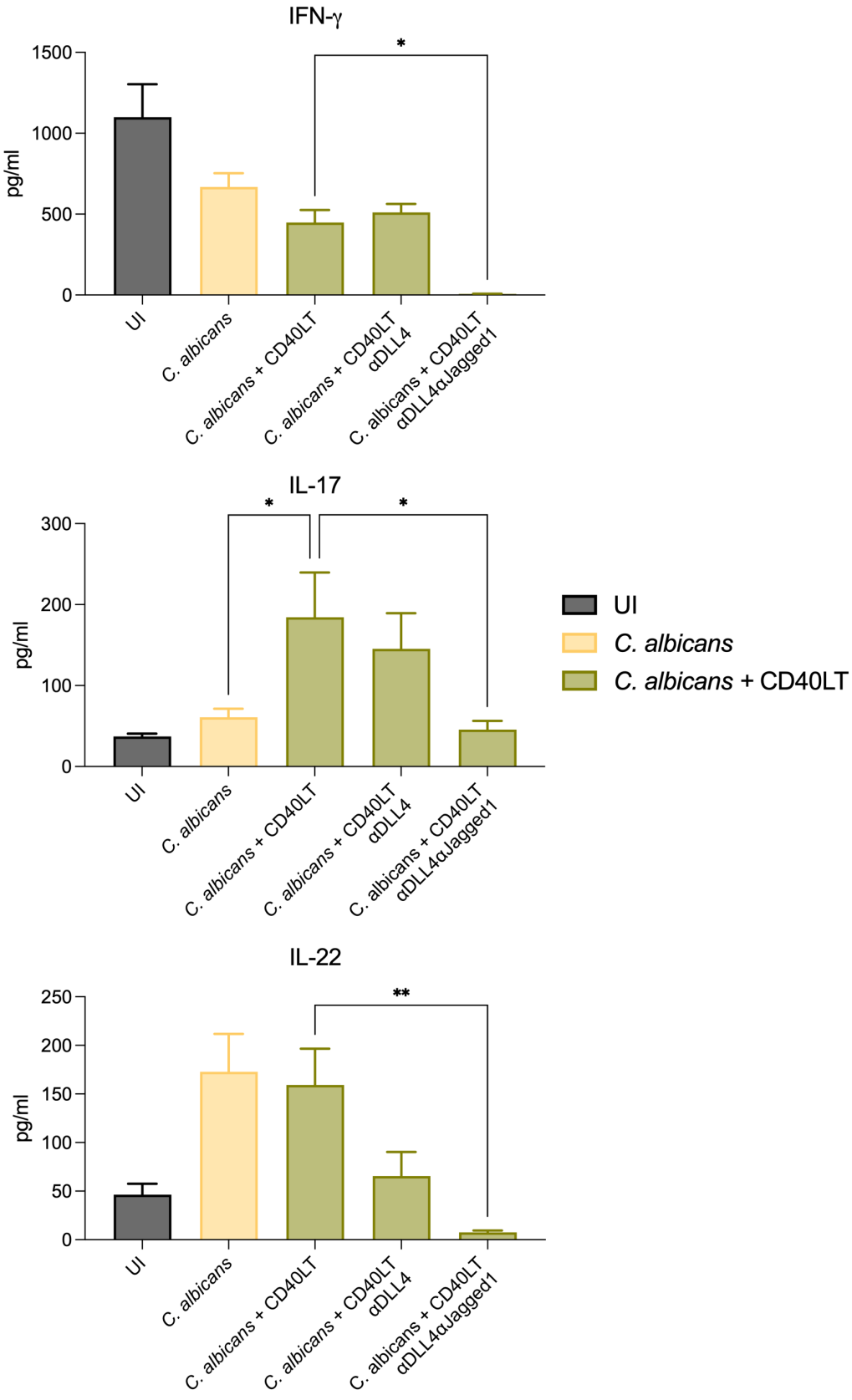
Supplemental Figure 4: Induction of *Dll4* following CD40 engagement of DCs is independent of Mtb viability. BMDCs were stimulated with either media (UI) or HK Mtb (MOI 30) with or without the addition of 1 μg/ml of CD40LT. At designated time points, cell free supernatants and RNA were collected to assay for protein and mRNA. A) ELISA measurements of IL-6 and IL-12p40 in supernatants. B) and C) qPCR analysis of genes was standardized to housekeeping gene GAPDH, analyzed using the ΔΔCt method, and presented as $2^{-\Delta\Delta Ct}$. Data are presented mean ± SD (a) or mean ± SEM (B-C). Data are representative of 3 independent experiments. Data were analyzed in A) using a one-way ANOVA with a correction for multiple comparisons and B-C) using a two-way ANOVA with a correction for multiple comparisons. Statistical significance p-value key is the following: * = ≤ 0.05, ** = ≤ 0.01, *** = ≤ 0.001, **** = ≤ 0.0001. ND = not-determined.



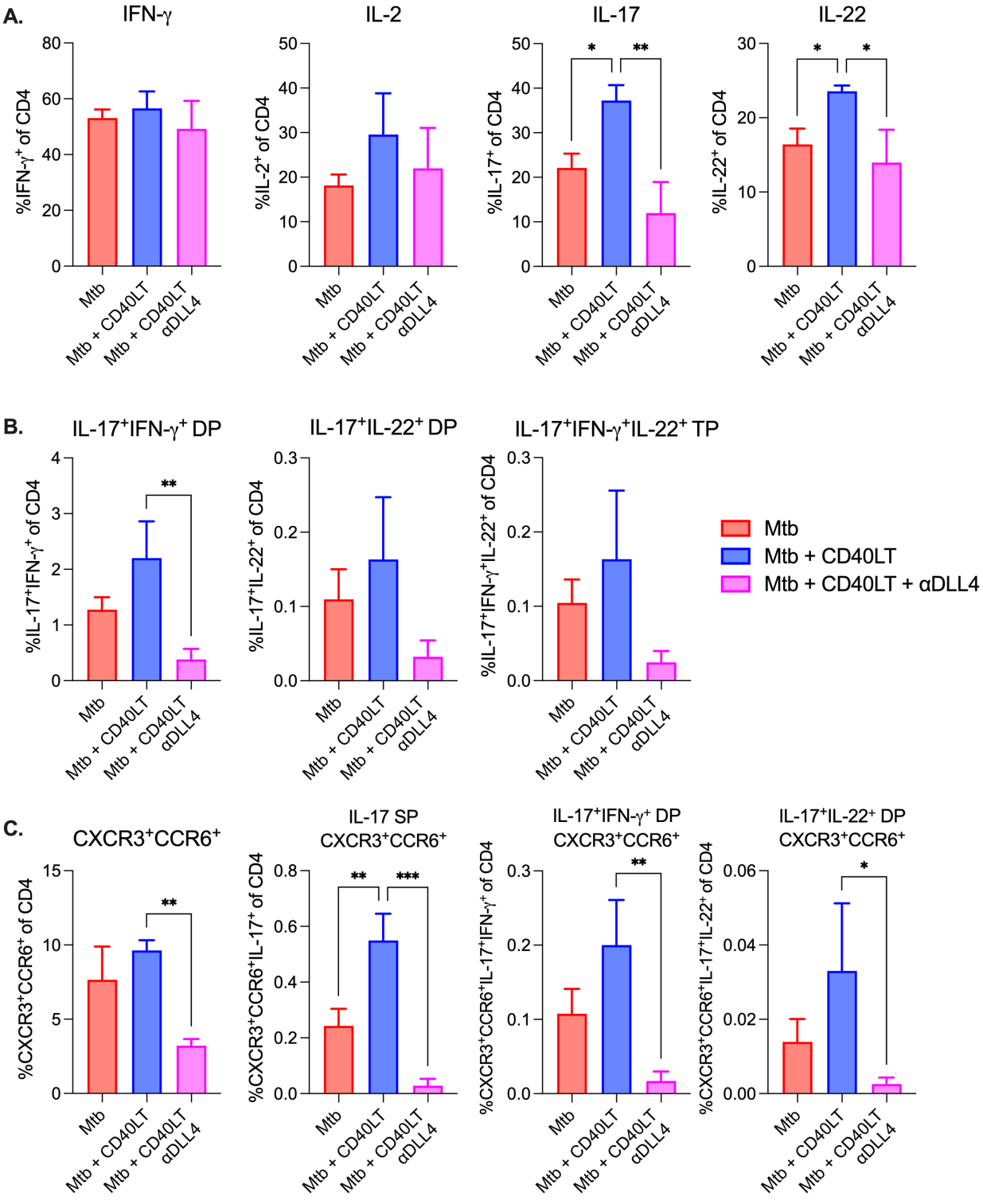
Supplemental Figure 5: DCs stimulated with CD40LT alone do not induce Th_{17} polarization. BMDCs were stimulated with either media alone (UI) or 1 μg/ml of CD40LT. Following 24H of stimulation, cells were pulsed with 10 μg/ml of cognate peptide ($OVA_{323-339}$) for one hour and then co-cultured with purified naïve CD4 OT-II Tg Thy1.1 T cells at a ratio of 4:1. After 72H of co-culture, cell-free supernatants were harvested and assayed for cytokines by ELISA. Data are presented as mean ± SD. Data are representative of 3 independent experiments. Data were analyzed using an unpaired Student's t-test. Statistical significance p-value key is the following: ns = no significance, *** = ≤ 0.001, **** = ≤ 0.0001.



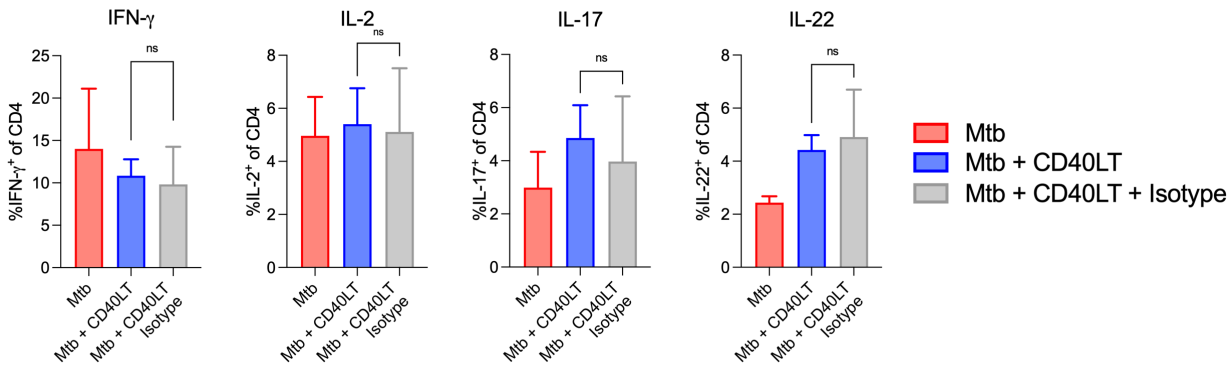
Supplemental Figure 6: Isotype control reveals specificity of DLL4 and Jagged1 blocking antibodies in DC-T cell co-culture experiment. BMDCs we stimulated with either media alone (UI), or heat-killed Mtb (MOI 30) with or without 1 μg/ml of CD40LT. Following 24H of stimulation, cells were pulsed with 10 μg/ml of cognate peptide ($OVA_{323-339}$) for one hour and then co-cultured with purified naïve CD4 OT-II Tg Thy1.1 T cells at a ratio of 4:1. For blockade conditions, blocking antibodies to either DLL4, Jagged1, or both DLL4 and Jagged1 (or corresponding isotypes) were added at the time of co-culture at a concentration of 30 μg/ml. After 72H of co-culture, cell-free supernatants were harvested and assayed for T cell cytokines using ELISA. A) IFN-γ ELISA. B) IL-17 ELISA. Data are presented as mean ± SD. Data are representative of 3 independent experiments. Data were analyzed using a one-way ANOVA with a correction for multiple comparisons. Statistical significance p-value key is the following: ns = no significance.


IFN-γ
1500
1000
500
0
pg/ml
*
UI
C. albicans
C. albicans + CD40LT
C. albicans + CD40LT αDLL4
C. albicans + CD40LT αDLL4αJagged1
IL-17
300
200
100
0
pg/ml
*
*
UI
C. albicans
C. albicans + CD40LT
C. albicans + CD40LT αDLL4
C. albicans + CD40LT αDLL4αJagged1
UI
C. albicans
C. albicans + CD40LT
IL-22
250
200
150
100
50
0
pg/ml
**
UI
C. albicans
C. albicans + CD40LT
C. albicans + CD40LT αDLL4
C. albicans + CD40LT αDLL4αJagged1

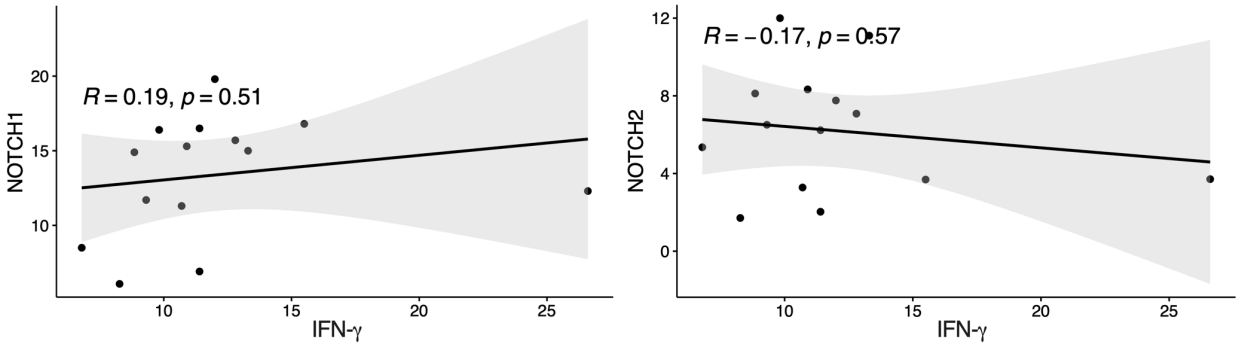
Supplemental Figure 7: Blocking Notch ligands reduces Th_{17} polarization following *C. albicans*-stimulated DC-T cell co-culture. BMDCs we stimulated with either media alone (UI) or heat-killed *C. albicans* at an MOI of 17 with or without 1 μg/ml of CD40LT. Following 24H of stimulation, cells were pulsed with 10 μg/ml of cognate peptide ($OVA_{323-339}$) for one hour and then co-cultured with purified naïve CD4 OT-II Tg Thy1.1 T cells at a ratio of 4:1. For blockade conditions, blocking antibodies to either DLL4, Jagged1, or both DLL4 and Jagged1 were added at the time of co-culture at a concentration of 60 μg/ml. After 72H of co-culture, cell-free supernatants were harvested and assayed for T cell cytokines using ELISA. Data are presented as mean ± SD. Data are representative of 2 independent experiments. Data were analyzed using a one-way ANOVA with a correction for multiple comparisons. Statistical significance p-value key is the following: * = ≤ 0.05, ** = ≤ 0.01.


A.
IFN-γ
IL-2
IL-17
IL-22
%IFN-γ+ of CD4
%IL-2+ of CD4
%IL-17+ of CD4
%IL-22+ of CD4
Mtb
Mtb + CD40LT
Mtb + CD40LT αDLL4
B.
IL-17+IFN-γ+ DP
IL-17+IL-22+ DP
IL-17+IFN-γ+IL-22+ TP
%IL-17+IFN-γ+ of CD4
%IL-17+IL-22+ of CD4
%IL-17+IFN-γ+IL-22+ of CD4
Mtb
Mtb + CD40LT
Mtb + CD40LT + αDLL4
C.
CXCR3+CCR6+
IL-17 SP CXCR3+CCR6+
IL-17+IFN-γ+ DP CXCR3+CCR6+
IL-17+IL-22+ DP CXCR3+CCR6+
%CXCR3+CCR6+ of CD4
%CXCR3+CCR6+IL-17+ of CD4
%CXCR3+CCR6+IL-17+IFN-γ+ of CD4
%CXCR3+CCR6+IL-17+IL-22+ of CD4

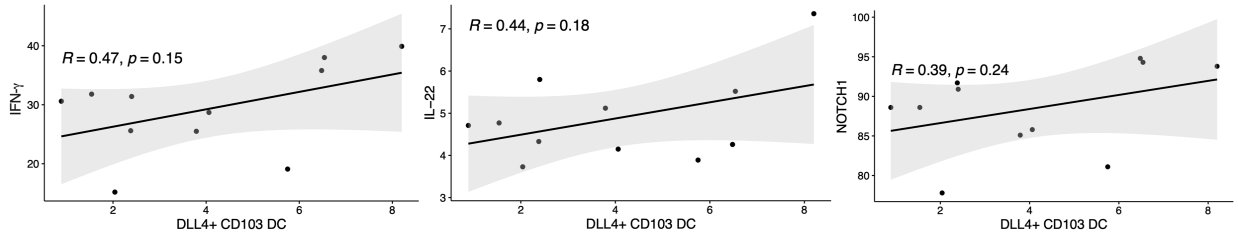
Supplemental Figure 8: Blocking DLL4 on Mtb-infected DCs reduces antigen-specific Th_{17} and multifunctional CD4 T cell responses *in vivo*. BMDCs were infected with Mtb at an MOI of 1, with or without CD40LT, for 48H. For antibody blockade studies, 60 μg/ml anti-DLL4 antibody was added at this time. DCs were then harvested and 1E6 were intratracheally (IT) transferred into the lungs of mice along with the additional blocking antibody. At 4 weeks post transfer, mice were euthanized and single-cell lung suspensions were isolated and stimulated with 10 μg/ml WCL to assess antigen-specific responses. Lung cells were then stained and markers measured using flow cytometry. A) Cytokine-positive frequency of CD4 T cells. B) Frequency of multiple-cytokine positive CD4 T cells as determined by Boolean analysis. C) Frequency of $CXCR3^{+}CCR6^{+}$ population and Boolean analysis of multiply marker/cytokine populations. Populations shown were pre-gated on singlets, live cells, and $CD3^{+}$ cells with data plotted from CD4 T cells. Experimental schema was made with BioRender.com. Data are presented as mean ± SD. Data are representative of 2 independent experiments. Data were analyzed using a one-way ANOVA with a correction for multiple comparisons. Statistical significance p-value key is the following: * = ≤ 0.05, ** = ≤ 0.01, *** = ≤ 0.001. SP = single-positive, DP = double-positive, TP = triple-positive.



Supplemental Figure 9: Isotype control reveals specificity of DLL4 blocking antibodies in Mtb-infected DC IT transfer experiment. BMDCs were infected with Mtb at an MOI of 1, with or without CD40LT, for 48H. For blockade, 60 μg/ml isotype antibody was added at this time. DCs were then harvested and 1E6 were intratracheally (IT) transferred into the lungs of mice with the addition of blocking antibody. At 4 weeks post transfer, mice were euthanized and lung *ex vivo* responses were measured using flow cytometry. Cytokine-positive frequency of CD4 T cells is plotted. Populations shown were pre-gated on singlets, live cells, and $CD3^+$ cells with data plotted from CD4 T cells. Data are presented as mean ± SD. Data are representative of 2 independent experiments. Data were analyzed using a one-way ANOVA with a correction for multiple comparisons. Statistical significance p-value key is the following: ns = no significance.



Supplemental Figure 10: IFN-γ does not correlate with NOTCH1 or NOTCH2. BMDCs were infected with Mtb at an MOI of 1, with or without CD40LT, for 48H. For blockade, 60 μg/ml DLL4 blocking antibody was added at this time. DCs were then harvested and 1E6 were intratracheally (IT) transferred into the lungs of mice with the addition of blocking antibody. At 4 weeks post transfer, mice were euthanized and lung *ex vivo* responses were measured using flow cytometry. Correlations between frequencies of markers on CD4 T cells were made using R package "ggscatter." All correlations presented are Pearson's correlations. Data are representative of 2 independent experiments.



Supplemental Figure 11: DLL4^{+} CD103^{+} DC does correlate with IFN-γ, IL-22, or NOTCH1. B6 mice were infected *via* the aerosol route with a low-dose of Mtb or *hip1* mutant. Following 2 weeks post-infection, mice were euthanized and lung *ex vivo* responses were measured using flow cytometry. Correlations between different CD4 T cell and innate immune populations were made using R package "ggscatter." All correlations presented are Pearson's correlations. Data are representative of 2 independent experiments.

Chapter III

Engaging CD40 enhances dendritic cell functions that augment T cell responses during *Mycobacterium tuberculosis* infection

Chapter adapted from:

Enriquez, A.B., Tharp, G.K., Boddapati, A.K., Dkhar, H.K., and Rengarajan, J. (2022). Engaging CD40 enhances dendritic cell functions that augment T cell responses during *Mycobacterium tuberculosis* infection. *In preparation*.

Abstract

Mycobacterium tuberculosis (Mtb) successfully impairs dendritic cell (DC) functions during infection, thereby impeding protective immunity against tuberculosis (TB). We previously demonstrated that Mtb is able to restrict early T helper 17 (Th_{17}) CD4 T cell responses by impairing CD40 expression on the surface of DCs and that engaging the CD40 signaling pathway enhances Th_{17} responses and pathogen control. However, the molecular mechanisms downstream of CD40 signaling on DCs that contribute to enhanced Th_{17} polarization during infection are not completely understood. Here we used RNA sequencing to dissect the transcriptional pathways downstream of CD40 on DCs that mediate enhanced T cell responses during infection. We now demonstrate that engaging CD40 on DCs results in global reprogramming of DC gene expression that reflects their enhanced functions, including pro-inflammatory functions early during infection and increases in cytokines that contribute to Th_1 and Th_{17} polarization. We found that CD40 engagement increased expression of genes that promote DC migration and led to an increase in the co-stimulatory molecules OX40L and CD70 on the surface of DCs. CD40-engaged DCs induced high frequencies of multifunctional IL-2^+ T cells in the lungs of mice and significantly augmented antigen-specific Th cell proliferation *in vitro* compared to Mtb infection of DCs alone. Notably, blocking OX40L or CD70 during DC-T cell co-culture reduced this effect. Overall, our results demonstrate that exogenous engagement of CD40 in the context of Mtb infection promotes robust DC activation, thus overcoming Mtb immune evasion of DC-T cell crosstalk, and resulting in enhanced T cell functions. Our studies identify important new targets for improving vaccine and adjuvant strategies against TB.

Introduction

Tuberculosis (TB), an infectious disease caused by the bacterial pathogen *Mycobacterium tuberculosis* (Mtb), remains among the leading causes of death worldwide[1]. While the only licensed vaccine against TB, *Mycobacterium bovis* Bacillus Calmette-Guérin (BCG), is able to prevent disseminated disease in children, it is unable to provide protection against pulmonary disease in either children or adults. Efforts to generate more efficacious vaccines, however, are hindered by our limited understanding of the host immune pathways that contribute to protective immunity. Therefore, studies that dissect the molecular mechanisms leading to the generation of protective immune responses are necessary to address these gaps in knowledge.

A critical component of protective immunity to TB are $CD4^+$ T helper (Th) cell responses. Infection with Mtb results in a robust IFN-γ-producing Th_1 response which is unable to provide sterilizing immunity. While Th_1 responses play a role in initiating antimicrobial effector functions in innate immune cells, emerging research studies, include our own, have suggested that additional Th responses such as IL-17-producing Th_{17} and multifunctional Th cells are also necessary for protection. However, Th_{17} responses are poorly induced and are actively suppressed during Mtb infection. To limit protective immunity, Mtb impairs the function of dendritic cells (DCs), which are critical for activating and polarizing Th cells into distinct subsets that proliferate and perform their effector functions. To generate these responses, DCs need to perform several functions such as antigen presentation, co-stimulatory molecule upregulation, and secretion of Th-polarizing cytokines. Research from our lab has found that one of the ways Mtb is able to limit Th_{17} responses is by restricting the expression co-stimulatory molecule CD40

on the surface of DCs[195,198] and that CD40-CD40L signaling is required for generating Th_{17} responses during infection[195]. Importantly, we showed that exogenously engaging CD40 on DCs, through use of a multimeric CD40L (CD40L) reagent, augments Th_{17} responses which correlated with reduced bacterial burdens in a DC vaccination and challenge model[195]. Extending these studies, we recently showed that Notch signaling, particularly the canonical Notch ligand Delta ligand-like 4 (DLL4), downstream of CD40 signaling on DCs is critical for Th_{17} polarization. We demonstrated that Mtb infection limits DLL4 expression on DCs and that exogenous CD40 engagement overcomes this restriction to increase DLL4 mRNA and surface expression on Mtb-infected DCs. Moreover, blocking DLL4 on Mtb-infected DCs significantly impaired Th_{17} and multifunctional T cell responses *in vitro* and *in vivo*. To gain a global understanding of how CD40 signaling impacts Mtb-infected DC gene expression and subsequent Th function, we sought to comprehensively identify additional pathways downstream of CD40 signaling that impact DC-T cell crosstalk and lead to increased Th cell functionality. We reasoned that these studies could also identify molecular targets for designing improved vaccination and adjuvant strategies.

Using RNA sequencing, we found that engaging CD40 on Mtb-infected DCs leads to significant differences in gene expression at early stages following Mtb infection. Differentially expressed genes that were upregulated following CD40 were revealed to encode Th_1 and Th_{17}--polarizing cytokines, molecules related to DC migration and increased expression of other co-stimulatory molecules ICOSL, OX40L, 41BBL and CD70. In a DC adoptive transfer model, we found that transfer of DCs with exogenous CD40 engagement led to significantly increased numbers of activated IL-2^+ T cells that also expressed Th_1, Th_{17}, Th_{22} and $Th_1/Th_{17}/Th_{22}$ cytokines. Finally,

we found that CD40 engagement is able to significantly increase the proliferative capacity of T cells, and this is partially dependent on OX40L and CD70. Overall, our data show that CD40 engagement reprograms Mtb-infected DCs to enhance their functions and promote T cell functions associated with protective immunity against TB.

Results

Bulk RNA seq reveals that CD40 engagement leads to reprogramming of the Mtb-infected DC transcriptome.

To gain a comprehensive understanding of the pathways downstream of CD40 signaling that enhance the ability of DCs to activate and differentiate Th cells, we used bulk RNA sequencing (RNA seq). To engage CD40 on DCs, we used our previously employed model wherein we provide multimeric CD40L reagent (CD40LT), which is able to cross-link and activate CD40 signaling[195]. We generated bone marrow-derived dendritic cells (BMDCs) from C57BL/6 mice and infected them with Mtb in the presence or absence of CD40 ligand trimer (CD40LT). After 8H and 24H of infection, infected cells in either condition were lysed and purified RNA was generated from these samples and submitted for sequencing (Figure 1A). Uninfected (UI) cells were also harvested at the 0H time point and served as a control. We found that Mtb infection alone and Mtb + CD40LT each led to thousands of upregulated and downregulated gene transcripts compared to the 0H UI time point (Figure 1B), with CD40 engagement leading to a greater number of unique differentially expressed genes (DEGs) (Figure 1C). A greater number of differences in gene expression was found at the 8H compared to the 24H time point (Figure 1C and 1D). We next wanted to investigate the DEGs between the Mtb + CD40LT and Mtb infection alone conditions. We found that addition of CD40LT led to a significant upregulation

of genes compared to Mtb infection alone, and that these differences were greater at the 8H time point (Figure 1D). The greatest differences in gene regulation were found in the upregulated genes compared to the downregulated genes, and this remained at both the 8H and 24H time point (Figure 1D). Overall, these data demonstrate that addition of CD40LT promotes substantial changes in the transcriptome of Mtb-infected DCs.

We next sought to further characterize the genes that are significantly upregulated in the Mtb + CD40LT condition compared to Mtb alone. We found 75 upregulated DEGs present with CD40 engagement (Figure 2A and 2B). We also found 15 downregulated DEGs in Mtb + CD40LT (Figure 2A and 2B). To gain further insight into the functions of the upregulated DEGs, we performed Gene-Set Enrichment Analysis (GSEA) from the molecular signatures database (MSigDB) and found that CD40 engagement led to a significant increase in pro-inflammatory functions (Figure 2C) compared to Mtb alone. Specifically, exogenous engagement of CD40 led to a significant increase in pathways that correspond to the production of cytokines, cytokine signaling, antigen presentation/activation, and signaling through recognition receptors (Figure 2C). Using functional enrichment analysis, we then sought to understand the functions of the 75 upregulated DEGs specifically present with CD40 engagement by identifying the gene ontology (GO), Kyoto Encyclopedia of Genes and Genomes (KEGG), and Reactome pathways in which these genes were enriched. Similar to our GSEA, we observed that exogenous CD40 engagement led to a significant increase in genes which function in cytokine production, cytokine signaling, and broad inflammatory responses (Figure 2D). The functions of 15 downregulated DEGs correspond to different functions such as Actin remodeling and maintaining DNA integrity (data not shown). Taken together, these data demonstrate that engaging CD40 leads to

greater differences in mRNA transcript and pathways that contribute to critical DC functions. Moreover, these data highlight pathways that are limited during Mtb infection.

CD40 engagement increases the ability of DCs to produce Th_1 and Th_{17} polarizing cytokines.

We know that Mtb impairs DC responses and restricts Th_{17} cell polarization. Consistent with this, we found that a subset of the genes that were significantly upregulated at the 8H time point corresponds to pro-inflammatory Th_1 and Th_{17}- polarizing cytokines (Figure 3A) *Il12b*, *Il1a*, *Il1b*, *Il23a*, *Il6*, *Tgfbi*, and *Tnf* (Figure 3A and 3B). *Il12b* and *Tnf* have traditionally been associated with the ability of DCs to polarize towards Th_1 responses, while *Ill6*, *Tgfbi*, *Il23a*, *Il1a* and *Il1b* have been associated with the ability of DCs to polarize and maintain Th_{17} responses. These results support our previous studies in which we showed that addition of CD40LT significantly increases the capacity of Mtb-infected DCs to produce IL-6, IL-23, and IL-12p40[195]. Moreover, we found CD40 engagement led to a significant increase in *Dll4* compared to Mtb infection alone, consistent with our previous data implicating a role for the Notch ligand DLL4 in Th_{17} polarization. Collectively, these data demonstrate that engaging CD40 on Mtb-infected DCs significantly upregulates DC gene expression programs associated with pro-inflammatory immune responses and Th_1/Th_{17} polarization.

CD40 engagement augments expression of migration-associated chemokines.

Several studies have demonstrated that Mtb infection induces delayed and suboptimal immune responses. Mtb-infected DCs exhibit delayed migration from the site of infection in the lungs to the mediastinal lymph nodes, where T cell responses are initiated[60]. We found that among the

significantly upregulated genes in the Mtb + CD40LT condition included those that function in DC migration. These include *Ccl17*, *Ccl22* and *Ccl5* (encoding chemokines that provide chemotactic signals for DC migration), *Ccr7* (encoding a chemokine receptor important for homing to lymph nodes), and *Fscn1* (encoding fascin actin bundling protein) (Figure 4A and 4B). To validate the finding that CD40 engagement enhances chemokines that promote DC migration, we infected BMDCs with Mtb in the presence or absence of CD40LT for up to 48H. Following infection, cell-free supernatants were harvested and protein expression was assayed using enzyme-linked immunoassay (ELISA). We found that CD40 engagement led to a significant increase in CCL5, CCL17, and CCL22 protein production during Mtb infection (Figure 4C). This effect was observed at both 24H and 48H and corroborates our results from RNA seq analysis (Figure 4A and 4B). Therefore, our data demonstrate that while Mtb likely impedes DC migration by restricting chemotactic signals associated with DC migration, exogenously engaging CD40 on DCs induces the secretion of chemokines known to promote DC migration.

CD40 engagement enhances expression of DC co-stimulatory molecules.

We found that among the significantly upregulated genes in the Mtb + CD40LT condition were those corresponding to co-stimulatory molecules, specifically, *Cd70*, *Icosl*, *Tnfsf4* (*Ox40l*), and *Tnfsf9* (*41bbl*) (Figure 5A and 5B), suggesting that CD40 engagement enhances the co-stimulatory capacity of DCs to activate T cells. We tested the cell surface expression of these molecules on DCs since co-stimulatory markers need to be present on the surface of DCs in order to interact with their cognate receptors on T cells. We infected BMDCs with Mtb in the presence of absence of CD40LT for up to 48H. At designated time points, cells were harvested

and stained for different markers (including those of the co-stimulatory molecules of interest) and samples were acquired for flow cytometry analysis (Figure 5C). We found that there was a significant increase in $CD70^+$ DCs in Mtb + CD40LT DCs compared to Mtb infection alone (Figure 5C). We did not observe a significant increase in the other co-stimulatory molecules measured, but did observe that 41BBL was increased in Mtb + CD40LT at 24H post-infection compared to Mtb alone (Figure 5C). These data therefore demonstrate that engaging CD40 on the surface of Mtb-infected DCs increases their ability to express co-stimulatory molecules, specifically CD70.

CD40-engaged DCs significantly enhance proliferation of CD4 T cells in a mechanism that is partially dependent on co-stimulatory molecule expression.

Having observed that engaging CD40 is able to enhance the ability of DCs to produce pro-inflammatory cytokines and upregulate co-stimulatory molecules (Figures 3 and 5), along with data from the lab showing an increase in multifunctional T cell responses, led us to hypothesize that the pathways induced by CD40 engagement result in better T cell activation. To test this, we used an intratracheal (IT) transfer model wherein we transferred Mtb-infected DCs directly into the lungs of mice. This model allows us to dissect DC-specific mechanisms and their effect on T cell responses *in vivo*. We generated BMDCs and infected them with Mtb with or without CD40LT for 48H. Following infection, these cells were collected and transferred into the lungs of mice through the trachea as previously described. Following 4 weeks post-transfer, mice were euthanized and single-cell suspensions were generated from the lungs (Figure 6A). Following stimulation, the cells were then harvested and stained for different markers and T cell responses measured using flow cytometry. Using Boolean analysis, we observed an increase in the

percentage of T cells that were expressing multiple cytokines (Figure 6B). Using IL-2 as a marker of activation, we found that transfer of Mtb + CD40LT DCs led to a significant increase in activated T cells that expressed IL-17, IL-22 and IFN-γ (Figure 6B). IL-22 is an additional cytokine produced by Th_{17} subsets as well as the recently-described Th_{22} subset. When we measured multiple-cytokine producing cells, we found that CD40 engagement led to an increase in activated T cells that co-expressed IL-17^{+}IFN-γ^{+} (Figure 6B). Additionally, we observed an increase in activated T cells that co-expressed IL-17^{+}IFN-γ^{+}IL-22^{+} (Figure 6B). Th_{17} cells that co-express multiple cytokines have been suggested to be protective in both human samples and in primate models of TB in work from our lab and others. We then wanted to test whether CD40 engagement enhanced T cell proliferation, as increased proliferation can account for an increase in activated and multiple cytokine-producing T cells. To directly test the impact of CD40 ligation on T cell proliferative responses, we used a DC-T cell co-culture *in vitro* assay. We stimulated BMDCs with Mtb in the presence or absence of CD40LT for 24H. We then pulsed DCs with cognate peptide (OVA peptide [$OVA_{323–339}$]) for 1H and then co-cultured them with cell trace violet (CTV)-stained OVA-specific Tg naïve CD4 T cells (at a 1:4 DC:T cell ratio) for 5 days. Labeling cells with CTV allow us to trace multiple generations of T cell division, which we quantify as proliferation. Following co-culture, cell-free supernatants were acquired for protein quantification assays and cells were harvested to measure CTV using flow cytometry. We observed an increase in IL-17 and IL-22 secretion, corresponding to Th_{17} polarization, as we have previously reported[195] (Supplementary Figure 1). We did not observe an increase in IFN-γ, or Th_{1} polarization, as have also previously found (Supplementary Figure 1). In addition, we found that co-culture of T cells with Mtb + CD40LT DCs led to a significant increase in the proliferative capacity of CD4 T cells at the different concentrations of OVA used for the assay

(Figure 6E and 6F). We hypothesized that the increase in co-stimulatory molecules downstream of CD40 engagement could account for the increase in T cell proliferation as these molecules have been shown to be important for this function in T cells. We therefore repeated our co-culture experiment and provided blocking antibodies ICOSL, OX40L, 41BBL, or CD70. We found that there was a reduction in proliferation in T cells in the OX40L and CD70 blockade conditions (Figure 6G). Taken together, these data demonstrate that engaging CD40 on Mtb-infected DCs increases the costimulatory capacity of DCs pathways that lead to better activation and proliferation and polarization of antigen-specific T cells during Mtb infection.

Discussion

In this study, we dissect the molecular mechanisms downstream of CD40 signaling on DCs that result in increased Th_{17} and multifunctional T cell responses during infection. We demonstrate that engaging CD40 on the surface of DCs, using CD40LT, results in greater differences in gene expression at early time points during infection. We found that these differentially expressed genes are part of pro-inflammatory DC function pathways. Specifically, CD40 engagement resulted in an increase in pro-inflammatory cytokines, Th_1/Th_{17} polarizing cytokines, chemokines, migration molecules, and upregulation of other co-stimulatory molecules. We also show that exogenous CD40 engagement increases the ability of DCs to activate multiple cytokine-producing T cells and T cell proliferation, and this is partially dependent on an increase in co-stimulatory molecules. Overall, our study provides insight into the molecular mechanisms that are necessary to activate T cells during Mtb infection which, can be harnessed to develop effective vaccines and adjuvants.

Th CD4 T cell responses are a critical component in protective immunity to TB[117,119,195,254], but protective subsets such as Th_{17} cells are limited during infection[195,198]. To dampen protective immunity, Mtb subverts the functions of DCs during infection. We previously demonstrated that Mtb is able to limit DC function during infection by restricting the expression of CD40 co-stimulatory molecule[195,198]. Moreover, we found CD40-CD40L pathway is necessary for generating Th_{17} responses and that exogenously engaging CD40LT on DCs is able to augment protective Th_{17} responses in a DC mucosal vaccination model[195]. While we found in a recent study that Notch signaling ligand DLL4 is downstream of CD40 signaling on DCs and plays a role in polarization of Th_{17} and multifunctional T cells, we wanted to uncover all of the mechanisms by which exogenously engaging CD40 of DCs leads to Th_{17} and other T cell responses. Using RNA sequencing, we found that CD40 engagement increases gene expression and pro-inflammatory DC functions (Figure 1 and 2). We observed an increase in pro-inflammatory cytokine as well as multiple Th subset polarization cytokines (Figure 3). We found an increase in chemokine molecules as well as those related to migration (Figure 4). We also observed an increase in the expression of other co-stimulatory molecules, and found a significant increase in CD70 on the surface of DCs following exogenous CD40 engagement during infection (Figure 5). In the lungs of mice *in vivo*, adoptive transfer of DCs with CD40 engagement resulted in a greater percentage of activated T cells that expressed Th_1, Th_{17}, Th_{22}, or multiple $Th_1/Th_{17}/Th_{22}$ cytokines (Figure 6). *In vitro* we observed that exogenously engaging CD40 led to a significant increase in proliferation, and that this effect was partially dependent on OX40L and CD70 (Figure 6). While the results presented here are in the context of Mtb infection, these insights are likely to be beneficial in other contexts where pathogen manipulation of DC

responses results in diminished protective immune responses. Importantly, the results presented here are important for furthering understanding how DCs are able to initiate T cell responses.

Our results on the modulation of immune responses by Mtb support previous studies which have demonstrated impairment of DC responses[195,198] and highlight additional immune evasion mechanisms by which Mtb impaired protective immunity. Early studies showed that Mtb is able to infect DCs[60,65], thereby demonstrating that Mtb is able to directly interact with these cells during infection. Subsequent studies demonstrated that these interactions result in suboptimal activation of DCs and delayed migration from the lungs to the lymph nodes, where T cell responses are intiated[60,201,289]. Mtb is able to impair the ability of DCs to upregulate MHC-II and present antigen, upregulate co-stimulatory molecules, and secrete cytokines[195,198]. Interestingly, we found that exogenous CD40 engagement is able to enhance the abilities of DCs to perform functions that have been demonstrated to be dampened by Mtb. While one of the mechanisms that contribute to delayed migration of DCs is attributed to reduced activation, our results suggest that this is also due to restriction of chemokines CCL5, CCL17, and CCL22 and chemokine receptor CCR7 (Figure 4), which has been previously observed[204]. Interestingly, we found that Mtb also limits the expression of other co-stimulatory molecules ICOSL, OX40L, 41BBL and CD70 (Figure 5). Previous studies have demonstrated that Mtb is able to limit CD80/CD86, two classical co-stimulatory markers of activation, but to our knowledge, this study is the first to highlight modulation of these co-stimulatory molecules in the context of Mtb infection. Taken together, our results demonstrate that CD40 engagement is able to overcome pathogen modulation to induce important DC functions.

While recent research in the field has allowed us to gain a better understanding of which immune responses are protective in the context of Mtb infection, the molecular mechanisms that contribute to these immune responses are poorly understood. Here we provide insight into the DC molecular mechanisms that contributes to these responses. Multifunctional T cells, or those that express cytokines from different Th subsets, have become an important immune subset in the context of vaccine-induced adaptive T cell responses. Research from our lab and others have highlighted the role of these subsets in protection in both human and primate models of TB[119,120,122]. In our recent study where we found that DLL4 is downstream of CD40 signaling, we also observed that blocking DLL4 in an adoptive transfer model led to a significant decrease in multifunctional T cells. While we found that DLL4 contributes to these important T cell subsets, the results presented here also highlight additional molecular cues that allow DCs to polarize multifunctional T cells. We observed that exogenous CD40 engagement led to an increase cytokine that participate in polarization of multiple Th subsets, particularly Th_1 and Th_{17}, suggesting that cytokines also contribute to activation of these T cells. Additionally, the increase in co-stimulatory molecules that we observed suggest these signals too function in multifunctional T cell polarization. ICOSL, 41BBL, OX40L and CD70 have all been shown to participate in the polarization of multiple Th subsets. We hypothesize that a combination of these co-stimulatory molecules along with CD40 on DCs synergize to aid in polarization of multifunctional T cells. Our study also provides insight into Th_{22} subsets, a newly-described Th subset that was recently shown to provide protection in human patients and was depleted following co-infection. The molecular cues on DCs that contribute to Th_{22} subsets are unknown. Our results suggest that similar DC pathways that contribute to Th_{17} generation also contribute to Th_{22} polarization as we observe an increase in generation of both subsets (Figure 6), though

future studies should aim to better characterize the induction of this subset. While it is important to generate specific T cell responses during infection, timing is also critical to dictating whether specific T cell responses are protective or pathogenic. Even though Th_{17} and multifunctional T cell subsets have been highlighted as pathogenic in other models such as autoimmunity, in the context of Mtb infection, these responses early in infection function in a protective capacity. Interestingly, our RNA seq results demonstrated that CD40 engagement results in greater differences in gene expression at earlier time points during infection (Figure 1 and 2). We therefore hypothesize that the pathways upregulated through CD40 signaling contribute to polarization of these early, protective T cell subsets. These results highlight the importance of early CD40 activation (though means such as vaccination or adjuvants) in order to generate immune responses that can contribute to protection.

In summary, our study demonstrates that engaging CD40 on DCs is able to overcome Mtb immune evasion to enhance DC functions that augment Th_{17} and multifunctional T cell responses during infection. Moreover, we provide insight into the molecular mechanisms that underlie protective immune responses and identify immune targets for vaccine and adjuvant strategies.

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Materials and Methods

Mice

C57BL/6 mice used in this study were purchased from The Jackson Laboratory. C57BL/6 OT-II $OVA_{323–339}$ Thy1.1+ mice (originally developed by Dr. F. Carbone, University of Melbourne) were kindly provided by Dr. Bali Pulendran (Stanford University, formerly Emory University) and bred in the Yerkes vivarium. All mice used for experiments were eight-to-twelve weeks of age. Mice were given food and water ad libitum and housed in the Yerkes National Primate Research Center animal BSL-1 and animal BSL-3 vivarium under sterile conditions. All animals were handled according to the Emory University Institutional Animal Care and Use Committee (IACUC) regulations.

Generating bone marrow-derived dendritic cells

Bone marrow-derived dendritic cells (BMDCs) were generated as previously described. Briefly, the femur and tibia of C57BL/6 mice was removed and the bones were flushed to remove progenitor cells using cold RPMI-1640 (Lonza). The red blood cells from this mixture were then lysed using Red Blood Cell Lysis (Sigma). The resultant cells were resuspended in R10 media (RPMI-1640 [Lonza] supplemented with 2mM L-glutamine [Lonza], 0.1 mM NEAA [Gibco], 10 mM HEPES [Corning], 1mM Sodium Pyruvate [Lonza], 10% heat-inactivated FBS [Gemini]) supplemented with 1:100 Penicillin/Streptomycin (Lonza), 1:1000 2-mercaptoethanol (BME;

Gibco) and 20 ng/ml murine rGM-CSF (R&D Systems) at a concentration of 1E6/ml in tissue culture plates and placed in the 37ºC incubator (with 5% CO_2) to grow. The cells were then "fed" on day 3 and 6 following culture using R10 media supplemented with 1:1000 BME and 20 ng/ml murine rGM-CSF. On day 8 of culture, cells were harvested and $CD11c^+$ microbeads (Miltenyi Biotec) were used to enrich for desired population (according to manufacturers' recommendations). Cell purity was confirmed using flow cytometry. The resulting DCs were then resuspended in R10 media supplemented with 1:1000 BME at a concentration of 6E5/ml and plated in 24-well tissue culture plates until infection. All cells were counted using trypan blue dye and a Countess automated cell counter (Invitrogen).

Bacterial strains

Mycobacterium tuberculosis (Mtb) strain H37Rv was used in this study. As previously described, Mtb strains were grown in Middlebrook 7H9 liquid media (BD) supplemented with 0.5% glycerol (Sigma), 10% oleic acid-albumin-dextrose-catalase (OADC) (BD) and 0.05% Tween 80 (VWR). Stocks were prepared by growing Mtb to an OD of 0.4 – 0.6, then filtered and resuspended in 7H9 media with 25% glycerol (Sigma) and stored at -80ºC.

Mtb in vitro infection of BMDCs

BMDCs were infected as previously described. Briefly, cell supernatants were removed from each well and replaced with infection inoculum consisting of bacteria resuspended in R10 media supplemented with 1:1000 BME (and 1 µg/ml CD40LT (Adipogen) for relevant conditions). All BMDCs were infected with H37Rv at an MOI of 1.0. Cells were then placed in the 37ºC incubator (with 5% CO_2) for 6 hours. Afterwards, 200 µg/ml of an Amikacin (Sigma) solution

(in R10 supplemented with 1:1000 Mercaptoethanol) was added to cells for 30 minutes to kill extracellular bacteria. After the Amikacin step, cell supernatants were removed and wells were washed X3 using PBS (Sigma). Cells were then resuspended in R10 media (supplemented with 2-Mercaptoethanol) with or without 1 μg/ml CD40LT for relevant conditions and plates were placed in the 37ºC incubator (with 5% CO_2) until desired time point. To enumerate CFU, certain wells were lysed after the Amikacin step with PBS + 0.5% TritonX (Fisher Scientific). Serial dilutions were plated on Middlebrook 7H10 agar plates (supplemented with 0.5% glycerol [Sigma], 10% OADC [BD] and 0.1 mg/ml cycloheximide solution [Sigma]) and placed in a 37ºC incubator to grow. At designated time points of the infection, supernatants were removed from each well and filtered using a 0.23 μm filter (to be removed from the BSL-3) for protein quantification assays. To collect samples for RNA purification, wells were washed X1 using PBS (Sigma) and 350 μl of Buffer RLT (Qiagen) (supplemented with 1:1000 BME [Sigma]) was added to each well and pipetted up and down to lyse the cells. Samples were then flash-frozen using 70% ethanol and dry ice and stored in the -80ºC until use.

Enzyme-linked immunosorbent assay

To enumerate protein secretion, Enzyme-Linked Immunosorbent Assay (ELISA) assays were carried out on cell-free supernatants. The following ELISA kits were used according to manufacturer's recommendation: murine CCL5 (R&D Systems), CCL17 (R&D Systems), CCL22 (R&D Systems), IFN-γ (Mabtech), IL-2 (BD), IL-17A (Invitrogen), and IL-22 (R&D Systems). Most plates were mechanically washed using a Biotek ELx405 machine or hand-washed according to protocol. All plates were measured using a BioTek ELx808 reader.

Bulk RNA sequencing and analysis

For bulk RNA seq, samples were purified using the RNeasy Micro Kit (Qiagen) according to manufacturer's recommendation. One nanogram of total RNA was used as input for cDNA synthesis using the Clontech SMART-Seq v4 Ultra Low Input RNA kit (Takara Bio) according to the manufacturer's instructions. Amplified cDNA was fragmented and appended with dual-indexed bar codes using the NexteraXT DNA Library Preparation kit (Illumina). Libraries were validated by capillary electrophoresis on an Agilent 4200 TapeStation, pooled at equimolar concentrations, and sequenced on an Illumina NovaSeq 6000 at 100SR, yielding 25 million reads per sample. Alignment was performed using STAR version 2.5.2b[290] and transcripts were annotated using mm10. Transcript abundance estimates were calculated internal to the STAR aligner using the algorithm of htseq-count[291]. DESeq2 was used for normalization[292], producing a both a normalized read count table and a regularized log expression table. Data from this experiment has been deposited in GEO.

Intratracheal instillation of DCs and mouse tissue harvest

Purified BMDCs were infected as outlined above in tissue culture plates for intratracheal (IT) transfer with either Mtb or Mtb + CD40LT. Following 48 hours of infection, BMDCs were harvested, washed X1 using PBS (Sigma), then spun down, counted, and resuspended at 20E6/ml in PBS. For IT transfer, mice were anesthetized using isoflurane in a closed isoflurane chamber (Med-Vet) and 1E6 infected BMDCs (in 50 μl volume in PBS) were instilled into the trachea of mice as previously described[195]. After four weeks post-IT, mice were euthanized using isoflurane overdose and lungs were harvested as previously described[195]. Briefly, lungs were placed into lung C-Tubes (MiltenyiBiotec) containing HBSS (Corning) supplemented with 2%

heat-inactivated FBS (Gemini) and 10mM HEPES (Corning). A mixture of 0.1% collagenase, type IV (Worthington) and 0.01% DNAse I (Worthington) was then added into each tube. The organs were homogenized using an automated gentleMACS Dissociator (MiltenyiBiotec) using the manufacturer's murine lung processing program. After addition of collagenase/DNAse mixture, lungs were dissociated and placed in a 37ºC (with 5% CO_2) incubator for 30 minutes. Following this time, lungs were placed in the dissociator again on the murine lung processing program. The tubes were then spun down and red blood cells were lysed from the mixture using RBC lysis buffer (Sigma). Lungs were resuspended at 10E6/ml in R10 supplemented with 1:1000 BME and 1E6 cells were plated per well in 96-well U-bottom propylene plates for assaying *ex vivo* immune responses. Plates were then placed in the 37ºC incubator (with 5% CO_2) and after 2 hours, a mixture of 5 μg/ml Brefeldin A (Sigma) and 1:1500 GolgiStop (BD) in R10 supplemented with 1:1000 BME was added. The plate was then returned to the incubator overnight and cells were stained the next day.

Flow cytometry

Lung suspensions and infected BMDC responses were assayed using flow cytometry. Cells were stained with Fixable Near-IR Dead Cell Stain Kit (Invitrogen) to distinguish between live and dead cells and mouse Fc block (BD) was used to prevent non-specific binding. The live/dead stain (1:500) and Fc block (1:50) mixture was diluted in PBS (Sigma). To stain BMDCs, the following antibodies were used (all of which are for surface staining): BUV395 anti-mouse CD155 (clone: 3F1, BD), BUV563 anti-mouse CD80 (clone: 16-10A1, BD), BUV737 anti-mouse DLL4 (clone: 9A1.5; BD), BUV805 anti-mouse CD45 (clone: 30-F11; BD), BV421 anti-mouse OX40L (clone: OX-86; BD), BV480 anti-mouse CD103 (clone: M290; BD), BV605 anti-

mouse CD226 (clone: 10E5; BD), BV650 anti-mouse CD11c (clone: N418; Biolegend), BV711 anti-mouse CD70 (clone: FR70; BD), BV786 anti-mouse CD47 (clone: miap301; BD), FITC anti-mouse CD11b (clone: M1/70; Biolegend), PerCP-eFluor710 anti-mouse 41BBL (clone: TKS-1; Invitrogen), PE anti-mouse ICOSL (clone: HK5.3; Biolegend), PE-CF594 anti-mouse PD-L1 (clone: 10F.9G2; Biolegend), PE-Cy5 anti-mouse CD40 (clone: 1C10; Invitrogen), PE-Cy7 anti-mouse CD64 and anti-mouse F4/80 (dump channel) [clone: X54-5/7.1; Biolegend, clone: BM8; Biolegend)], APC anti-mouse Jagged1 (clone: HMJ1-29; Biolegend), Alexa700 anti-mouse I-A/I-E (clone: M5/114.15.2; Biolegend). To stain lung T cells, the following surface stain antibodies were used: FITC anti-mouse CD14 (clone: Sa14-2, Biolegend), FITC anti-mouse NK-1.1 (clone: PK136, Biolegend), FITC anti-mouse TER-119/Erythroid (clone: TER-119, Biolegend), FITC anti-mouse CD19 (clone: 1D3/CD19, Biolegend), FITC Rat anti-mouse IL-2 (clone: JES6-5H4, BD), PE Rat anti-mouse Vβ6 T-Cell (clone: RR4-7, BD), PerCP anti-mouse CD45 (clone: 30-F11, Biolegend), Alexa 700, Hamster anti-mouse CD3ε (clone: 500A2, BD), APC-Cy7 anti-mouse CD8α (clone: 53-6.7, Biolegend), BV650 anti-mouse/human CD44 (clone: IM7, Biolegend), BV650 anti-mouse CD183 (CXCR3) (clone: CXCR3-173, Biolegend), BV711 anti-mouse CD8α (clone: 53-6.7, Biolegend), BV785 anti-mouse CD196 (CCR6) (clone: 29-2L17, Biolegend), APC-Cy7 Rat anti-mouse CD44 (clone: IM7, BD). To stain lung T cells, the following intracellular stain antibodies were used: PE-CF594 Rat anti-mouse IL-17A (clone: TC11-18H10, BD), PE-Cy7 Rat anti-mouse TNF (clone: MP6-XT22, BD), BV786 Rat anti-mouse CD4 (clone: RM4-5, BD), APC anti-mouse IFN-γ (clone: XMG1.2, eBioscience), BV421 Rat anti-mouse IL-2 (clone: JES6-5H4, BD), PE Rat anti-mouse NOTCH1 (clone: 22E5.5, BD), PerCP-Cy5.5 anti-mouse IL-22 (clone: Poly5164, Biolegend), PE-Cy7 anti-mouse CD40L (clone: SA047C3, Biolegend), V450 Hamster anti-mouse CD3ε (clone: 500A2, BD), BV605 Rat

anti-mouse NOTCH2 (clone: 16F11, BD), Alexa700 Rat anti-mouse CD4 (clone: RM4-5, BD). All antibodies were titrated before use to determine optimal concentration. The surface stain antibodies were diluted in FACS buffer (PBS [Sigma], 2% heat-inactivated FBS [Gemini], and 2 mM EDTA [Corning]). For intracellular staining, the BD Cytofix/Cytoperm kit and buffers were used according to manufacturer's instructions. Following staining, cells were fixed in 1:1 FACS buffer to 4% paraformaldehyde (PFA) (Electron Microscopy Sciences) for BSL-3 samples, or 2% PFA for non-BSL-3 samples. For compensation, Anti-Rat and Anti-Hamster Ig κ /Negative Control Compensation Particles (BD), UltraComp eBeads compensation beads (Invitrogen), or ArC Amine Reactive Compensation Bead Kit (Invitrogen) were used. All samples were acquired using an LSR-II machine (BD) or an A5 Symphony (BD) using FACSDiva (BD) software. All data were analyzed using FlowJo software (FlowJo LLC). For DC experiments, the gating strategy is the following: singlets, live cells, $CD11c^+$ MHC-II High. For T cell experiments, the gating strategy is the following: singlets, live cells, $CD3^+CD4^+$. All gates were set using FMO controls.

DC-T cell co-culture proliferation assays

C57BL/6 OT-II $OVA_{323–339}$ $Thy1.1^+$ naïve CD4 T cells were purified using the mouse naïve $CD4^+$ T cell isolation kit (StemCell) according to manufacturers' instructions. After isolation, purified T cells were stained with CellTrace Violet (CTV) dye (Invitrogen) at a concentration of 10 μM according to manufacturer's instruction. Purified BMDCs were plated in 96 well U-bottom polypropylene plates at a concentration of 4E5 cells/ml and stimulated with 100 μg/ml of gamma-irradiated Mtb (H37Rv strain, BEI) with or without 1 μg/ml CD40LT in R10 supplemented with 1:1000 BME for 24 hours. Following this time period, cell-free supernatants

were removed and DCs were "pulsed" with 0.1, 1, or 10 μg/ml of cognate peptide ($OVA_{323-339}$, Invivogen). For co-stimulatory blockade experiments, 60 μg/ml of anti-murine ICOSL (clone: HK5.3; BioXCell), anti-murine OX40L (clone: RM134L; BioXCell), anti-murine 41BBL (clone: TKS-1; BioXCell), or anti-murine CD70 (clone: FR70; BioXCell) was added at the time of pulsing. Afterwards, CTV-stained OT-II $OVA_{323–339}$-specific $Thy1.1^+$ CD4 T cells were added at a ratio of 4:1 T cell: DCs to each well. The plates were then placed in a 37ºC incubator (with 5% CO_2) for 5 days. Following this time, cell-free supernatants were harvested for protein enumeration. Cells were then collected and proliferation was measured using flow cytometry.

Statistical analyses

Statistical analyses of data and graphs were mostly conducted using Prism (GraphPad). Figures were also generated using R. The experimental schema figures were generated using BioRender.com. All data presented are representative of 2 to 4 independent experiments and are presented as mean ± S.D Statistical significance p-value key is the following: ns = no significance, * = ≤ 0.05, ** = ≤ 0.01, *** = ≤ 0.001, **** = ≤ 0.0001. Statistical tests performed for each figure are noted in individual figure key.

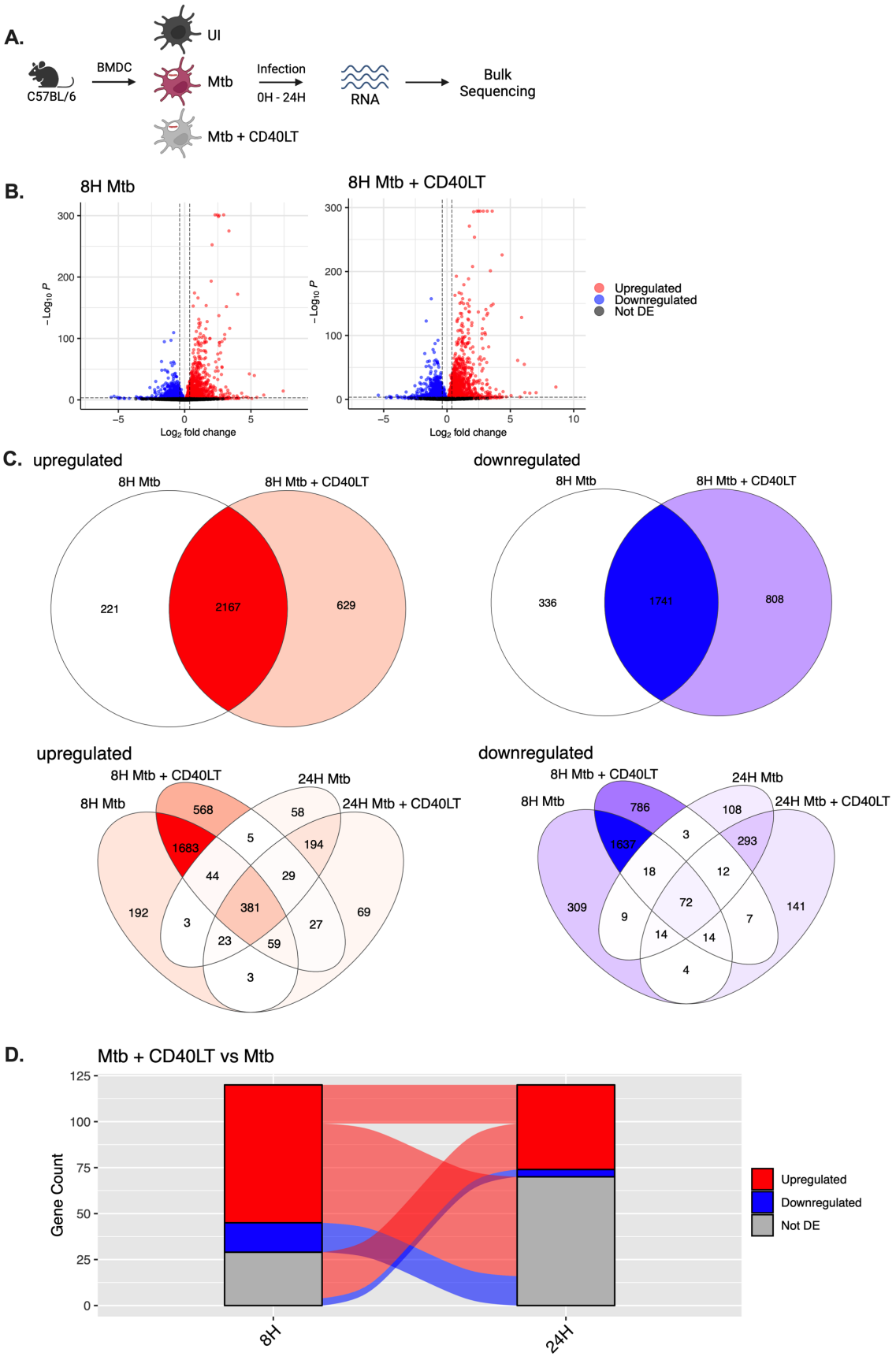
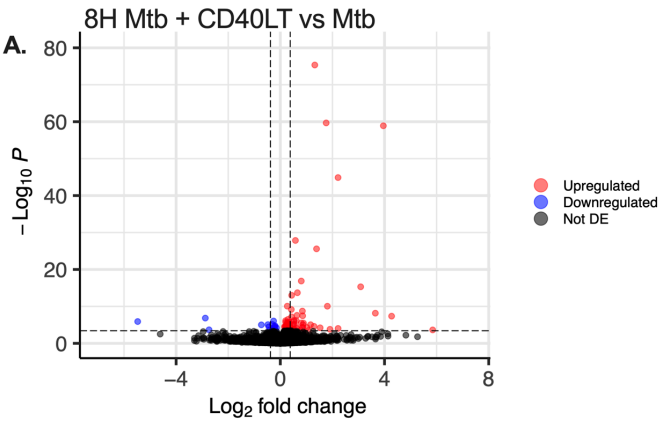
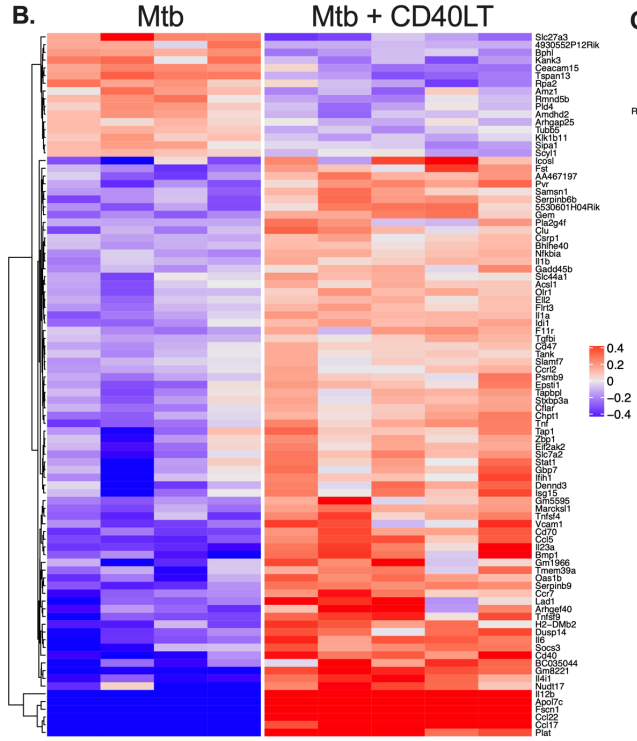
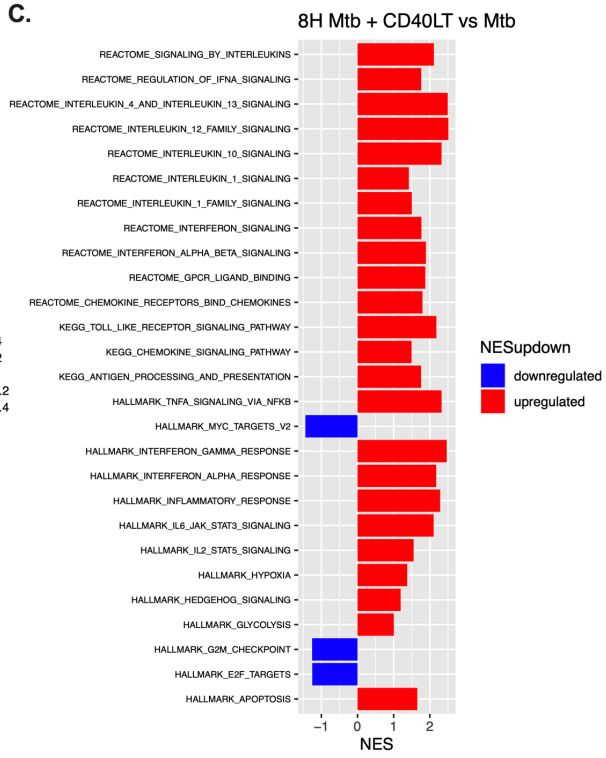
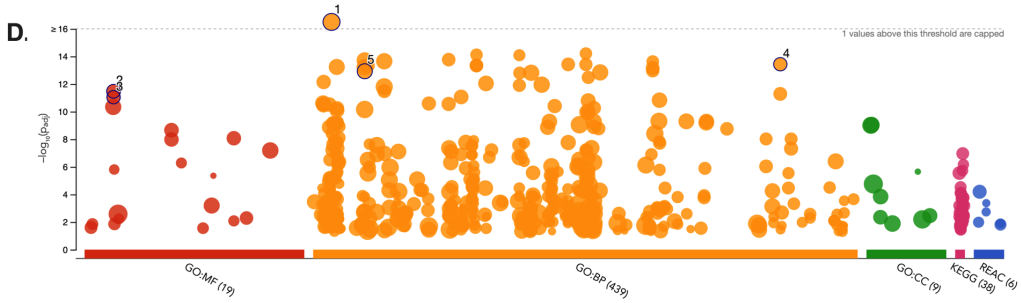

A.
UI
Mtb
Mtb + CD40LT
C57BL/6
BMDC
Infection
0H - 24H
RNA
Bulk
Sequencing
B.
8H Mtb
8H Mtb + CD40LT
−Log10 P
Log2 fold change
Upregulated
Downregulated
Not DE
C. upregulated
8H Mtb
8H Mtb + CD40LT
221
2167
629
downregulated
336
1741
808
upregulated
24H Mtb
24H Mtb + CD40LT
568
58
1683
5
194
44
29
381
192
3
27
69
23
59
3
downregulated
786
108
1637
3
293
18
12
72
309
9
7
141
14
14
4
D.
Mtb + CD40LT vs Mtb
Gene Count
125
100
75
50
25
0
8H
24H
Upregulated
Downregulated
Not DE

Figure 1: Bulk RNA seq reveals that CD40 engagement leads to reprogramming of the Mtb-infected DC transcriptome. C57BL/6 (B6) BMDCs were infected with either no bacteria (UI), Mtb (H37Rv, MOI 1), or Mtb and multimeric CD40L reagent (CD40LT, 1 μg/ml) for up to 24H. At 0H, 8H, and 24H, UI and infected cells were harvested, lysed, and RNA generated for submission for bulk RNA sequencing. A) Experimental schema. B) Volcano plot representing differentially expressed genes 8H Mtb/0H UI or 8H Mtb + CD40LT/0H UI. C) Venn diagrams comparing upregulated and downregulated genes between Mtb/0H UI and Mtb + CD40LT/0H UI comparisons. D) Alluvial plot representing changes in differentially expressed genes between Mtb + CD40LT at 8H and 24H. Experimental schema was made with BioRender.com. Not DE = not differentially expressed.









...	Sour...	Term ...	Term Na...	p_{adj} (query_1)
1	GO:BP	GO:0002376	immune system process	4.408×10^{-17}
2	GO:MF	GO:0005126	cytokine receptor binding	3.334×10^{-12}
3	GO:MF	GO:0005125	cytokine activity	8.986×10^{-12}
4	GO:BP	GO:1903039	positive regulation of leukocyte cell-cell adhesi...	3.671×10^{-14}
5	GO:BP	GO:0006954	inflammatory response	1.186×10^{-13}

Figure 2: CD40 engagement increases pro-inflammatory pathways in Mtb-infected DCs. BMDCs were infected with either no bacteria (UI), Mtb (H37Rv, MOI 1), or Mtb and multimeric CD40L reagent (CD40LT, 1 μg/ml) for up to 24H. At 0H, 8H, and 24H, UI and infected cells were harvested, lysed, and RNA generated for submission for bulk RNA sequencing. A) Volcano plot representing differentially expressed genes for 8H Mtb + CD40LT/8H Mtb comparison. B) Heat map of top differentially expressed genes (p value <0.05); scale was generated from the log2 foldchange from mean 8H Mtb +CD40LT/Mtb. C) Selected mSigDB pathways from GSEA analysis that were significantly upregulated in the 8H Mtb + CD40LT condition compared to 8H Mtb. D) Functional gene enrichment analysis of the top significantly upregulated genes in 8H Mtb + CD40LT condition compared to 8H Mtb generated in g:GOSt/g:Profiler. Not DE = not differentially expressed. NES = normalized enrichment score.

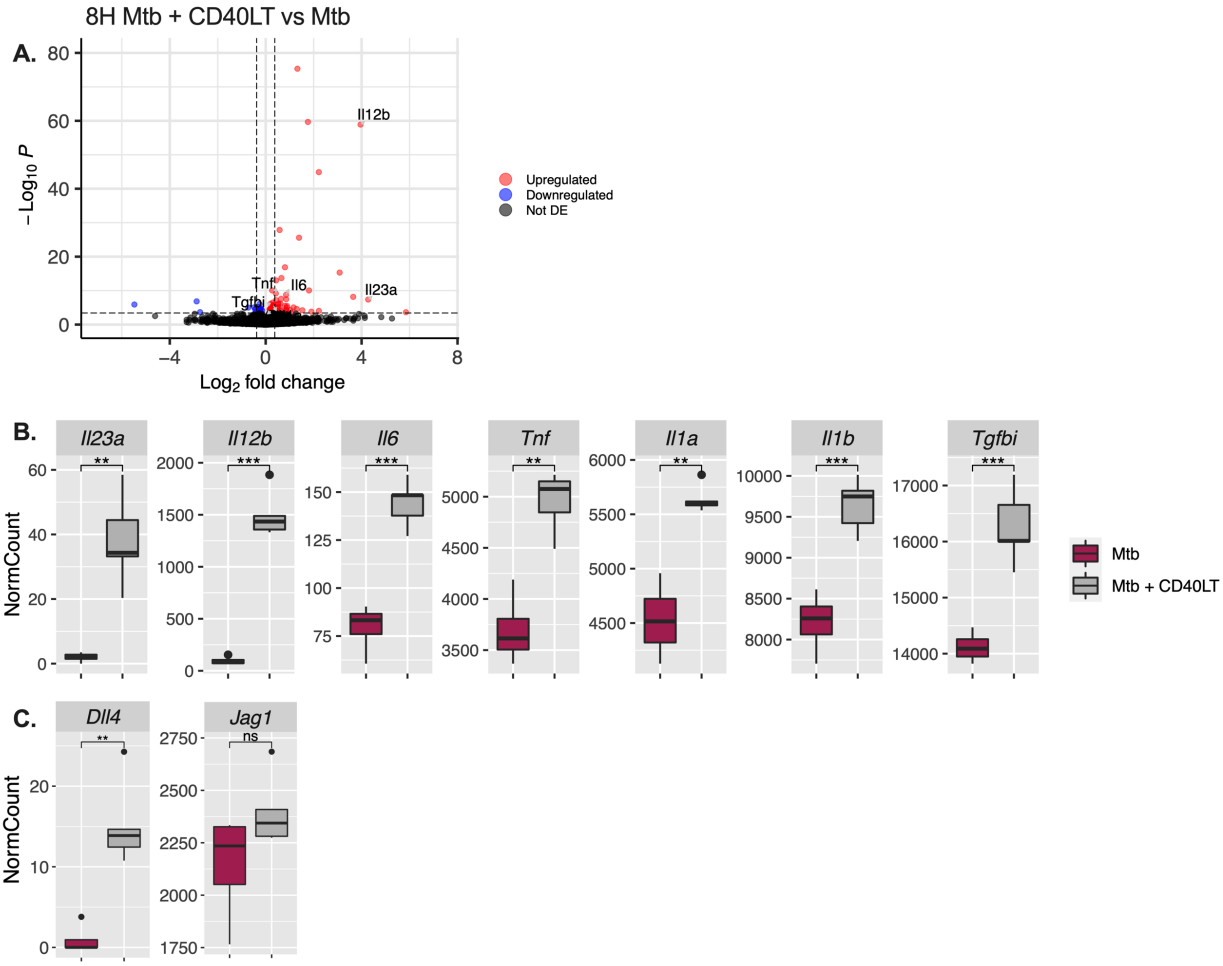

8H Mtb + CD40LT vs Mtb
A.
−Log10 P
Log2 fold change
Il12b
Tnf
Il6
Tgfbi
Il23a
Upregulated
Downregulated
Not DE
B.
Il23a
Il12b
Il6
Tnf
Il1a
Il1b
Tgfbi
NormCount
Mtb
Mtb + CD40LT
C.
Dll4
Jag1
ns

Figure 3: CD40 engagement increases the ability of DCs to produce Th_1 and Th_{17} polarizing cytokines. BMDCs were infected with either no bacteria (UI), Mtb (H37Rv, MOI 1), or Mtb and multimeric CD40L reagent (CD40LT, 1 μg/ml) for up to 24H. At 0H, 8H, and 24H, UI and infected cells were harvested, lysed, and RNA generated for submission for bulk RNA sequencing. A) Volcano plot representing differentially expressed genes for 8H Mtb + CD40LT/8H Mtb comparison with the following genes highlighted: *Il12b*, *Il1a*, *Il1b*, *Il23a*, *Il6*, *Tgfbi*, *Tnf*. B) Normalized counts plotted for *Il12b*, *Il1a*, *Il1b*, *Il23a*, *Il6*, *Tgfbi*, *Tnf*. C) Normalized counts plotted for *Dll4* and *Jag1*. Data in B) and C) are presented as mean ± SD. Data were analyzed using an unpaired Student's t-test. Statistical significance p-value key is the following: ns = no significance, * = ≤ 0.05, ** = ≤ 0.01, *** = ≤ 0.001, **** = ≤ 0.0001. Not DE = not differentially expressed.

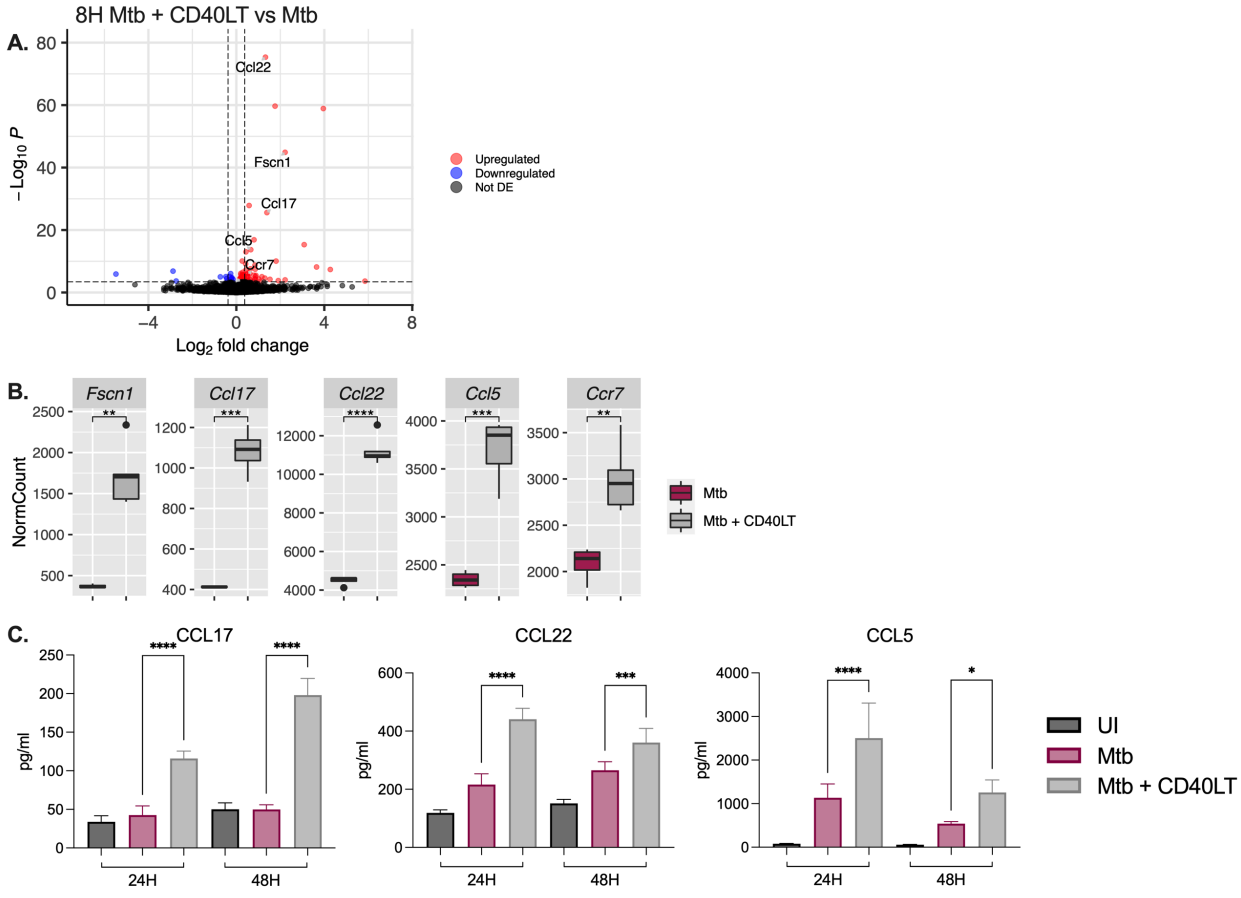

A.
8H Mtb + CD40LT vs Mtb
Ccl22
Fscn1
Ccl17
Ccl5
Ccr7
Upregulated
Downregulated
Not DE
Log2 fold change
B.
Fscn1
Ccl17
Ccl22
Ccl5
Ccr7
NormCount
Mtb
Mtb + CD40LT
C.
CCL17
CCL22
CCL5
pg/ml
24H
48H
UI
Mtb
Mtb + CD40LT

Figure 4: CD40 engagement augments expression of migration-associated chemokines. BMDCs were infected with either no bacteria (UI), Mtb (H37Rv, MOI 1), or Mtb and multimeric CD40L reagent (CD40LT, 1 μg/ml) for up to 24H. At 0H, 8H, and 24H, UI and infected cells were harvested, lysed, and RNA generated for submission for bulk RNA sequencing. A) Volcano plot representing differentially expressed genes for 8H Mtb + CD40LT/8H Mtb comparison with the following genes highlighted: *Ccl17*, *Ccl22*, *Ccl5*, *Ccr7*, *Fscn1*. B) Normalized counts plotted for *Ccl17*, *Ccl22*, *Ccl5*, *Ccr7*, *Fscn1*. D) BMDCs were infected for up to 48H and cell-free supernatants were harvested for protein enumeration. ELISA data for CCL5, CCL17, and CCL22. Data are presented as mean ± SD. Data in D) are representative of 2 independent experiments. Data were analyzed in C) using an unpaired Student's t-test and D) using a one-way ANOVA with a correction for multiple comparisons. Statistical significance p-value key is the following: ns = no significance, * = ≤ 0.05, ** = ≤ 0.01, *** = ≤ 0.001, **** = ≤ 0.0001. Not DE = not differentially expressed.

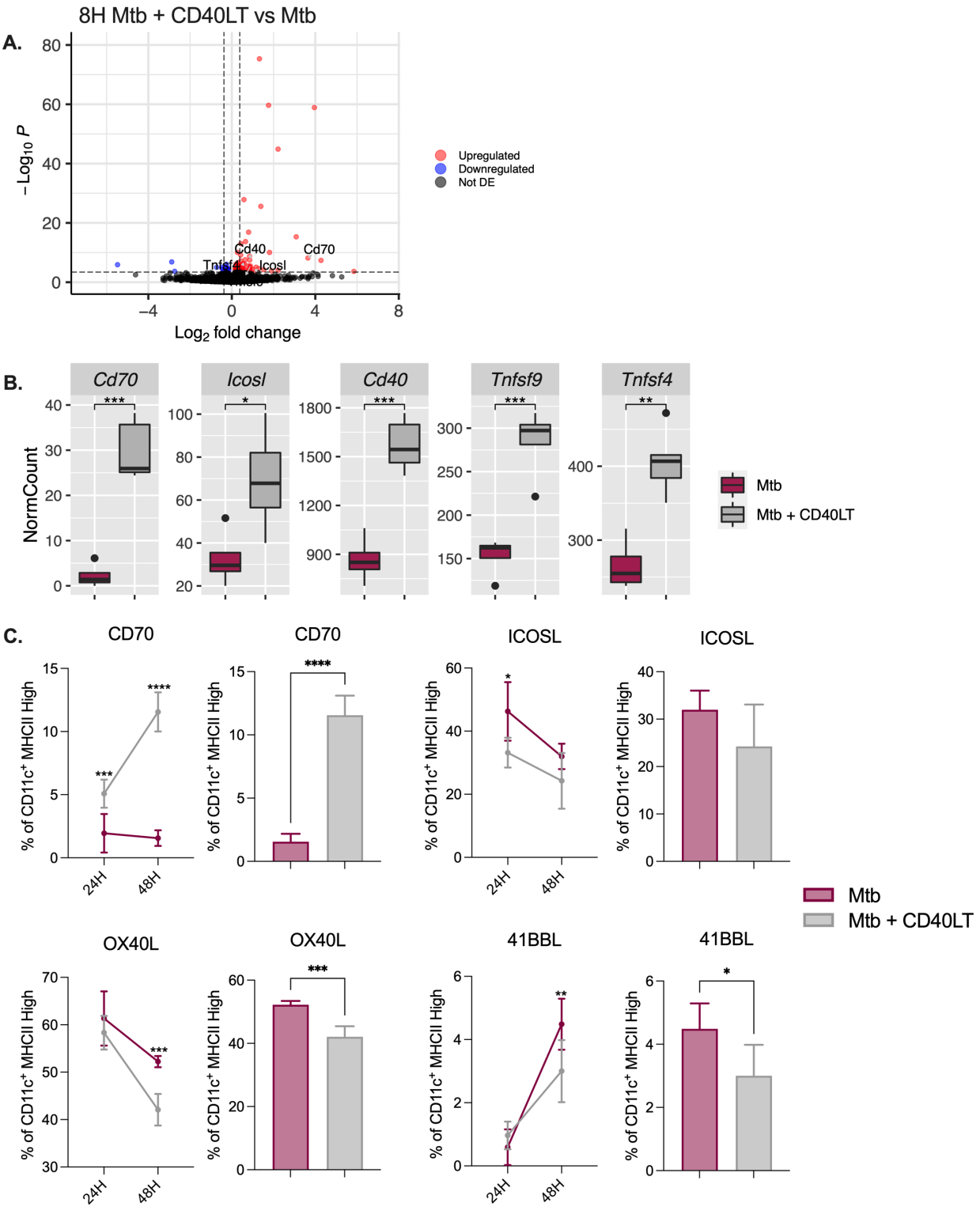

A.
8H Mtb + CD40LT vs Mtb
−Log10 P
Log2 fold change
Upregulated
Downregulated
Not DE
Cd40
Cd70
Tnfsf4
Icosl
B.
Cd70
Icosl
Cd40
Tnfsf9
Tnfsf4
NormCount
Mtb
Mtb + CD40LT
C.
CD70
ICOSL
OX40L
41BBL
% of CD11c+ MHCII High
24H
48H
Mtb
Mtb + CD40LT

Figure 5: CD40 engagement enhances expression of DC co-stimulatory molecules. BMDCs were infected with either no bacteria (UI), Mtb (H37Rv, MOI 1), or Mtb and multimeric CD40L reagent (CD40LT, 1 μg/ml) for up to 24H. At 0H, 8H, and 24H, UI and infected cells were harvested, lysed, and RNA generated for submission for bulk RNA sequencing. A) Volcano plot representing differentially expressed genes for 8H Mtb + CD40LT/8H Mtb comparison with the following genes highlighted: *Cd40*, *Cd70*, *Icosl*, *Tnfsf4 (Ox40L)*, *Tnfsf9 (41bbl)*. B) Normalized counts plotted for *Cd40*, *Cd70*, *Icosl*, *Tnfsf4 (Ox40L)*, *Tnfsf9 (41bbl)*. C) BMDCs were infected for up to 72H and cells were harvested and surface expression of co-stimulatory markers was measured using flow cytometry. Populations shown were pre-gated on singlets, live cells, and $CD11c^+$ MHC-II High populations. Frequency of $CD70^+$, $ICOSL^+$, $OX40L^+$, and $41BBL^+$ populations are represented. Bar plots are from 48H time point. Data are presented as mean ± SD. Data in D) are representative of 2 independent experiments. Data were analyzed in C) using an unpaired Student's t-test and D) using a one-way ANOVA with a correction for multiple comparisons. Statistical significance p-value key is the following: ns = no significance, * = ≤ 0.05, ** = ≤ 0.01, *** = ≤ 0.001, **** = ≤ 0.0001. Not DE = not differentially expressed.

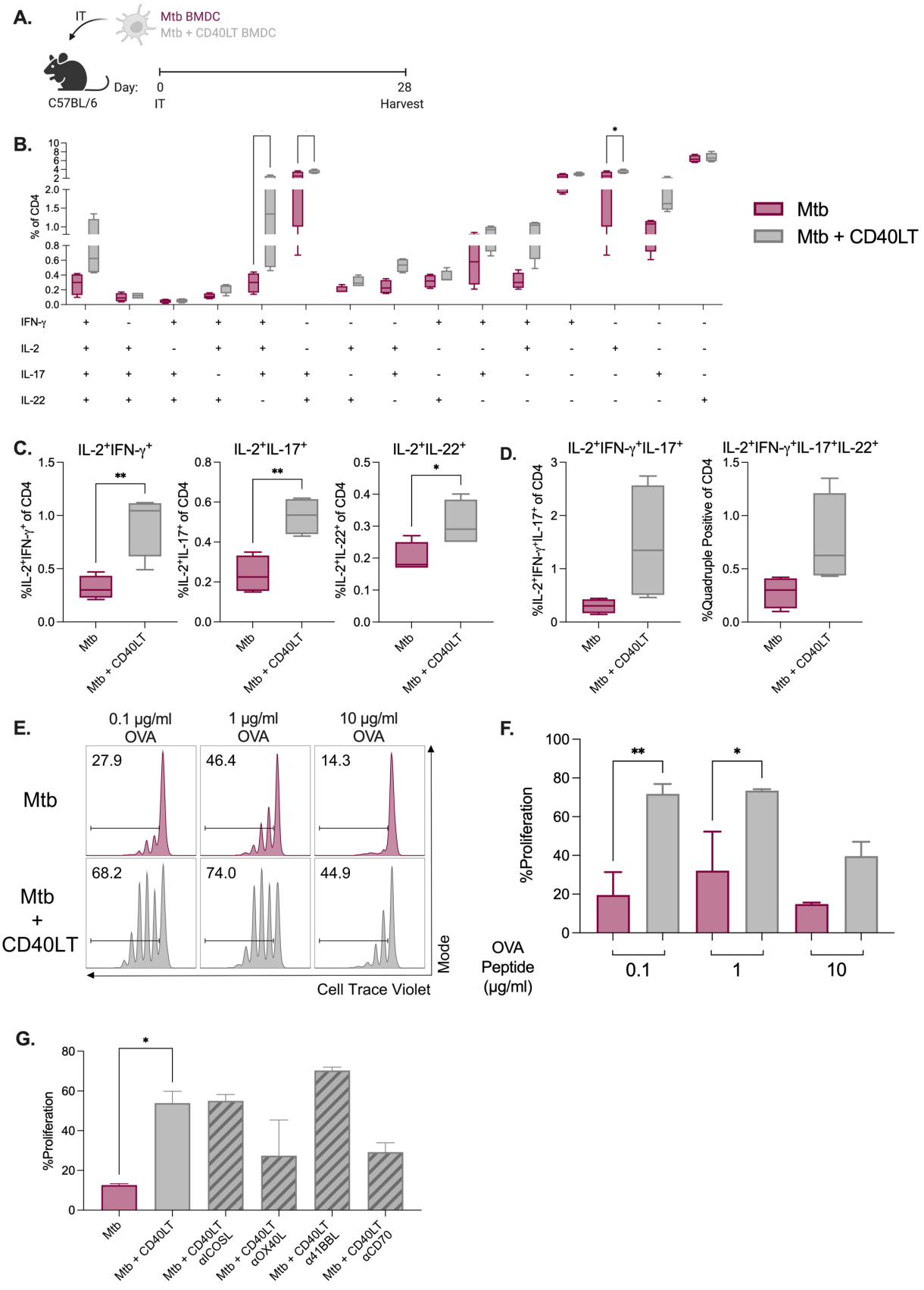
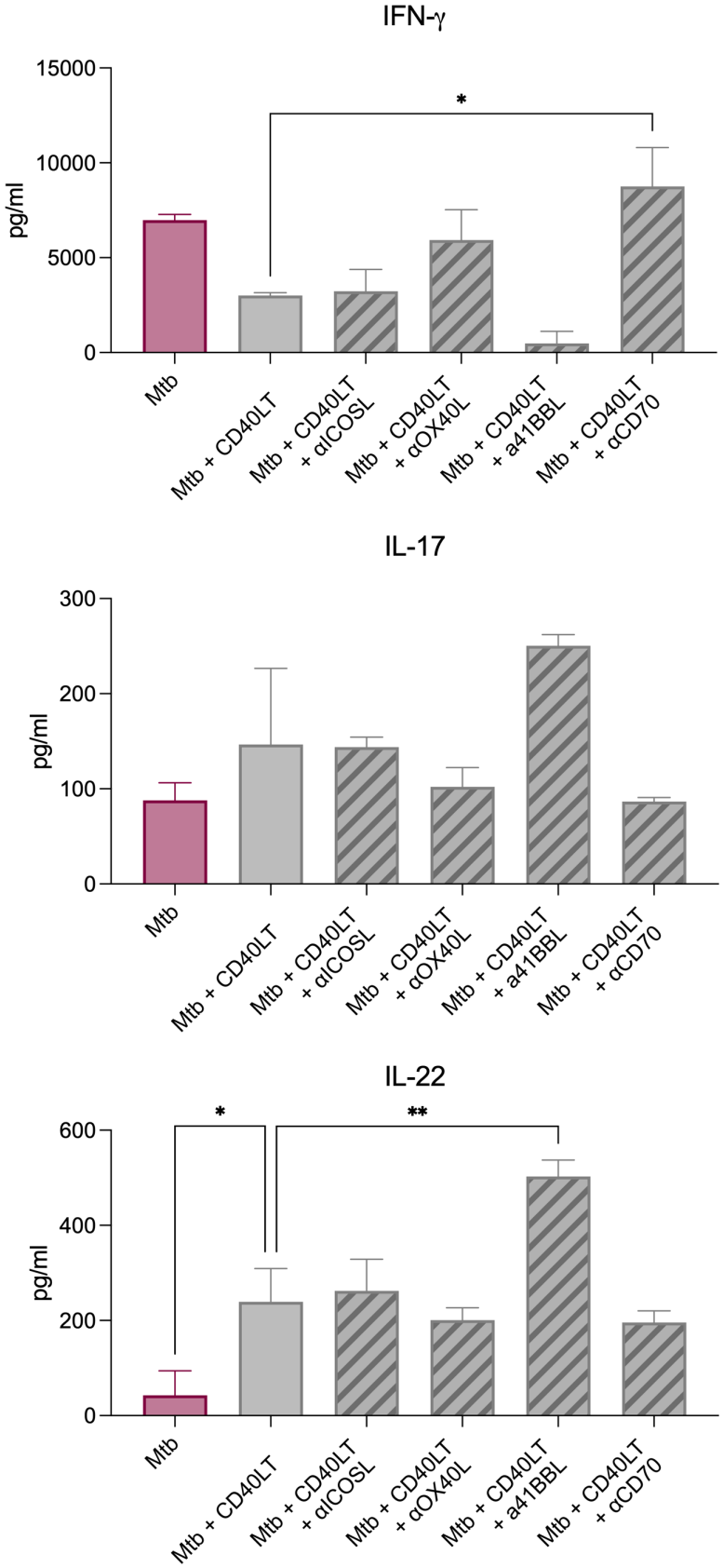

A.
IT
Mtb BMDC
Mtb + CD40LT BMDC
C57BL/6
Day:
0
IT
28
Harvest
B.
% of CD4
IFN-γ
IL-2
IL-17
IL-22
Mtb
Mtb + CD40LT
C.
IL-2+IFN-γ+
IL-2+IL-17+
IL-2+IL-22+
%IL-2+IFN-γ+ of CD4
%IL-2+IL-17+ of CD4
%IL-2+IL-22+ of CD4
D.
IL-2+IFN-γ+IL-17+
IL-2+IFN-γ+IL-17+IL-22+
%IL-2+IFN-γ+IL-17+ of CD4
%Quadruple Positive of CD4
E.
0.1 μg/ml OVA
1 μg/ml OVA
10 μg/ml OVA
27.9
46.4
14.3
68.2
74.0
44.9
Mtb
Mtb + CD40LT
Mode
Cell Trace Violet
F.
%Proliferation
OVA Peptide (μg/ml)
0.1
1
10
G.
%Proliferation
Mtb
Mtb + CD40LT
Mtb + CD40LT αICOSL
Mtb + CD40LT αOX40L
Mtb + CD40LT α41BBL
Mtb + CD40LT αCD70

Figure 6: Engaging CD40 on DCs augments polyfunctional and proliferating CD4 T cells responses. A) Experimental schema. BMDCs were infected with Mtb (MOI 1) with or without CD40LT (1μg/ml) for 48H. Following infection, DCs were harvested and 1E6 were transferred into the lungs of mice. After four weeks post-transfer, mice were euthanized and lungs immune responses *ex vivo* were assayed using flow cytometry. B) Boolean analysis of % of CD4 T cells that expressed IFN-γ, IL-2, IL-17 and IL-22. Populations shown were pre-gated on singlets, live cells, and $CD3^{+}CD4^{+}$. C) and D) Frequency of select multiple-cytokine producing T cells from Boolean analysis. E) BMDCs were stimulated with γ-irradiated Mtb (H37Rv strain, 100 μg/ml) in the presence or absence of CD40LT (1 μg/ml) for 24H. Following this time, DCs were pulsed with cognate peptide OVA (0.1 - 10 for 1 hour and co-cultured with cell-trace violet-stained OT-II naïve CD4 Tg T cells at a 4:1 T cell-DC ratio for 5 days. Representative flow plots of proliferating T cells between Mtb and Mtb + CD40LT condition for 0.1, 1, and 10 μg/ml of OVA. F) Frequency of proliferating T cells. G) At the time of co-culture, 60 μl of anti-mouse ICOSL, anti-mouse OX40L, anti-mouse 41BBL, anti-mouse CD70 was added in the Mtb + CD40LT condition. Frequency of proliferating T cells. Data are presented as mean ± SD. Data are representative of 2 independent experiments. Data were analyzed in B) using a two-way ANOVA with correction for multiple comparisons, C)-D) using an unpaired Student's t-test, and F)-G) using a one-way ANOVA with a correction for multiple comparisons. Statistical significance p-value key is the following: ns = no significance, * = ≤ 0.05, ** = ≤ 0.01, *** = ≤ 0.001, **** = ≤ 0.0001.


IFN-γ
15000
10000
5000
0
pg/ml
*
Mtb
Mtb + CD40LT
Mtb + CD40LT + αICOSL
Mtb + CD40LT + αOX40L
Mtb + CD40LT + α41BBL
Mtb + CD40LT + αCD70
IL-17
300
200
100
0
pg/ml
Mtb
Mtb + CD40LT
Mtb + CD40LT + αICOSL
Mtb + CD40LT + αOX40L
Mtb + CD40LT + α41BBL
Mtb + CD40LT + αCD70
IL-22
600
400
200
0
pg/ml
*
**
Mtb
Mtb + CD40LT
Mtb + CD40LT + αICOSL
Mtb + CD40LT + αOX40L
Mtb + CD40LT + α41BBL
Mtb + CD40LT + αCD70

Supplemental Figure 1: Blocking CD70 reduces Th_{17} but not Th_1 polarization. BMDCs were stimulated with γ-irradiated Mtb (H37Rv strain, 100 μg/ml) in the presence or absence of CD40LT (1 μg/ml) for 24H. Following this time, DCs were pulsed with cognate peptide OVA 0.1 μg/ml with or without blocking antibodies for 1 hour and co-cultured with cell-trace violet-stained OT-II naïve CD4 Tg T cells at a 4:1 T cell-DC ratio for 5 days. Following this time, cell-free supernatants were harvested for protein enumeration using ELISA. Data are presented as mean ± SD. Data are representative of 2 independent experiments. Data were analyzed using a one-way ANOVA with a correction for multiple comparisons (all comparisons were to Mtb + CD40LT). Statistical significance p-value key is the following: ns = no significance, * = ≤ 0.05, ** = ≤ 0.01.

Chapter IV

Discussion

Although tuberculosis (TB) has been present for thousands of years, it continues to be a burden to public health worldwide[1]. Despite modest efforts to curtail TB in the past decade[1], the current COVID-19 pandemic has set-back progress[2] and has highlighted the need to develop preventative strategies to limit TB incidence and death. Specifically, it has highlighted the need to develop efficacious vaccines and adjuvants. Vaccine-mediated immunity is necessary as *Mycobacterium tuberculosis* (Mtb), the causative agent of TB, is adept at subverting the function of immune cells to limit protective immune responses during infection. Current efforts to develop more effective vaccines and adjuvants, however, have been hindered by a lack of fundamental insight into key aspects of TB immunology. While we have gained an appreciation of the role of various immune cells in controlling infection, we lack comprehensive knowledge of protective immunity to TB. We lack insight into the ability of Mtb to modulate the function of different immune cells during infection. Moreover, we lack knowledge on strategies to overcome Mtb immune evasion in order to augment protective immunity. Insights into these critical aspects of TB pathogenesis and immunity are therefore necessary to develop effective vaccines and adjuvants in order to limit TB deaths and cases worldwide.

This dissertation aims at addressing these critical gaps in knowledge by providing mechanistic insights into the immune responses that contribute to protective immunity, the pathways that promote these responses, how Mtb subverts immune cell function, and the strategies that can be employed to overcome immune evasion to augment protective immunity. Dendritic cells (DCs) are a critical innate immune cell subset that bridges the innate/adaptive interface. DCs are considered “professional” antigen-presenting cells due to their enhanced ability to initiate T cell responses. Specifically, DCs present antigen, upregulate co-stimulatory molecules, and secrete

cytokines, three functions which are necessary for activating and polarizing naïve T cells. While the pivotal role of DCs in initiating T cell responses during Mtb infection is well-established[60,72,73], the molecular mechanisms that dictate optimal DC-T cell crosstalk and T cell polarization, along with insights into how Mtb impedes these responses during infection, were poorly understood. These mechanisms are important to elucidate as protective T cell responses, including $CD4^+$ T helper 17 (Th_{17}) responses, are limited during infection while Th_1 are readily present. Our laboratory has previously demonstrated that Mtb can restrict Th_{17} responses during infection by dampening the ability of DCs to upregulate CD40 co-stimulatory molecule during infection through the Hip1 immunomodulatory serine protease protein[195,198]. We also found that CD40-CD40L signaling is necessary for Th_{17} polarization during infection and that exogenously engaging CD40 through the use of a multimeric CD40L reagent (CD40LT) augmented Th_{17} responses in the lung[195]. However, the pathways downstream of CD40 on DCs that contributed to these responses remained unknown. In Chapter II, we sought to test whether the Notch signaling pathway is present downstream of CD40 signaling on DCs and whether this contributes to Th_{17} polarization during infection. We found that exogenously engaging CD40 on DCs led to an increase in transcript for *Dll4*, a canonical ligand of the Notch signaling pathway. Engaging CD40 also increased surface expression of DLL4 on DCs during infection, and blocking DLL4 in a DC-T cell co-culture led to a significant decrease in Th_{17} polarization while Th_1 polarization remained intact. To understand how the CD40/DLL4 axis functions in Th_{17} polarization *in vivo*, we used an adoptive DC transfer model wherein we transferred Mtb-infected DCs *via* the intratracheal (IT) route into the lungs of mice. We found that we were able to recapitulate our *in vitro* results *in vivo* and demonstrated that blocking DLL4 on DCs led to a significant decrease in IL-17 and IL-22 (another Th_{17} cytokine) responses while IL-2 and IFN-γ (Th_1) remained intact.

We also observed that blocking DLL4 led to a significant decrease in the ability of DCs to polarize multifunctional (or multiple cytokine-producing) T cells in the lung. Interestingly, we found that CD40 engagement also led to the increase in double- and triple-cytokine-producing T cells as well as $CXCR3^{+}CCR6^{+}$ Th_1/Th_{17} cells (which are important for protection in human and primate models of TB[119,122]), and that blocking DLL4 impaired the ability of DCs to induce these subsets. Our results also showed that transfer of Mtb + CD40LT DCs led to reduced bacterial burdens in the lung of mice and that reduced CFU correlated with increase IL-17. We then wanted to elucidate the mechanism by which Mtb evades these responses during infection. We found that infection of mice with a *hip1* mutant of Mtb led to a significant increase in the populations of DCs in the lung that expresses $DLL4^{+}$ along with a significant increase in Th_1 and Th_{17} populations at an early time point compared to wild type Mtb. Moreover, infection of $CD40^{-/-}$ mice demonstrated that Hip1 impairs the ability of lung DCs to express DLL4 by impairing upregulation of CD40. Overall, the results in Chapter II highlight a role for the CD40/DLL4 axis in Th_{17} and multifunctional T cell responses *in vivo* and demonstrate that Mtb dampens these responses to subvert protective immunity during infection.

While we found a mechanism that is necessary for polarizing Th_{17} and multifunctional T cell subsets, we wanted to uncover additional pathways downstream of CD40 signaling on DCs that contribute to the generation of functional T cell responses during infection. Therefore, in Chapter III we used bulk RNA sequencing (RNA seq) to dissect the pathways downstream of CD40 signaling on DCs during Mtb infection. We found that exogenously engaging CD40 on DCs led to a significant increase in differentially expressed genes (DEGs) and a re-programming of the DC transcriptome compared to Mtb infection alone. We then characterized the functions of these

DEGs and found that CD40 engagement led to a significant increase in pathways related to broad pro-inflammatory processes such as cytokine secretion/signaling and antigen presentation. Among the significantly upregulated genes in the Mtb + CD40LT condition were pro-inflammatory cytokines, specifically the cytokines that contribute to Th_1 and Th_{17} polarization. We observed a significant increase in genes that correspond to migratory functions of DCs and confirmed *via* ELISA that CD40 engagement significantly increases the ability of DCs to secrete chemokines CCL5, CCL17, and CCL22 during infection. We also found that exogenous engagement of CD40 on DCs led to a significant increase in genes for other co-stimulatory molecules and confirmed *via* flow cytometry that CD40 engagement significantly increases the ability of DCs to express CD70 during infection. When we measured T cell proliferation, we found that Mtb + CD40LT DCs led to a significant increase in the capacity of T cells to proliferate *in vitro* and led to more activated and multifunctional T cells *in vivo*. This effect is partially dependent on CD70 and OX40L expression, as blocking these two co-stimulatory molecules *in vitro* during co-culture reduced T cell proliferation. Collectively, these data presented in Chapter III highlight additional pathways downstream of CD40 signaling on DCs and demonstrate that exogenously engaging CD40 on DCs augments the ability of DCs to induce functional T cells during infection.

The work in this dissertation provides key mechanistic insights into the molecular pathways that contribute to optimal DC-T cell crosstalk and polarization of protective T cell subsets. While the Notch signaling pathway, specifically ligand DLL4, has been implicated in Th_{17} polarization in other experimental models such as autoimmunity[270,271,293], here we show that it is necessary for Th_{17} and multifunctional T cell polarization during Mtb infection. Studies in Chapter III have

similarly generated valuable insights into the mechanisms that contribute to functional T cell responses which have not been previously explored in Mtb infection. In addition to our work on Th_{17} polarization, our results also suggest that CD40 signaling on DCs contributes to the polarization of yet another Th subset: Th_{22}. In Chapter II, we observed a significant increase in IL-22^+ T cells following transfer of Mtb + CD40LT DCs, which was significantly reduced upon DLL4 blockade. In Chapter III, we similarly found that the transfer of Mtb + CD40LT DCs led to a significant increase in IL-2^+IL-22^+ T cells in the lungs of mice. Th_{22} cells are characterized by high expression of IL-22 in the absence of other Th_{17} cytokines[294]. While IL-22 has been shown to play a protective role during chronic stages of Mtb infection[55,142], the role of Th_{22} cells, specifically, in TB is poorly understood[146]. Our studies suggest that these responses can contribute to protective immunity as IL-22 cytokine was significantly upregulated following CD40 engagement and is expressed by multifunctional T cells (Chapter II). Characterizing the DC signals that contribute to the polarization of Th_{22} subsets is likely to be important as these insights will also inform the generation of protective immune responses. Given that excessive Th_{17} cells can be pathogenic[133,134], it would be interesting to test whether IL-22 expression defines protective Th_{17} cells in TB or if the presence of Th_{22} cells concurrently with Th_{17} cells helps fine-tune pathology/protection (which is supported by studies in other contexts presented in this recent review[295]). Although studying Th_{22} cells can be difficult given the lack of unique transcriptional lineage commitment marker[294,296], the inclusion of IL-22 in multiparameter flow cytometry experiments will provide insights that can serve to better define the role of these cells during TB.

The data presented here highlight the importance of engaging CD40 during Mtb infection as it increases the pathways necessary for augmenting Th_{17} and multifunctional T cell responses. While we have specifically characterized CD40 on DCs, CD40 signaling should also be studied in other immune cells. Macrophages also express CD40, which can contribute to pathology in autoimmunity and atherosclerosis[297]. It has been demonstrated that macrophages have the capacity to upregulate CD40 following Mtb infection[58], although the implications of this for macrophage function or macrophage-T cell crosstalk remain to be known. CD40 is also present on B cells and is well-known to be important for B cell activation and class-switch of antibody subtypes[298]. As the role of B cells and antibodies in protective immunity to TB is becoming more appreciated, it would be interesting to study how engaging CD40 in these cells may augment humoral immunity during infection. Interestingly, CD40 signaling also seems to dictate myeloid-derived suppressor cells (MDSC) function; CD40 downregulation was found to lead to an accumulation of MDSCs in gastric tumors[299,300] but in a hepatic mouse tumor model, CD40 expression on MDSCs led to an increase in suppressive capacity[301]. Studying the CD40 signaling pathway in different immune cell types is likely to be important as TB immunity requires a coordinated response between different facets of the immune response (which is best represented by the formation of granulomas). Future studies should characterize whether engaging CD40 is also able to enhance the function of other immune cells in order to limit infection, and importantly, whether this can overcome immune evasion of different cell types to augment protective immunity.

Our work here and previous studies from our laboratory have demonstrated that dissecting immune responses downstream of co-stimulatory molecules on DCs is a viable strategy for

elucidating the molecular mechanisms that contribute to protective immunity. In addition to this, our data also highlight additional co-stimulatory molecules of interest for TB immunity. In Chapter III, we observed a significant increase in CD70 co-stimulatory molecule expression on DCs following CD40 engagement both in RNA seq transcript and flow cytometry. Moreover, when we blocked CD70 *in vitro* in a DC-T cell co-culture proliferation assay, we observed a reduction in proliferation as well as IL-17 and IL-22 secretion. While CD70 has been demonstrated to be upregulated on DCs following CD40 stimulation[302-304], the role of this co-stimulatory signaling pathway in Mtb infection has yet to be fully characterized. We have more, yet limited, insight into the receptor with which CD70 interacts. During DC-T cell crosstalk, CD70 on DCs binds to CD27 on T cells[305]. Our laboratory previously showed that CD27 expression on T cells was detected in BCG-vaccinated individuals but not in LTBI individuals[28], suggesting that CD27 expression is limited during infection. Another study found that $CD27^{-/-}$ knockout mice had increased susceptibility to Mtb and reduced IFN-γ responses, although CD27 is not necessary for protection at chronic stages of infection[306]. While a study in *M. avium*-infected mice found CD70 was dispensable for protection[307], similar experiments have not been carried out for Mtb infection. Taken together, these data suggest a protective role for CD27 and potentially CD70 and highlight the need to characterize this signaling pathway further to understand how it can shape DC-T cell crosstalk and T cell responses during infection. Our data also emphasize the need to study another co-stimulatory/inhibitory signaling pathway. Extended analysis of the Mtb + CD40LT and Mtb RNA seq dataset (Chapter III) also demonstrated that DCs significantly upregulate *Pvr* (PVR) transcript following CD40 engagement as well as surface expression (Appendix Figure 1). CD155 (also known as PVR or poliovirus receptor) on DCs binds to TIGIT or CD226 receptors on T cells[308]. TIGIT and CD226 have competing

functions; binding TIGIT results in dampened responses whereas CD226 stimulates responses[309]. The presence of TIGIT/CD226 signaling during Mtb infection likely has important implications for the regulation of immune responses during infection. A study in humans found that CD226 is expressed on CD4 T cells that co-express IFN-γ^{+}IL-17^{+}, and blocking this receptor prevents dual secretion of these cytokines[310]. TIGIT can also regulate cytokine secretion in T cells as a study found that T regulatory (T_{reg}) cells that express TIGIT do not express IFN-γ^{+}IL-17^{+}[311]. In the context of Mtb, less is known about TIGIT/CD226/CD155, especially in DC-T cell crosstalk. What is known is that natural killer (NK) cells from patients that test positive for TB exposure and live in a TB-endemic setting had increased TIGIT expression[312]. Interestingly, when we measured the expression of TIGIT on CD4 T cells during aerosol infection of mice we found that *hip1* mutant resulted in greater TIGIT expression than wild type Mtb infection, though this difference is not significant (CD226 was not assayed; Appendix Figure 1). This, coupled with single-cell RNA sequencing data in the lab demonstrating that *hip1* mutant infection upregulates TIGIT/CD226/CD155 signaling pathways in various cells in the lung (Dkhar *et. al*. unpublished) makes these pathways a necessary focus of future studies. Further characterization of CD70 and CD155 on DCs, using similar approaches used in this dissertation, can provide valuable insight for the field. As these molecules are activated downstream of CD40, it is reasonable to hypothesize that pathways downstream of CD70 and CD155 could dictate protective T cell responses. Future studies should also test whether exogenously engaging CD70 and/or targeting TIGIT/CD226/CD155 through vaccines and adjuvants is beneficial for protective immunity.

Dissecting DC responses is necessary for uncovering mechanistic insights into protective immunity during Mtb infection. The work in Chapter II highlights how sequencing DC responses

has the potential to accelerate discovery of the mechanisms that contribute to protective immunity by uncovering the pathways that contribute to functional T cell responses during infection. This approach is particularly powerful when coupled with Mtb mutants of important immune evasion proteins. To gain a better understanding of how Mtb modulates DC responses during infection, we also infected DCs with a *hip1* mutant and performed RNA seq following infection (Enriquez *et. al.* unpublished). Preliminary analysis of this dataset reveals that infection with *hip1* mutant results in thousands of significantly upregulated genes compared to infection with wild type Mtb (Appendix Figure 2). We have also observed that *hip1* mutant upregulates the same pathways as exogenous engagement of CD40 along with a significant number of genes which all participate in diverse DC functions. Future studies should also sequence and compare DC responses following infection with different clinical isolates/Mtb strains. The HN878 "hypervirulent" strain is better at eliciting Th_{17} responses during infection compared to the H37Rv lab-adapted strain[117], but exactly how DCs respond differently to HN878 versus other strains is yet to be known. RNA sequencing of these different infections will likely provide important insights that allow us to better characterize host-pathogen interactions. While infection with other mutants and Mtb strains will yield critical mechanistic insights for vaccine development, we can also gain these insights from studying the Bacillus Calmette-Guérin (BCG) vaccine strain itself. While macrophages and other monocyte responses have been sequenced and characterized following BCG infection, these studies have yet to be carried out in DCs. We lack necessary information on the effect of BCG vaccination on DC-T cell crosstalk and T cell polarization. Importantly, as BCG is a live-attenuated strain, it still encodes immune evasion proteins[26]. Our lab found that Hip1 is also present in BCG and developed a knockout strain (BCGΔ*hip1*) to better characterize the role of Hip1 during infection[26]. In a DC mucosal

vaccination model, transfer of DCs stimulated with BCGΔ*hip1* led to more Th_{17} responses in the lung and afforded more protection against Mtb challenge compared to wild type BCG[26]. Sequencing the RNA from BCG or BCGΔ*hip1*-infected DCs will therefore provide insights that have direct implications for vaccine-mediated immunity.

The insights presented here provide a framework for developing effective vaccines and adjuvants. Specifically, our data demonstrate which pathways should be targeted in order to increase protective immunity during infection. In Chapter II, we demonstrated that DLL4 is necessary for polarization of Th_{17} and multifunctional T cells. In Chapter III, we found that increased expression of pro-inflammatory and Th_1/Th_{17} polarization cytokines, expression of other co-stimulatory molecules (especially CD70), and secretion of chemokines and migratory molecules are DC functions which are augmented following CD40 engagement. These pathways not only highlight the mechanisms necessary for inducing functional T cell responses but also serve as targets for developing future vaccines and adjuvants. While increasing these functions singly can contribute to polarization of protective T cell subsets, these data presented here and previous work in the lab[195] demonstrate that CD40 itself should be a target of vaccines and adjuvants as engaging this receptor is able to re-program the DC transcriptome and enhance function. In a DC mucosal vaccination model, exogenously engaging CD40 on DCs increases responses that contribute to protection following Mtb challenge[195]. While others have also demonstrated that mucosal transfer of activated DCs is able to augment T cell responses during Mtb infection[193-195], these studies serve as a proof of concept as transfer of DCs is unlikely to be used as a vaccination strategy in humans. Instead, future vaccine studies should focus on developing constructs that are able to engage CD40, especially on DCs. It would be interesting to

test if providing CD40LT reagent itself as a vaccine is able to augment protective immunity. This approach, however, would likely activate CD40 on other immune and non-immune cells. To target CD40 on DCs specifically, bivalent antibodies or MVA vaccines that can induce CD40 on DCs should be developed. Targeting CD40 directly (albeit not always on DCs) has already been used successfully in other disease models. A recent study in mice demonstrated that using a CD40 agonist which is able to target conventional DCs induces antitumor responses that are important for protection in bladder cancer[313]. Other CD40 and CD40L agonists have also shown promise in increases protective immunity to various cancers[314]. Collectively, our data supports targeting CD40 as it is able to induce functions which we have demonstrated are critical for augmenting T cell responses during infection.

To conclude, this dissertation addresses critical gaps in knowledge in the TB field. We demonstrated that DLL4 is downstream of CD40 signaling on DCs and is necessary for polarizing Th_{17} and multifunctional T cells *in vitro* and *in vivo* and have elucidated the mechanism by which Mtb impedes these responses. We have also dissected the DC pathways which contribute to optimal DC-T cell crosstalk and polarization of functional T cell responses during infection. While the mechanistic insights generated here can be harnessed to develop efficacious TB vaccines and adjuvants, this work also highlights the importance of characterizing other Mtb-immune cell interactions in order to gain a comprehensive understanding of TB immunity.

Chapter V

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Appendix

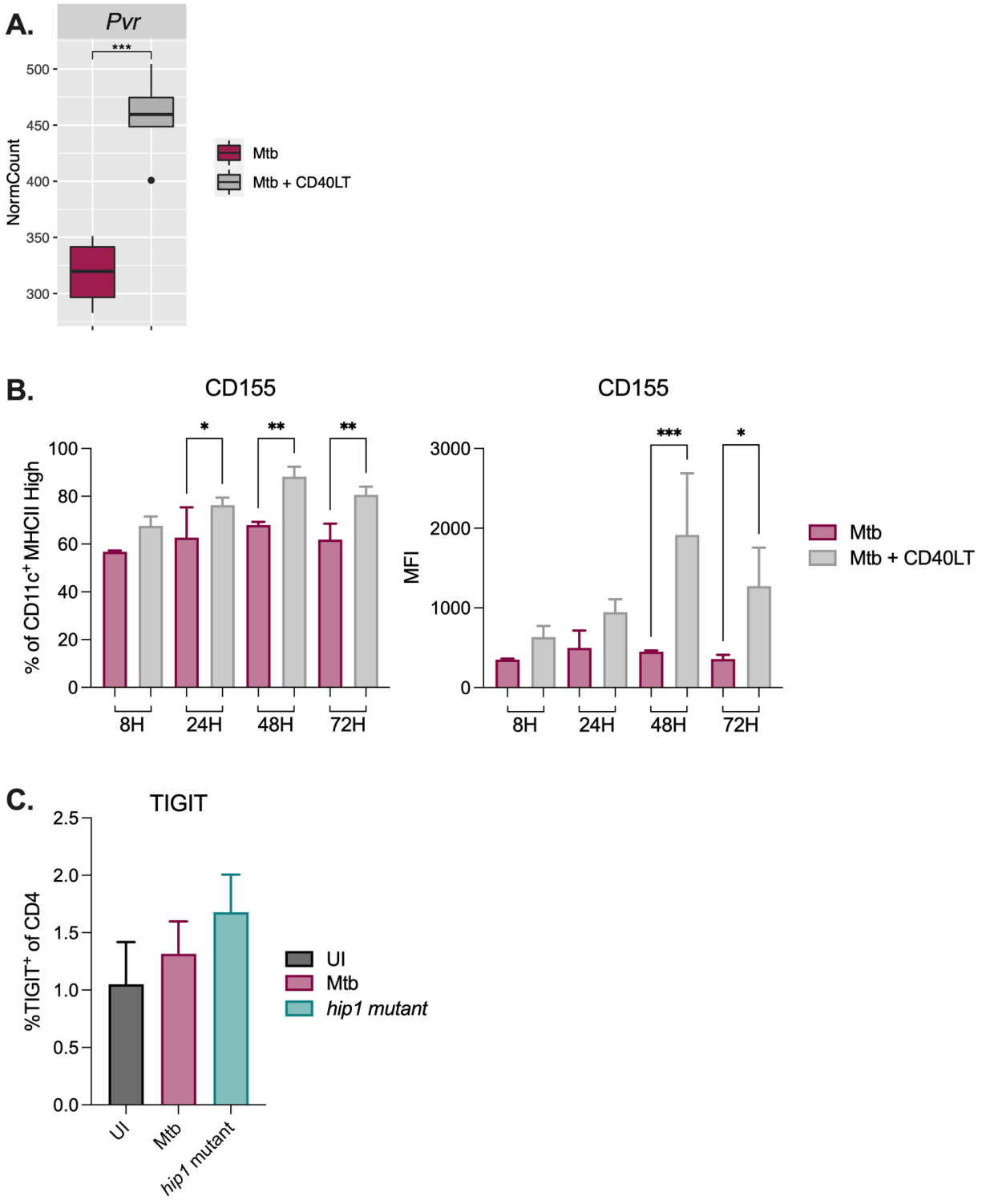
A.
Pvr
***
NormCount
500
450
400
350
300
Mtb
Mtb + CD40LT
B.
CD155
% of CD11c+ MHCII High
100
80
60
40
20
0
*
**
**
8H
24H
48H
72H
CD155
MFI
3000
2000
1000
0
***
*
8H
24H
48H
72H
Mtb
Mtb + CD40LT
C.
TIGIT
%TIGIT+ of CD4
2.5
2.0
1.5
1.0
0.5
0.0
UI
Mtb
hip1 mutant
UI
Mtb
hip1 mutant

Figure 1: Engaging CD40 on Mtb-infected DCs significantly increases expression of CD155. A) BMDCs were infected with either no bacteria (UI), Mtb (H37Rv, MOI 1), or Mtb and multimeric CD40L reagent (CD40LT, 1 μg/ml) for up to 24H. At 0H, 8H, and 24H, UI and infected cells were harvested, lysed, and RNA generated for submission for bulk RNA sequencing. Normalized counts plotted for *Pvr* (PVR; CD155) for the 8H timepoint is represented. B) BMDCs were infected with Mtb (H37Rv, MOI 1), or Mtb and multimeric CD40L reagent (CD40LT, 1 µg/ml) for up to 48H. Following this time, cells were harvested and surface expression of co-stimulatory markers was measured using flow cytometry. Frequency and MFI of CD155 is presented. Population shown was pre-gated on singlets, live cells, and $CD11c^+$ MHC-II High populations. C) B6 mice were infected via the aerosol route with a low-dose of wild type Mtb or *hip1* mutant. Following 2 weeks post-infection, mice were euthanized and lung *ex vivo* responses were measured using flow cytometry. Frequency of TIGIT is shown. T cell data were pre-gated on singlets, live cells, and $CD3^+$ cells with data plotted from CD4 T cells. Data were analyzed in A) unpaired Student's t-test B) using a one-way (right) and two-way (left) ANOVA with a correction for multiple comparisons and C) using a one-way ANOVA with a correction for multiple comparisons. Data are presented as mean ± SD. Data are representative of 2 independent experiments. Statistical significance p-value key is the following: * = ≤ 0.05, ** = ≤ 0.01, *** = ≤ 0.001.

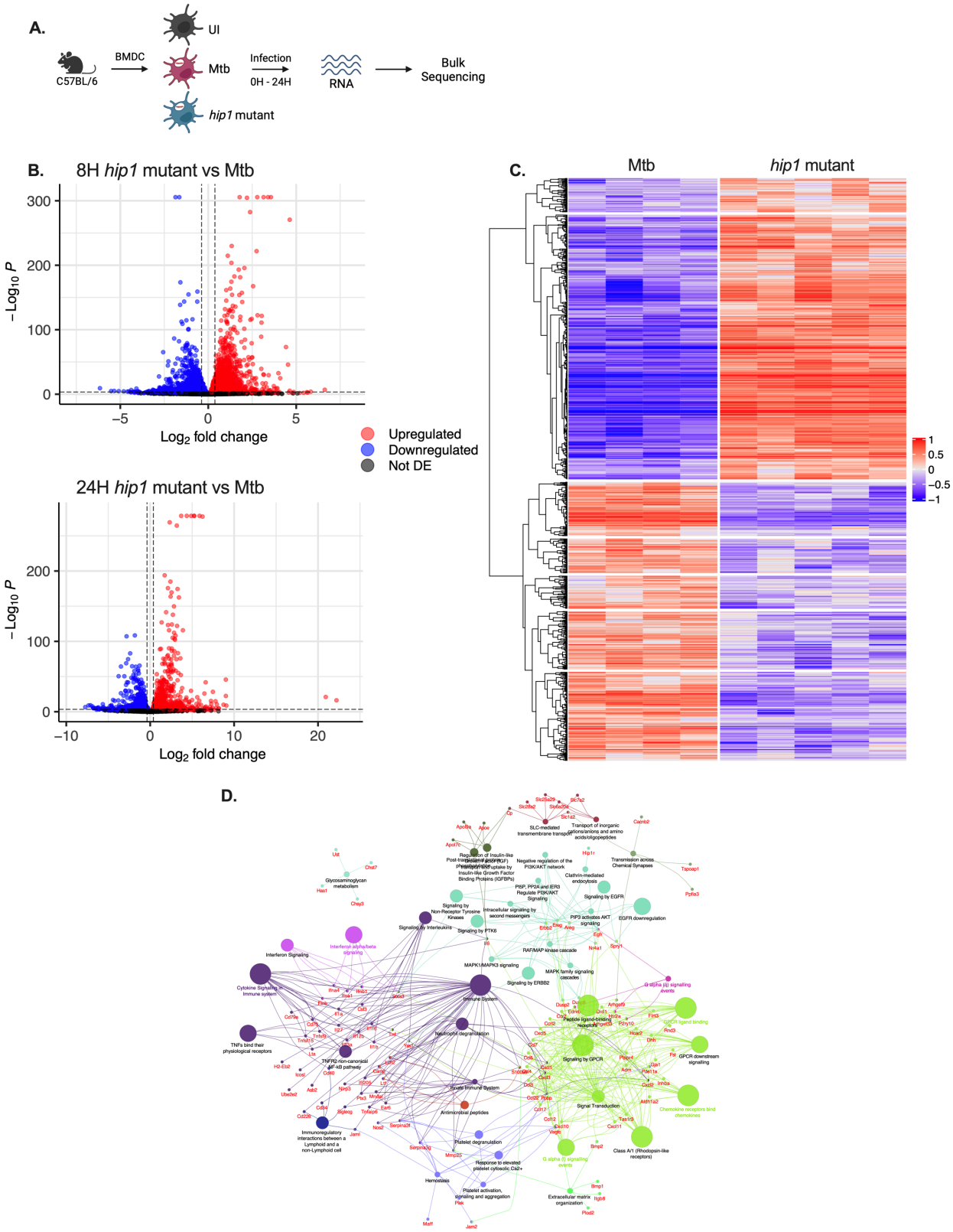
A.
UI
Mtb
hip1 mutant
C57BL/6
BMDC
Infection
0H - 24H
RNA
Bulk
Sequencing
B.
8H hip1 mutant vs Mtb
−Log10 P
Log2 fold change
Upregulated
Downregulated
Not DE
24H hip1 mutant vs Mtb
C.
Mtb
hip1 mutant
1
0.5
0
−0.5
−1
D.

Figure 2: Bulk RNA seq reveals infection with *hip1* mutant induces broad changes in gene expression in DCs during infection. C57BL/6 (B6) BMDCs were infected with either no bacteria (UI), wild type Mtb (H37Rv, MOI 1), or *hip1* mutant for up to 24H. At 8H, and 24H, infected cells were harvested, lysed, and RNA generated for submission for bulk RNA sequencing. A) Experimental schema. B) Volcano plot representing differentially expressed genes 8H *hip1*/8H Mtb or 24H *hip1*/24H Mtb. C) Heat map of top differentially expressed genes (p value <0.01); scale was generated from the log2 foldchange from mean 8H hip1/Mtb. D) Cytoscape ClueGO + CluePedia analysis of significantly upregulated genes in *hip1* compared to Mtb at the 8H time point. The experimental schema was made with BioRender.com. Not DE = not differentially expressed.

Advancing Adjuvants for *Mycobacterium tuberculosis* Therapeutics

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Contribution(s):

I contributed to the "immune evasion mechanisms" question. Moreover, I was responsible for re-writing significant portions of the manuscript in order to address reviewer recommendations.

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REVIEW
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Advancing Adjuvants for *Mycobacterium tuberculosis* Therapeutics

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Tuberculosis (TB) remains one of the leading causes of death worldwide due to a single infectious disease agent. BCG, the only licensed vaccine against TB, offers limited protection against pulmonary disease in children and adults. TB vaccine research has recently been reinvigorated by new data suggesting alternative administration of BCG induces protection and a subunit/adjuvant vaccine that provides close to 50% protection. These results demonstrate the need for generating adjuvants in order to develop the next generation of TB vaccines. However, development of TB-targeted adjuvants is lacking. To help meet this need, NIAID convened a workshop in 2020 titled "Advancing Vaccine Adjuvants for *Mycobacterium tuberculosis* Therapeutics". In this review, we present the four areas identified in the workshop as necessary for advancing TB adjuvants: 1) correlates of protective immunity, 2) targeting specific immune cells, 3) immune evasion mechanisms, and 4) animal models. We will discuss each of these four areas in detail and summarize what is known and what we can advance on in order to help develop more efficacious TB vaccines.

Keywords: tuberculosis, vaccines, adjuvants, M72, BCG, TLR, Th17, CD4+ T cells

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INTRODUCTION

Mycobacterium tuberculosis (Mtb) is the causative agent of tuberculosis (TB), an infectious disease that led to the death of 1.4 million individuals in 2019 (1). The current licensed vaccine against TB, a live attenuated strain of *Mycobacterium bovis* known as Bacillus Calmette-Guérin (BCG), is able to provide protection against disseminated forms of disease but is ineffective at providing protection

against pulmonary TB in children and adults. Therefore, in order to lessen TB burden worldwide, a more efficacious vaccine and improved vaccine delivery strategies are urgently needed.

The development of more robust TB vaccines has unique requirements that have made progress challenging. Vaccines traditionally provide durable protection by prophylactically inducing neutralizing antibodies that can serve as a first line of defense against pathogens (2). Although numerous pathogens require antibodies for protection, this is less clear in the case of TB. While research on the role of antibodies in TB is ongoing and is of interest due to new data (3), no evidence has yet to suggest that neutralizing antibodies are required for protection against Mtb. Therefore, TB vaccine research has focused on identifying antigens and delivery strategies that maximize the generation of T cell responses. In particular, research has shown that $CD4^+$ T cells are a critical component of protective immunity. Mouse studies, however, have demonstrated that T cells are not recruited to the lungs until weeks after infection is established (4). Mtb is able to suppress T cell recruitment and responses by utilizing several immune evasion mechanisms (IEM) to impede antigen-presenting cell (APC) function, thereby dampening adaptive responses. Researchers have attempted to bypass the delayed T cell response by targeting specific APCs such as dendritic cells (DCs), but there is limited evidence that this can preclude the immunosuppressive effects of Mtb upon challenge (5, 6).

Despite these challenges, recent developments have reinvigorated interest in TB vaccine research. A study in humans found that BCG revaccinated adults have increased protection compared to control groups (7). Moreover, while BCG is administered through the intradermal route, a recent study found that administering BCG intravenously can induce robust T cell responses and afford non-human primates (NHPs) protection against Mtb challenge (8, 9). Recent clinical trials of novel vaccine candidates have also yielded promising results. The Phase IIB clinical trial results of the $M72/AS01_E$ subunit adjuvanted vaccine demonstrated 49.7% protection against Mtb (10). M72 is a recombinant fusion protein consisting of antigens Mtb32A and Mtb39A and $AS01_E$ is an adjuvant that combines monophosphoryl lipid (MPL) with QS-21 (a purified saponin fraction). The AS01 adjuvant system success extends to other infectious disease vaccines as it is used in the FDA approved shingles vaccine "SHINGRIX" and is currently in clinical development for use in malaria (11). These successes set an important threshold for the clinical development of future TB vaccines.

To develop effective antigen-specific T cell responses, host immunity requires non-specific innate cell activation. In the case of a subunit vaccine, priming is accomplished with adjuvants. Modern adjuvants prime host immunity through binding of receptors that recognize pathogen-associated and damage-associated molecular patterns, such as toll like receptors (TLRs), C-type lectin receptors (CLRs), and NOD like receptor (NLRs) on the surface of APCs. Adjuvants are able to activate these receptors and induce downstream signaling pathways such as NFκB signaling, which in turn enables the activation of adaptive immune responses. Given the expense and time-consuming nature of vaccine development, adjuvant compounds and formulations that have been shown to be safe and effective are often repurposed for testing novel vaccine candidates. The majority of current TB vaccine candidates contain adjuvants (**Table 1**). However, a TB-specific adjuvant that is able to induce strong immune responses in the lung but minimize corresponding tissue damage is required. Adjuvanted vaccines delivered directly to the upper or lower respiratory tract may have increased efficacy compared with parenterally administration against respiratory pathogens, such as Mtb. Therefore, development of new adjuvants with defined modes of action will be necessary in order to generate improved vaccination strategies that can provide protective immunity to TB.

To address the needs of TB vaccine development and lack of TB-targeted adjuvants, the National Institute of Allergy and Infectious Diseases (NIAID) held a workshop in July 2020 titled "Advancing Vaccine Adjuvants for *Mycobacterium tuberculosis* Therapeutics." This workshop brought together vaccine and adjuvant developers from industry and academia and researchers in fields beyond TB. It is here that we identified research in four areas that will be essential for development of optimal adjuvants for TB vaccines: 1) correlates of protective immunity, 2) targeting specific immune cells, 3) immune evasion mechanisms, and 4) animal models. In this review article, we discuss these four areas in detail and highlight priority areas which the broader TB research community can address in order to develop efficacious adjuvants and vaccination strategies.

TABLE 1 | Adjuvants Used in Recent TB Vaccine Candidates.

Adjuvant/Vaccine	Antigen	Adjuvant target	Immune response	Ref
IC31	H56, H1	TLR-9, endocytosis	Th_1	(12–14)
$AS01_E$	M72	TLR-4, lysosomal disruption	B, Th_1, Th_2, NK, CTL, DC	(10, 15–20)
GLA-SE	ID93	TLR-4	Th_1	(21–25)
BCG	Whole cell		Trained innate immunity, Th_1	(8, 26–31)
MVA85A	Ag85A		Th_1	(32)
CAF01	H1	Mincle	Th_1, Th_{17}	(33–36)
Advax-CpG	CysVac2	TLR-9	Th_{17}	(33, 37, 38)
Lipokel		TLR-2	DC	(39)

Table lists of adjuvant-containing TB vaccine candidates that have recently been tested clinically in humans and pre-clinically in animal models. Known adjuvant targets are presented though other mechanisms of action may also be employed. Abbreviations: AS01, adjuvant system; GLA-SE, Glucopyranosyl Lipid A-stable oil-in-water nano-emulsion; BCG, Bacillus Calmette-Guèrin; MVA85A, Modified vaccinia Ankara expressing antigen 85A; CAF, Cationic Adjuvant Formulation; TLR, toll-like receptor, Th, T-helper cell; NK, natural killer cell; CTL, cytotoxic T lymphocyte; DC, dendritic cell.

CORRELATES OF PROTECTIVE IMMUNITY

Polarisation of CD4$^+$ T Cells

Targeting specific immune cells through passive or active vaccine modalities requires understanding the basic mechanisms of protective immunity to Mtb. Although thorough understanding of protective immunity to Mtb remains incomplete, research has shown that conventional T cells (particularly CD4$^+$ T-helper [Th] cells) play a vital role in protection (40). Early mouse studies and clinical data demonstrated that functional Th_1 CD4$^+$ T cells are crucial for protection against Mtb (41–45). Th_1 immunity is characterised by the secretion of IFN-γ, which aids in microbial clearance by enhancing processes such phagocytosis and secretion of reactive oxygen species in macrophages (46). While these responses have long been a major focus of TB vaccine design, studies have shown that there is a requirement for broader immunity as Th_1 responses alone are insufficient for protection (47). The MVA85A vaccine trial in humans demonstrated that despite robust induction of 'multifunctional' CD4$^+$ T cells (producing IFN-γ, TNF and IL-2 cytokines), these immune responses did not translate to additional protection from TB compared to placebo vaccination (32). Some studies even found that excessive Th_1 polarisation may hinder effective memory responses by producing terminally differentiated T cells that are unable to effectively migrate into the lung parenchyma during Mtb infection (48, 49). More recent studies demonstrated that IL-17 and Th_{17} responses, in addition to Th_1 responses, are necessary for protective immunity to Mtb (50, 51). Th_{17} cells have the capacity to differentiate into resident memory T cells while IL-17A is a key cytokine required for protection in several pre-clinical models of candidate TB vaccines (33, 34, 37). Th_{17} cells can also secrete additional cytokines that direct recruitment of neutrophils and IFN-γ-producing protective memory CD4$^+$ Th_1 cells during Mtb infection (52, 53). Other studies have also found that CCR6$^+$CXCR3$^+$ Th_1/Th_{17} cells responses were present in latently-infected individuals (compared to active infection) and were important for protection in an NHP model of TB (54, 55). But while Th_{17} responses are beneficial, excessive Th_{17} responses are detrimental to the host (56, 57). Therefore, adjuvants for TB vaccines should strive to induce early and balanced Th_1/Th_{17} as these are more likely to be necessary for protection.

Other Lymphocytes

While there is evidence that CD8$^+$ T cells contribute to protection against Mtb, their role remains debated due to variable findings and inherent differences in human and NHP immune function compared to mice. CD8$^+$ T cells can produce cytokines such as TNF, IFN-γ and IL-2 and produce cytolytic granzymes, a feature not shared by CD4$^+$ T cells. Of these key cytolytic granzymes is granulysin, which is expressed in human but not murine CD8$^+$ T cells, and is capable of direct mycobacterial killing (58). CD8$^+$ depletion studies in animal models, however, agree on the role of these T cells in protection. In NHPs, CD8$^+$ depletion reduces the protective efficacy of BCG vaccination and infection-induced immunity (59). CD8$^+$ knockout mice are also unable to contain Mtb infection, particularly at extended timepoints, suggesting a role for CD8$^+$ T cells in protection during chronic stages of infection (60).

Similarly, the function of B cells and humoral immunity in protection against TB has become of particular interest as broader immune parameters are investigated (61). Recent studies indicate the presence of antibodies in humans that are protective against Mtb infection (3, 62). The use of alternative vaccination routes, such as mucosal or intravenous, lead to the generation of pulmonary IgA and antibody-producing lymphoid follicles (iBALT), which has been associated with reduced bacterial burden (8, 26, 33, 37). The generation of inducible lymphoid structures is crucial as they can harbour cells such as CXCR5$^+$ CD4$^+$ T cells which were found to correlate with a better prognosis of TB disease (63, 64).

While not previously a focus of TB vaccine candidates, CD8$^+$ T cells and B cells are often measured as a readout of adjuvant function and may be the contributing factors to vaccine-induced protective immunity (6). The mechanism of the AS01 adjuvant, as determined by pre-clinical animal studies, is thought to be due in part to early IFN-γ production by NK and CD8$^+$ T cells (15). In humans, however, a review of TB vaccine candidates tested in the clinic revealed that CD8$^+$ T cell responses were relatively poor when compared to CD4$^+$ T cells responses (65). Until vaccine efficacy studies in humans become more available, the contributions of B and CD8$^+$ T cells cannot be discounted and should be considered in adjuvant development.

Lung-Localised Immunity

Research has suggested that generating immune responses at the site of infection is crucial for protective immunity. As Mtb is spread *via* the aerosol route, it can use lung-specific cell types to its advantage. The microfold cells found in the nasal associated lymphoid tissue (NALT) and iBALT have been identified as the entry site of the bacterium and granulomas can serve as a niche for the persistence of Mtb (66–68). The need for lung-localized immunity is supported by the fact that local immune cells can respond quickly to infection. The generation of T resident memory (T_{RM}) cells is a correlate of protection against Mtb and is an active target in mucosal vaccination strategies (33, 37, 69). Mucosal adoptive transfer of T_{RM} cells from BCG-vaccinated mice into naïve mice revealed that both CD8$^+$ and CD4$^+$ T_{RM} subsets could afford partial protection against Mtb infection (26). Furthermore, both CD4$^+$ and CD8$^+$ human T_{RM} cells have been characterised as capable of limiting intracellular Mtb survival *ex vivo* (70). A recent study using human samples also found that the frequency of Mtb-specific T_{RM}-like cells that produce IL-17 in the lungs negatively correlated with IL-1ß levels in the blood, suggesting an important role for controlling Mtb growth (71). Additionally, mucosal vaccination could help activate other resident cells, such as γδ-T cells and mucosal-associated invariant T cells (MAIT), that also produce IL-17A (53). While the generation of lung-localized immunity appears to be a significant correlate of protection against Mtb, the challenge remains to validate the safety and efficacy of novel

administration methods that generate lung-local immune memory in clinical trials.

Other Correlates of Protection

Interest in trained immunity as a potential correlate of protection has been driven by the hypothesis that it may be responsible for some of the protective characteristics of BCG. This is supported by studies demonstrating that BCG is able to provide protection against multiple respiratory diseases in addition to TB (27), and the observation that some individuals are capable of early clearance of Mtb without requiring an adaptive immune response (72). While there are multiple vaccine candidates that have been reported to be capable of stimulating systemic innate immune responses, there is emphasis on the generation of lung-resident trained immunity which has been observed after pulmonary and intravenous vaccine administration (6, 28).

Certain cytokines, in addition to those secreted by T cells, also play critical roles in protection. In particular, IL-23 expression was found to be essential for IL-17A-mediated responses against Mtb. Upon aerosol infection with Mtb, naïve murine lungs showed increased expression of IL-17A, which was ablated in the absence of IL-23 (73). IL-23, and to a lesser extent IL-17A and IL-22, is able to lead to CXCL13 production and generation of lymphoid follicles (63). Other studies reported that unvaccinated IL-23-deficient mice are still able to control mycobacterial growth in a fashion similar to wild type animals after exposure to Mtb and BCG as local IFN-γ responses are able to compensate for the loss of IL-17A (73–75). IL-23 plays a compensatory role in the absence of IL-12p70, a key Th_1 polarising factor, as the addition of IL-23 can also enhance protective immunity against Mtb in the absence of a functional Th_1 immune response (73, 76). Thus, there is evidence that IL-23 plays a significant role in protective responses which will be important for vaccine-induced immunity.

Clinical Studies

Insights from clinical trials can help address the knowledge gaps of efficacious TB vaccine design by helping identify correlates of protection. A small number of TB vaccines are currently undergoing clinical trials, including three vaccines that incorporate adjuvants. The most advanced of these TB vaccines is the M72/$AS01_E$ construct. The MPL components of the adjuvant, a derivative of the lipopolysaccharide from *Salmonella minnesota*, is commonly used in adjuvant formulations due to its ability to engage TLR4, activate NF-kB, and induce pro-inflammatory cytokines (16, 17). QS-21 can cause lysosomal disruption and consequent Syk activation, as well as NLRP3 inflammasome activation, which is thought to enhance cross-presentation with $CD8^+$ T cells and promote inflammatory cytokine production (18, 19). Preclinical studies suggest that the Th_1 polarising effects of AS01 are the result of MPL and QS-21 synergy. At early timepoints post-vaccination, it was observed that subcapsular sinus macrophages (SSM) in the draining lymph nodes promoted early IFN-γ production by resident NK cells and $CD8^+$ T cells in a process mediated by IL-18 (15). In humans, the peripheral immune response has been examined by analysing blood RNA expression and antigen-specific PBMC profiles during a two dose M72/$AS01_E$ regimen (77). PBMC restimulation showed that the vaccine induced $CD4^+$ T cells and multifunctional T cells after stimulation, though IL-17A was not detected. RNA analysis identified the upregulation of blood transcription modules associated with IFN signalling, innate activation including TLR and inflammatory signalling, as well as modules related to various chemotactic and cell adhesion processes.

The cationic peptide adjuvant IC31 is a component of two TB vaccine candidates undergoing clinical trials, H4:IC31 and H56:IC31 (7, 12). In a Phase IIB trial, which tested the efficacy of H4:1C31 and BCG revaccination, H4:IC31 did not demonstrate significant protection (30.5% efficacy) against either initial or sustained Mtb infection (7). However, BCG revaccination led to a 45.4% reduction in sustained infection (7). IC31 is made up of the antimicrobial peptide $KLKL_5KLK$ (KLK) combined with ODN1a, a TLR9 binding single stranded oligodeoxynucleotide (ODN) that activates the MyD88 pathway (13). The cationic peptide component is also an immunostimulant, hypothesised to enhance intracellular TLR access of ODN1a *via* stimulating endocytosis (14). H56:IC31 induces antigen-specific IgG responses and Th_1 cytokine expressing $CD4^+$ T cells (12). Low-dose vaccine administration induced more polyfunctional memory T cells than high dose vaccination, an observation in line with pre-clinical studies that identified lower antigen dose as conducive to more protective immune responses (78).

Comparative analysis of human immune responses to six TB vaccine candidates observed that a shared feature of the systemic immune responses induced by TB vaccine candidates was the enhanced production of IFN-γ expressing $CD4^+$ T cells, with M72/$AS01_E$ inducing the greatest response (65). Furthermore, little to no IL-17A expression was induced by the candidate vaccines. This study did not include analysis of CAF01, a cationic liposomal formulation consisting of DDA liposomes and the Mincle agonist trehalose-6,6-dibehenate (TDB), which is known to initiate a Th_{17} response when administered parenterally in mice (79). Vaccination of humans with H1-CAF01, however, induced strong antigen-specific Th_1 responses while IL-17 responses were low and were not significantly increased (35). This may reflect a requirement to examine mucosal immune responses in humans to better reflect vaccine immunogenicity, as was performed with the CTH522/CAF01 chlamydia vaccine candidate (80). Overall, there was a lack of diversity in T cell responses generated by the different TB vaccine candidates, reinforcing the argument for a requirement to develop and test more novel adjuvants that induce distinct immune responses.

Adjuvating Strategies for Inducing $CD4^+$ T Cells

Differential receptor activation, with adjuvants, have been demonstrated to be important for dictating specific T cell responses. Th_1 differentiation *via* TLR activation and downstream IL-12 secretion is believed to play a primary role in the protective efficacy of TLR4-targeting adjuvants such as AS01 (15). Similarly, the major driver of immunogenicity of the vaccine candidate ID93 + GLA-SE vaccine is the synthetic TLR4 agonist, GLA; its delivery in a squalene emulsion is also necessary

for adjuvanticity (21). The Th_1 polarising properties of GLA-SE is MyD88- and TRIF- dependent, and type I and II IFN expression is also critical for the adjuvant mode of action (21, 22). Furthermore, IL-18 and Caspase1/11 expression is required for T cell activation by GLA-SE, but not the NLRP3 inflammasome (23). In humans, ID93 + GLA-SE vaccination results in the generation of multi-cytokine producing T cells (TNF, IFN-γ and IL-2), with little IL-17A detected, and IgG1 and IgG3 antibody production (24, 25). Thus, both MPL adjuvant (in AS01) and GLA (in a squalene emulsion) have demonstrated the ability to enhance T cell immunity to Mtb in combination with different antigens, mainly through TLR4-mediated Th_1 polarisation.

Some adjuvants are innately capable of stimulating Th_{17} polarisation, often by activating non-TLR pattern recognition receptors such as Mincle (81). It is known that some TLR4 and TLR7/8 agonists can induce IL-23 expression, and Mincle-activating adjuvants such as TDB, used in the cationic liposome formulation CAF01, can also shift the balance towards IL-17A producing T cells (36). It is thought that TDB, a derivative of the mycobacterial cord factor (TDM), is the major contributor to the Th_{17} polarisation in this vaccine formulation. Mincle activation, as well as MyD88 and the inflammasome component ASC, have all been identified as a requisite for the Th_{17} generating characteristics of TDB (79, 82, 83). Thus, there are novel adjuvant strategies focused on generating synthetic aryl-trehalose derivatives that afford the best Th_1 and Th_{17} polarisation (84, 85). Similarly, it has been observed that adjuvants such as cyclic dinucleotides and chitosan that activate the cGAS-STING (cyclic GMP-AMP synthase-stimulator of interferon genes) pathway also stimulate Th_1 and Th_{17} responses (86–88). As mentioned above, mucosal delivery is another effective strategy for the generation of Th_{17} responses, with many vaccines displaying enhanced Th_{17} polarisation upon mucosal vaccination that was not observed with parenteral administration. Evaluation of these new Th_{17}-inducing adjuvants alone and in combination with other established adjuvant systems in relevant animal models is a critical next step in the advancement of improved vaccination strategies for Mtb.

In summary, data supports the development of adjuvants and vaccines that elicit both local (tissue resident) and systemic antigen-specific Th_1 and Th_{17} cells as these responses have been demonstrated to be critical for protection. However, research should continue to elucidate correlates of protection to identify new pathways that can be targeted by adjuvants to induce protective immunity against Mtb.

TARGETING SPECIFIC IMMUNE CELLS

Active or passive targeting of specific immune cells through vaccination is an important step in the development of a safe and effective vaccine for TB. Mtb is primarily transmitted *via* inhalation and establishes infection in the lung through phagocytic uptake of the bacilli by tissue-resident macrophages. The initial recognition and immune activation by innate immune subsets sets the stage for either clearance or persistent containment within a granuloma or active disease through suppression of the immune system (89). Cells involved in lung-specific innate and adaptive immune responses are important frontline targets for Mtb vaccination approaches. The majority of Mtb vaccines are delivered *via* the intramuscular (such as $M72/AS01_E$) or intradermal (BCG) route, creating additional challenges in the recruitment of tissue resident memory cells to the site of initial infection. Targeting vaccines to specific cell types could help to overcome some of the shortfalls of current vaccine approaches while improving both safety and efficacy. Several groups have worked to overcome these challenges through targeting specific immune cells *via* passive (adjuvants, delivery systems) or active (mucosal vaccination, prime-pull, receptor targeting) immunization strategies with great success in pre-clinical and early clinical investigations. Coordination of the innate and adaptive immune response is important for resolution of Mtb infection and targeting of specific immune cells that orchestrate this response at the site of infection is critical in the development of an effective vaccine.

The context in which antigens are presented to the immune system controls the immunological outcome of antigenic exposure (90). The innate immune system uses pathogen recognition receptors (PRRs) to decode the nature of the antigen (*e.g.*, viral, bacterial, fungal) and to translate this into an appropriate adaptive immune response. Central to the idea of cell-targeting is activation of the innate immune response by pathogen associated molecular patterns (PAMPs) and the specific cellular targets they encounter are crucial for early control of infection and for the subsequent development of protective long-term adaptive immunity. The use of passive and active cell targeting strategies opens the door towards the rational design of vaccines for Mtb that could lead to more durable and protective mediated immune responses.

Cell Targeting *via* PRR Expression and Adjuvant Use

To drive the desired adaptive immune response, the innate immune response must be properly activated to provide the correct signals for differentiation of antigen-specific T and B cells. Different adjuvants activate specific cell types as a result of differential PRR expression. The directed, rational use of adjuvants is therefore one way in which specific innate immune cells can be targeted in the context of Mtb vaccination. Many adjuvants being explored pre-clinically or in early clinical trials for Mtb vaccines target and activate DCs. In the context of Mtb, several studies have shown that directly targeting DCs can lead to protective immune responses during infection (5, 91–93). As previously discussed, MPL of AS01 activates TLR4 (15). DCs express high levels of TLR4 and are highly responsive to MPL, driving downstream antigen-specific T cell differentiation (11, 20). Both myeloid DCs (mDCs) from blood and monocyte-derived DCs (moDCs) express TLR4 and are able to upregulate co-stimulatory markers and secrete Th_1 polarising cytokines (in particular IL-12p70) after TLR4

stimulation (94). DCs also express high levels of TLR2 (94). Adjuvants that engage TLR2 on DCs have the potential to augment early production of pro-inflammatory cytokines such as IL-6, IL-1β, TNF-α and IL-12 that are necessary for generating balanced Mtb-specific Th_1/Th_{17} responses (95, 96). This is demonstrated by studies using Lipokel, an adjuvant which stimulates TLR2 through binding of the ligand Pam2Cys. A protein-Lipokel vaccine conjugate was able to reduce Mtb CFU in the lungs of mice and increase the frequency of DCs in lymph nodes following vaccination (39). PRR-targeting adjuvants, including some TLR agonists, can also preferentially target and activate monocytes and macrophages in addition to DCs. Monocytes and macrophages are specialized phagocytic cells that are capable of interacting with and activating antigen-specific T cells (97). TLR2, TLR4, and TLR8 are highly expressed on monocytes (98). In addition to activating DCs, $AS01_E$ was also able to activate SSM innate immune cells (15). The ability of $AS01_E$ to target both DCs and monocytes/macrophages concurrently may contribute to success as an adjuvant in vaccine strategies for a variety of pathogens, including Mtb. Additionally, non-TLR PRR agonists, such as Mincle or Dectin-1 ligands, preferentially target monocytes and macrophages due to their high Mincle expression (99). CAF01 has demonstrated efficacy as an Mtb vaccine adjuvant in pre-clinical mouse models and induces a strong Th_{17} polarised immune response in mice (36). Interestingly, when used as an adjuvant in human clinical trials, CAF01 elicited an antigen-specific Th_1 response instead of a Th_{17} response (35). TDB, the main component of CAF01 believed to be responsible for Th17 polarisation, does not appear to be a particularly potent human Mincle agonist relative to its potency in mice, therefore it is possible that more potent human Mincle agonists may be required to elicit antigen-specific Th_{17} immunity (84). Given the complex nature of various PRRs and their cellular targets in natural Mtb infection and resolution, it is likely that a combination adjuvant and delivery system approach (similar to AS01) and innovative antigen design may be necessary to improve vaccine efficacy. Promising approaches include combining CLR/TLR adjuvants (presented at this workshop) and other novel antigen and adjuvant combination approaches, such as $CysVac2/Advax^{CpG}$, have shown promise in pre-clinical models (38).

Cell Targeting *via* Mucosal Administration

Most vaccines are injected into muscle although BCG is given as an intradermal injection. However, mucosal immune subsets that have the ability to quickly transport antigen to stimulate an immune response may be important targets for mucosal Mtb vaccines (66, 100). T_{RM} can be induced by mucosal vaccine administration (9, 26, 101, 102). Following mucosal vaccine administration or natural infection, lung resident T cells acquire a polyfunctional phenotype and are more likely to reside in the airway lumen and lungs, where they can rapidly respond to Mtb (40, 103–106). These lung-resident T cells are not typically generated as a result of parenteral vaccination, and it seems likely that mucosal or lung-resident innate cells are crucial for the development and differentiation of Mtb-specific lung-resident T cells. These important innate immune effector cells can be uniquely targeted *via* intranasal and/or intrapulmonary vaccination. Several groups have explored a "prime-pull" strategy which takes advantage of both parenteral vaccine (prime) and mucosal vaccine boost (pull) to direct antigen-specific T_{RM} to the lungs (107, 108).

There are a small number of mucosal vaccines currently in clinical use and all but one (FluMist, intranasal) are delivered orally (109, 110). However, there are many preclinical adjuvants that have been tested for either intranasal or intrapulmonary administration, with a particular bias towards intranasal administration due to improved safety profile compared to intrapulmonary administration and lower requirement for specialised equipment (111, 112). Particulate adjuvants such as carbohydrate or PLGA have also been used extensively for mucosal vaccination, often chosen for their mucoadhesive properties (113). Naturally-derived carbohydrate particles such as delta inulin (Advax), chitosan, and *Bacillus subtilis* spores have all been utilised as mucosal adjuvants in TB vaccine candidates, all producing a Th_1/Th_{17} phenotype alongside increased pulmonary IgA (37, 114–116). CAF01 has been tested intranasally and has also been spray-dried for intrapulmonary administration (107). Th_{17} immunity is often achieved *via* mode of administration, as it has been observed across multiple vaccine platforms that mucosal vaccination, particularly intranasal, favours Th_{17} differentiation (117, 118). Many adjuvants have the capacity to be Th_1 or Th_2 polarising when administered parenterally but promote Th_{17} differentiation when delivered mucosally (37, 119, 120). Some TB vaccines, such as BCG or the CysVac2/Advax candidate, have been observed to be more protective after intranasal or intrapulmonary administration, a quality attributed to local IL-17 production and the establishment of T_{RM} (37, 119). This was observed when the clinical candidate vaccine ID93 + GLA-SE was administered through a different route; parenteral administration induces a Th_1 responses but intranasal administration induces a Th_{17} responses (121).

Receptor-Mediated Active Cell Targeting

DCs and macrophages are a primary focus of vaccine design due to their role as APCs and critical function in orchestrating long-term cell-mediated immunity. The tissue heterogeneity in both DC and macrophage populations creates challenges for both passive and active targeting of the various systemic and tissue-specific APC subsets. Active targeting to specific APC subsets through endocytic receptors is a promising approach to improve vaccine efficacy and reduce unintended effects (122). Such receptors include DEC-205, Clec9A, Clec12A, and DC-SIGN, among others (122–126). Early work demonstrating active targeting by DEC-205, a cell surface receptor involved in the uptake of dying cells and cross-presentation of antigens, led to the evaluation of an anti-DEC-205-Ag85B vaccine conjugate for Mtb (125). While strong Ag85B-specific humoral immunity was noted following vaccination, cell-mediated immunity was lacking without BCG priming of vaccinated mice and the vaccine failed to improve protection from Mtb challenge (125). A similar approach was used to target DC-SIGN; anti-DC-SIGN

antibodies conjugated to Ag85B were used to vaccinate an hSIGN transgenic mouse (human DC-SIGN under control of the murine CD11c promotor) in combination with various adjuvants. This innovative vaccine design induced strong antigen-specific $CD4^+$ T cell responses. However, similar to the DEC-205 approach, enhanced protection from Mtb challenge was not achieved (126). Improved humoral and/or cell-mediated immunity to Ag85B was measured using both endocytic receptor APC targeting strategies demonstrating proof-of-concept for improving immunity using this approach. Additional research efforts are required to identify the appropriate combination of antigen(s), targeting mechanisms, and adjuvants to drive durable immunity and protection in Mtb animal challenge models.

Targeting Cell Specific Responses Through Trained Immunity

In the previous section, we briefly discussed the potential of trained immunity to serve as a correlate of protection. The contribution of trained immunity to vaccine-mediated protection against Mtb is currently being investigated (127, 128). PRR agonists, among other factors, can drive functional and epigenetic reprogramming in innate immune cells in order to increase Th-polarising cytokines and phagocytic and cytotoxic killing capacity. BCG, when administered intravenously, provides both short and long-term protection from subsequent Mtb challenge in NHPs (8, 28–31). A recently discovered mechanism of protection by intravenous BCG is the generation of trained immunity in hematopoietic stem cells (HSCs). Following intravenous administration, BCG is able to enter the bone marrow (BM) where it can be detected for up to 7 months after vaccination in mice (which is not observed in subcutaneous vaccination) (28). Once in the BM, BCG promotes myelopoiesis and induces trained monocytes and macrophages (28). These BM monocytes have a particular transcriptional and epigenetic program, allowing them to differentiate into trained lung macrophages and mount a rapid protective response against Mtb challenge (28). A similar trained immunity effect on HSCs was found through the use of β-glucan, a Dectin-1 agonist, administered *via* intraperitoneal injection (129). Subsequent work showed that mice with β-glucan-induced trained HSC-derived monocytes and macrophages were significantly better protected from Mtb challenge (130). Therefore, targeting trained immunity through vaccination will require unique adjuvants capable of eliciting trained lung resident macrophages either locally or through HSC-derived cells. Data suggests this may be accomplished through the use of live-attenuated vaccines with endogenous adjuvants (*e.g.*, BCG) or exogenous adjuvants in combination with a subunit vaccine (*e.g.*, β-glucan or other CLR agonist). Additionally, it may be possible to elicit trained immunity specifically in lung macrophages and DCs *via* mucosal vaccination routes (131).

In summary, using adjuvants that activate PRR and other key receptors on innate immune cells and are administered through the mucosal route hold the most promise in inducing adaptive immune responses. Additionally, being able to generate adjuvants that activate processes such as trained immunity could be beneficial for inducing protective immunity to TB.

IMMUNE EVASION MECHANISMS

An important reason for the poor efficacy of natural immunity to Mtb or BCG vaccine-induced immunity is immune evasion mechanisms that lead to ineffective crosstalk between innate and adaptive immunity (95, 132). By identifying critical Mtb factors that prevent optimal innate immune responses and elucidating the molecular basis for how host pathways are subverted by Mtb, we can engineer vaccines that target specific pathogen and host pathways to improve the immunogenicity and efficacy.

Mtb has evolved multiple strategies to evade innate immunity and impede T cell responses. By impairing DC functions (i.e., co-stimulation, cytokine production and antigen presentation) during early stages of infection in the lung, Mtb induces delayed and suboptimal antigen-specific T cell responses that fail to eradicate infection or provide lasting protection (4, 133–136). Vaccine strategies need to augment the induction of additional Mtb-specific T cell subsets, such as Th_{17} cells, that will work in concert with Th_1 responses to enhance protective immunity (137). Mtb also inhibits macrophage microbicidal functions and dampens production of early proinflammatory cytokines and chemokines critical for shaping the nature and magnitude of T cell subsets that home to the site of infection (95, 138–140). Moreover, emerging evidence that myeloid-derived suppressor cells (MDSC) (141) and/or neutrophils (142) suppress T cell responses to Mtb infection suggests that targeting these cell types may improve vaccine-induced immunity.

Several Mtb genes have been implicated in evading DC and macrophage responses and, when deleted in BCG or Mtb, show enhanced vaccine-induced protection against TB in animal models. Thus, removing immune evasion genes shared by Mtb and BCG is an important approach for improving live attenuated vaccination strategies. The following studies are examples of applying knowledge gained from mechanistic studies of Mtb immune evasion genes and the host pathways that they modulate towards designing better vaccines for TB.

Inhibition of apoptosis as an immune evasion strategy is exemplified by the *nuoG* gene in Mtb, which encodes a subunit of NADH dehydrogenase and inhibits macrophage apoptosis (143, 144). Infection of mice with Mtb*ΔnuoG* led to an increase in apoptosis along with earlier activation of T cells compared to WT, suggesting that *nuoG* dampens the ability of innate cells to initiate T cell responses (145). Moreover, deleting *nuoG* in BCG*ΔureC::hly*, a recombinant vaccine strain that is more protective than BCG in animal models, led to increased apoptosis following vaccination and significantly lowered bacterial burdens in Mtb-challenged mice (146). Deletion of Mtb *sigH*, which regulates multiple stress-induced proteins in Mtb, also led to increased apoptosis and chemokine responses in infected macrophages relative to wild type (147, 148). Mucosal vaccination with Mtb*ΔsigH* in NHPs resulted in increased survival and reduced lung pathology following challenge compared to BCG, with higher central and effector memory T cells in the lung (9).

Cell surface proteins are well positioned to modulate innate immune functions. The Mtb serine protease Hip1 is present in

the cell wall of Mtb and dampens macrophage and DC functions *via* proteolytically cleaving its substrate, Mtb GroEL2 (138, 149). Hip1 inhibits TLR2 and inflammasome-dependent macrophage proinflammatory responses, impairs CD40-mediated costimulatory responses in DCs, and restricts Th_{17} polarisation during infection (91, 136, 138). Both an Mtb *hip1* mutant and BCGΔ*hip1* strain augmented CD40 expression on DCs, enhanced macrophage and DC functions and led to higher lung Th_{17} responses (150). In a mucosal DC vaccination model, BCGΔ*hip1* induced immune responses that significantly reduced Mtb burden following challenge (150). These studies suggest that impeding CD40-CD40L interactions allows Mtb to induce suboptimal immunity, and that adjuvants that augment CD40 during vaccination are likely to improve efficacy. Another cell surface protein implicated in dampening innate immunity is LprG, a lipoprotein that binds to TLR2 on macrophages and has been implicated in inhibiting MHC class II antigen presentation and phagosome/lysosome fusion (151–153). Deleting *lprG* in BCG led to higher levels of pro-inflammatory cytokines, lower bacterial burdens and higher Th_{17} responses following vaccination compared to BCG in murine models (154).

Many Mtb secreted proteins have also been implicated in evading host immune responses. SapM is secreted extracellularly *via* the SecA2 pathway and is involved in preventing phagosome maturation in macrophages (155, 156). Vaccination of mice with BCGΔ*sapM* increased protection following challenge and led to increased activation and recruitment of DCs (157, 158). Together, these studies illustrate that deleting immune evasion genes in the context of live attenuated vaccine strains can significantly improve efficacy and suggest that sequential deletion of multiple immune evasion genes in a single vaccine strain is likely to lead to synergistic effects that improve vaccine efficacy.

Studies focused on innate immune pathways modulated by Mtb may also provide key insights into strategies for enhancing innate immunity during vaccination. Adjuvants that trigger and engage specific DC responses during Mtb infection may be of particular interest as a way to combat Mtb induced delays in DC activation and suppression of antigen presentation to $CD4^+$ T cells. Approaches to enhancing Th_{17} responses include adjuvants that engage CD40 on DCs (91) and MPL/chitosan formulations that induce Th_{17} polarising cytokines in DCs (114). Ligands that stimulate TLR7 and TLR9 on DCs have also been shown to upregulate MHCII and reduce anti-inflammatory IL-10 responses following BCG vaccination (159). Other receptors to target include inflammasome components and other cytosolic recognition receptors known to play a role in protective immunity to Mtb infection (160). Developing adjuvants that target additional immune evasion pathways in DCs, such as autophagy, can enhance antigen presentation (93). Targeting costimulatory and coinhibitory molecules on DCs has the potential to be beneficial in improving the immunogenicity of candidate vaccine by fine-tuning the pro- and anti-inflammatory pathways necessary for optimal immunity (91, 161–163). Additionally, adjuvants that limit early induction of IL-10 and T-regulatory cell expansion may be effective given the role of these responses in dampening immune responses during infection (164–166). Finally, we need a deeper understanding of the suppressive functions of neutrophils and MDSCs in order to develop strategies that target these cell types using adjuvant-like approaches or by targeted depletion.

In summary, studies on Mtb immune evasion mechanisms have given us valuable insights, not only on how Mtb manipulates host immunity, but have also identified pathogen and host targets for designing live attenuated vaccine strains and novel vaccine adjuvants that enhance vaccine efficacy.

ANIMAL MODELS

TB is a complex disease and no specific animal model perfectly mimics or recapitulates Mtb infection in humans. However, harnessing the strength of different animal models available will prove useful in developing and testing new adjuvants for TB.

NHPs have played a significant role in TB research and have been increasingly used for vaccine and adjuvant development. The strength of this NHP model lies in its ability to recapitulate disease similar to that in humans. NHPs can develop a latent version of Mtb infection, produce granulomatous lesions, and be used as a model to study HIV/TB co-infection. The use of primates has provided key insights such as the enhanced protective effect of intravenous BCG vaccination and the safety and efficacy of trial TB vaccines including $M72/AS01_E$ and ID93 + GLA-SE (8, 167). Different routes of vaccination can be tested in NHPs, as the intravenous BCG study also included comparisons to intradermal, aerosol, and intradermal & aerosol routes of administration (8). NHPs have been used to test different vaccination strategies such as the prime-boost approach in which macaques are primed with BCG and then boosted with experimental vaccines and adjuvants 3-4 months after priming (168, 169). Additionally, NHP studies on protective vaccines can help inform immune correlates of protection, such as the study which observed protection using pulmonary-delivered BCG and the intravenous BCG study (8, 51). Given their similarity to humans, the NHP model is best equipped to study the priority areas outlined in this review. However, due to the prohibitive costs and challenges of working with primates (along with limited number of animals available for research), the use of small animal models should continue to be used to perform initial pre-clinical studies of potential adjuvants.

Historically, small animal models (mice and guinea pigs) have been used by developers to test adjuvant suitability for inducing protective immunity under rigorous *in vivo* conditions. The mouse model represents an excellent screen for first time *in vivo* assessment of a test vaccine. Using mice affords greater feasibility in testing of different variables such as the effect of different doses, alternative routes of inoculation and different inoculation times post-vaccination in a relatively short time frame. Additionally, the ability to use knockout and transgenic mice provides an additional level of interrogation for vaccine formulations and a better understanding of mechanisms involved in inducing protective immune responses (37). For

instance, a recent analysis of the effect of intranasal vaccination using ODN as a molecular adjuvant showed that immunity could be achieved in a type I interferon-dependent manner (170). Another study demonstrated that intranasal vaccination was the best route for inducing protective immunity compared to intraperitoneal, subcutaneous, or intragastric routes (170). The cost of mouse models also allows for long-term immune profiling in vaccination studies. Extended timepoints have been previously been used to identify improved protective efficacy of subunit vaccine candidates compared to BCG (171). New approaches are also being used that allow mouse models to better mimic the heterogenous immune responses observed in humans. The diversity outbred (DO) mice, where initial breeding is done with inbred and wild-caught strains, have been developed with a level of genetic diversity similar to humans and NHPs. In a BCG vaccine study with DO mice, a diverse response was observed in which some vaccinated mice were protected and others were highly susceptible to Mtb challenge (172). Another attempt to improve the mouse model is the recent development of the ultra-low dose model. Infecting with 1-2 CFU of Mtb *via* aerosol, instead of the conventional ~100 CFU dose, causes mice to produce a heterogenous immune response and granulomatous structure in the lung similar to humans (173). The use of these and other mouse models will therefore continue to be important for testing novel adjuvants and vaccination strategies against TB.

Guinea pigs serve as an additional model and is traditionally employed after a vaccine formulation has demonstrated success in mice. Guinea pigs were originally used in TB research to understand disease pathogenesis due to their susceptibility to Mtb infection as well as their use in distinguishing "mammalian" bacilli from "avian type" bacilli due to their resistance to the latter (174). Subsequent studies by Smith and colleagues (175, 176) demonstrated that aerosol infection of guinea pigs with virulent Mtb resulted in pulmonary pathology similar to that observed in humans as well as a lethal course of disease (177). This model therefore allow researchers to test the ability of a vaccine formulation to limit disease, reduce Mtb burden, and prolong survival. The success of the guinea pig to identify vaccine candidates that have the potential to succeed in humans was demonstrated by a study which found the M72 vaccine candidate was able to boost BCG responses in the model (178). The long-term guinea pig model was used until recently to determine the efficacy of a vaccine as a low dose aerosol infection with virulent Mtb able to induce progressive pulmonary and extra-pulmonary disease similar to acute TB in humans (179). Vaccination with BCG resulted in the significant reduction of pathology and prolonged survival in guinea pigs, therefore validating the use of this model to test vaccines that can be efficacious in humans. Guinea pigs can therefore provide valuable insight into the ability of different formulations to significantly limit disease progression.

Together, the use of both mouse and guinea pig models of TB have contributed to a better understanding of adjuvant mechanisms in vaccines. The NIH/NIAID TB Vaccine testing program (180) has used both the C57BL/6 short-term mouse model and the Hartley guinea pig as a short- and long-term model to test vaccines and novel adjuvants. Both models have similar experimental strategies; animals are vaccinated with the test vaccine formulation, rested, and then infection is established by aerosol challenge with a low dose of Mtb (approximately 100 CFU for the mouse and 10 CFU for the guinea pig). The transition of testing adjuvants from the mouse to the guinea pig model has resulted in down selection of candidate vaccines under the NIH testing contracts and few have been able to reach the clinical testing stage. Of note has been the M72/AS01$_E$ vaccine, which demonstrated activity in the mouse and guinea pig models (178). Using both models also reconcile differences observed experimentally. Recent investigations with ODN adjuvants have highlighted these discrepancies; when the same formulation and inoculation route were used in both models, the adjuvant proved less effective in guinea pigs. These results have been observed with other adjuvants and emphasize the need to test vaccines in both the mouse and guinea pig models While adjuvanted subunit vaccines have in general been less effective at limiting disease in the guinea pig model, it is not clear whether this is a limitation of the model or whether guinea pigs are selecting out formulations that may perform poorly in clinical trials. Unfortunately, there are only a small number of adjuvanted vaccines currently in clinical trials, some of which have been successfully tested in the guinea pig model (181, 182). An increased understanding of immune mechanisms in the guinea pig would be useful to determine if adjuvants were providing the appropriate signals to generate protective immunity. With the limited number of immunological tools available for the guinea pig to perform in-depth analyses (compared to mice), it is difficult to determine why such differences occur. Developing novel reagents for the guinea pig is an area that must be supported to achieve a better understanding of immunological responses to infection and vaccination. Additionally, a systematic analysis of cell phenotypes, expression of PRRs and other innate immune factors will be required to determine why adjuvants have provided limited protective immunity in the guinea pig model. This will help strengthen the use of this model for use in adjuvant studies for TB.

In summary, while NHP models more closely mimic human disease, small animal models provide feasability in testing vaccine adjuvants in a pre-clinical setting. With the rapid increase in the development of new molecular adjuvants, the mouse and guinea pig models provide the capacity to test them rigorously in an infectious disease setting. Harnessing the strength of all three of these animal models will therefore be crucial for developing new and improved adjuvants for TB vaccines.

CONCLUSION

Recent advances in vaccine development, including an adjuvanted TB vaccine providing nearly 50% protection and observations on improving BCG, have reinvigorated the field. As our understanding of the complex interplay between Mtb and

the host immune system is improved, new strategies for adjuvant development may be employed to further boost efficacy and develop a protective vaccine against TB.

In this review, we have highlighted four areas of importance for researchers in the TB field to address in order to generate more efficacious adjuvants and vaccination strategies. Defining correlates of protection is necessary to dictate which pathways should be targeted in order to induce protective immune responses. Similarly, learning how to target cells that are responsible for inducing those protective immune responses serves as a direct access to immune responses that are often dampened during infection. This is critical as Mtb is able to encode immune evasion proteins that serve to actively impede key immune cell subsets. Further characterizing these proteins give us direct insight into pathways that can be activated to overcome pathogen inhibition. Additionally, having appropriate animal models is necessary to test whether adjuvants and vaccines can be beneficial to humans while at the same time serving as a tool to further study infection and disease. While research into these topics can contribute directly to the generation of new adjuvants and vaccines, current adjuvants and vaccines are instructive in determining the requirements for protective immunity to TB. While intravenous delivery of BCG in humans is controversial, the study in NHPs (8) served as a model for identifying correlates of protection that can be targeted through safer, more practical means. Similarly, the M72/AS01$_E$ vaccine study (10) demonstrated the benefit of adjuvants in TB vaccine strategies and contributed potential insights into mechanisms of protection. Cross-talk between basic TB science research and TB translational research should be encouraged as each of these inform the other. Additionally, insights from within the field are able to inform other fields that require generation of similar immune responses for their own models of protection. Therefore, advancing research in these four areas identified at the workshop has the potential to improve immunology in general while working to reduce TB burden worldwide.

AUTHOR CONTRIBUTIONS

ABE, AI, SMM, ELS, RNM, DJF, JTE, JR and JAT developed, wrote, and edited sections of the review as well as edited the entire manuscript. ABE provided significant revisions to the manuscript. All authors contributed to the article and approved the submitted version.

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ARTICLE

Deletion of BCG Hip1 protease enhances dendritic cell and CD4 T cell responses

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Abstract

Dendritic cells (DCs) play a key role in the generation of CD4 T cell responses to pathogens. *Mycobacterium tuberculosis* (Mtb) harbors immune evasion mechanisms that impair DC responses and prevent optimal CD4 T cell immunity. The vaccine strain *Mycobacterium bovis* Bacille Calmette-Guérin (BCG) shares many of the immune evasion proteins utilized by Mtb, but the role of these proteins in DC and T cell responses elicited by BCG is poorly understood. We previously reported that the Mtb serine protease, Hip1, promotes sub-optimal DC responses during infection. Here, we tested the hypothesis that BCG Hip1 modulates DC functions and prevents optimal antigen-specific CD4 T cell responses that limit the immunogenicity of BCG. We generated a strain of BCG lacking *hip1* (BCGΔ*hip1*) and show that it has superior capacity to induce DC maturation and cytokine production compared with the parental BCG. Furthermore, BCGΔ*hip1*-infected DCs were more effective at driving the production of IFN-γ and IL-17 from antigen-specific CD4 T cells in vitro. Mucosal transfer of BCGΔ*hip1*-infected DCs into mouse lungs induced robust CD4 T cell activation in vivo and generated antigen-specific polyfunctional CD4 T cell responses in the lungs. Importantly, BCGΔ*hip1*-infected DCs enhanced control of pulmonary bacterial burden following Mtb aerosol challenge compared with the transfer of BCG-infected DCs. These results reveal that BCG employs Hip1 to impair DC activation, leading to attenuated lung CD4 T cell responses with limited capacity to control Mtb burden after challenge.

KEYWORDS

immune evasion, immunogenicity, tuberculosis, vaccine

1 | INTRODUCTION

Critical to the success of *Mycobacterium tuberculo*sis (Mtb) as a pathogen is its ability to evade host innate and adaptive immunity. Mtb dampens macrophage functions and impairs the ability of dendritic cells (DCs) to induce optimal antigen-specific CD4 T cell responses. As the major APC in the immune system, DCs are central to the generation of CD4 T cell responses after infection and vaccination. However, immunomodulatory factors expressed by Mtb promote sub-optimal DC maturation, cytokine production, and antigen presentation to CD4 T cells, which adversely affects T cell immunity and impedes control of Mtb infection.[1,2] Bacille Calmette-Guérin (BCG) is an attenuated strain of *Mycobacterium bovis* and the only licensed vaccine for tuberculosis (TB) in humans. Although BCG vaccination protects children under the age of 5 from disseminated forms of TB disease, BCG has limited efficacy against pulmonary TB in children and adults.[3,4] However, the immunologic basis for sub-optimal immunity induced by BCG remains unclear. The genome of the BCG parent strain, *M. bovis* shares over 99.95% sequence identity with the Mtb genome[5] and BCG retains many of the genes shown to encode immune evasion proteins in Mtb. We therefore reasoned that retention of immune evasion strategies that are present in virulent mycobacteria by BCG may impede generation of effective innate and adaptive immune responses induced by the vaccine. Thus, we hypothesized that deleting immune evasion genes in BCG that impair DC functions has the potential to improve innate and adaptive immune responses induced by BCG.

Abbreviations: BCG, bacille Calmette-Guérin; BMDC, bone marrow-derived dendritic cell; BMDM, bone marrow-derived macrophage; DC, dendritic cell; Hip1, hydrolase important for pathogenesis 1; MOI, multiplicity of infection; Mtb, *Mycobacterium tuberculosis*; OADC, oleic acid-albumin-dextrose-catalase; TB, tuberculosis; Th, T-helper; WCL, whole cell lysate

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We have previously demonstrated that an Mtb cell wall-associated serine protease, Hip1 (Hydrolase important for pathogenesis 1, Rv2224c), is involved in impairing DC functions.[6] Because Hip1 from BCG and Mtb are 100% identical, we hypothesized that BCG Hip1 may contribute to sub-optimal DC and CD4 T cell responses and that deletion of *hip1* from BCG would augment innate and adaptive immune responses. In this study, we generated a BCG (Danish) strain lacking *hip1* (BCGΔ*hip1*) to investigate whether deletion of *hip1* in BCG enhances DC functions and improves CD4 T cell responses in vitro and in vivo. We show that DCs infected with BCGΔ*hip1* produce significantly enhanced levels of pro-inflammatory cytokines and express higher levels of MHC class II and costimulatory molecules compared with the DCs infected with the parent BCG strain. Additionally, deletion of *hip1* from BCG augmented DC antigen presentation to CD4 T cells in vitro. Moreover, mucosal priming of immune responses via intratracheal instillation of BCGΔ*hip1*-infected DCs improved antigen-specific CD4 T cell responses in the lungs and enhanced control of Mtb burden following aerosol challenge compared with the transfer of BCG-infected DCs. Our results demonstrate that BCG subversion of DC functions through Hip1 impedes the generation of robust CD4 T cell responses and provides a rationale for targeting *hip1* to improve BCG immunogenicity.

2 | MATERIALS AND METHODS

2.1 | Bacterial strains and culture conditions

BCG (Danish), BCGΔ*hip1*, and BCGΔ*hip1* complemented with *hip1* (BCGΔ*hip1* comp) were grown at 37°C in Middlebrook 7H9 broth supplemented with 10% oleic acid-albumin-dextrose-catalase (OADC), 0.2% glycerol, and 0.05% Tween 80 or on Middlebrook 7H10 agar supplemented with 10% OADC, 0.5% glycerol, and 0.2% Tween 80. Media for complemented BCGΔ*hip1* was supplemented with 20 μg/mL of streptomycin (Sigma–Aldrich, St. Louis, MO). For growth curves, bacterial strains were inoculated into supplemented 7H9 medium at OD_{600} 0.05, and the OD_{600} measurements were taken daily.

2.2 | Construction of BCGΔ*hip1* and complemented strains

BCG was transformed via electroporation with 3 μg of pEBOP-2 (pYUB657 suicide vector containing a Δ*hip1* allele, a selectable hygromycin resistance marker, and a counter selectable *sacB* marker). Resulting transformants that were resistant to hygromycin were then patched onto 7H10 plates containing 2% sucrose and 50 μg/mL of hygromycin (Roche Diagnostics, Indianapolis, IN). Colonies that displayed hygromycin resistance and sucrose sensitivity were considered to have undergone a single crossover event resulting in incorporation of pEBOP-2 into the BCG genome. These colonies were then grown to saturation for a week in 5 mL of 7H9 broth, and then serial dilutions were plated in duplicate onto 7H10 plates supplemented with 2% sucrose. Colonies arising on these plates were patched onto hygromycin-containing plates. Colonies that were both hygromycin sensitive and sucrose resistant were grown in 7H9 broth, and genomic DNA was extracted using the protocol adapted from Belisle and Sonnenberg.[7] Genomic DNA was then subjected to Southern blot analysis. DNA was digested with *Nco*I, and then probed with a DIG-labeled DNA amplicon corresponding to a 1 kb region present in both the genome and pEBOP-02. Deletion of *hip1* was also confirmed via amplification of the deleted region using primers upstream (forward primer 5′-CGGCCACCCGCTCACCGCCCTCG-3′) and downstream (reverse primer 5′-GCACGGCGAATGTCAGATAGGG-3′) of the 1 kb regions of homologous recombination, resulting in a 4.5 kb amplicon from the BCGΔ*hip1* genome, and a 6 kb amplicon from the wild-type BCG genome (Supplemental Fig. 1C). These amplicons were then sequenced for further confirmation of gene deletion. BCGΔ*hip1* was complemented with *hip1* expressed from its natural promoter on an integrated plasmid.

2.3 | Mice

All mice were housed under specific pathogen-free conditions in filter-top cages within the vivarium at the Yerkes National Primate Center, Emory University, and provided with sterile water and food ad libitum. C57BL/6J mice were purchased from The Jackson Laboratory (Bar Harbor, ME). OT-II TCR transgenic mice specific for $OVA_{323–339}$ peptide were obtained from Dr. Bali Pulendran (originally generated in the laboratory of Dr. F. Carbone, University of Melbourne), and bred at the Yerkes animal facility.

2.4 | BMDM and BMDC generation and infection

Bone marrow-derived macrophages (BMDMs) were generated as previously described.[8] Bone marrow cells were isolated from C57BL/6J mice and differentiated for 7 days at 37°C with 5% CO_2 in DMEM/F-12 medium (Lonza, Walkersville, MD) containing 10% FBS (HyClone, Logan, UT), 2 mM L-glutamine, and 10% L-cell conditioned medium (LCM). Adherent cells were collected, and macrophages were plated onto 24-well plates at 3×10^5 per well and rested overnight. For heat-killed BCG infections, bacteria suspended in DMEM/F-12 medium containing 10% FBS, 2 mM L-glutamine, and 5% LCM were added to differentiated BMDMs in 24 well plates at indicated MOIs. Murine bone marrow-derived dendritic cells (BMDCs) were generated as previously described.[9] Bone marrow cells isolated from C57BL/6J mice were grown and differentiated for 8 days in RPMI medium (Lonza) supplemented with 10% FBS (HyClone), 2 mM L-glutamine (Gibco, Grand Island, NY), 20 ng/mL rmGM-CSF (R&D systems, Minneapolis, MN), 50 μM beta-mercaptoethanol (Gibco), 1X non-essential amino acids (Gibco), 10 mM HEPES buffer (Lonza), and 1 mM sodium pyruvate (Lonza). Non-adherent cells were harvested after 8 days and purified using CD11c microbeads (Miltenyi, Gladbach, Germany), plated at 3×10^5 per well, and rested overnight. For heat-killed BCG infections, bacteria were suspended in DC media without rmGM-CSF and added to differentiated BMDCs in 24 well plates at indicated MOIs and incubated at 37°C. For live infections, BCG strains suspended in DC media without rmGM-CSF were added to BMDCs at indicated MOIs and allowed to incubate at 37°C for 4 hours. Monolayers were then treated

with 200 μg/mL amikacin for 45 minutes. Cells were then washed thrice with PBS, and DC media without rmGM-CSF was then added to infected cells. To determine the CFU of bacteria within infected cells, one set of BMDCs was first washed with PBS then lysed using PBS containing 0.5% TritonX, and serial dilutions of the lysate were plated onto 7H10 plates. To assess BCG growth within BMDCs, cell lysates were plated on days 1, 3, and 5 after initial infection. CFU were enumerated after 21 days of incubation. Supernatants from infected BMDCs or BMDMs were collected at indicated timepoints and analyzed via ELISA for cytokine levels according to the manufacturers' instructions: BD OptEIA kits for IL-6, IL-1β, IL-12p70, and IL-12p40 (BD Biosciences, San Jose, CA) and IL-23 (Biolegend, San Diego, CA) per manufacturer's instructions.

2.5 | BMDC-T cell cocultures

BMDCs were cocultured with CD4 T cells 24 hours after infection with BCG strains. Briefly, CD4 T cells were isolated from splenocytes collected from OT-II TCR transgenic mice and purified using CD4 magnetic microbeads (Miltenyi). Purified CD4 T cells were suspended at 1×10^6/mL in supplemented RPMI and cocultured with BMDCs to achieve a 1:4 DC:T cell ratio. Supernatants from cocultures were collected after 72 hours, spun down to remove cells, and frozen. Cytokine levels were analyzed via ELISA according to manufacturers' instrucutions: IFN-γ (Mabtech, Cincinnati, OH), IL-2 (BD Biosciences), and IL-17 (eBioscience, San Diego, CA).

2.6 | Intratracheal instillation of BMDCs

BMDCs were generated as described, purified using CD11c microbeads (Miltenyi), and stimulated with either BCG or BCGΔ*hip1* at an MOI of 30 or left unstimulated in media for 24 hours. BMDCs were then washed, resuspended in PBS, and intratracheally instilled (1×10^6 per mouse in 50 μL PBS) into isoflurane-anesthetized C57BL/6J hosts.

2.7 | Assessment of antigen-specific responses

Six days after intratracheal BMDC transfer, lungs were harvested and processed for further analysis. Briefly, organs were minced and placed in harvest medium consisting of HBSS containing 10 mM HEPES, 2% FBS, 0.1% collagenase type IV (Worthington, Lakewood, NJ), and 0.01% DNase I (Worthington) for 30 min at 37°C. Following incubation, organs were processed into single cell suspension utilizing the gentleMACs tissue dissociator (Miltenyi). Cells were thoroughly washed, counted, and 1×10^6 cells were plated for phenotypic analysis or antigen restimulation. Cells were exposed to media (unstimulated), PMA/ionomycin (80 and 500 ng/mL, respectively), or 10 μg/mL whole cell lysate (WCL). Cells were then incubated at 37°C for 1.5 hours before addition of BFA (5 μg/mL) and monensin (1:1500) followed by a further incubation at 37°C for 4.5 hours (for media and PMA/ionomycin stimulations) or overnight (for WCL stimulations). Cells were then spun down, washed, and stained with the following fluorophore conjugated antibodies purchased from BD Biosciences, Biolegend, or eBioscience for flow cytometric analysis: anti-CD8 PerCP (clone 53–6.7; BD), anti-CD44 APC-Cy7 (clone IM7; BD), anti-TCR $\gamma\delta$ BV605 (clone GL3; Biolegend), anti-CD3e V450 (clone 500A2; BD), anti-CD4 Alexa700 (clone RM4-5; BD), anti-TNF-α PE-Cy7 (clone MP6-XT22; BD), anti-IL-2 FITC (clone JES6-5H4; BD), anti-IL-17 PECF594 (clone TC11-18H10; BD), and anti-IFN-γ APC (clone XMG1.2; eBioscience). Live cells were determined by exclusion of amine-reactive dye (Live/Dead Fixable Aqua Dead Cell Stain kit; Life Technologies, Carlsbad, CA). Samples were acquired using an LSRII flow cytometer and analyzed by FlowJo (FlowJo, LLC, Ashland, OR).

2.8 | Mtb aerosol challenge and enumeration of bacteria

Groups of five mice were intratracheally instilled with 1×10^6 BMDCs, rested for 14 days, and then challenged with a low dose (~100 CFU) of Mtb H37Rv using an Intox aerosol apparatus. Lungs from infected mice were harvested 28 days post-challenge, homogenized, plated on 7H10 agar plates, and incubated for 21 days in 37°C prior to CFU enumeration.

3 | RESULTS

3.1 | Construction of a BCGΔ*hip1* strain

To determine the role of *hip1* in BCG-induced DC responses, we generated an in-frame, unmarked deletion of *hip1* in the BCG Danish strain. We utilized the suicide vector, pYUB657, which expresses a hygromycin resistance cassette and a counter-selectable marker, to introduce an allelic exchange-based deletion of *hip1* (Supplemental Fig. 1A). The resulting BCGΔ*hip1* strain harbored a complete deletion of *hip1* from its genome, which we verified via PCR amplification of the genomic region as well as through Southern blot analysis (Supplemental Figs. 1B and C). Next, we sought to determine the effect of deleting *hip1* on BCG growth kinetics. We observed no significant differences between BCG, BCGΔ*hip1*, or a BCGΔ*hip1* strain complemented with *hip1* (BCGΔ*hip1* comp) on growth in 7H9 broth (Fig. 1A). Additionally, these strains grew comparably in BMDCs over 5 days of culture (Fig. 1B).

3.2 | BCGΔ*hip1* elicits robust DC cytokine responses compared with BCG

DC cytokine production is a canonical signal driving differentiation of naïve CD4 T cells to specific CD4 T-helper (Th) subsets. We therefore sought to compare cytokine responses from DCs infected with BCG or BCGΔ*hip1*. We infected BMDCs from C57BL/6J mice with BCG, BCGΔ*hip1*, or BCGΔ*hip1* comp and measured DC cytokine production by ELISA. DCs infected with BCGΔ*hip1* produced significantly higher levels of IL-6 and IL-12p40 than DCs infected with BCG. Importantly, cytokine levels induced by BCGΔ*hip1* were restored to BCG levels after infection with the complemented strain (Fig. 2A), indicating that BCG limits DC cytokine production through *hip1*. We next infected DCs with BCG or BCGΔ*hip1* at multiplicities of infection (MOI) of 10

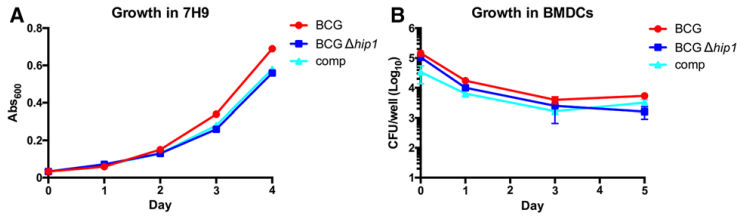

FIGURE 1 **Deletion of *hip1* from BCG does not affect growth of BCG in broth or in dendritic cells.** (A) BCG Danish, BCGΔ*hip1*, or complemented BCGΔ*hip1* (comp) were inoculated into 7H9 liquid medium supplemented with OADC and glycerol at a starting OD_{600} of 0.05, were incubated at 37°C shaking, and the absorbance of the cultures at OD_{600} was recorded daily. (B) BMDCs from C57BL/6J mice were infected with BCG, BCGΔ*hip1*, or complemented BCGΔ*hip1* (comp) and grown at 37°C. DCs were collected and lysed at the indicated time points, and bacteria were plated on 7H10 plates for CFU determination. Results are representative of 3 independent experiments. Values are presented as means ± SD

and 20 and assessed cytokine levels in the supernatant at 24, 48, and 72 hours after infection by ELISA. DCs infected with BCGΔ*hip1* produced significantly higher levels of IL-6 and IL-12p40 relative to BCG infection at all MOIs and time points tested (Fig. 2B). Notably, incubation of DCs with heat-killed BCGΔ*hip1* also resulted in significantly higher levels of cytokines compared with heat-killed BCG, indicating that enhanced cytokine production by DCs infected with BCGΔ*hip1* was not dependent on viability of the bacteria (Fig. 2C). Further, we found that BMDMs infected with BCGΔ*hip1* produced higher levels of IL-6 and IL-1β compared with BCG-infected BMDMs at all MOIs tested (Fig. 2D). These data demonstrate that deletion of *hip1* in BCG results in significantly augmented pro-inflammatory cytokine production from both infected DCs and macrophages.

3.3 | BCGΔ*hip1* enhances expression of costimulatory molecules on infected DCs

Following infection, DCs undergo maturation, which is required for optimal antigen presentation and initiation of antigen-specific CD4 T cell responses. DCs present antigens via MHC class II complexes and provide critical costimulatory signals to CD4 T cells through upregulation of molecules such as CD40 and CD86. We determined the expression levels of MHC class II and the costimulatory molecules CD40 and CD86 on DCs infected with BCG or BCGΔ*hip1* by flow cytometry. DCs infected with BCGΔ*hip1* expressed higher levels of MHC class II, CD40, and CD86 when compared with BCG-infected DCs (Fig. 3). These data suggest that deletion of *hip1* in BCG enhances DC maturation and expression of costimulatory molecules.

3.4 | Enhanced polarization of IFN-γ and IL-17-producing antigen-specific CD4 T cells by DCs infected with BCGΔ*hip1*

IL-12 is known to drive the polarization of IFN-γ-producing Th_1 subsets, whereas IL-6, IL-1β, TGF-β, and IL-23 drive the polarization and expansion of IL-17-producing Th_{17} subsets.[10] Since DCs infected with BCGΔ*hip1* induced higher levels of pro-inflammatory cytokines (Fig. 2) and displayed an enhanced maturation profile (Fig. 3) compared with BCG infection, we hypothesized that BCGΔ*hip1*-infected DCs would more effectively polarize antigen-specific CD4 T cells toward Th_1 and Th_{17} subsets compared with BCG-infected DCs. To test this, we cocultured DCs infected with BCG or BCGΔ*hip1* with naïve ovalbumin-specific TCR transgenic CD4 T cells (OT-II) for 3 days in the presence of cognate peptide ($OVA_{323–339}$). Levels of IFN-γ, IL-17, and IL-2 were measured via ELISA (Fig. 4A). DCs infected with BCGΔ*hip1* promoted significantly higher levels of IFN-γ and IL-17 from antigen-specific CD4 T cells after coculture (Fig. 4A). This was consistent with higher levels of the Th_1-polarizing cytokines IL-12p40 and IL-12p70 induced by BCGΔ*hip1*-infected DCs as well as higher levels of Th_{17}-polarizing cytokines IL-6 and IL-1β (Fig. 4B). We do not detect significant levels of TGF-β under any of our culture conditions. Further, compared with BCG-infected DCs, BCGΔ*hip1*-infected DCs produced higher levels of IL-23, which is a cytokine known to maintain Th_{17} lineage commitment.[11] These data demonstrate that BCGΔ*hip1*-infected DCs have an enhanced capacity to induce antigen-specific IFN-γ and IL-17 responses compared with BCG-infected DCs.

3.5 | Intratracheal instillation of DCs infected with BCGΔ*hip1* enhances lung CD4 T cell responses in vivo and improves control of Mtb burden following aerosol challenge

Mucosal administration of DCs has been utilized to assess early antigen-specific T cell responses to mycobacteria in the lungs of mice,[12] and antigen-loaded DCs have previously been shown to confer protection against Mtb challenge.[9,13–15] Therefore, we used a tractable DC intratracheal instillation model to assess antigen-specific CD4 T cell responses in vivo following transfer of BCG or BCGΔ*hip1*-infected DCs, or control uninfected DCs, into the lungs of mice. After 6 days, we harvested lungs and assessed, by flow cytometry, antigen-specific CD4 T cell responses upon ex vivo stimulation of lung cells with Mtb WCL. We measured the number of activated $CD44^+$ CD4 T cells in the lungs following intratracheal instillation of BCG or BCGΔ*hip1*-infected DCs, which reflects levels of CD4 T cell activation. We found higher numbers of $CD44^+$ CD4 T cells in the lungs of mice that received DCs infected with BCGΔ*hip1* compared with mice that received BCG (Fig. 5B), indicating that BCGΔ*hip1*-infected DCs induced better activation of CD4 T cells in vivo. Next, we assessed the functionality of antigen-specific CD4 T cells in the lungs by measuring cytokine responses by intracellular cytokine staining and flow cytometry

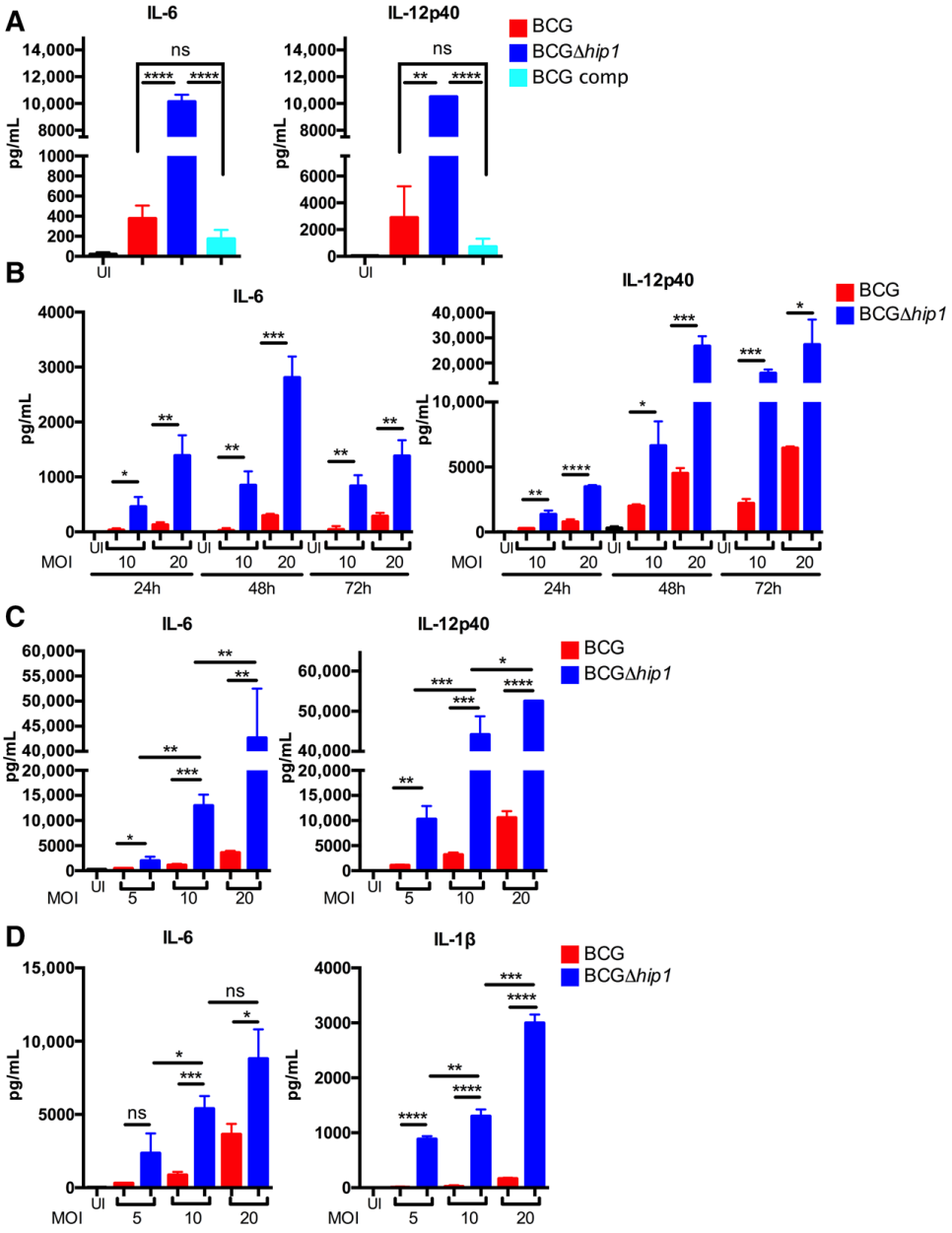

FIGURE 2 **BCGΔ*hip1* induces stronger pro-inflammatory cytokine production from innate immune cells.** (A) BMDCs from C57BL/6J mice were infected with BCG, BCGΔ*hip1*, or BCGΔ*hip1* complemented with *hip1* (BCG comp) at a multiplicity of infection (MOI) of 10 and incubated at 37°C. After 24 hours, the culture supernatants were assessed for levels of IL-6 and IL-12p40 via ELISA. (B) BMDCs were infected with varying MOIs (10 and 20) of BCG or BCGΔ*hip1*, and culture supernatants were assayed for IL-6 and IL-12p40 at 24, 48, and 72 hours post-infection. Heat-killed BCG or BCGΔ*hip1* were exposed to BMDCs (C) or BMDMs (D) at MOIs of 5, 10, and 20 for 24 hours at 37°C. Culture supernatants were tested for IL-6 and IL-12p40 (C) or IL-6 and IL-1β (D) via ELISA. Results are representative of 3 independent experiments. Statistical significance was determined by unpaired two-tailed Student's *t*-test. Values are presented as mean ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$; ns, no significance; ui, uninfected

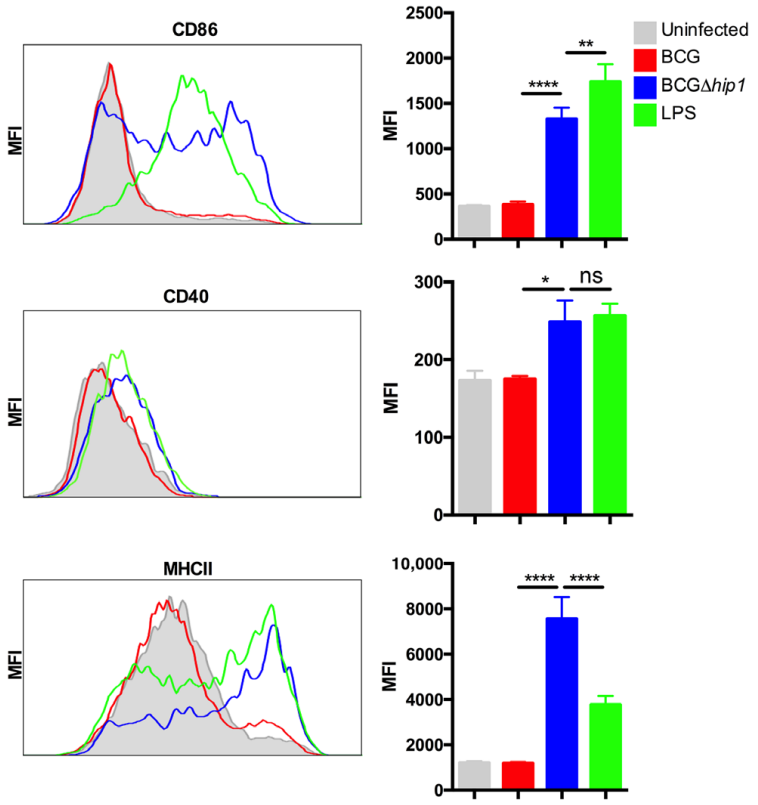

FIGURE 3 **BCGΔ*hip1* enhances expression of costimulatory molecules on infected DCs.** BMDCs from C57BL/6J mice were either uninfected, infected with BCG or BCGΔ*hip1* (MOI 10), or stimulated with LPS (1 μg/mL) for 24 hours. BMDCs were then stained for maturation markers CD86 (top), CD40 (middle), and MHC class II (bottom). Representative histograms (left) and summary graphs (right) of median fluorescence intensities of each marker is shown. Cells were pre-gated on live, $CD11c^+CD11b^+$ singlets. Results are representative of 3 independent experiments. Statistical significance was determined by unpaired two-tailed Student's *t*-test. Values are presented as mean ± SD. $^*P < 0.05$, $^{**}P < 0.01$, $^{****}P < 0.0001$; ns, no significance

following restimulation with WCL. We found higher numbers of antigen-specific CD4 T cells producing IL-2, IFN-γ, TNF-α, and IL-17 in the lungs of mice that received BCGΔ*hip1* DCs compared with BCG DCs (Fig. 5C). Triple cytokine-producing CD4 T cells, conventionally termed polyfunctional CD4 T cells, are thought to be indicative of a more protective adaptive immune response.[16] Interestingly, we observed higher frequencies of CD4 T cells producing IFN-γ, IL-2, and TNF-α in animals that received BCGΔ*hip1* DCs compared with BCG DCs (Fig. 5D). To investigate whether mucosal administration of DCs exposed to BCGΔ*hip1* would provide enhanced bacterial control compared with BCG after low dose aerosol Mtb challenge, we intratracheally instilled BCG DCs or BCGΔ*hip1* DCs, rested mice for 2 weeks prior to aerogenic challenge with low-dose Mtb H37Rv, and determined lung Mtb bacterial burden 4 weeks post-challenge. As shown in Fig. 5E, mice that received BCGΔ*hip1*-infected DCs harbored significantly less Mtb CFU post-challenge compared with mice that received BCG-infected DCs (Fig. 5E). These results demonstrate that mucosal-targeted approaches using BCGΔ*hip1* can augment antigen-specific CD4 T cell responses compared with BCG and lead to enhanced control of Mtb burden.

4 | DISCUSSION

The interplay between mycobacteria and DCs is a critical consideration for rational development of efficacious vaccines for TB. In this study, we demonstrate that deletion of the BCG serine protease, Hip1, promotes robust DC activation and enhances antigen-specific lung CD4 T cell responses. We have shown that BCGΔ*hip1*-infected DCs produce higher levels of cytokines, express elevated levels of costimulatory molecules, and enhance CD4 T cell responses both in vitro and in vivo compared with DCs infected with BCG. These data provide insight into the sub-optimal immunogenicity of BCG and demonstrate that deletion of the immune evasion gene, *hip1*, in BCG

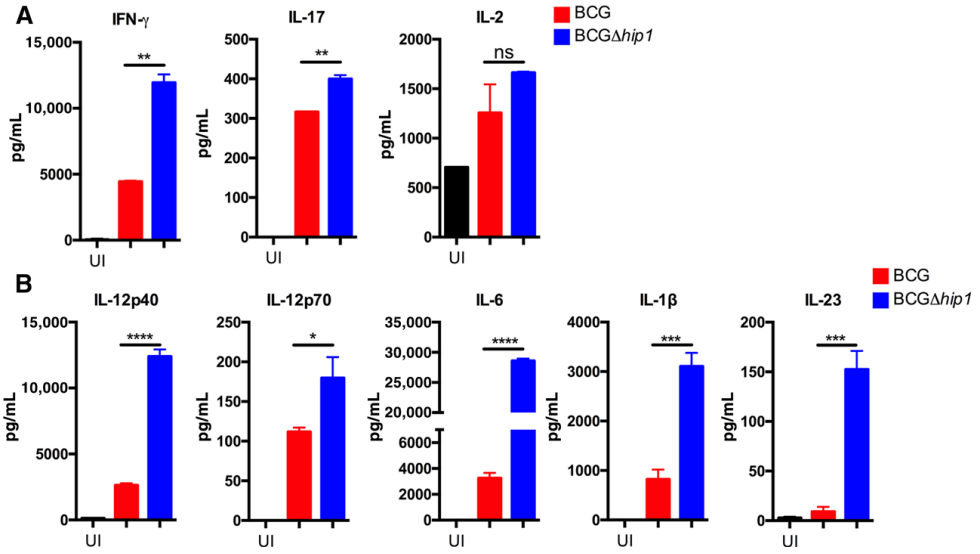

FIGURE 4 **DCs infected with BCGΔ*hip1* induce higher levels of IFN-γ and IL-17 production from CD4 T cells compared with BCG.** BMDCs from C57BL/6J mice were pulsed with $OVA_{323-339}$ peptide, and 6 hours later, infected with BCG or BCGΔ*hip1*. After 24 hours, culture supernatants were removed and purified OT-II CD4 T cells were added to the adherent BMDC monolayer at a 4:1 T cell to DC ratio. Three days after addition of CD4 T cells, culture supernatants were collected and IL-2, IFN-γ, and IL-17 levels were assessed via ELISA (A). Levels of IL-12p40, IL-12p70, IL-6, IL-1β, and IL-23 from culture supernatants containing solely BMDCs were assessed via ELISA (B). Results are representative of 3 independent experiments. Statistical significance was determined by unpaired two-tailed Student's *t*-test. Values are presented as mean ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$; ns, no significance

promotes enhanced DC-T cell crosstalk that leads to better control of Mtb burden.

The underlying reasons for the variable efficacy of BCG as a TB vaccine are unclear. As DCs are the key cells linking innate and adaptive immunity, the interaction between DCs and BCG is a critical factor in generating anti-mycobacterial T cell responses. Our results are consistent with a growing body of literature suggesting that BCG impairs innate and adaptive immune responses. BCG has been shown to adversely impact antigen-specific CD4 T cell activation through the up-regulation of PD-L1 and PD-L2,[17] and diminish activation of antigen-specific CD8 T cells by inducing DC death.[18] Furthermore, BCG-infected DCs produce significantly lower levels of IL-23, IL-1β, TNF-α, and IL-12 than Mtb-infected DCs,[19] indicating that BCG stimulates weaker innate immune responses than Mtb. Interestingly, a study by Satchidanandam et al.[20] showed that overexpression of an Mtb glycosylated protein, Rv1860, in BCG impaired DC maturation, attenuated Th_1 and Th_{17} polarization, and led to subsequent loss of protection against Mtb challenge conferred by BCG vaccination, suggesting that Mtb proteins that negatively impact DC responses can attenuate the protective effect of BCG vaccination. Conversely, relatively little is known about BCG genes that retain immunomodulatory properties and thus promote impaired DC responses and sub-optimal T cell immunity. Our data showing that BCG Hip1 contributes to impaired DC cytokine production and maturation demonstrates that BCG retains immunomodulatory factors that negatively impact DC and T cell responses, leading to impaired control of Mtb after challenge.

Several avenues have been explored for improving BCG immunogenicity and efficacy, including introduction of immunodominant proteins from Mtb,[20–23] and expression of host proteins.[24–30] The majority of studies utilizing recombinant or mutant strains of BCG are primarily focused on enhancing macrophage driven responses and functions, such as phagasomal maturation, apoptosis of infected macrophages, and bacterial escape from phagasomal compartments.[31–36] For instance, a recombinant strain of BCG lacking urease production and expressing listeriolysin from *Listeria monocytogenes* (BCGΔ*ureC::hly)* was shown to enhance apoptosis of infected macrophages,[33] leading to increased central memory T cell responses,[37] enhanced Th_1 and Th_{17} immunity,[38] and cross-presentation to CD8 T cells.[34] Interestingly, deletion of anti-apoptotic gene *nuoG* in BCGΔ*ureC::hly* showed enhanced efficacy over BCGΔ*ureC::hly*.[39] Notably, we show evidence that BCGΔ*hip1*-infected macrophages display enhanced cytokine production relative to BCG-infected macrophages (Fig. 2). As we utilized a mucosal transfer approach that exclusively utilized DCs, it will be important to address the role that macrophages may play at priming antigen-specific CD4 and CD8 T cell responses after vaccination with BCGΔ*hip1*.

Relatively few studies have elaborated on BCG factors that can be targeted to improve DC responses. However, targeting DCs has proven to be a viable approach to improve Mtb-specific CD4 T cell responses after vaccination. H56, a subunit vaccine incorporating Ag85B, ESAT-6, and Rv2660, was shown to provide enhanced protection relative to BCG,[40] and utilizes a liposome-based adjuvant (CAF01)

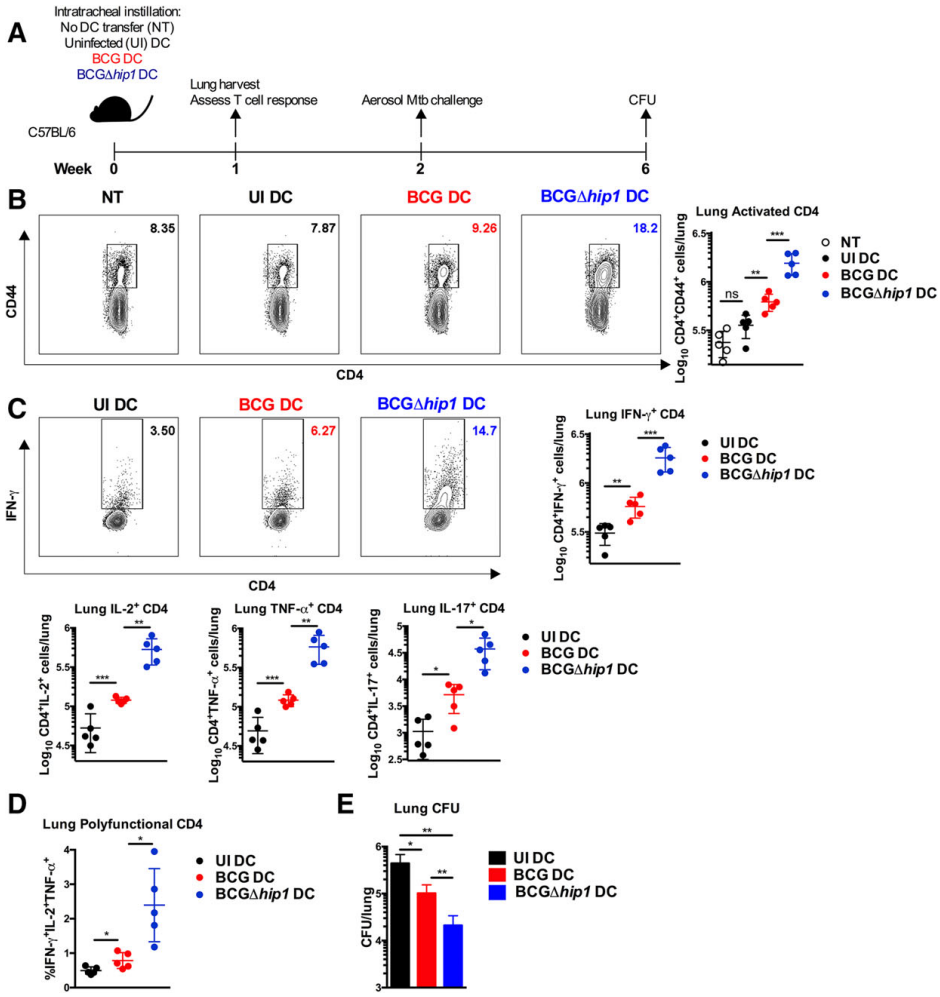

FIGURE 5 **Intratracheal instillation of DCs infected with BCGΔ*hip1* enhances mucosal CD4 T cell responses and improves control of Mtb burden after aerosol challenge.** (A) Diagram of experimental design. BMDCs were exposed to BCG or BCGΔ*hip1* or were left uninfected for 24 hours prior to being intratracheally instilled (1×10^6 per mouse). Mice that did not receive any DCs were used as no transfer (NT) controls. Lung immune responses were assessed 1 week post-intratracheal instillation. Remaining animals were challenged 2 weeks post-intratracheal instillation with low dose aerosolized Mtb (H37Rv) and lung bacterial burden was assessed 4 weeks post-challenge. (B) Representative plots of the frequencies (left) and summary graph of the absolute counts (right) of $CD4^+CD44^+$ cells in the lungs 1 week post-intratracheal instillation. (C) Lung IFN-γ^+ $CD4^+$ cells (top) and IL-2^+, TNF-α^+, IL-17^+ $CD4^+$ cells (bottom) responding to whole cell lysate restimulation 1 week post-intratracheal instillation. (D) Summary graph of the frequency of lung polyfunctional (IFN-γ^+IL-2^+TNF-α^+) $CD4^+$ T cells responding to whole cell lysate restimulation 1 week post-intratracheal instillation. Cells were pre-gated on live, $CD3^+CD8^-$TCR $\gamma\delta^-$ singlets. (E) Lung bacterial burden at 4 weeks post-challenge from animals that received uninfected BMDCs or BMDCs stimulated with BCG or BCGΔ*hip1*. Results are representative of 2 independent experiments. Statistical significance was determined by unpaired two-tailed Student's *t*-test. Values are presented as mean ± SD. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$; ns, no significance

that targets DCs.[41] Furthermore, improving DC antigen-presentation by induction of autophagy has been shown to improve BCG immunogenicity and improve control of Mtb burden after challenge.[42] Our data show that transfer of BCGΔ*hip1*-DCs leads to enhanced lung CD4 T cell responses compared with the transfer of BCG-DCs, including higher frequencies of antigen-specific, polyfunctional CD4 T cells contributing to better control of Mtb burden after challenge (Fig. 5). Thus, our studies suggest that deleting BCG *hip1* alone, or in concert with deleting additional immune evasion genes, is a feasible approach to enhance DC functions for the rational improvement of BCG as a vaccine. A growing number of studies indicate that vaccination through the mucosal route induces robust antigen-specific responses that

confer better protection at mucosal surfaces, such as the lung, relative to parenteral routes.[9,13,14,43–48] Using an intratracheal DC installation model, we observed that transfer of DCs infected with BCGΔ*hip1* into mouse lungs more effectively activated CD4 T cells, induced higher numbers of antigen-specific mucosal CD4 T cells secreting IFN-γ, IL-2, TNF-α, and IL-17 in vivo, and led to enhanced Mtb control after challenge compared with the transfer of BCG-DCs. These studies provide proof of principle data and reveal insights into BCG interactions with DCs, but may not mirror mucosal vaccination using bacteria alone. Bacteria encounter a wider variety of myeloid cells in the lungs, including alveolar macrophages and lung DC subsets, that may differ from BMDCs. Therefore, further studies examining the effects of BCGΔ*hip1* utilizing more traditional vaccination approaches are of interest.

In summary, our work supports a growing body of evidence that enhancing DC functions will improve BCG-induced immunity. Deletion of *hip1* in BCG augmented DC functions, improved antigen-specific CD4 T cell responses in the lungs, and promoted enhanced control of Mtb burden after challenge. These data indicate that strategies targeting BCG immune evasion genes, such as *hip1*, are a viable avenue for improving innate and adaptive immunity to provide enhanced control of Mtb.

AUTHORSHIP

J.R., E.B., and J.K.S. contributed to the experimental design and conception of this study. E.B., J.K.S., M.Q., A.E., and M.G. did the experimentation. J.R., E.B., and J.K.S. analyzed the data. J.R., E.B., and J.K.S. wrote the manuscript. J.R., E.B., J.K.S., and A.E. reviewed and edited the manuscript.

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DISCLOSURES

The authors declare no conflicts of interest.

AUTHOR CONTRIBUTIONS

Erica Bizzell and Jonathan Kevin Sia contributed equally to this work.

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SUPPORTING INFORMATION

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