A novel STING-activating vaccine for *M. tuberculosis* induces pathogen-specific Th17 cells and provides significant IL-17-dependent protection against infection

By

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Abstract

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Before the emergence of SARS-CoV-2 and the ensuing COVID-19 pandemic, the pathogenic bacteria *Mycobacterium tuberculosis* was the world's leading infectious disease killer. In 2020, an estimated 1.6 – 1.8 million people worldwide died of tuberculosis (TB) disease, comparable to the ~1.9 million lives claimed by COVID-19. However, while highly effective vaccines were rapidly developed for COVID-19, the sole approved TB vaccine, Bacillus Calmette-Guérin (BCG), is only moderately effective at blocking infection and has not prevented the TB pandemic despite 100 years of use and more widespread distribution that any vaccine in history. This highlights the urgent need for improved vaccines to prevent TB, which begins with a more complete understanding of the protective immune response to TB and the development of new strategies to effectively induce this immune response in patients. Here, we demonstrate a novel TB vaccine strategy using small molecule cyclic di-nucleotide (CDN) adjuvants to activate the STING pathway of the innate immune system.

Subcutaneous administration with a CDN adjuvanted protein subunit vaccine for TB leads to protection in mice that is equivalent to that afforded by BCG. This protective efficacy is STING-dependent but Type I IFN-independent and correlates with a significant population of Th17 cells that are not present in the lungs of unvaccinated mice. Similarly, intranasal immunization leads to a multifaceted pathogen-specific CD4 T cell response characterized by both Th1 and Th17 cells, as well as a non-classical Th1-Th17 population that co-expresses IFN-γ and IL-17. Intranasally vaccinated mice also benefit from enhanced CD4 T cell localization to the lung parenchyma and homing to granulomatous lung lesions compared to unvaccinated mice. This distinct CD4 T cell response leads to protection against infection that is significantly greater than BCG and is more effective than any protein subunit vaccine tested against *M. tuberculosis* in mice to date. Full protective efficacy after intranasal immunization is dependent on both IFN-γ and IL-17 and, in contrast to subcutaneous administration, relies on Type I IFN signaling. Importantly, intranasal immunization with a CDN vaccine is also capable of boosting BCG-mediated immunity. These results demonstrate that stimulation of the STING pathway is sufficient to induce a protective CD4 T cell response to an

intracellular pathogen and contribute to our knowledge of CD4 T cell subsets that can mediate protection against *M. tuberculosis* infection.

This work is dedicated to Neil Van Dis, Marilyn Van Dis,
Gary Skidmore, and in memory of Margaret Skidmore

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Chapter One: The innate immune response to *Mycobacterium tuberculosis* infection

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Abstract

Infection with *Mycobacterium tuberculosis* causes >1.5 million deaths worldwide annually. Innate immune cells are the first to encounter *M. tuberculosis*, and their response dictates the course of infection. Dendritic cells (DCs) activate the adaptive response and determine its characteristics. Macrophages are responsible both for exerting cell-intrinsic antimicrobial control and for initiating and maintaining inflammation. The inflammatory response to *M. tuberculosis* infection is a double-edged sword. While cytokines such as TNF-α and IL-1 are important for protection, either excessive or insufficient cytokine production results in progressive disease. Furthermore, neutrophils—cells normally associated with control of bacterial infection—are emerging as key drivers of a hyperinflammatory response that results in host mortality. The roles of other innate cells, including natural killer cells and innate-like T cells, remain enigmatic. Understanding the nuances of both cell-intrinsic control of infection and regulation of inflammation will be crucial for the successful development of host-targeted therapeutics and vaccines.

Recognition of *M. tuberculosis* by pattern recognition receptors of the innate immune system

The first step in initiating an immune response to *Mycobacterium tuberculosis* is detection by pattern recognition receptors (PRRs). Several classes of PRRs, including Toll-like receptors (TLRs), nucleotide-binding domain and leucine-rich repeat–containing receptors (NLRs), C-type lectin receptors (CLRs), and cyclic GMP-AMP synthase (cGAS)/stimulator of interferon genes (STING), have been proposed to contribute to recognition of *M. tuberculosis* (**Figure 1**). Studies of mouse models have identified TLR2, which recognizes lipoproteins and lipoglycans from *M. tuberculosis*, and TLR9, which recognizes unmethylated CpG DNA, as the most important TLRs for control of *M. tuberculosis* infection (Bafica et al., 2005; Feng et al., 2003; Reiling et al., 2002; Sugawara et al., 2003). Mice lacking both TLR2 and TLR9 are more susceptible than $TLR2^{-/-}$ or $TLR9^{-/-}$ single knockout mutants, suggesting that each TLR makes a nonredundant contribution to the immune response (Bafica et al., 2005). The importance of TLR sensing stems from production of inflammatory cytokines, in particular IL-12, which is necessary for priming IFN-γ-producing T cells that mediate control of *M. tuberculosis* infection (Bafica et al., 2005; Flynn et al., 1993; Scanga et al., 2004). In addition to TLRs, several CLRs have been proposed to play a role in immune recognition of *M. tuberculosis.* The cell wall glycolipid mannose-capped lipoarabinomannan can be recognized by DC-SIGN, mannose receptor, or Dectin-2 (Doz et al., 2007; Kang et al., 2005; Maeda et al., 2003; Yonekawa et al., 2014), and trehalose dimycolate can be recognized by Mincle or Marco (Bowdish et al., 2009; Yonekawa et al., 2014). However, experiments using mutant mice have suggested a limited role for individual CLRs (Court et al., 2010; Heitmann et al., 2013; Marakalala et al., 2011; Yonekawa et al., 2014), which may be partially explained by redundancy in function. Finally, the NLR NOD2, which senses small muramyl peptides derived from bacterial cell wall peptidoglycan, contributes to cytokine responses to *M. tuberculosis* in myeloid cells cultured in vitro (Coulombe et al., 2009; Ferwerda et al., 2005; Gandotra et al., 2007; Girardin et al., 2003; Juárez et al., 2012; Pandey et al., 2009; Yang et al., 2007). However, mice lacking NOD2 are largely resistant to infection, exhibiting modest susceptibility only six months after infection (Divangahi et al., 2008; Gandotra et al., 2007). The NLR NLRP3, a component of the inflammasome, is reviewed in the Section titled IL-1.

cGAS is a cytosolic DNA sensor that produces cyclic GMP-AMP (cGAMP) upon DNA binding (Ablasser et al., 2013; Wu et al., 2013). STING signaling is initiated by binding of cGAMP or other cyclic dinucleotides exported by pathogenic bacteria (Burdette et al., 2011; Diner et al., 2013; Woodward et al., 2010). STING induces expression of type I interferons, a family of cytokines that are detrimental to host control of *M. tuberculosis* infection (Ji et al., 2019; Manca et al., 2005; Mayer-Barber et al., 2011; Stanley et al., 2007). Activation of STING by *M. tuberculosis* and production of type I interferon require perforation of the vacuolar membrane by the ESX-1 type VII secretion system(Manzanillo et al., 2012). Three independent reports demonstrated that cGAS is required for type I interferon induction, suggesting that DNA is the pathogen-associated molecular pattern (PAMP) that leads to STING activation (Collins et al.,

2015; Wassermann et al., 2015; Watson et al., 2015). However, it was also reported that *M. tuberculosis* induces type I interferon by direct STING recognition of cyclic-di-AMP produced by the bacterium (Dey et al., 2015). Whereas TLRs, CLRs, and NLRs have been proposed to benefit the immune response to *M. tuberculosis* by promoting the production of proinflammatory cytokines and chemokines, the cGAS-STING pathway may be an example in which a bacterial pathogen engages an antiviral pathway to promote pathogenesis.

Innate cytokines

TNF-α. TNF-α was one of the first cytokines associated with tuberculosis and is crucial for control of infection. Macrophages and DCs are the primary producers of TNF-α during infection; however, TNF-α is also produced abundantly by CD4 T cells (Dorhoi and Kaufmann, 2014). Mice lacking TNF-α or the TNF receptor are highly susceptible to infection and exhibit poor activation of myeloid cells, a defect in chemokine production, and diffuse inflammation that lacks organized structure (Bean et al., 1999; Flynn et al., 1995; Kaneko et al., 1999; Roach et al., 2002). Evidence for the importance of TNF-α in human tuberculosis infection comes primarily from patients treated with anti-TNF agents for inflammatory disorders, who have a high propensity for reactivation of tuberculosis disease (Galloway et al., 2013; Keane et al., 2001; Xie et al., 2014). Nonhuman primate and mouse models support the idea that TNF-α is important for granuloma formation, structure, and integrity (Egen et al., 2008; Kindler et al., 1989; Reece et al., 2010). However, studies using the zebrafish model of infection with *Mycobacterium marinum*, which is particularly well-suited to studying granuloma formation (Myllymäki et al., 2016), have suggested that TNF-α maintains granuloma structure indirectly by restricting mycobacterial growth (Clay et al., 2008; Tobin et al., 2010); this has also been suggested by mouse studies (Di Paolo et al., 2015). Furthermore, the zebrafish model has demonstrated that excess TNF-α can lead to increased macrophage cell death, which promotes hyperinflammation and death of the host. This finding illustrates the concept that in innate immunity to tuberculosis, excessive production of protective factors can be detrimental (Roca and Ramakrishnan, 2013; Roca et al., 2019) (**Figure 2**).

GM-CSF. The cytokine granulocyte-macrophage colony-stimulating factor (GM-CSF) was originally implicated in myeloid cell and granulocyte differentiation. However, mice lacking the GM-CSF gene, *Csf2,* have normal steady-state myelopoiesis but lack alveolar macrophages (AMs) (Guilliams et al., 2013). Lungs of *Csf2*$^{-/-}$ mice exhibit a buildup of pulmonary surfactant due to impaired catabolism by AMs, as well as pulmonary lymphoid hyperplasia at baseline (Dranoff et al., 1994). GM-CSF levels rise in the lungs of wild-type mice for at least 60 days after *M. tuberculosis* infection, and *Csf2*$^{-/-}$ mice are highly susceptible to *M. tuberculosis*, succumbing rapidly after infection (Gonzalez-Juarrero et al., 2005). While nonhematopoietic cells are the primary producers of GM-CSF, *Csf2*$^{-/-}$ mice are partially rescued by adoptive transfer of wild-type but not *Csf2*$^{-/-}$ CD4 T cells, implying a minor role for T cell–derived GM-CSF (Rothchild et al., 2017). *Csf2*$^{-/-}$ mice have a defect in their production of inflammatory cytokines and chemokines in response to infection, resulting in impaired recruitment of both myeloid cells and T cells to the lungs (Gonzalez-Juarrero et al., 2005). *Csf2*$^{-/-}$

infected mice also exhibit a significant increase in bacterial burden in the lungs compared with wild-type mice, suggesting a potential antibacterial role for GM-CSF (Gonzalez-Juarrero et al., 2005). Indeed, addition of exogenous GM-CSF to *M. tuberculosis*–infected peritoneal macrophages and human monocytes results in enhanced control of infection (Rothchild et al., 2014, 2017). However, whereas treatment of wild-type mice with anti-GM-CSF neutralizing antibodies results in significant weight loss and larger granulomas in the lungs, it induces no change in lung colony-forming units, suggesting a role for GM-CSF in immune regulation rather than control of *M. tuberculosis* growth (Benmerzoug et al., 2018). A clear role for GM-CSF in activating the microbicidal capabilities of macrophages in vivo has yet to be demonstrated. Furthermore, the fact that *Csf2*$^{-/-}$ mice have baseline alterations in lung function complicates the interpretation of results from these mice (Bryson et al., 2019; Chroneos et al., 2009; Rothchild et al., 2014).

IL-1. The first interleukin to be described was IL-1, discovered as a potent modulator of innate immunity. The IL-1 family members IL-1α and IL-1β, are produced during infection with *M. tuberculosis* by inflammatory monocyte-macrophages, inflammatory DCs, and neutrophils (Juffermans et al., 2000; Mayer-Barber et al., 2011). They play critical and nonredundant protective roles early during infection, despite signaling through the same receptor. Neutralization of both IL-1α and IL-1β has a more significant impact on morbidity after infection than neutralization of either protein individually (Guler et al., 2011). Similarly, mice doubly deficient for IL-1α and IL-1β (*Il1a*$^{-/-}$/ *Il1b*$^{-/-}$) are more susceptible to *M. tuberculosis* infection and show higher bacterial burdens in the lung compared to mice lacking the individual cytokines (Bourigault et al., 2013; Di Paolo et al., 2015; Mayer-Barber et al., 2011). The protective function of IL-1 is further confirmed by blocking receptor signaling with anti-IL-1R antibodies or in an *Il1r*$^{-/-}$ mouse model; the mice become highly susceptible to *M. tuberculosis* infection and show increased bacterial burden in the lungs (Guler et al., 2011; Ji et al., 2019; Juffermans et al., 2000; Mayer-Barber et al., 2010, 2011). Interestingly, loss of IL-1 signaling does not result in diminished TNF-α, IL1-2p40, inducible nitric oxide synthase (iNOS), or IFN-γ responses (Mayer-Barber et al., 2011). In other bacterial infections, the protective function of IL-1 is often ascribed to recruitment of neutrophils; however, neutrophils are not known to be protective in the context of tuberculosis, and it remains unclear why IL-1 signaling is critical for resistance. IL-1R signaling in *trans* by infected bystander cells is sufficient to induce restriction of intracellular bacterial growth in infected myeloid cells that lack IL-1R (Bohrer et al., 2018), suggesting that IL-1 promotes production of a soluble protective factor. Finally, a protective role of IL-1 during human tuberculosis infection has been suggested based on case studies in which rheumatoid arthritis patients treated with IL-1R antagonist anakinra occasionally showed reactivation of tuberculosis (Brassard et al., 2006; Settas et al., 2007).

IL-1β is produced as a precursor protein and is cleaved into a mature form by inflammasome and caspase-1 activation and then released to act systemically. Unlike the case of IL-1β, IL-1α activity does not require proteolytic processing by caspase-1. The main inflammasome that becomes activated upon in vitro infection with *M. tuberculosis* appears to be NLRP3. This requires the ESX-1 secretion system (Abdalla et al., 2012; Carlsson et al., 2010; Dorhoi et al., 2012; Mishra et al., 2010; Wong and Jacobs, 2011), although the exact mechanism remains controversial. Despite detection

of *M. tuberculosis* by the inflammasome, *Nlrp3*$^{-/-}$, *Asc*$^{-/-}$, and *Casp1*$^{-/-}$ mice are not nearly as susceptible to infection as mice deficient in IL-1α and/or IL-1β or IL-1R (Dorhoi et al., 2012; Fremond et al., 2007; Mayer-Barber et al., 2010; McElvania Tekippe et al., 2010; Saiga et al., 2012; Walter et al., 2010). Furthermore, IL-1β production is still present in *Nlrp3*$^{-/-}$ or *Casp1*$^{-/-}$ mice (Dorhoi et al., 2012; Mayer-Barber et al., 2010), indicating that pro-IL-1β can be processed and released through an inflammasome-independent mechanism (Greten et al., 2007; Guma et al., 2009; Joosten et al., 2009; Karmakar et al., 2012). An excess of IL-1 has been linked to an increased influx of neutrophils and lung inflammation, which result in high bacterial burden and mortality (Mishra et al., 2013) (**Figure 2**). However, IL-1α and IL-1β regulation is complex, and postsecretion, their activity is controlled further by the presence of IL-1R antagonist (IL-1Ra), complicating the interpretation of IL-1 protein levels. For example, *Sst1*^{S} mice, which are highly susceptible to *M. tuberculosis* infection, have elevated levels of IL-1 protein in the lungs during infection. However, they also have high levels of IL-1Ra, which limit IL-1 activity, causing a functional deficiency in IL-1 signaling and increased *M. tuberculosis* susceptibility (Ji et al., 2019).

Type I interferons. Type I interferons comprise a family of cytokines that signal through the interferon receptor to induce interferon-stimulated genes. Most cell types produce type I interferon upon stimulation of cytosolic DNA or RNA sensors that normally sense cytosolic viruses or through signaling via specific TLRs. In the case of *M. tuberculosis* infection, type I interferon is induced when the ESX-1 secretion system perforates the vacuolar membrane, leading to activation of the cGAS/STING pathway (Manzanillo et al., 2012; Stanley et al., 2007; Watson et al., 2012). Although type I interferon is critical for resistance to viral infections, the effect of type I interferon during *M. tuberculosis* infection is primarily detrimental. Tuberculosis patients with active disease show a distinct upregulation of type I interferon–inducible transcripts in blood neutrophils and monocytes. This gene expression profile correlates with disease severity and may predict the transition from active to latent disease (Berry et al., 2010; Maertzdorf et al., 2011; Moreira-Teixeira et al., 2020; Scriba et al., 2017; Singhania et al., 2018; Zak et al., 2016). In mice, type I interferon is detrimental to *M. tuberculosis* infection; however, the severity of the phenotype appears to be background dependent. In C57BL/6 wild type mice, loss of the type I IFN receptor or other signaling components results in only modest enhancement of control of infection (Cooper et al., 2000a; Desvignes et al., 2012; Dorhoi et al., 2014; Ji et al., 2019; Stanley et al., 2007; Yamashiro et al., 2020). However, if these mice are stimulated to produce higher levels of type I interferon than are naturally produced during *M. tuberculosis* infection by administration of intranasal TLR3 ligand poly-ICLC, increased lung pathology and mortality during *M. tuberculosis* infection is observed, demonstrating that increasing type I interferon levels in the C57BL/6 background results in highly impaired immunity (Antonelli et al., 2010). Furthermore, the susceptibility of B6.*Sst1*^{S} congenic mice that carry the tuberculosis susceptibility allele of the *Sst1* locus derived from the highly susceptible C3H/HeBFeJ strain was recently shown to be primarily driven by type I interferon, as crossing these mice with *Ifnar*$^{-/-}$ mice alleviated the exacerbated disease (Ji et al., 2019). Type I interferons inhibit IL-1 signaling indirectly through strong upregulation of IL-1Ra expression during *M. tuberculosis* infection (Ji et al., 2019). Blocking IL-1Ra in B6.*Sst1*^{S} mice restores IL-1 protective signaling and rescues the

type I interferon–induced susceptibility to infection, suggesting that the type I interferon–based susceptibility observed in these mice is almost entirely explained by inhibition of IL-1 signaling (Ji et al., 2019). Although several mechanisms by which type I interferons inhibit host defenses have been proposed, including modulating eicosanoids, iNOS production, and IL-10 (Ji et al., 2019; Mayer-Barber et al., 2011, 2014; McNab et al., 2014; Novikov et al., 2011), it is likely that the primary impact of type I interferon on *M. tuberculosis* immunity is to impair the production of IL-1, which is critical for protection against infection. Despite deleterious effects of high levels of type I interferon on the host immune response, it is possible that type I interferon is protective in some contexts, particularly in the absence of IFN-γ. The balance of deleterious and protective responses of type I interferon is further reviewed by Moreira-Teixeira et al. (Moreira-Teixeira et al., 2018).

IL-10. IL-10 is an anti-inflammatory cytokine that downregulates both innate and adaptive immune responses. Pulmonary tuberculosis patients have elevated levels of plasma IL-10, and their T cells exhibit both enhanced *Il10* expression and evidence of IL-10 stimulation (Harling et al., 2018; Olobo et al., 2001). Studies of the role of IL-10 in mice have yielded mixed results, likely reflecting the complex role of IL-10 and other immunosuppressive cytokines in infection. One study showed that *Il10*$^{-/-}$ C57BL/6 mice experience a significant increase in bacterial numbers in the lungs and increased mortality starting late in infection (Higgins et al., 2009), while another study found that *Il10*$^{-/-}$ mice on the C57BL/6 and BALB/C backgrounds have reduced bacterial burdens in the lungs during the late stage of infection (Redford et al., 2010). CBA/J mice, which are highly susceptible to *M. tuberculosis*, are clearly impacted by IL-10 deficiency, as *Il10*$^{-/-}$ mice on this background exhibit lower bacterial numbers in the lungs and spleen throughout the course of infection when compared with wild-type (Cyktor et al., 2013). Furthermore, treatment of CBA/J mice with an anti-IL-10R blocking antibody during the chronic stage of *M. tuberculosis* infection lowers bacterial numbers in the lungs and improves survival compared to untreated CBA/J mice (Beamer et al., 2008). The seemingly contradictory results in the mouse model likely reflect the fact that IL-10 has a context-dependent role in infection; while it can contribute to restraining detrimental inflammation in the context of a potential hyperinflammatory response (such as CBA/J mice), it can also harm the host by suppressing effective responses.

TGF-β. Transforming growth factor β (TGF- β) is an immunosuppressive cytokine that plays a crucial role in immune homeostasis and peripheral tolerance. TGF-β is known to have a suppressive effect on cells that play a key role in regulating *M. tuberculosis* infection, including macrophages, dendritic cells, neutrophils, and T cells (reviewed in (Batlle and Massagué, 2019). High levels of TGF-β are found in the lungs of patients with active pulmonary tuberculosis (Bonecini-Almeida et al., 2004; Toossi et al., 1995) and serum levels of TGF-β correlate with disease severity (Fiorenza et al., 2005). Similarly, high TGF-β levels are associated with active diseases in murine and in non-human primate models, where successful antibiotic therapy is results in diminished TGF-β levels (DiFazio et al., 2016; Rook et al., 2007). Although production of TGF-β is crucial to prevent hyper-inflammation and autoimmunity (Batlle and Massagué, 2019), several lines of evidence suggest that TGF-β may suppress effective immune responses to *M. tuberculosis* to the detriment of the host. In mice, blocking TGF-β signaling using neutralizing antibody, recombinant TGF-β receptor, or small molecule

inhibitors results in increased control of disease as measured by bacterial burden in the lungs (Hernández-Pando et al., 2006; Jayaswal et al., 2010). A recent study suggests that the specific mechanism by which TGF-β suppresses host immunity is by preventing CD4 T cells from producing IFN-γ in granuloma cores, thereby limiting effective macrophage activation (Gern et al., 2021). Thus, pharmacological inhibition of TGF-β may be an attractive strategy for managing patients with active TB disease.

Macrophage-based mechanisms of innate control

Macrophages are programmed to detect invading pathogens, activate microbicidal mechanisms, and coordinate the subsequent immune response. However, in the absence of adaptive immunity, macrophages are not capable of controlling *M. tuberculosis* infection. Although for many years it was speculated that *M. tuberculosis* resisters (individuals whose purified protein derivative (PPD) and IFN-γ release assay (IGRA) results never convert despite considerable exposure to *M. tuberculosis*) were able to clear infection via innate immunity, deeper immunological analysis of these individuals revealed the existence of class-switched antibodies, solid evidence of an adaptive response to infection (Li et al., 2017). Indeed, in both mouse and nonhuman primate models, growth of *M. tuberculosis* is unrestricted in macrophages until the arrival of CD4 T cells in the lungs (Cadena et al., 2016; Mogues et al., 2001). The primary role of CD4 T cells in macrophage activation is understood to be the production of IFN-γ, which can directly activate macrophages to control infection (Flynn et al., 1993). In addition, there appear to be IFN-γ-independent mechanisms that have yet to be identified (Gallegos et al., 2011). Although several decades of research have focused on understanding the cell-intrinsic mechanisms of bacterial killing downstream of macrophage activation, recent revisions in our understanding of the functions of antimicrobial responses have left holes in our knowledge of effectors that have direct antimicrobial activity (**Figure 2**).

Autophagy. Autophagy (self-eating) is a conserved cellular process with important roles in homeostasis, development, and metabolism. In addition, it is well-established that a form of selective autophagy known as xenophagy is a major contributor to innate immune defense against microbial infections (reviewed in (Bah and Vergne, 2017). The first evidence for an antimycobacterial effect of autophagy was the observation that starvation or rapamycin treatment leads to restriction of *M. tuberculosis* growth in RAW macrophages (Gutierrez et al., 2004). Subsequently it was found that autophagic targeting of *M. tuberculosis* occurs as a response to perforation of the phagosome by the bacterial ESX-1 secretion system and stimulation of cGAS-STING (Collins et al., 2015; Dey et al., 2015; Watson et al., 2012, 2015). TBK1 activation downstream of STING leads to ubiquitin-mediated autophagic targeting of the *M. tuberculosis*–containing phagosome. The E3 ubiquitin ligases Parkin and Smurf promote autophagic targeting of *M. tuberculosis*, and mice deficient in these factors are susceptible to *M. tuberculosis* (Franco et al., 2017; Manzanillo et al., 2013). In addition, *Atg5*$^{fl/fl}$*Lyz2Cre* mice, which lack the core autophagy effector ATG5 in myeloid cells, are hypersusceptible to *M. tuberculosis* (Castillo et al., 2012; Watson et al., 2012). However, subsequent detailed analyses of autophagy-deficient mice have shown the role of autophagy to be complex. While *Atg5*$^{fl/fl}$*Lyz2Cre* mice succumb rapidly to *M.*

tuberculosis, mice deficient in other core autophagy effectors have no significant weight loss or inability to restrict bacterial replication through several months of infection (Kimmey et al., 2015). The susceptibility of *Atg5*$^{fl/fl}$*Lyz2Cre* mice is rescued by depletion of neutrophils, and much of the susceptibility is recapitulated in *Atg5*$^{fl/fl}$*Mrp8Cre* mice, which lack ATG5 specifically in neutrophils (Kimmey et al., 2015). This suggests there is a unique role for ATG5 in the regulation of inflammation and neutrophil recruitment, discrete from its role in autophagic targeting of bacteria. Taken together, these findings provide significant evidence that autophagy plays a role in *M. tuberculosis* infection but not all effects are intrinsic to the macrophage.

Vitamin D and cathelicidin. Vitamin D has been used to treat tuberculosis since the mid-1800s. Multiple cohort studies show an association between low serum vitamin D levels and tuberculosis disease risk (Aibana et al., 2019; Wilkinson et al., 2000). However, clinical trials have not clearly demonstrated that vitamin D treatment of tuberculosis patients already receiving antibiotics improves outcomes (Jolliffe et al., 2019; Wu et al., 2018). In vitro treatment of *M. tuberculosis*–infected cells with vitamin D restricts growth of the bacteria, indicating that vitamin D leads to cell-intrinsic control of *M. tuberculosis* (Campbell and Spector, 2012; Liu et al., 2006). A major effect of vitamin D treatment in *M. tuberculosis*–infected human monocytes is expression of the cathelicidin antimicrobial peptide LL-37 (Liu et al., 2006; Rao Muvva et al., 2019). LL-37 has antibacterial activity against *M. tuberculosis* in liquid culture (Rivas-Santiago et al., 2013), and administration of LL-37 to *M. tuberculosis*–infected mice starting 60 days postinfection significantly reduced the bacterial load in the lungs (Rivas-Santiago et al., 2013). *Cramp*$^{-/-}$ mice, which lack the gene for murine cathelicidin, have enhanced mortality and a defect in controlling bacterial growth after *M. tuberculosis* infection compared to wild-type mice (Gupta et al., 2017). Importantly, in other studies researchers have observed no effect of exogenous vitamin D on *M. tuberculosis* growth in human monocytes (Fabri et al., 2011; Vogt and Nathan, 2011). Thus, although low vitamin D levels may correlate with tuberculosis disease, whether the main function of vitamin D is to activate microbicidal mechanisms remains unclear.

Reactive oxygen species. The production of reactive oxygen species (ROS) is a crucial defense against phagocytosed pathogens. The production of ROS is initiated by the NADPH oxidase complex, which catalyzes the production of superoxide. Through a series of reactions, multiple other ROSs are then produced, including hydrogen peroxide, hypochlorous acid, and hydroxyl radicals. Data suggest that NADPH oxidase is required for control of tuberculosis in humans. Patients with chronic granulomatous disease (CGD), who have inherited defects in NADPH oxidase, have presented with active tuberculosis or disseminated bacillus Calmete-Guérin (BCG) disease upon vaccination (Bustamante et al., 2011). Macrophages isolated from a CGD patient were unable to control growth of *M. tuberculosis*, suggesting that ROSs are important for cell-intrinsic control of *M. tuberculosis* infection in humans (Khan et al., 2016). However, studies on the role of ROSs in control of *M. tuberculosis* in the mouse model are inconclusive. Mice lacking components of NADPH oxidase display no increase in bacterial burden in the lungs or, at most, a mild and transient increase (Cooper et al., 2000b; Olive et al., 2018). A recent study in the mouse model revealed a potential immunoregulatory role for ROSs, independent of bactericidal potential. While *Cybb*$^{-/-}$ mice, which lack the NADPH oxidase component gp91, are able to control *M.*

tuberculosis growth similar to wild-type mice, they experience greater weight loss and have a significant increase in mortality associated with excessive neutrophil recruitment (Olive et al., 2018). Blocking IL-1 signaling in *Cybb*$^{-/-}$ mice reduces neutrophil infiltration and rescues susceptibility, demonstrating that ROSs may limit harmful inflammation (Olive et al., 2018).

iNOS. The importance of IFN-γ during *M. tuberculosis* infection has been attributed to its ability to activate microbicidal mechanisms of macrophages, most importantly expression of the enzyme iNOS, encoded by the gene *Nos2* (MacMicking et al., 1997a). iNOS catalyzes the production of the bactericidal/static radical nitric oxide (NO). Human tuberculosis patients exhibit iNOS expression in the lungs and are known to exhale NO, confirming that this molecule is produced during human *M. tuberculosis* infection (Idh et al., 2008; Nathan, 2002; Nicholson et al., 1996). The importance of NO for control of *M. tuberculosis* infection is clear, as *Nos2*$^{-/-}$ mice are extremely susceptible to infection (MacMicking et al., 1997b). However, studies of mixed bone marrow chimeras that examined different genotypes in the same inflammatory environment have demonstrated no difference in *M. tuberculosis* burden in wild-type and *Nos2*$^{-/-}$ cells, raising the possibility that NO does not function in a cell intrinsic manner for control of bacterial numbers. Indeed, it has been proposed that NO limits IL-1β production by two mechanisms. First, by nitrosylation and inhibition of the NLRP3 inflammasome, NO may limit neutrophil recruitment and subsequent destruction of host tissue (Mishra et al., 2013, 2017). Depleting neutrophils in *Nos2*$^{-/-}$ mice rescues the increase in bacterial burden in the lungs at 24 days after infection (Mishra et al., 2013). Second, NO may also limit IL-1β transcription by inhibiting NF-κB signaling (Braverman and Stanley, 2017). However, the facts that the ability to resist NO is an important virulence trait for *M. tuberculosis* (Darwin et al., 2003) and that iNOS-deficient macrophages suffer from increased bacterial burdens in vitro make it clear that NO can impact cell-intrinsic antimicrobial activity of macrophages, independent of the inflammatory context. Thus, a role for NO in cell-intrinsic control of infection in vivo cannot be ruled out, and there is likely more to learn about the contribution of NO to control of infection in vivo.

Interferon-inducible GTPases. Interferon-inducible GTPases are a family of proteins that encompass myxovirus resistance proteins (Mxs), guanylate-binding proteins (GBPs), immunity-related guanosine triphosphatases (IRGs), and very large inducible GTPase proteins (VLIGs). Both GBPs and IRGs are IFN-γ-inducible proteins that have been implicated in mycobacterial infections. Almost a decade ago, it was demonstrated that Gbp1 is required for control of *Mycobacterium bovis* BCG infection in vivo (Kim et al., 2011). However, mice with a chromosomal deletion that removes six GBPs, including GBP1, are only mildly susceptible to *M. tuberculosis*, with a modest increase in bacterial burden emerging at 100 days after infection (Ahmed et al., 2020). A gene expression signature associated with the transition from latent to active disease contains *GBP1*, providing some relevance to human disease (Plumlee et al., 2020; Zak et al., 2016). Although the IRG family member Irgm1 was shown to mediate host resistance to *M. tuberculosis* in mice (MacMicking et al., 2003), the significance of this finding is difficult to interpret in light of the emerging understanding that these knockout mice exhibit baseline alterations in immunity (Hunn and Howard, 2010). Nonetheless, results from human studies suggest a role for IRGM1 in resistance to *M. tuberculosis*

(reviewed in (Kim et al., 2012). Therefore, more research is needed into a potential role for these proteins in antituberculosis immunity.

Aerobic glycolysis and metabolic regulation of infection. The metabolic program of aerobic glycolysis is associated with differentiation of macrophages into the M1 phenotype. It is now understood that changes in levels of metabolites during aerobic glycolysis impact specific programs of gene expression and cellular differentiation. *M. tuberculosis*–infected macrophages transition to aerobic glycolysis, and this transition is required for effective control of bacterial growth (Braverman et al., 2016; Gleeson et al., 2016). During *M. tuberculosis* infection, aerobic glycolysis impacts gene expression by promoting the activity of the transcription factor hypoxia-inducible factor 1α (HIF-1α) (Tannahill et al., 2013). HIF-1α is a crucial mediator of IFN-γ-dependent immunity required for host defense against *M. tuberculosis* and is essential for expression of inflammatory cytokines, production of host-protective eicosanoids, and cell-intrinsic control of bacterial replication (Braverman et al., 2016). How HIF-1α and/or aerobic glycolysis promote cell-intrinsic control of infection is yet unknown.

Cell death and eicosanoids

There are multiple mechanisms by which the host cell can undergo cell death during *M. tuberculosis* infection, and the field has coalesced around a paradigm in which apoptotic death benefits the host whereas necrotic death benefits the bacterium. However, this paradigm is based largely on in vitro experiments and is difficult to establish conclusively, as there is no experimental means to selectively eliminate either form of death in vivo without also affecting other parameters of the immune response. In general, several attenuated strains and mutants of *M. tuberculosis* have been found to induce apoptotic cell death in macrophages (Chen et al., 2006; Duan et al., 2001; Lee et al., 2011). Apoptotic cells can be phagocytosed by DCs and subsequently stimulate T cell priming and activation (Blomgran et al., 2012; Divangahi et al., 2010; Hinchey et al., 2007; Schaible et al., 2003; Winau et al., 2006). Efferocytosis of apoptotic cells by uninfected macrophages is thought to result in killing of bacteria through fusion of the efferocytic phagosome with lysosomes, and macrophage apoptosis is therefore considered beneficial for host survival (Blomgran et al., 2012; Martin et al., 2012). In contrast, macrophages infected with virulent *M. tuberculosis* undergo necrosis (Chen et al., 2006; Divangahi et al., 2009). Recent findings with a zebrafish model of *Mycobacterium marinum* infection showed that excess TNF-α can induce necrosis through interaction of multiple signaling pathways, including activation of RIP kinases, production of mitochondrial ROSs, and subsequent activation of cyclophilin D(Roca et al., 2019). TNF-α was also implicated in apoptosis induced by eicosanoid synthesis (Chang et al., 1992). Production of the eicosanoid PGE_2 promotes apoptosis in macrophages infected with avirulent *M. tuberculosis* (Chen et al., 2008). In contrast, PGE_2 production is inhibited by LXA_4, which is induced during infection with virulent strains and leads to necrosis. Mice lacking prostaglandin E synthase (*Ptges*$^{-/-}$) show a higher bacterial burden in the lungs, whereas *Alox5*$^{-/-}$ mice, which are unable to synthesize certain eicosanoids (including LXA_4 and LTB_4), are more resistant to *M. tuberculosis* infection (Divangahi et al., 2009). This suggests that PGE_2 has a protective effect against virulent *M. tuberculosis*. However, whether this effect is mediated through

regulation of cell death or regulation of inflammation is unclear. PGE_2 has both pro- and anti-inflammatory functions, including the regulation of cytokine expression in DCs and T cell differentiation (Ricciotti and FitzGerald, 2011). In addition, lipoxins have been described as negative regulators of acute inflammatory processes and together with PGE_2 regulate neutrophil recruitment (Levy et al., 2001; Mishra et al., 2017). Interestingly, polymorphisms in the promoter region of leukotriene A4 hydrolase (*lta4h*), which catalyzes the production of eicosanoid LTB_4, have been associated with mortality and response to anti-inflammatory treatment in patients with tuberculosis meningitis, further supporting the notion that eicosanoids are important for regulating inflammatory processes (Thuong et al., 2017; Tobin et al., 2012).

Other innate cells

Neutrophils in host defense. Polymorphonuclear neutrophils are short-lived cells of the innate immune response that are highly abundant during bacterial infections. Neutrophils possess a potent antimicrobial arsenal effective against many bacterial and fungal pathogens (Segal, 2005). In the case of *M. tuberculosis*, while there is some evidence that neutrophils participate in protective immunity, a clear role in host defense has yet to be defined. In some settings, they may promote *M. tuberculosis* infection. Recruitment of neutrophils to the lung after *M. tuberculosis* infection is rapid and is mediated through multiple chemokines, including IL-17, CXCL5, and KC, and by eicosanoids produced by 12/15-lipoxygenase (Gopal et al., 2013a; Mishra et al., 2017; Nouailles et al., 2014). Neutrophils take up bacteria in vivo (Blomgran and Ernst, 2011; Blomgran et al., 2012; Mishra et al., 2017). However, studies examining whether neutrophils are able to effectively kill phagocytosed *M. tuberculosis* are inconclusive and contradictory (Lowe et al., 2012), in part because of the difficulty of working with primary neutrophils ex vivo and the paucity of appropriate cells lines for neutrophil research. Studies of neutrophil function in vivo are also inconclusive. Neutrophils harboring bacteria may die by apoptosis, which is followed by efferocytosis by resident macrophages, possibly facilitating control of infection (Martin et al., 2012). Alternatively, it has also been proposed that neutrophils are a permissive niche for growth and persistence in vivo (Eruslanov et al., 2005; Lovewell et al., 2020). Separate from their ability to kill bacteria, neutrophils may have an influence on priming of adaptive immunity. Depletion of neutrophils at early stages of infection in resistant mouse strains has yielded differing results, with some studies finding no impact and other studies finding that depletion of neutrophils compromises host defense (Blomgran and Ernst, 2011; Scott et al., 2020).

Destructive inflammation mediated by neutrophils. In human tuberculosis, neutrophils are generally associated with active disease, caseous necrosis, and exacerbated pathogenesis (Hunter, 2011), and neutrophils may be drivers of the pathology associated with active disease. Indeed, animal models established that excessive accumulation of neutrophils in the lungs drives destructive inflammation and susceptibility to infection. Furthermore, the phenotypes of many mice known to be susceptible to *M. tuberculosis* infection, including *Nos2*$^{-/-}$, *Atg5*$^{-/-}$, *Irg1*$^{-/-}$, and *Card9*$^{-/-}$ mice, can be at least partially rescued by depletion of neutrophils (Dorhoi et al., 2010; Kimmey et al., 2015; Mishra et al., 2017; Nair et al., 2018). These data suggest that

defects in immunity resulting from disparate perturbations lead to a common pathway of neutrophil-driven susceptibility (**Figure 2**). However, many questions remain. First, it is unclear whether neutrophils are a common driver of susceptibility in humans. Second, the mechanisms by which neutrophils are recruited to excess under specific conditions, and how they drive destructive inflammation, are unclear. Finally, it is possible that neutrophils are in fact more heterogeneous in tuberculosis disease than is currently appreciated and that specific subsets of neutrophils participate in host defense, whereas others contribute to pathology.

Alveolar macrophages and innate cells during early infection. AMs are a subset of tissue-resident macrophages that reside within the lung airspace and play crucial roles in lung homeostasis, surfactant metabolism, and tissue repair (Puttur et al., 2019). AMs are the first cell type to encounter *M. tuberculosis*. Studies of human AM infections are difficult, as the AM phenotype is programmed and maintained in the tissue niche and is rapidly lost in cell culture (Papp et al., 2018). In mice, a productive *M. tuberculosis* infection starts with infection of AMs that reside in the lung alveoli (Cohen et al., 2018; Rothchild et al., 2019). Depletion of AMs with liposome-encapsulated dichloromethylene diphosphanate prior to infection reduces the bacterial burden in the lungs and increases survival, suggesting that AMs form a replicative niche early after infection (Huang et al., 2018; Leemans et al., 2001). Indeed, infected AMs initially exhibit an anti-inflammatory NRF2-depdendent antioxidant response (Guirado et al., 2018; Huang et al., 2018; Rothchild et al., 2019). Approximately 10 days after infection is initiated, AMs exhibit a more proinflammatory transcriptional state that precedes their transition from the airway into the pulmonary interstitium at approximately 14 days postinfection (Cohen et al., 2018; Rothchild et al., 2019). AMs in the interstitium localize in infectious foci, a process that is mediated by IL-1R signaling in nonhematopoietic cells (Cohen et al., 2018). At two weeks, AMs appear to be the predominant infected cell type in the lung (Repasy et al., 2013; Wolf et al., 2007). Shortly thereafter, however, *M. tuberculosis* disseminates to monocyte-derived cells and neutrophils (Huang et al., 2018; Lee et al., 2020). Interstitial macrophages show a glycolytic transcriptional profile, express iNOS and IL-1, and restrict intracellular growth of *M. tuberculosis* more efficiently than AMs (Huang et al., 2018). In addition, mycobacterial growth in the lungs appears to be sustained by a constant influx of new monocytes into the lungs(Norris and Ernst, 2018). Thus, although airway AMs are a more permissive niche for growth early after infection, *M. tuberculosis* replication in the lungs can be sustained through dynamic infection of new monocytes that provide *M. tuberculosis* with new cellular niches that become rapidly infected.

Dendritic cells. DCs bridge innate and adaptive immunity, traveling from sites of infection and inflammation to secondary lymphoid tissues for activation of T cells. Both classical/resident and monocyte-derived DCs are present in the lungs during *M. tuberculosis* infection (Marino et al., 2004). Antibody-based depletion of $CD11c^{+}$ cells, which transiently eliminates both classical and monocyte-derived DCs, results in defective CD4 T cell priming and increased susceptibility to *M. tuberculosis* infection, demonstrating the importance of DCs for host defense (Tian et al., 2005). Several studies using $CCR2^{-/-}$ mice have suggested that inflammatory monocytes, and not DCs, may be responsible for trafficking *M. tuberculosis* to the draining lymph nodes for activation of T cell responses (Peters et al., 2001, 2004). However, a more recent study

using diphtheria toxin to selectively ablate CCR2 at different stages of infection found that while interstitial macrophages traffic bacteria to the draining lymph nodes, classical DCs are largely responsible for priming CD4 T cell responses (Samstein et al., 2013). Human data have suggested that the onset of adaptive immunity to *M. tuberculosis* is significantly delayed (Miller et al., 2008; Poulsen, 1950; Wallgren, 1948). Indeed, data from both mice and nonhuman primates have clearly demonstrated that the priming of T cells in draining lymph nodes is delayed during *M. tuberculosis* infection relative to other infections (Blomgran et al., 2012; Garcia-Romo et al., 2013; Madan-Lala et al., 2014; Marino et al., 2004; Wolf et al., 2008), although limited antigen availability due to *M. tuberculosis* slow replication rate and low infectious dose may be a confounding factor. Importantly, experimental perturbations that result in more rapid priming of effector T cells, either through BCG vaccination, dendritic cell vaccination, or adoptive transfer, results in more effective control of *M. tuberculosis* infection (Gallegos et al., 2008; Griffiths et al., 2016; Khader et al., 2007).

Natural killer cells. Natural killer (NK) cells are innate lymphocytes present in both lymphoid and nonlymphoid tissues that play a major role in defense against viral infection. In human tuberculosis, a reduction in the number of NK cells or in their expression of activation markers correlates with loss of control and the transition to active disease (Bozzano et al., 2009; Roy Chowdhury et al., 2018). Furthermore, changes in peripheral blood NK cell levels correlate with disease progression and treatment response, and they inversely correlate with lung inflammation in tuberculosis patients across multiple independent cohorts (Roy Chowdhury et al., 2018). However, whether these studies indicate a functional role for NK cells in the immune response is unclear. Although the exact ligands are unknown, NK cells are capable of detecting *M. tuberculosis*–infected macrophages through activating receptors (e.g., NKp46, NKG2D) (Vankayalapati et al., 2005). Human and mouse NK cells produce perforin and granulysin, are capable of killing *M. tuberculosis*–infected cells through a contact-dependent mechanism (Brill et al., 2001; Denis, 1994; Lu et al., 2014), and produce IFN-γ during infection. *M. tuberculosis*–infected mice show an increase in NK cell numbers in the lungs within 21 days (Desvignes et al., 2012; Junqueira-Kipnis et al., 2003). NK cell depletion does not result in an increase in bacterial growth in the lungs in C57BL/6 mice (Junqueira-Kipnis et al., 2003), indicating that these cells are not critical for restricting the bacterial burden. However, depletion of NK cells or IFN-γ in $RAG^{-/-}$ mice further increases the susceptibility of these mice to *M. tuberculosis* infection (Feng et al., 2006).

Nonclassical T cells. Nonclassical T cells, including mucosal-associated invariant T (MAIT) cells and γδ T cells, span innate and adaptive immunity. Their T cell receptor repertoire is highly limited, often recognizing PAMPs, and they participate in rapid innate-like effector responses. MAIT and γδ T cells have been frequently associated with tuberculosis; however, their role during infection remains unclear. MAIT cells are activated by intermediates of bacterial riboflavin biosynthesis that bind to the highly conserved major histocompatibility complex–related 1 (MR1) molecule(Howson et al., 2015). Most bacterial species, including *M. tuberculosis*, synthesize riboflavin and therefore activate MAIT cells. Once activated, individual MAIT cell subsets can produce different combinations of inflammatory/T helper 1 (Th1) cytokines and can kill infected cells through the release of cytotoxic granules (Howson et al., 2015). In nonhuman

primates, tetramer-restricted MAIT cells accumulate in the airways but not inside granulomas and only show minimal expression of granzyme B or the proliferation marker Ki76, suggesting that MAIT cells are not essential contributors to *M. tuberculosis* restriction in macaques (Ellis-Connell et al., 2020; Kauffman et al., 2018a). Mice lacking MR1 are susceptible to infection with BCG and *M. tuberculosis* (Sakala et al., 2015). BCG induces MAIT cell formation in BCG-vaccinated humans and nonhuman primates (Greene et al., 2017; Suliman et al., 2019). Furthermore, MAIT cells have activity against BCG-infected macrophages (Chua et al., 2012). However, it is unclear whether induction of MAIT cells contributes to the efficacy of BCG and what role MAIT cells play in human *M. tuberculosis* infection.

γδ T cells expand early during *M. tuberculosis* infection(Janis et al., 1989; Vorkas et al., 2018). Furthermore, tuberculosis patients have a higher proportion of IL-17-producing γδ T cells compared to healthy controls (Peng et al., 2008). Human γδ T cell clones derived from peripheral blood mononuclear cells respond to live *M. tuberculosis* and to *M. tuberculosis* lysate in vitro (Tanaka et al., 1994). Both AMs and monocytes activate and induce expansion of γδ T cells (Balaji et al., 1995). Activated γδ T cells can produce IFN-γ in response to *M. tuberculosis* and are cytotoxic to infected monocytes, macrophages, and extracellular bacteria due to release of perforin and granulysin (Dieli et al., 2001; Tsukaguchi et al., 1995). C57BL/6 mice deficient for T cell receptor (TCR) δ chain lack γδ T cells and show a transient higher bacterial burden early in infection compared to control mice. Interestingly, TcR-$\delta^{-/-}$ mice show control of low-dose infection at later time points but eventually succumb to high-dose infections (Ladel et al., 1995). The most abundant population of γδ T cells in humans are Vγ9Vδ2 T cells that recognize HMBPP, an intermediate of the nonmevalonate pathway of isoprenoid biosynthesis (Chen, 2013; De Libero et al., 1991; Panchamoorthy et al., 1991). Vγ9Vδ2 T cells activated by BCG are able to protect against *M. tuberculosis* infection in a macaque model (Shen et al., 2002). Furthermore, using *Listeria monocytogenes* as a vaccine platform to stimulate Vγ9Vδ2 T cells effectively protects against *M. tuberculosis* infection in primates (Shen et al., 2019), demonstrating the potential of γδ T cells for vaccine-elicited control of infection. However, whether they play an important role in containing natural human infection remains unclear.

Harnessing the innate immune response

Innate immunity and adjuvant development for protein subunit vaccines.

One of the most important practical applications of understanding innate immunity to *M. tuberculosis* is the rational design of novel vaccines. The current vaccine strain BCG is widely administered due to its efficacy in preventing severe manifestations of childhood tuberculosis; however, it has limited efficacy against adult pulmonary tuberculosis. Recently, the M72/AS01$_E$ protein subunit vaccine demonstrated 50% efficacy in preventing reactivation disease in previously BCG-vaccinated adults, providing some of the first concrete evidence that vaccines other than BCG can enhance naturally acquired immunity to tuberculosis (Van Der Meeren et al., 2018). Formulating novel vaccines with optimized adjuvant and antigen combinations could improve upon this efficacy, raising the exciting possibility of a truly effective vaccine for tuberculosis. Recent years have witnessed a major leap forward in the development of novel

adjuvant systems, including alum and emulsions, TLR agonists, STING agonists, and several lipids derived from *M. tuberculosis* (Nanishi et al., 2020; Stewart et al., 2019). Although these adjuvants all elicit inflammatory responses, the balance of specific cytokines produced can be adjuvant specific, suggesting that adjuvant selection may be important for fine-tuning the innate, and therefore adaptive, response to vaccination. In the context of *M. tuberculosis*, adjuvants under development that have shown efficacy in preclinical animal studies include agonists of TLR2, TLR3, TLR4, TLR7/8, Mincle, and the inflammasome (reviewed in (Stewart et al., 2019). Thus far, the development of vaccines and selection of specific adjuvants have been largely empirical, due to the lack of immune correlates of protection to guide tuberculosis vaccine design. However, several key lessons have emerged from vaccine development. First, whereas traditional vaccination strategies have sought to maximize the development of IFN-γ-producing Th1 and polyfunctional T cells, it is now appreciated that excessive Th1 development may inhibit the development of other (as yet unidentified) protective T cell subsets (Billeskov et al., 2017; Sallin et al., 2017). Furthermore, mucosal delivery of vaccines for tuberculosis can promote enhanced protective immunity relative to parenteral immunization, promoting the development of antigen-specific Th17 cells (Hart et al., 2018; Van Dis et al., 2018). Therefore, it is crucial that adjuvants for tuberculosis vaccines be selected not purely for their ability to elicit strong inflammatory responses but also for their capacity to elicit balanced Th1/Th17 immunity and for mucosal efficacy. Finally, because the effect of adjuvants can differ based on genetic and epigenetic factors, care must be taken in the selection of the appropriate adjuvant for for tuberculosis vaccination in the target population (e.g., infant versus adult) (Nanishi et al., 2020).

Trained immunity. Soon after the introduction of the BCG vaccine in Europe in the early twentieth century, it was noted that BCG reduces childhood mortality in a manner that could not be explained by a reduction in tuberculosis incidence. Subsequent studies have confirmed this phenomenon and have attributed the efficacy to a reduction in mortality from childhood respiratory diseases (reviewed in (Shann, 2010). The ability of BCG to protect against nonmycobacterial infections is attributed to trained immunity—the long-term functional reprogramming of innate immune cells resulting in enhanced responses to other pathogens. BCG vaccination protects mice from viral infections including influenza and herpes simplex virus 2 via nonspecific trained immunity (Spencer et al., 1977; Starr et al., 1976). Intriguingly, the observation that coronavirus disease 2019 (COVID-19) cases and fatalities are fewer in regions of the world with universal BCG vaccination has prompted speculation that BCG vaccination may be protective against COVID-19 (O'Neill and Netea, 2020). However, this has not been established through rigorous clinical trials. Intravenously injected BCG elicits an expansion and reprogramming of hematopoietic stem cells in the bone marrow that promote the production of macrophages primed to respond to *M. tuberculosis* infection. This trained immunity is induced via epigenetic changes that result in enhanced responsiveness of innate immunity genes in macrophages and other innate cells. In the mouse model this results in a modest reduction in bacterial titers after infection with *M. tuberculosis* of ~0.5–1 log—comparable to standard vaccination with BCG (Kaufmann et al., 2018). BCG infection of bone marrow results in changes that persist for many weeks after eradication of BCG using antibiotics. However, as the

timing of infection in the mouse model is necessarily compressed due to a short life span, it is unclear how long-lived trained immunity can be in humans. Importantly, intravenous BCG results in almost complete protection against *M. tuberculosis* infection in macaques (Barclay et al., 1970; Darrah et al., 2020); however, there is no evidence of a contribution of trained immunity to this remarkable protective efficacy (Darrah et al., 2020). Although early exposure to *M. tuberculosis* in humans induced a protective state in circulating monocytes that limited *M. tuberculosis* outgrowth, this effect was modest in BCG-vaccinated individuals (Joosten et al., 2018). Although it is unclear whether innate immunity alone, even when trained, can ever completely protect against *M. tuberculosis* infection, future vaccination strategies should consider eliciting trained immunity as a contributor to other mechanisms.

Conclusions and perspectives

The original view of innate immunity to tuberculosis primarily focused on resistance—the ability of the cells and cytokines of the immune system to prevent infection or eliminate infectious microbes. Thus, much of the first few decades of tuberculosis research focused on identifying mechanisms by which activated macrophages kill or prevent the proliferation of *M. tuberculosis* bacilli in a cell-intrinsic manner and inflammatory cytokines that are important for control of disease. However, there are still major gaps in our understanding of resistance mechanisms. It remains unclear exactly how macrophages control infection with *M. tuberculosis* at the cell-intrinsic level. Furthermore, we lack an understanding of how cytokines like IL-1 contribute to control of infection. The roles of many innate cells, including NK cells and nonclassical T cells, remain enigmatic. The idea that tolerance—limiting the collateral damage caused by the immune response to infection—determines the outcome of infection has more recently become a major focus of research. In the mouse model of infection, it appears that disturbing tolerance may be a major pathway to host susceptibility. This corresponds with our understanding that death from human tuberculosis results from inflammatory destruction of host lung tissue. However, in most susceptible strains of mice rescued by neutrophil depletion, there is an increase in bacterial burden in the lungs, leaving open the question of whether a failure of resistance drives the excessive inflammation that results in death. Furthermore, simply suppressing the immune response using nonspecific anti-inflammatory drugs does not clearly benefit patients with active pulmonary tuberculosis (Schutz et al., 2018). Human tuberculosis is a remarkably heterogeneous disease, both during different stages of disease within an individual patient and from patient to patient. The design of novel therapeutics that modulate inflammation appropriately for individual patients, or that enhance resistance mechanisms, will require a deeper understanding of the innate pathways that contribute to progression of disease.

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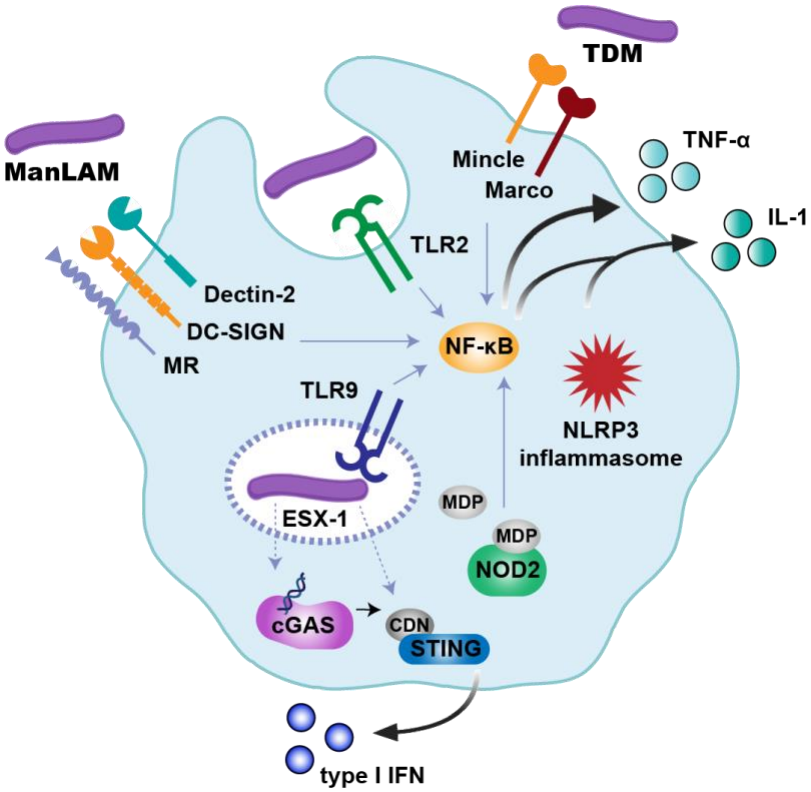

Figure 1: PRRs implicated in detecting *M. tuberculosis* infection and initiating the production of important innate cytokines. *M. tuberculosis* is detected by multiple classes of PRRs. The CLRs MR, DC-SIGN, and Dectin-2 have been proposed to recognize the glycolipid ManLAM, whereas Mincle and Marco recognize TDM on the surface of bacteria. TLR2 recognizes lipoproteins and/or lipoglycans on the surface, whereas TLR9 recognizes DNA released in the phagolysosome. The NLR NOD2 recognizes MDP released from bacterial peptidoglycan. NLRP3 triggers inflammasome activation upon *M. tuberculosis* infection. The ESX-1 secretion system promotes detection by cytosolic sensors by perforating the phagosomal membrane and allowing bacterial pathogen-associated molecular patterns to enter the cytosol, resulting in activation of cGAS/STING. CLRs, TLRs, and NOD2 signal through NF-κB to activate transcription of inflammatory cytokines including IL-1 and TNF-α. Processing and activation of IL-1β are promoted by the NLRP3 inflammasome. The cGAS-STING pathway leads to the expression of type I interferon, which is detrimental to the host. Abbreviations: CDN, cyclic dinucleotide; cGAS, cyclic GMP-AMP synthase; CLR, C-type lectin receptor; ManLAM, mannose-capped lipoarabinomannan; MDP, muramyl dipeptide; MR, mannose receptor; NLR, nucleotide-binding domain and leucine-rich repeat–containing receptor; PRR, pattern recognition receptor; STING, stimulator of interferon genes; TDM, trehalose dimycolate; TLR, Toll-like receptor.

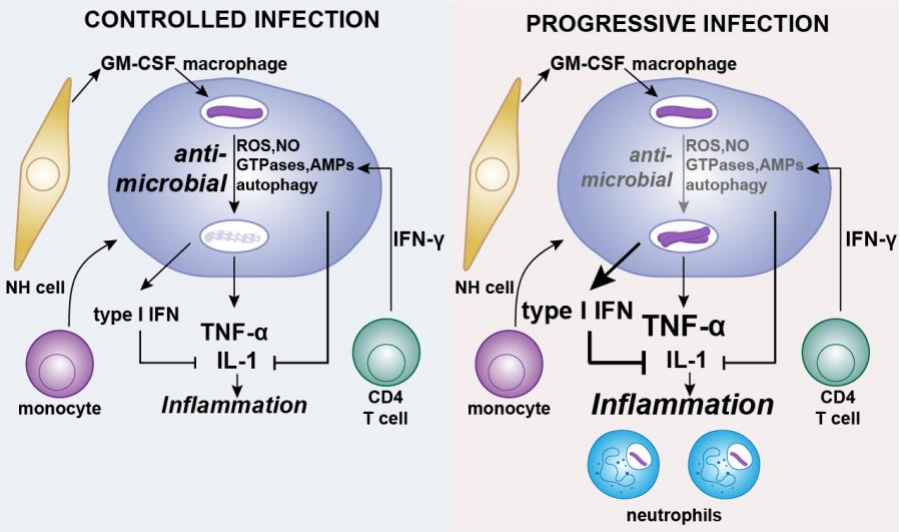

Figure 2: A combination of antimicrobial function and regulation of inflammation is required for successful control of *M. tuberculosis* infection. Successful control of *M. tuberculosis* infection is associated with robust macrophage-based control of bacterial replication by antimicrobial mechanisms. Mechanisms that have been proposed to contribute to cell-intrinsic control of infection include autophagy, interferon-inducible GTPases, ROS, NO, and antimicrobial peptides. Cytokines such as GM-CSF produced by nonhematopoietic cells and IFN-γ produced by CD4 T cells promote the microbicidal functions of macrophages. In controlled infection, there is appropriate production of inflammatory cytokines including TNF-α and IL-1; type I interferon, which blocks IL-1 function, is produced at low levels. Indeed, some of the susceptibility of mice lacking factors formerly assumed to be directly antimicrobial may be attributed to inflammatory imbalances. In contrast, uncontrolled infection may result from either a failure of antimicrobial control or imbalanced cytokine production. If antimicrobial mechanisms fail, the increased bacterial burden can drive the excessive production of inflammatory cytokines, leading to the recruitment of neutrophils that contribute to excessive inflammation. Alternatively, increased type I interferon production can functionally block IL-1 signaling, leading to immune failure. In most cases in mice, susceptible strains can be rescued by depletion of neutrophils, suggesting that in the mouse model diverse failures of immunity converge on a single neutrophil-driven mechanism of mortality. Abbreviations: AMP, antimicrobial peptide; GM-CSF, granulocyte-macrophage colony-stimulating factor; NH, nonhematopoietic; NO, nitric oxide; ROS, reactive oxygen species.

Chapter Two: STING-activating adjuvants elicit a Th17 immune response and protect against *M. tuberculosis* infection

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Abstract

Vaccines against intracellular pathogens that require cell-based immunity for control have been difficult to develop. A common strategy is to vaccinate with pathogen protein antigens in combination with an adjuvant, often a bacterial-derived product. However, there are a limited number of adjuvants that elicit effective cell-based immunity. Here we report that STING-activating cyclic dinucleotides (CDNs) formulated in a protein subunit vaccine (5Ag/RR-CDG) elicit long-lasting protective immunity to *Mycobacterium tuberculosis* in the mouse model. Protection afforded by subcutaneous administration of this vaccine was equivalent to protection by the live attenuated vaccine strain Bacille Calmette-Guérin (BCG). This protective efficacy was STING-dependent but type I IFN-independent, and correlated with an increased frequency of a recently described subset of CXCR3-expressing T cells that localize to the lung parenchyma. Furthermore, intranasal delivery of 5Ag/RR-CDG resulted in superior protection compared to BCG, significantly boosted BCG-based immunity, and elicited both Th1 and Th17 immune responses, the latter of which correlated with enhanced protection. Finally, ML-RR-cGAMP, a universal human STING agonist, has equivalent protective efficacy as RR-CDG in a protein subunit vaccine. Thus, a CDN adjuvanted protein subunit vaccine has the capability of eliciting a multi-faceted immune response that results in protection from infection by an intracellular pathogen.

Introduction

Infection with *Mycobacterium tuberculosis* continues to be a leading cause of death worldwide, in part due to the lack of an effective vaccine (Young and Dye, 2006). The current vaccine for *M. tuberculosis*, Bacille Calmette-Guérin (BCG), is widely administered(Floyd, 2016), yet its protective efficacy against adult pulmonary tuberculosis (TB) is variable, ranging from 0-80% in clinical trials (Andersen and Doherty, 2005). Additionally, as a live attenuated vaccine, BCG is not recommended for individuals with a compromised immune system, including infants with HIV (Marais et al., 2016). Significant effort has focused on developing vaccines that can either replace or boost BCG to generate a protective immune response against pulmonary TB. Currently, there are 12 vaccine candidates for TB in clinical trials, 8 of which are novel protein subunit vaccines (Kaufmann et al., 2017). One benefit of subunit vaccines is that they generally exhibit better safety profiles than live attenuated vaccines that cannot always be given to immunocompromised individuals. However, subunit vaccines require an adjuvant to elicit a strong memory immune response to the vaccine antigen, and there is a lack of clinically approved adjuvants that elicit antigen-specific effector and long-lived memory CD4+ and CD8+ T cells (Iwasaki and Medzhitov, 2010).

Cyclic dinucleotides (CDNs) were initially characterized as ubiquitous second messengers in bacteria (Tamayo et al., 2007) and were found to be pathogen-associated molecular patterns (PAMPs) recognized by the cytosolic surveillance pathway (Burdette et al., 2011; McWhirter et al., 2009). CDNs activate the cytosolic receptor Stimulator of Interferon Genes (STING), leading to signaling through multiple immune pathways: TBK1/IRF3 leading to type I IFN, classical inflammation via NF-κB, and STAT6-dependent gene expression (Burdette and Vance, 2013; Burdette et al., 2011; Chen et al., 2011; McWhirter et al., 2009). Treatment with CDNs stimulates innate immune cells to control *Klebsiella pneumoniae* and *Staphylococcus aureus* infection *in vivo* (Karaolis et al., 2007b, 2007a). Additionally, immunizing with model antigens in conjunction with CDNs results in distinct immune responses depending on the route of delivery, with subcutaneous administration leading to a Th1/Th2 response and mucosal administration leading to a Th17 response (Ebensen et al., 2011). CDNs have also been shown to elicit protective antibody-based immunity when used as a vaccine adjuvant against the extracellular bacterial pathogens *S. aureus* and *Streptococcus pneumoniae* (Dubensky et al., 2013; Ebensen et al., 2007b, 2007a; Hu et al., 2009; Libanova et al., 2010; Madhun et al., 2011; Ogunniyi et al., 2008; Yan et al., 2009). Finally, CDNs are under investigation as promising agents for cancer immunotherapy (Chandra et al., 2014; Hanson et al., 2015; Woo et al., 2014). No study has yet demonstrated that a CDN adjuvant can elicit T cell-based protective immunity against an intracellular bacterial pathogen.

CDNs activate the same cytosolic surveillance pathways as *M. tuberculosis* and other intracellular pathogens (Dey et al., 2015; Wassermann et al., 2015; Watson et al., 2015) suggesting that CDNs may induce an immune response effective against these pathogens. Importantly, other vaccine adjuvants under development for TB utilize TLR agonists or TB cell wall lipids (Agger, 2016) that are not known to activate STING or any other cytosolic surveillance pathway. In addition, BCG does not activate STING due to the loss of a key virulence mechanism (Watson et al., 2015). Furthermore, the fact that

CDNs can elicit Th17 responses may be important in the context of *M. tuberculosis*, where Th17 T cells are important for the protection conferred by BCG in mice (Khader et al., 2007). Collectively, these data provide a compelling rationale for the use of CDNs as a clinical TB vaccine adjuvant. To this end, we tested whether CDNs would be a suitable adjuvant for a protein subunit vaccine to protect against *M. tuberculosis* challenge in a mouse model. We found that subcutaneous (SQ) administration of a synthetic analogue of cyclic diguanylate (CDG) with a fusion protein containing five *M. tuberculosis* proteins (5Ag) conferred 1 log of protection against challenge with virulent *M. tuberculosis* and elicited a population of parenchymal homing T cells. Furthermore, intranasal (IN) delivery of 5Ag/RR-CDG resulted in 1.5 – 2 logs of protection at 12 weeks post challenge when administered as a sole vaccine or as a booster to BCG, and elicited a robust Th17 response that correlated with enhanced protection. This level of sustained protection is better than has been observed with any protein subunit vaccine for *M. tuberculosis* to date, and these results provide the first demonstration that CDNs can elicit T cell responses that elicit protection against infection with an intracellular bacterial pathogen.

Materials and Methods

Ethics statement
All procedures involving the use of mice were approved by the University of California, Berkeley, Institutional Animal Care and Use Committee (Protocol R353-1113B). All protocols conform to federal regulations, the National Research Council's Guide for the Care and Use of Laboratory Animals, and the Public Health Service's Policy on Humane Care and Use of Laboratory Animals.

Reagents
RR-CDG and ML-RR-cGAMP were synthesized at Aduro Biotech as described previously (Corrales et al., 2015; Gaffney et al., 2010). Synthesis of CDN molecules utilized phosphoramidite linear coupling and H-phosphonate cyclization reactions. Both steps were followed by sulfurization reactions to yield phosphorothioate internucleotide linkages. Addavax (Invivogen, San Diego, CA) was used for formulation of antigen and adjuvant as directed by manufacturer. 5Ag fusion protein and peptide pools were provided by Aeras.

Mice
CB6F1 (used in Figure 2-3) and C57BL/6 mice (Figures 4-10) were obtained from The Jackson Laboratory (Bar Harbor, ME). *Ifnar* $^{-/-}$ mice were obtained from the Vance lab (UC Berkeley) and were bred in house. *Sting*$^{gt/gt}$ mice were a gift from the Raulet lab (UC Berkeley) and were bred in house. Both *Sting*$^{gt/gt}$ and *Ifnar* $^{-/-}$ are on the C57BL/6 background, and sex and age matched wild-type C57BL/6 mice were used as controls for these experiments.

Phosphodiesterase assay
CDG, RR-CDG, or saline alone was incubated overnight with or without 1 mg snake venom phosphodiesterase (SVPD; Sigma) at 37 °C. Samples were boiled for 10 minutes to inactivate the SVPD. 1 x 10^5 DC2.4 murine cells were incubated with 100 μM of CDG, RR-CDG or saline pretreated with or without SVPD in triplicate for 30 min at 37 °C. After 30 minutes, cells were washed and incubated with RPMI containing 10% FBS at 37 °C with 5% CO_2. Supernatants were collected after 4 hours and added to L929 cells expressing luciferase under control of an interferon-stimulated response element (ISRE). After 4 hours incubation, cells were lysed with lysis buffer (Promega) and luciferin was added. Luminescence was measured on a SpectraMax L microplate reader (Molecular Devices).

Bacterial culture
The *M. tuberculosis* strain Erdman was used for all challenges and *M. bovis* BCG (Pasteur) was used for all vaccinations. *M. tuberculosis* and BCG were grown in Middlebrook 7H9 liquid media supplemented with 10% albumin-dextrose-saline (*M. tuberculosis*) or 10% OADC (BCG), 0.4% glycerol, and 0.05% Tween 80 or on solid 7H10 agar plates supplemented with 10% Middlebrook OADC (BD Biosciences) and 0.4% glycerol. Frozen stocks of BCG were made from a single culture and used for all experiments.

Vaccinations
RR-CDG (5 μg) and 5Ag (3 μg) were formulated in PBS for IN delivery or in 2% Addavax in PBS for SQ delivery. Groups of 6 to 10 week old mice were vaccinated three times at 4 week intervals with 100 μL subcutaneously at the base of the tail (50 μL on each flank) or with 20 μL intranasally. BCG vaccinated mice were injected once with 2.5-5 x 10^5 CFU/mouse in 100 μL of PBS subcutaneously in the scruff of the neck. At the indicated week post immunization, mice were bled (retro-orbital; 200 μL) for immunological assays (IFN-γ ELISPOT and/or ICS).

Challenge experiments with *M. tuberculosis*
Twelve weeks after the initial vaccine injection, mice were infected via the aerosol route with *M. tuberculosis* strain Erdman. Aerosol infection was done using a Nebulizer and Full Body Inhalation Exposure System (Glas-Col, Terre Haute, IN). A total of 9 mL of culture was loaded into the nebulizer calibrated to deliver ~100 bacteria per mouse as measured by CFU in lungs one day following infection (data not shown). Unless stated otherwise, groups of five mice were sacrificed 4 and 12 weeks post challenge to measure CFU and immune responses in the lungs (4 weeks only). For bacterial enumeration, one lung lobe (the largest, 4 weeks post challenge) or all lung lobes (12 weeks post challenge) was homogenized in PBS plus 0.05% Tween 80, and serial dilutions were plated on 7H10 plates. CFUs were counted 21 days after plating. Remaining lung lobes were used for ICS at 4 weeks post challenge.

Pre-challenge ELISPOT and ICS assays
Heparinized blood from five mice was analyzed separately (Figure 10) or pooled (Figures 2, 6-8) and lymphocytes were isolated (Lympholyte-Mammal, Cedar Lane, cat #CL5115). For ELISPOTs, the lymphocytes (1x10^5 cells/well or 1 x10^4 cells/well for Ag85B and ESAT-6) were put in plates pre-coated with IFN-γ capture antibody (BD Biosciences #551881) containing splenocytes (1 x10^5 cells/well) and peptide pools (2 μg/mL). Plates were incubated overnight, then washed and developed as per the BD Biosciences kit protocol. Spots were enumerated on a CTL Immunospot Analyzer. For ICS, cells were re-stimulated with no peptide, ESAT-6 peptide pools (2 μg/mL), or Ag85B peptide pools (2 μg/mL), CFSE-labeled splenocyte feeder cells from an uninfected mouse (1 x10^5 cells/well), and GolgiPlug and GolgiStop for 5 hours at 37 °C. Cells were kept at 4 °C overnight and then washed and stained with Live/Dead stain (Thermofisher, L34970), CD4 (BD, #564933), CD8 (BD, #563898), CD90.2 (BD, #561616), MHCII (Biolegend, #107606), Ly6G (BD, #551460), IFN-γ (eBioscience, #12-73111-81), TNF-α (BD, 506324), and IL-17 (Biolegend, #506904). Data were collected using a BD LSR Fortessa flow cytometer with FACSDiva Software (BD) and analyzed using FlowJo Software (Tree Star Inc., Ashland, OR).

Post challenge Intracellular Cytokine Staining
Lungs (the four smallest lobes) were harvested 4 weeks post challenge into cRPMI (RPMI-1640, 10% FBS, 1% Sodium pyruvate, 1% Hepes, 1% L-glutamine, 1% Non-essential amino acids, 1% pen/strep, 50 μM 2-ME), dissociated and strained through a

40 μm strainer. Cells were re-stimulated with no peptide, ESAT-6 peptide pools (2 μg/mL), or Ag85B peptide pools (2 μg/mL), and GolgiPlug and GolgiStop for 5 hours at 37 °C. Cells were washed and stained with antibodies used for pre-challenge ICS and CXCR3 (Biolegend, #126522) and KLRG1 (Biolegend, #107606). Cells were fixed and permeabilized at RT for 20 minutes and removed from the BSL3. Data were collected and analyzed as outlined above.

Results

A STING-activating RR-CDG adjuvanted protein subunit vaccine protects against *M. tuberculosis* infection.

The efficacy of CDNs as an adjuvant for *M. tuberculosis* antigens was tested with a synthetic form of CDG in which the non-bridging oxygen atoms were replaced with sulfur atoms in the R,R stereochemical configuration (RR-CDG) to prevent cleavage and inactivation by host cell phosphodiesterases (Corrales et al., 2015) (Figure 1). RR-CDG was combined with the antigen 5Ag, a fusion of five *M. tuberculosis* proteins: Antigen-85B (Ag85B), ESAT-6, Rv1733c, Rv2626c, and RpfD(Zvi et al., 2008). Ag85B and ESAT-6 are established immunogenic TB antigens that have been tested in a variety of subunit vaccines and have been shown to elicit T cell responses in humans (Baldwin et al., 1998; Brandt et al., 2000; Horwitz et al., 1995; Langermans et al., 2005; Olsen et al., 2004; Weinrich Olsen et al., 2001). Rv1733, Rv2626c, and RpfD were identified in a bioinformatics analysis that identified potential T cell epitopes based on *M. tuberculosis* gene expression data (Zvi et al., 2008). RR-CDG and 5Ag were formulated in Addavax, a commercially available squalene-based oil-in-water nano-emulsion(Ott et al., 1995), to yield the experimental vaccine 5Ag/RR-CDG. Mice were vaccinated according to a standard vaccine schedule, receiving three immunizations with 5Ag/RR-CDG at 4 week intervals or one immunization with BCG 12 weeks prior to a low-dose aerosol challenge with the virulent Erdman strain of *M. tuberculosis* (Figure 2A).

To determine whether 5Ag/RR-CDG elicits Th1 immunity, IFN-γ ELISPOT was performed using peripheral blood mononuclear cells (PBMCs) after each boost. 5Ag/RR-CDG generated T cell specific responses to Ag85B, ESAT-6, and Rv1733c that were dependent on RR-CDG (Figure 2B) and increased in magnitude after the second boost (Figure 2C). BCG elicited significantly lower antigen specific T cell responses than 5Ag/RR-CDG (Figure 2B). Twelve weeks after the initial vaccination, mice were challenged with *M. tuberculosis*. At 4 weeks post challenge, 5Ag/RR-CDG vaccinated mice had 1 log fewer bacteria in the lungs when compared with PBS vaccinated mice, protection equivalent to that afforded by BCG (Figure 2D). Importantly, this level of protection was durable out to 12 weeks post challenge (Figure 2E), indicating that 5Ag/RR-CDG vaccinated mice may maintain elevated numbers of memory-derived CD4+ T cells(Carpenter et al., 2017).

To facilitate comparison to other vaccine adjuvants, RR-CDG was formulated with a fusion protein of ESAT-6 and Ag85B, antigens commonly used together in vaccine studies (Agger et al., 2008; Weinrich Olsen et al., 2001). At 12 weeks post infection, the protection afforded by RR-CDG and the ESAT-6/Ag85B fusion protein was equivalent to 5Ag/RR-CDG (Figure 3). Thus, when combined with TB proteins, RR-CDG provides significant protective efficacy against *M. tuberculosis* challenge that is as effective as any other adjuvant tested in the context of a *M. tuberculosis* protein subunit vaccine to date (Aagaard et al., 2011; Baldwin et al., 2012; Bertholet et al., 2010; Billeskov et al., 2012; Ma et al., 2017; Skeiky et al., 2004).

5Ag/RR-CDG vaccine increases the percentage of parenchymal-homing T cells in the lungs relative to PBS or BCG vaccinated mice.

At the peak of the immune response, 4 weeks post challenge, mice vaccinated with 5Ag/RR-CDG had a significantly higher percentage of CD4+ T cells in the lungs compared to mice vaccinated with PBS (Figure 4A) and a corresponding decrease in the percentage of CD8+ T cells (Figure 4B), suggesting that 5Ag/RR-CDG specifically promotes the recruitment and/or expansion of CD4+ T cells after infection. To examine antigen-specific T cell responses, cells from infected lungs were re-stimulated *ex vivo* with antigenic peptide pools. Due to the robust responses elicited by Ag85B and ESAT-6, only peptides from these antigens were used for post challenge intracellular cytokine staining (ICS) analyses. Ag85B-specific CD4+ IFN-γ+ T cell responses were only observed in 5Ag/RR-CDG immunized mice (Figure 5A). A robust ESAT-6-specific CD4+ IFN-γ+ T cell population was observed in 5Ag/RR-CDG immunized mice (Figure 5A), although it was lower than PBS immunized mice. A similar trend was observed for poly-functional T cells (Figure 5B). In total, while 5Ag/RR-CDG vaccinated mice exhibited an increased frequency of total CD4+ T cells, there was not a strong correlation between protection and the presence of Ag85B- or ESAT-6-specific IFN-γ-producing CD4+ T cells in the lungs.

Previous studies have identified two functional categories of CD4+ T cells during TB infection: CXCR3- KLRG1+ cells that localize to the lung vasculature and produce abundant levels of IFN-γ, and CXCR3+ KLRG1- cells that localize to the lung parenchyma and, despite producing lower levels of IFN-γ, are better at controlling *M. tuberculosis* infection (Sakai et al., 2014; Woodworth et al., 2017). At 4 weeks post challenge, there was no significant difference between the percentage of CXCR3-KLRG1+ vascular CD4+ T cells among the groups (Figure 2F). However, there was a significant increase in the percentage of CXCR3+ KLRG1- parenchymal CD4+ T cells in the lungs of 5Ag/RR-CDG vaccinated mice compared to PBS controls (Figure 2G). Although the percentage of CXCR3+ KLRG1- CD4+ T cells was higher in lungs of mice immunized with 5Ag/RR-CDG, a lower percentage of these cells produced IFN-γ when re-stimulated with Ag85B or ESAT-6 compared to PBS immunized mice (Figure 5C). Thus, the 5Ag/RR-CDG vaccine elicits an increased frequency of CD4+ T cells and CXCR3+ KLRG1- T cell populations in the lungs, both of which are known to be protective against *M. tuberculosis.*

5Ag/RR-CDG vaccine-induced protection requires STING but not type I IFN signaling.

To determine whether the antigen-specific T cell response and protective efficacy elicited by 5Ag/RR-CDG was dependent on STING and/or type I IFN signaling through the type I IFN receptor (IFNAR), mice lacking a functional copy of STING (*Sting*$^{gt/gt}$) (Sauer et al., 2011) or IFNAR (*Ifnar* $^{-/-}$) were immunized according to the schedule outlined in Figure 2A. Seven days after the 2nd boost, both Ag85B- and ESAT-6-specific T cell responses were undetectable in PBMCs from *Sting*$^{gt/gt}$ mice, indicating that antigen-specific T cell responses promoted by 5Ag/RR-CDG are STING-dependent (Figures 6A and 6B). Interestingly, antigen-specific T cell responses were equivalent in wild-type and *Ifnar* $^{-/-}$ mice PBMCs (Figures 6A and 6B), suggesting that 5Ag/RR-CDG responses are not dependent on IFNAR signaling.

Sting$^{gt/gt}$ mice immunized with 5Ag/RR-CDG had equivalent CFU in the lungs at 4 and 12 weeks post challenge with *M. tuberculosis* compared to *Sting*$^{gt/gt}$ mice

immunized with PBS (Figures 6C and 6D), demonstrating that the protective efficacy of RR-CDG is dependent upon STING. In contrast, *Ifnar* $^{-/-}$ mice immunized with 5Ag/RR-CDG had equivalent protection to wild-type 5Ag/RR-CDG vaccinated mice (Figures 6C and 6D). Thus, while 5Ag/RR-CDG protection is STING-dependent, signaling through IFNAR is not necessary for the development of a protective immune response to *M. tuberculosis* challenge in 5Ag/RR-CDG vaccinated mice.

Intranasal, but not subcutaneous, boosting of BCG with 5Ag/RR-CDG significantly enhances protection from *M. tuberculosis* challenge.

We next sought to determine whether the 5Ag/RR-CDG vaccine could boost BCG vaccination to provide enhanced protection in mice. Following the vaccination schedule outlined in Figure 7A, BCG primed mice received two boosts of 5Ag/RR-CDG or 5Ag alone via SQ injection and were compared to mice that received three SQ injections of 5Ag/RR-CDG as outlined in Figure 2A. After the 2nd boost, IFN-γ ELISPOT using PBMCs showed that BCG immunized mice boosted SQ with 5Ag/RR-CDG had increased Ag85B- and ESAT-6-specific T cell responses compared to mice that were immunized only with BCG (Figure 7B). However, there was no difference in IFN-γ levels between mice immunized with BCG and boosted with SQ 5Ag/RR-CDG compared to mice that received three SQ administrations of 5Ag/RR-CDG alone (Figure 7B). Additionally, boosting BCG with SQ 5Ag/RR-CDG did not result in enhanced protection against *M. tuberculosis* aerosol challenge (Figures 7C and 7D).

We next tested whether mucosal administration of 5Ag/RR-CDG via the IN route would enhance protection against *M. tuberculosis* infection using the vaccination schedule outlined in Figure 2A, or as outlined in Figure 7A for IN boosting of BCG. As Addavax is not suitable for IN vaccination, 5Ag/RR-CDG was formulated in PBS. Seven days after the 2nd boost, IN administration of 5Ag/RR-CDG resulted in an increase in IFN-γ-producing Ag85B-specific CD4+ T cells in PBMCs compared to PBS vaccinated mice (Figure 8A). However, significantly fewer IFN-γ producing cells were elicited by IN vaccination than by SQ vaccination (Figure 8A). In contrast, IN administration of 5Ag/RR-CDG produced a robust IL-17 response from CD4+ T cells upon re-stimulation with Ag85B peptide pools (Figure 8B), a response that was not observed with SQ administration of 5Ag/RR-CDG or with BCG vaccination.

Vaccinated mice were challenged with *M. tuberculosis* to determine the protective efficacy of IN delivered CDN vaccines. As expected, ~1 log of pulmonary protection was seen in mice vaccinated with either BCG or SQ 5Ag/RR-CDG (Figures 8C and 8D). However, IN administration of 5Ag/RR-CDG resulted in an additional ~0.5 log of control at 4 weeks post challenge (Figure 8C) and a trend towards increased control that was not statistically significant at 12 weeks (Figure 8D). Remarkably, BCG vaccinated mice receiving IN boosts of 5Ag/RR-CDG had significantly lower CFU in the lungs at 12 weeks post challenge compared with BCG vaccination alone, resulting in 2 logs of protection against infection (Figure 8D). As with SQ vaccination, the percentage of CD4+ IFN-γ+ T cells in the lungs of IN vaccinated mice was not enhanced beyond infection-induced responses exhibited in PBS immunized mice at 4 weeks post challenge (Figure 8E). However, the pre-challenge increase in Th17 cells noted in the blood (Figure 8B) was reflected post challenge with a large fraction of CD4+ T cells in the lungs producing IL-17 (Figure 8F). IN immunization or IN-based boosting of BCG

with 5Ag/RR-CDG resulted in significantly more IL-17+ CD4+ T cells than BCG vaccination or SQ administration of 5Ag/RR-CDG, both alone and as a booster vaccine (Figure 8F, Figure 9). Thus, IN delivery of 5Ag/RR-CDG resulted in robust protection against infection and had an additive effect when combined with BCG. Additionally, protection elicited via the IN route correlated not with increases in Th1 cells, but with increases in Th17 T cells.

ML-RR-cGAMP, a universal human STING agonist, elicits a Th17 response and protects against challenge with *M. tuberculosis.*

RR-CDG efficiently activates murine STING; however, it does not engage all five common STING alleles in the human population (Corrales et al., 2015; Yi et al., 2013). We therefore tested the adjuvant activity of ML-RR-cGAMP, a dithio-substituted diastereomer of cGAMP with both a non-canonical 2'-5' and a canonical 3'-5' phosphodiester linkage (denoted mixed-linkage, ML) that is both resistant to hydrolysis by phosphodiesterases and a potent activator of all human STING alleles (Corrales et al., 2015). Mice were immunized via the IN or SQ route with either 5Ag/RR-CDG or 5Ag/ML-RR-cGAMP, and the frequency of Ag85B-specific CD4+ T cells in the blood that produce either IL-17 or IFN-γ was measured 7 days after the 1st boost by ICS. Both 5Ag/RR-CDG and 5Ag/ML-RR-cGAMP vaccines elicited IFN-γ-producing and IL-17-producing CD4+ T cells when administered IN (Figures 10A, 10B and 10C). SQ administration of 5Ag/ML-RR-cGAMP did not elicit IL-17-producing CD4+ T cells (Figure 5C), but elicited more IFN-γ-producing CD4+ T cells than IN immunization (Figure 10B). This is similar to the trend seen with SQ vs. IN immunization of 5Ag/RR-CDG (Figures 8A and 8B).

Mice vaccinated with 5Ag/ML-RR-cGAMP were challenged with virulent *M. tuberculosis* and protection was evaluated by CFU in the lungs at 4 weeks post challenge. Importantly, IN immunization with 5Ag/ML-RR-cGAMP provided ~1.5 logs of protection when used as a sole vaccine (Figure 10D), equivalent to 5Ag/RR-CDG. These data demonstrate that ML-RR-cGAMP, a STING-activating compound with translational potential to human vaccines, behaves similarly to RR-CDG when used as an adjuvant in a protein subunit vaccine.

Discussion

Here we report that STING-activating adjuvants elicit antigen-specific Th1 and Th17 responses, recruitment of CXCR3+ KLRG1- parenchymal-homing T cells, and protection against *M. tuberculosis*. RR-CDG in combination with the 5Ag fusion protein provided 1.5 logs of protection against challenge with virulent *M. tuberculosis* when used as a sole vaccine and 2 logs when used as a booster to BCG. In contrast to a similar protein subunit vaccine formulated with the Th1 adjuvant dimethyldioctadecylammonium liposomes with monophosphoryl lipid A (DDA/MPL) (Carpenter et al., 2017) the protection afforded by CDN adjuvanted experimental vaccines was durable through 12 weeks post challenge. This level of sustained efficacy is better than any vaccine adjuvant evaluated for use as a protein subunit vaccine for *M. tuberculosis* to date (Aagaard et al., 2011; Baldwin et al., 2012; Bertholet et al., 2010; Billeskov et al., 2012; Skeiky et al., 2004), and suggests that CDNs are capable of eliciting longer lived memory T cells than other vaccine adjuvants. Finally, the demonstration that a CDN adjuvanted vaccine can reduce TB disease in mice, presumably through T cell-dependent mechanisms, suggests that CDN adjuvants may be suitable for vaccination against other intracellular pathogens.

CDN activation of STING results in signaling via three distinct innate immune pathways(Burdette and Vance, 2013), the best described being TBK1/IRF3-mediated induction of type I IFNs (Ishikawa and Barber, 2008). We found that the efficacy of CDNs as a vaccine adjuvant is dependent on STING but not on type I IFN in mice immunized SQ. Although we did not test whether the protection of mice immunized with CDNs delivered IN is dependent on type I IFN, others have shown that the immune response to mucosally delivered CDG does not require type I IFN (Blaauboer et al., 2014). STING also activates NF-κB which induces classical pro-inflammatory cytokines including TNF-α, IL-1, IL-23 and IL-12 which may contribute to the efficacy of CDN adjuvants (Blaauboer et al., 2014). Furthermore, STING activates STAT-6-dependent expression of chemokines that are required for the antiviral responses of STING (Chen et al., 2011). Moving forward, it will be important to determine which innate immune signaling mechanisms promote the development of protective T cells against challenge with *M. tuberculosis* in CDN vaccinated mice.

The 5Ag experimental vaccine fusion protein contains five *M. tuberculosis* proteins including Ag85B and ESAT-6, two well characterized immunodominant antigens (Baldwin et al., 1998; Brandt et al., 2000; Horwitz et al., 1995; Langermans et al., 2005; Olsen et al., 2004; Skjøt et al., 2000; Weinrich Olsen et al., 2001). In addition, 5Ag contains Rv1733c, Rv2626c, and RpfD, putative T cell antigens hypothesized to play a role in latency and/or reactivation from latency (Zvi et al., 2008). In these relatively short-term studies, we only observed significant T cell responses to ESAT-6 and Ag85B. Furthermore, we found that a fusion protein of only ESAT-6 and Ag85B provided equivalent protective efficacy to that afforded by 5Ag. However, it is possible that in longer-term experiments, or in animal models that better mimic human latency, Rv1733c, Rv2626c, and RpfD may play a role in protection with a CDN adjuvanted vaccine.

Although both CD4+ T cells and IFN-γ are required for control of *M. tuberculosis* infection (Flynn et al., 1993; Green et al., 2013), it has been difficult to establish whether

these factors are sufficient to establish protective immunity(Fletcher et al., 2016; Kagina et al., 2010). Recently, the recombinant vaccine strain Modified Vaccinia Ankara virus expressing Ag85A (MVA85A) became the first new TB vaccine candidate to be tested for efficacy in infants in a clinical trial since BCG (Tameris et al., 2013). Despite eliciting antigen-specific Th1 T cell responses, MVA85A did not protect against the development of active TB disease in infants as a booster vaccine for BCG. It is not clear whether the elicited Th1 response was too weak/narrow or if in fact a Th1 response is not sufficient to confer protective immunity (Kaufmann, 2014). Since the basis for sterilizing immunity against *M. tuberculosis* in humans and animal models is not mechanistically understood, it is difficult to explain the negative result observed in the MVA85A trial or make progress towards the rational design of an effective vaccine. Thus, studies of novel vaccine formulations may be useful both for ultimately developing an effective vaccine and for clarifying correlates of protection. Although both SQ and IN vaccination with RR-CDG conferred protection and production of IFN-γ-producing T cells, the enhanced performance of 5Ag/RR-CDG when delivered via the IN route correlated with the production of Th17 cells. The role of Th17 cells in protective immunity to *M. tuberculosis* is unclear. In one study, IL-17 was shown to be dispensable for primary immunity to *M. tuberculosis* (Khader et al., 2005). In contrast, Th17 T cells were shown to protect against challenge with a highly virulent *M. tuberculosis* isolate (Gopal et al., 2014), and adoptive transfer of Th17 cells was shown to enhance control of *M. tuberculosis* infection *in vivo* (Gallegos et al., 2011). In a vaccination setting, IL-17 was required for full efficacy of BCG and correlated with a more rapid recruitment of IFN-γ-producing T cells into the lungs upon challenge (Khader et al., 2007). It is possible that in 5Ag/RR-CDG vaccinated mice Th17 cells play a critical role by recruiting protective T cells earlier during infection, at time points not examined in this study. Alternatively, it is also possible that the Th17 cells observed in IN vaccinated mice at 4 weeks post challenge are themselves capable of suppressing bacterial replication. Future work will focus on discerning the mechanism by which Th17 responses could contribute to the enhanced protection observed in mice receiving IN immunizations.

An ideal vaccine for *M. tuberculosis* would elicit memory T cells that traffic into the lung tissue, as these populations of T cells are protective when adoptively transferred to mice infected with *M. tuberculosis* (Sakai et al., 2014). We observed that vaccination with 5Ag/RR-CDG resulted in an increase in CD4+ CXCR3+ KLRG1- T cells, previously described to home to the lung parenchyma (Sakai et al., 2014), at 4 weeks post challenge. Despite inducing higher levels of parenchymal homing T cells, vaccination with 5Ag/RR-CDG resulted in a lower percentage of these cells producing IFN-γ compared to PBS immunized animals. Previous studies have suggested that there exists a population of T cells that can control infection in the lung independent of IFN-γ production (Gallegos et al., 2011; Green et al., 2013; Sakai et al., 2014). The fact that the majority of parenchymal homing T cells elicited by the vaccine do not produce IFN-γ raises the intriguing possibility that a previously undescribed T cell subset may mediate control in 5Ag/RR-CDG vaccinated mice.

Development of a vaccine adjuvant that elicits an effective T cell response has been challenging, and there are currently no clinically approved adjuvants that induce a T cell memory response (Rappuoli et al., 2011). CDNs have significant potential as a vaccine adjuvant for intracellular pathogens (Dubensky et al., 2013). Unlike other

bacterial products under development, CDNs are small molecules amenable to targeted and precise modification and optimization through chemical synthesis (Dubensky et al., 2013). Indeed, the immunostimulatory properties of different CDN molecules vary significantly (Libanova et al., 2010), potentially facilitating optimization based on the type of T cell immunity required for protection against a given pathogen. A synthetic, human STING-activating CDN (ADU-S100) is currently in Phase I clinical trials as a cancer therapeutic alone and in combination with checkpoint inhibition (Clinical trials.gov #NCT02675439 and #NCT03172936). Thus, there is an ongoing effort to develop CDN analogs with improved translational capacity, including longer half-life, less toxicity, and improved STING binding affinity (Corrales et al., 2015, 2016). In addition, optimization of formulation and delivery holds great potential to maximize the therapeutic efficacy of CDNs.

We have shown that CDNs are an effective adjuvant for a TB subunit vaccine in mice. CDN adjuvanted vaccines are promising candidates to help achieve the goal of developing an effective TB vaccine in humans. Furthermore, having an effective protein subunit vaccine provides an important tool to mechanistically dissect immune responses required for protection against *M. tuberculosis* in mice and other model organisms. With this information, rational design of a safe and effective vaccine to combat *M. tuberculosis* infection is possible.

Acknowledgements

Ifnar-/- mice were a gift from Russell Vance's laboratory and *Sting*$^{gt/gt}$ mice were a gift from David Raulet's laboratory at UC Berkeley. We thank Gwenyth Davis, Robyn Jong, Katie Lien and Jonathan Braverman for help with sample preparation. We thank members of the Cox lab for helpful discussions and Ellen Robey for review of the manuscript. This work was supported by NIH grant T32 GM 7232-40 and NSF Graduate Research Fellowship DGE-1106400 to EVD; NIH grant T32 AI 100829-4 to KMS, NIH grant 1R56AI091976-01 to DAP; and funding from Aeras and the Immunotherapeutics and Vaccine Research Initiative to SAS.

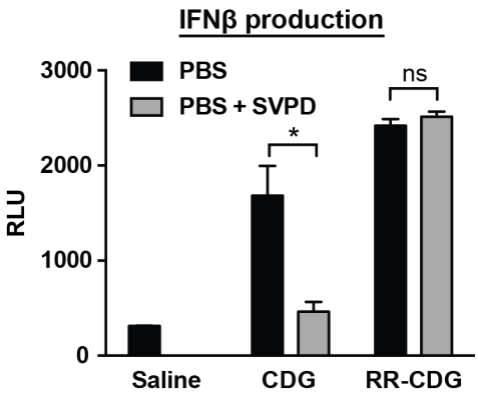

Figure 1: RR-CDG is resistant to inactivation by phosphodiesterases. RLU from L929 cells expressing luciferase under control of an interferon-stimulated response element (ISRE) after addition of supernatants from DC2.4 cells incubated overnight with saline, CDG or RR-CDG with or without 1 mg snake venom phosphodiesterase (SVPD). Data are expressed as mean (±SD) and are representative of experiments done in triplicate. Mann Whitney t-test p value * < 0.05

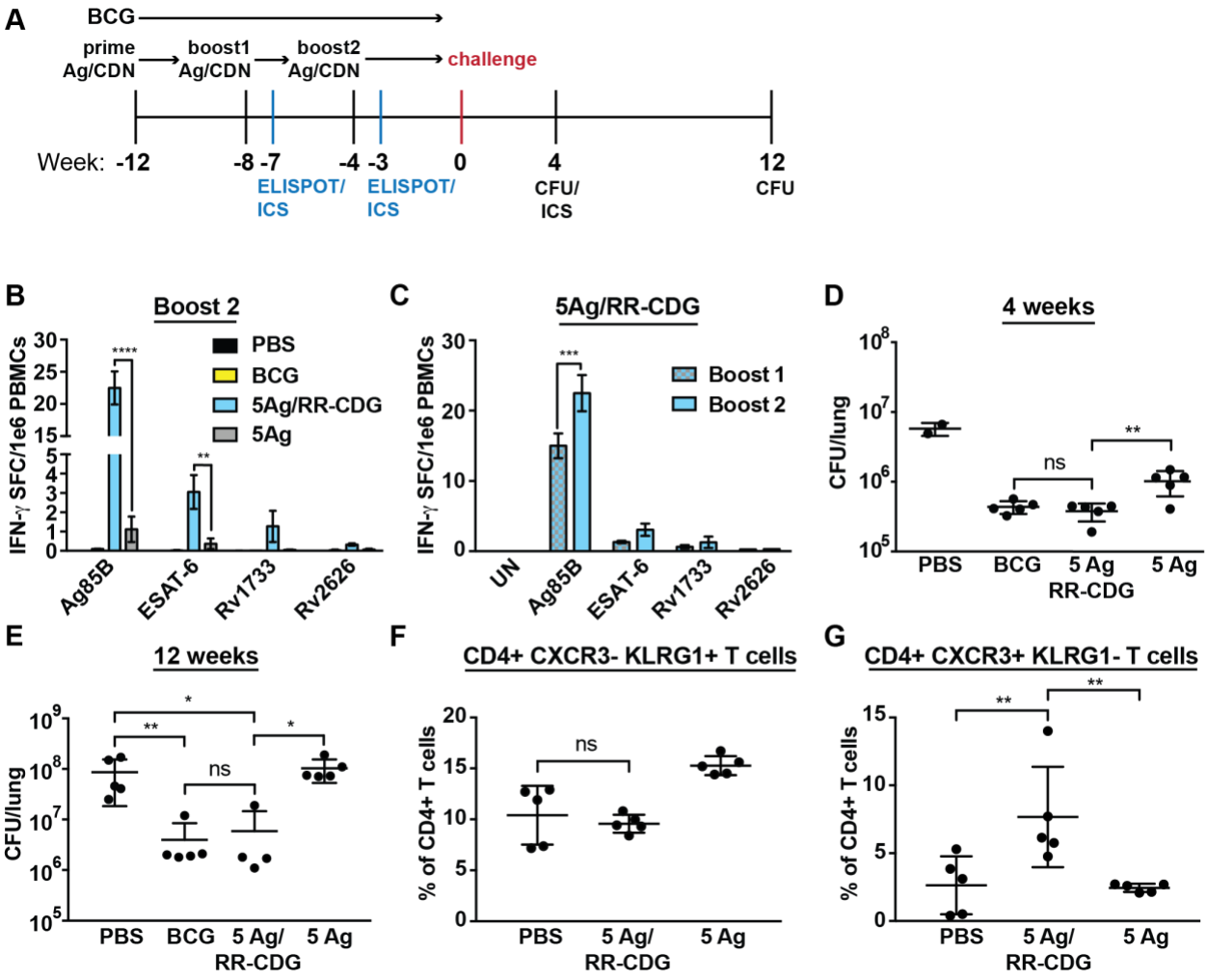

Figure 2: RR-CDG adjuvanted vaccine protects equivalently to BCG vaccination and induces T cell populations known to protect against *M. tuberculosis* infection. (A) Experimental timeline for vaccine experiments. Mice were vaccinated using the indicated schedule and challenged with ~100 CFU of *M. tuberculosis* strain Erdman. **(B)** IFN-γ ELISPOT from PBMCs harvested seven days after the 2nd boost with PBS, BCG, 5Ag/RR-CDG or 5Ag and re-stimulated *ex vivo* with the indicated peptide pools. **(C)** IFN-γ ELISPOT from PBMCs harvested seven days after each boost from mice vaccinated with 5Ag/RR-CDG and re-stimulated *ex vivo* using the indicated peptide pools or left unstimulated (UN). For **(B** and **C)**, data are expressed as the mean (±SD) of 10 animals assayed in two pools of five. Two-way ANOVA with Tukey's post-hoc p value ** < 0.002, *** <0.001, **** <0.0001. **(D** and **E)** CFU counts from lungs of vaccinated mice at **(D)** 4 weeks and **(E)** 12 weeks post challenge. Mann Whitney t-test p value * < 0.02; ** < 0.002. **(F)** ICS for percentage of CD4+ T cells in the lungs of mice that are CXCR3- KLRG1+ at 4 weeks post challenge. **(G)** ICS for percentage of CD4+ T cells in the lungs of mice that are CXCR3+ KLRG1- at 4 weeks post challenge. One-way ANOVA with Tukey's post-hoc p value ** < 0.002. For **(D** – **G)**, data are expressed as mean (±SD) and each symbol represents an individual animal. Data are representative of experiments done at least in duplicate.

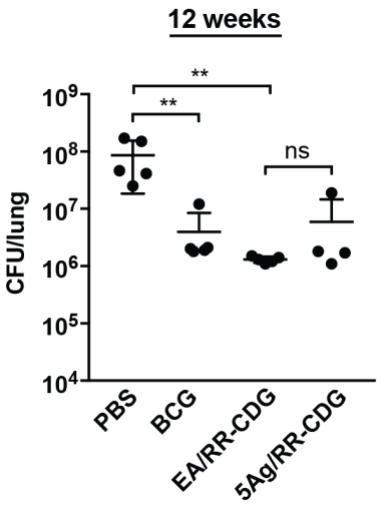

Figure 3: ESAT-6/Ag85B antigen formulated with RR-CDG in Addavax provides protection equivalent to 5Ag/RR-CDG against challenge with *M. tuberculosis*. F1 hybrid mice were immunized once with BCG, or three times with ESAT-6/Ag85B/RR-CDG (EA/RR-CDG) or 5Ag/RR-CDG following the vaccine schedule in Figure 1A. CFUs were enumerated in the lungs at 12 weeks post challenge. Each symbol represents an individual animal. Mann Whitney t-test p value ** < 0.002. Data are expressed as mean (±SD) and are representative of experiments done in duplicate.

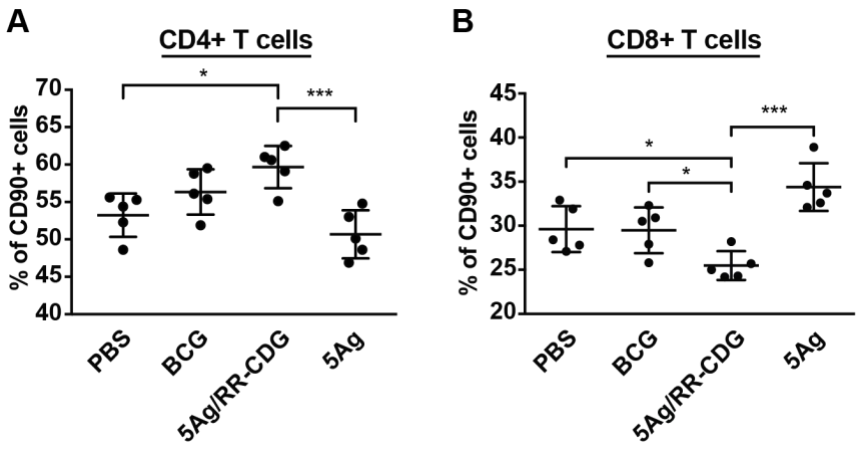

Figure 4: RR-CDG adjuvanted vaccine alters T cell populations in the lungs of infected mice. (A and **B)** ICS for percentage of **(A)** CD4+ T cells and **(B)** CD8+ T cells in the lungs of mice at 4 weeks post challenge. Mann Whitney t-test p value * < 0.05, *** < 0.0002. Data are expressed as mean (±SD) and each symbol represents an individual animal. Data are representative of experiments done in duplicate.

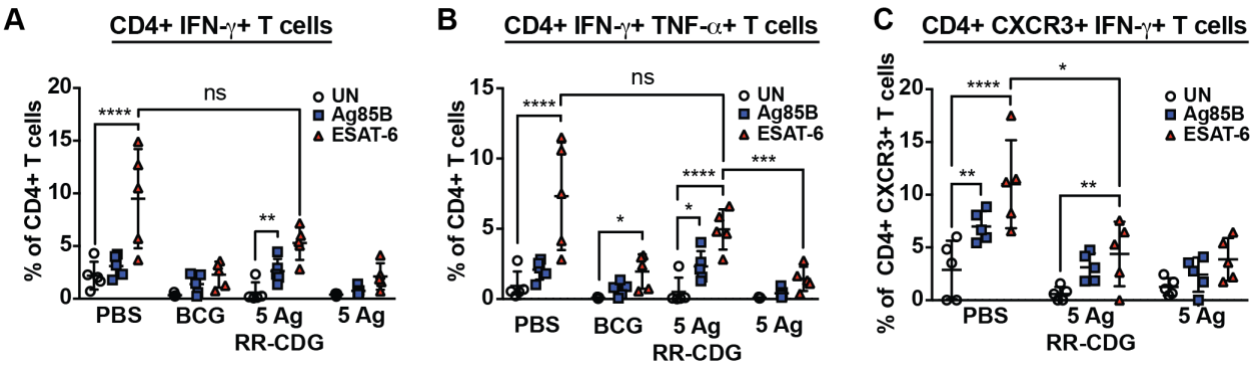

Figure 5: RR-CDG adjuvanted vaccine leads to antigen-specific T cell responses. **(A** and **B)** ICS for percentage of antigen-specific CD4+ T cells in the lungs of mice that produce **(A)** IFN-γ or **(B)** both IFN-γ and TNF-α, upon re-stimulation ex vivo with indicated peptide pools at 4 weeks post challenge. **(C)** ICS for percentage of antigen-specific CD4+ CXCR3+ KLRG1- T cells in the lungs of mice that produce IFN-γ upon re-stimulation ex vivo with indicated peptide pools at 4 weeks post challenge. Two-way ANOVA with Tukey's post-hoc p value * < 0.05, ** < 0.002, *** < 0.0002, **** < 0.0001. Data are expressed as mean (±SD) and each symbol represents an individual animal. Data are representative of experiments done in duplicate.

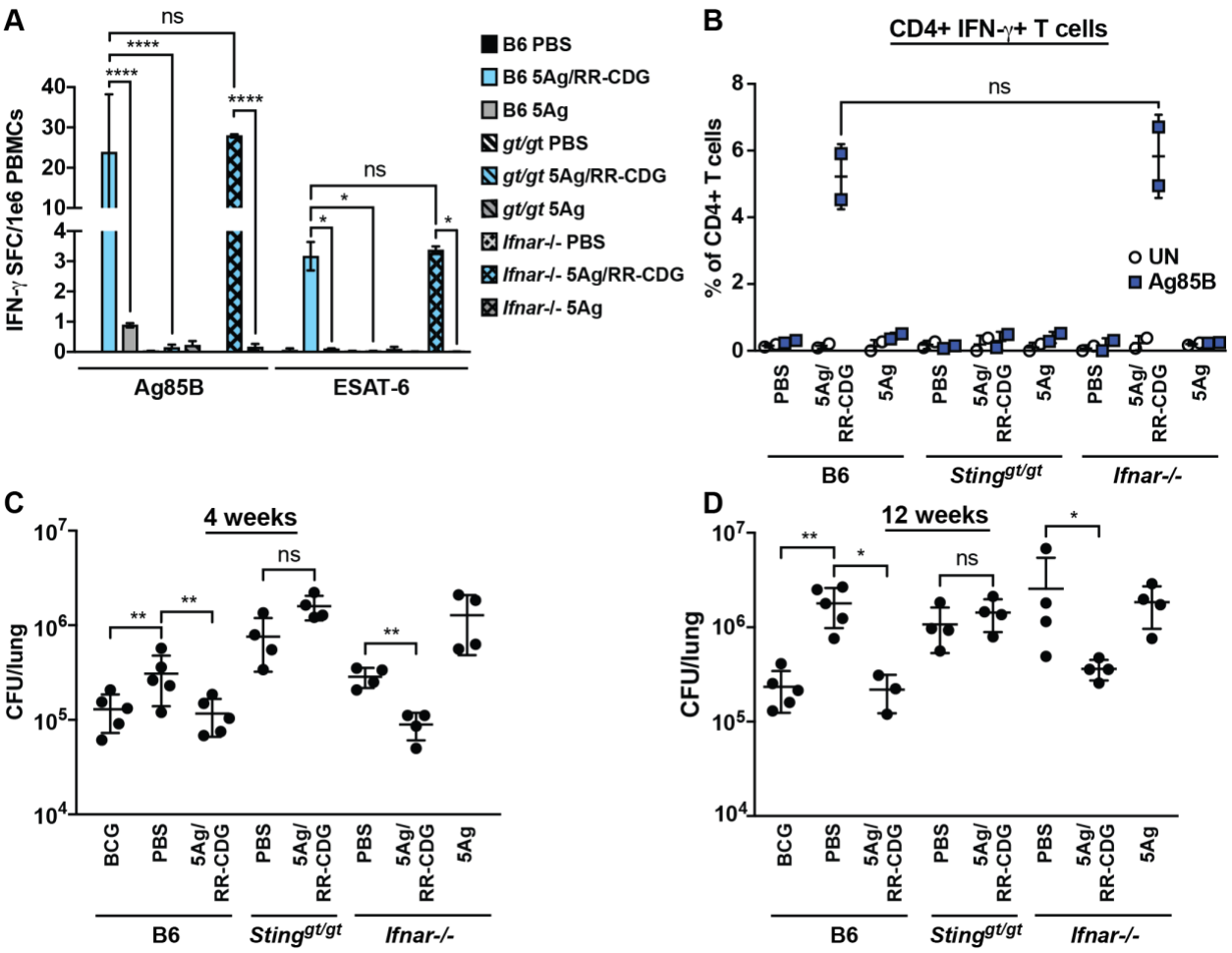

Figure 6: 5Ag/RR-CDG vaccine-induced protection requires STING but not type I IFN signaling. (A) IFN-γ ELISPOT from PBMCs re-stimulated *ex vivo* with Ag85B and ESAT-6 peptide pools seven days after the 2nd boost. Data are expressed as the mean (±SD) of 10 animals assayed in two pools of five. **(B)** ICS for percentage of Ag85B-specific CD4+ T cells that produce IFN-γ seven days after the 2nd boost. Data are expressed as mean (±SD) and each symbol represents blood pooled from five animals. Two-way ANOVA with Tukey's post-hoc p value * < 0.05, **** < 0.0001. **(C** and **D)** CFU counts from lungs of vaccinated mice at **(C)** 4 weeks and **(D)** 12 weeks post challenge. For CFU experiments, data are expressed as mean (±SD) and each symbol represents an individual animal. Mann Whitney t-test p value * < 0.05, ** < 0.002. Data are representative of experiments done in duplicate.

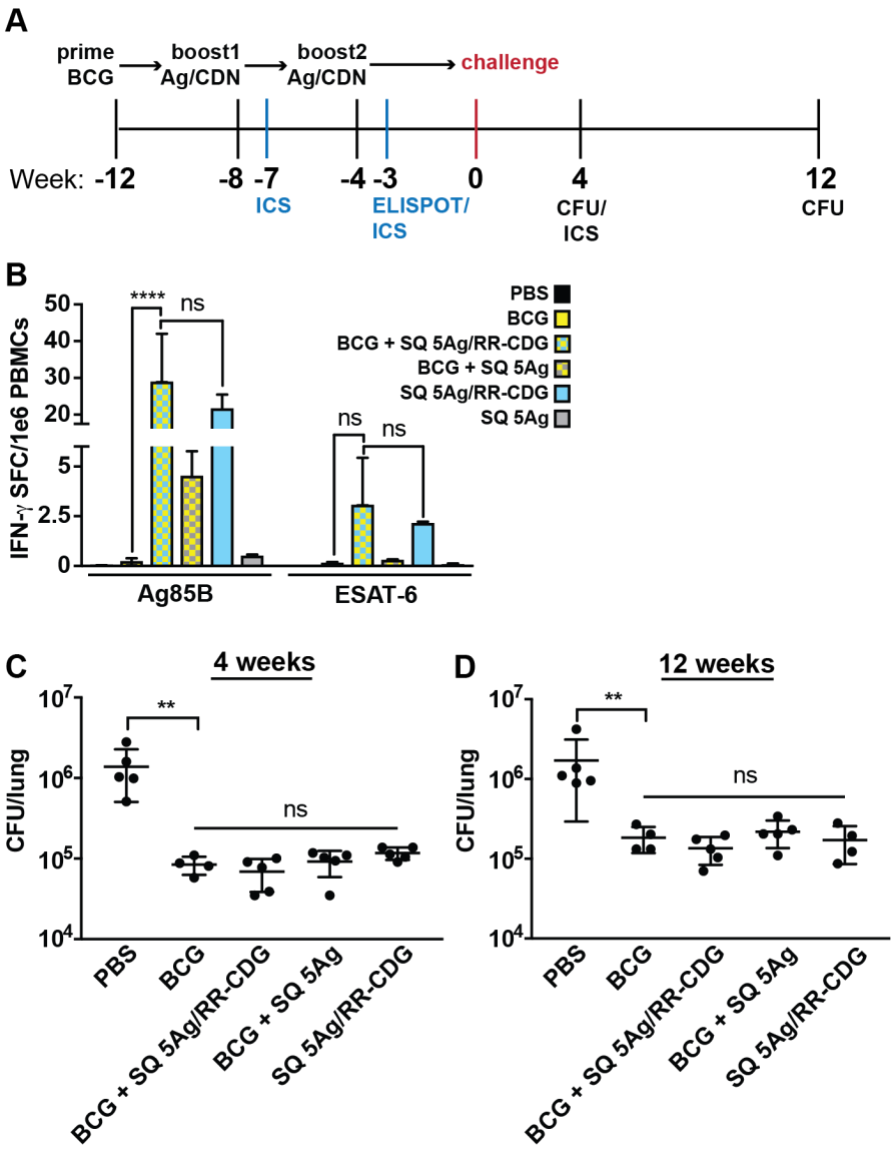

Figure 7: Subcutaneous (SQ) boosting of BCG with 5Ag/RR-CDG does not enhance protection from *M. tuberculosis* challenge. (A) Experimental timeline for BCG boosting experiments. Mice were vaccinated using the indicated schedule and challenged with ~100 CFU of *M. tuberculosis* strain Erdman. **(B)** IFN-γ ELISPOT from PBMCs re-stimulated *ex vivo* with Ag85B and ESAT-6 peptide pools seven days after the 2nd boost. Data are expressed as the mean (±SD) of 10 animals assayed in two pools of five. Two-way ANOVA with Tukey's post-hoc p value **** < 0.0001. **(C** and **D)** CFU counts from lungs of vaccinated mice at **(C)** 4 weeks and **(D)** 12 weeks post challenge. For CFU experiments, data are expressed as mean (±SD) and each symbol represents an individual animal. Mann Whitney t-test p value ** < 0.002. Data are representative of experiments done in duplicate.

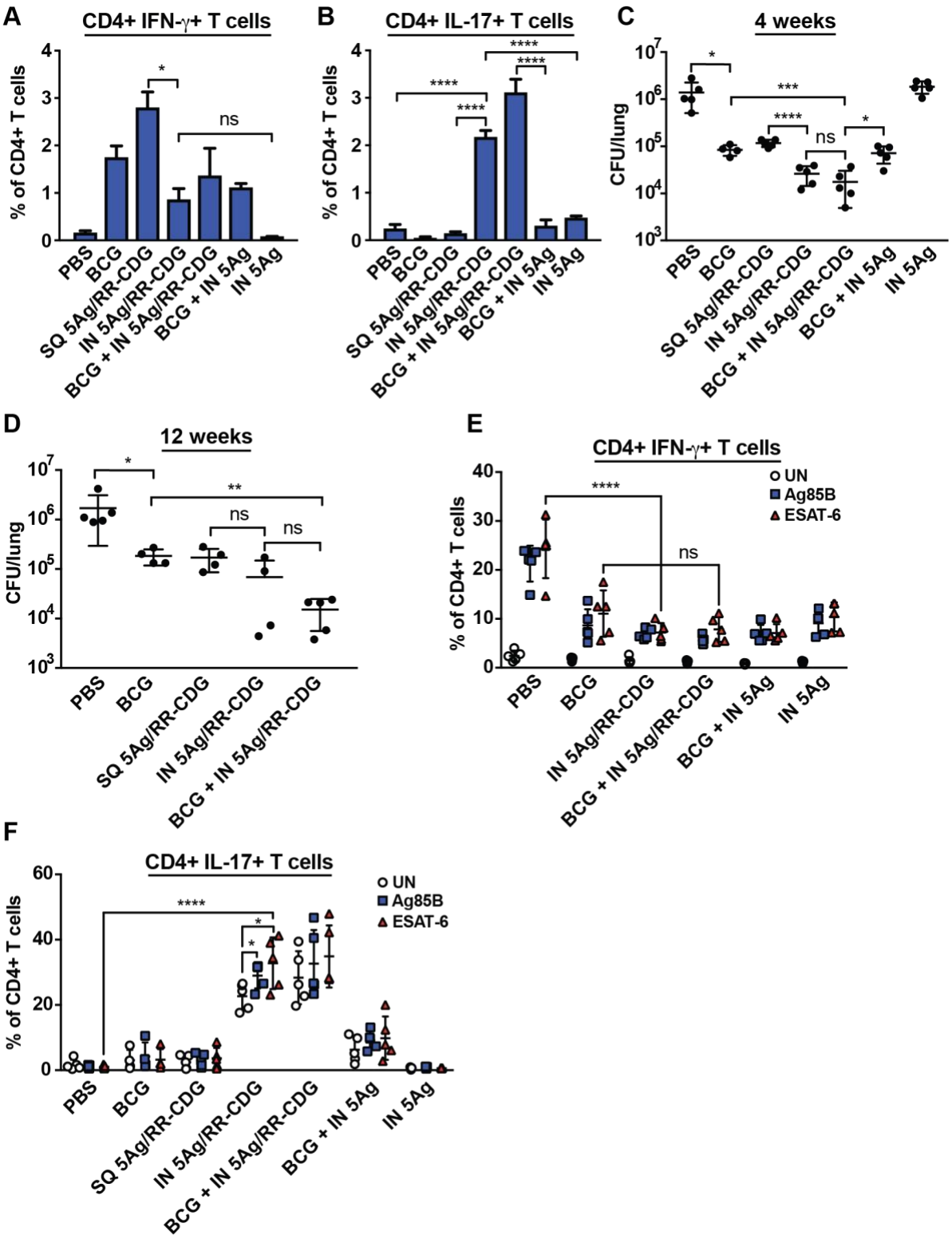

Figure 8: Intranasal (IN) administration of 5Ag/RR-CDG induces Th17 cells and enhances protection when used as a booster vaccine for BCG. (A and **B)** ICS for percentage of Ag85B-specific CD4+ T cells that produce **(A)** IFN-γ or **(B)** IL-17 seven days after the 2nd boost. Data are expressed as the mean (±SD) of 10 animals assayed

in two pools of five. One-way ANOVA with Tukey's post-hoc p value * < 0.05, **** < 0.0001. **(C** and **D)** CFU counts from lungs of vaccinated mice at **(C)** 4 weeks and **(D)** 12 weeks post challenge. Mann Whitney t-test p value * < 0.05, ** < 0.002, *** < 0.0002, **** < 0.0001. **(E** and **F)** ICS for percentage of antigen-specific CD4+ T cells from the lungs of infected mice that produce **(E)** IFN-γ or **(F)** IL-17 upon re-stimulation *ex vivo* with Ag85B or ESAT-6 peptide pools at 4 weeks post challenge. Two-way ANOVA with Tukey's post-hoc p value * < 0.05, **** < 0.0001. For **(C** – **F)**, data are expressed as mean ($\pm$SD) and each symbol represents an individual animal. Data are representative of experiments done in duplicate. See also Figure S5.

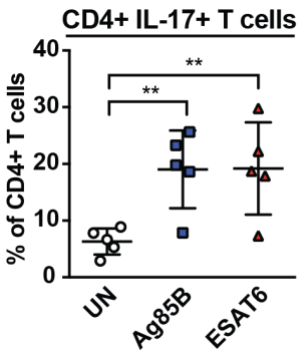

Figure 9: Intranasal (IN) administration of 5Ag/RR-CDG induces antigen-specific Th17 cells. ICS for percentage of antigen-specific CD4+ T cells from the lungs of 5Ag/RR-CDG vaccinated mice that produce IL-17 upon re-stimulation ex vivo with indicated peptide pools at 4 weeks post challenge. Two-way ANOVA with Tukey's post-hoc p value ** < 0.002. Data are expressed as mean (±SD) and each symbol represents an individual animal.

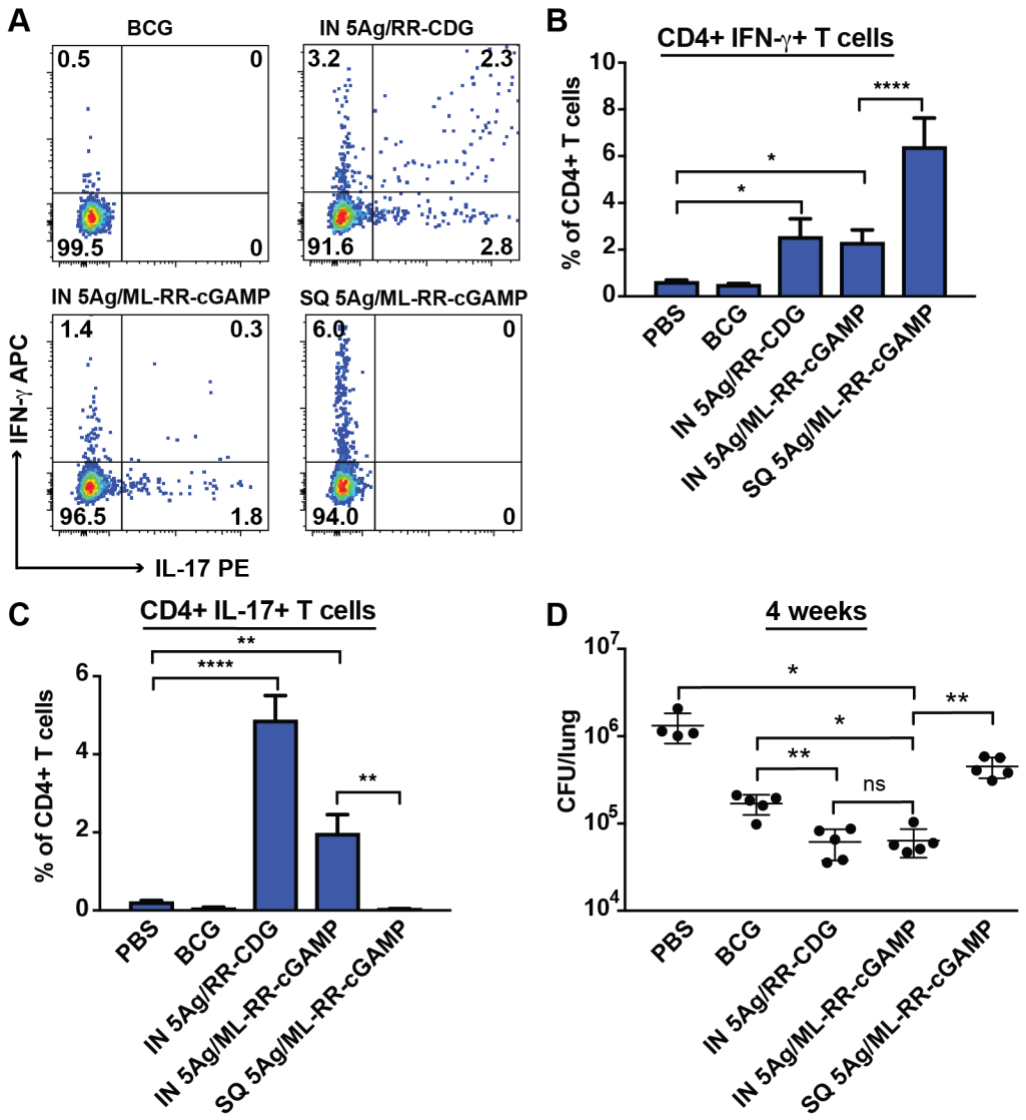

Figure 10: ML-RR-cGAMP adjuvanted vaccine elicits a Th17 response and protects against challenge with *M. tuberculosis.* (A) Representative flow plots showing log10 fluorescence and percentage of Ag85B-specific CD4+ T cells that produce IFN-γ, IL-17 or both 7 days after the 1st boost. **(B** and **C)** ICS for percentage of Ag85B-specific CD4+ T cells that produce **(B)** IFN-γ or **(C)** IL-17 seven days after the 1st boost. Data are expressed as mean (±SD) of five mice per group. Two-way ANOVA with Tukey's post-hoc p value * < 0.05, ** < 0.002, **** < 0.0001. **(D)** CFU counts from lungs of vaccinated mice at 4 weeks post challenge. Data are expressed as mean (±SD) and each symbol represents an individual animal. Mann Whitney t-test p value * < 0.05, ** < 0.002. Data are representative of experiments done in duplicate.

Chapter Three: Mucosal vaccination with cyclic-di-nucleotide adjuvants induces effective T cell homing and IL-17 dependent protection against *M. tuberculosis* infection

This work has been uploaded to bioRxiv:

Jong RM*, Van Dis E*, Nguyenla X, Baltodano A, Pastenkos G, Xu C, Yosef N, McWhirter SM, Stanley SA. Mucosal vaccination with cyclic-di-nucleotide adjuvants induces effective T cell homing and IL-17 dependent protection against *M. tuberculosis* infection. doi: https://doi.org/10.1101/2020.11.25.398651

*These authors contributed equally

Abstract

Tuberculosis consistently causes more deaths worldwide annually than any other single pathogen, making new effective vaccines an urgent priority for global public health. However, progress is limited by a lack of vaccine adjuvants that have been shown to be effective for vaccines against intracellular pathogens such as *Mycobacterium tuberculosis*. STING-activating cyclic-di-nucleotides (CDNs) uniquely activate a cytosolic sensing pathway activated by virulent pathogens. Recently, we demonstrated that a CDN adjuvanted protein subunit vaccine robustly protects against tuberculosis infection in mice. Here we delineate the mechanistic basis of efficacy of CDN vaccines for tuberculosis. CDN adjuvants appear to be uniquely effective in eliciting the production of T cells that home to lung parenchyma and penetrate into the core of bacteria containing macrophage lesions in the lung. In contrast to what is known about primary immunity to tuberculosis, protection is partially dependent on IL-17 and type I interferon (IFN). Single cell transcriptomics reveals mucosal vaccination with a CDN vaccine increases transcriptional heterogeneity in CD4 cells, including a significant population of non-classical IFN-γ and IL-17 co-expressing Th1-Th17 cells, as well as markers of memory and activation. Th1-Th17 cells in vaccinated mice are enriched for expression of the T cell functional markers *Tnfsf8* and *Il1r1* relative to more conventional Th1 cells. These data provide critical insight into the immune mediators and diversity of T cell responses that can contribute to vaccine efficacy against *M. tuberculosis* infection.

Introduction

Tuberculosis (TB) causes more deaths worldwide than any other single infectious agent(Organization, 2020). The existing live-attenuated vaccine against TB, *M. bovis* Bacille Calmette-Guérin (BCG), is widely administrated in many countries with endemic TB and is effective against severe forms of childhood disease (Organization, 2020). However, efficacy wanes over time and BCG provides minimal protection against adult pulmonary TB (Colditz, 1994; Fine, 1995; Mangtani et al., 2014). There is a clear need for an improved vaccine to combat this global health threat. One of the barriers to the development of effective vaccines for TB is the lack of reliable immune correlates of protection. Immunity to TB has long been considered to be dependent on the production of IFN-γ by CD4 T cells (Cooper, 2009), as defects in IFN-γ signaling are linked to susceptibility to mycobacterial infection (Caruso et al., 1999; Flynn et al., 1993; Green et al., 2013). Thus, the development of new TB vaccines to date has focused on eliciting IFN-γ-producing CD4 T cells and polyfunctional CD4 T cells that produce IFN-γ, TNF-α, and IL-2 (Lewinsohn et al., 2017; Rodo et al., 2019). A highly anticipated clinical trial of the BCG booster protein subunit vaccine MVA-85A showed successful induction of *M. tuberculosis*-specific polyfunctional CD4 T cells in infants(Tameris et al., 2013). However, this immune response did not translate into significant protection against TB infection or disease (Tameris et al., 2013). More recently, the protein subunit vaccine candidate M72/AS01E stimulated IFN-γ producing T cells and demonstrated 50% efficacy in preventing the transition from latent to active disease over a 3-year period (Tait et al., 2019). Why two vaccines that both elicit IFN-γ-producing CD4 T cells bore different results is unclear, however these results suggest that alternate biomarkers of efficacy may more clearly predict vaccine efficacy.

Animal studies have suggested the existence of IFN-γ-independent mechanisms of CD4 T cell-mediated control of *M. tuberculosis* infection (Caruso et al., 1999; Cowley and Elkins, 2003; Gallegos et al., 2011; Mittrücker et al., 2007), however, the basis of this control has remained elusive (Nunes-Alves et al., 2014). IL-17 producing Th17 T cells do not contribute to control of primary TB infection in mice, possibly because they are not robustly elicited during primary infection. However, intranasal administration of BCG (Aguilo et al., 2016; Perdomo et al., 2016) or protein subunit vaccines (Gopal et al., 2013b; Monin et al., 2015) elicits IL-17-producing memory T cells that correlate with enhanced protection against *M. tuberculosis* challenge in rodent models. Th17 cells are a heterogenous effector subset of CD4 T cells that can range in function from inflammatory to regulatory and can even transdifferentiate into Th1 cells(Bystrom et al., 2019). Although TB vaccine-elicited memory Th17s can persist in lung-draining lymph nodes and acquire Th1 characteristics after challenge (Lindenstrøm et al., 2012), it is unclear which Th17 subtype is most important for vaccine-elicited control of TB, or how this subtype enhances control of *M. tuberculosis* infection.

In order to exert control of infection, T cells must both differentiate into a protective effector state and adopt an effector phenotype that allows them to migrate to the site of infected macrophages in the lung. In mouse models, lung parenchyma-homing $CXCR3^+$ $CD153^+$ $KLRG1^-$ CD4 T cells mediate superior protection against *M. tuberculosis* compared to vasculature-associated $KLRG1^+$ CD4 T cells that do not migrate into the lung tissue (Sakai et al., 2014; Sallin et al., 2018). Furthermore, T cells

that are blocked from entering the granuloma core are unable to provide as effective protection as T cells that penetrate into the central core of infected macrophages (Gern et al., 2021). However, it is unclear how to rationally tailor a vaccine that will elicit CD4 T cell homing to granuloma cores.

We recently demonstrated in mice that an experimental protein subunit vaccine formulated with STING-activating cyclic-di-nucleotide (CDN) adjuvants is highly efficacious in mice, particularly when delivered by a mucosal route (Van Dis et al., 2018). Here we find that protective efficacy correlates with the production of parenchymal homing T cells, penetration of T cells into lung lesions, and a T cell response dominated by Th17 at early timepoints. We further show that type I IFN, IL-17, and IFN-γ are required for complete intranasal CDN-adjuvanted vaccine-induced protection. While intranasal vaccine delivery elicits IFN-γ-producing CD4 T cells, vaccine-mediated protection is partially IFN-γ-independent and does not correlate with increased Th1 responses. Single cell transcriptomics (scRNA-seq) shows that naïve infected mice predominantly produce KLRG1^{+} CX3CR1^{+} effector Th1s that do not infiltrate lung lesions, while mucosal vaccination promotes activation and memory marker-expressing CD4 T cells. Intranasal vaccination induces T cell heterogeneity, particularly within the *Il17a*-expressing subset, and a large number of mixed Th1-Th17 cells. These results demonstrate that CDN-adjuvanted protein subunit vaccines generate a multifaceted CD4 T cell response that leads to enhanced protection against TB disease.

Materials and Methods

Ethics Statement. All procedures involving the use of mice were approved by the University of California, Berkeley Institutional Animal Care and Use Committee (protocol 2015-09-7979). All protocols conform to federal regulations, the National Research Council Guide for the Care and Use of Laboratory Animals, and the Public Health Service Policy on Humane Care and Use of Laboratory Animals.

Vaccine Reagents. ML-RR-cGAMP was synthesized at Aduro Biotech as described previously (Gaffney et al., 2010). AddaVax (InvivoGen, San Diego, CA) was used for the subcutaneous formulation of antigen and adjuvant as directed by the manufacturer. 5Ag fusion protein was provided by Aeras, H1 fusion protein was provided by Aeras and Statens Serum Institut, and peptide pools were provided by the NIH BEI Resources Repository.

Mice. C57BL/6J (#000664), *Il17a*^{Cre} (#016879, referred to here as *Il17*$^{-/-}$), *Ifng*$^{-/-}$ (#002287) and *Stat6*$^{-/-}$ (#005977) mice were obtained from The Jackson Laboratory (Bar Harbor, ME) and bred in-house. *Ifnar1*$^{-/-}$ (*Ifnar*$^{-/-}$, Jackson Laboratory strain #028288) mice were obtained from the Vance lab (University of California, Berkeley). Mice were sex- and age-matched for all experiments.

Bacterial Culture. *M. tuberculosis* strain Erdman and *M. bovis* BCG (Pasteur) were grown in Middlebrook 7H9 liquid medium supplemented with 10% albumin- dextrose-saline (*M. tuberculosis*) or 10% oleic acid, albumin, dextrose, catalase (OADC) (BCG), 0.4% glycerol, and 0.05% Tween 80 or on solid 7H10 agar plates supplemented with 10% Middlebrook OADC (BD Biosciences) and 0.4% glycerol. Frozen stocks of *M. tuberculosis* and BCG were made from single cultures and used for all experiments.

Vaccinations. 5 µg ML-RR-cGAMP and 3 µg fusion antigen protein (5Ag or H1) were formulated in PBS for intranasal (i.n.) delivery or in 2% AddaVax in PBS for subcutaneous (s.c.) delivery. Mice were vaccinated three times at 4-week intervals with 20 µL i.n or with 100 µL s.c. at the base of the tail (50 µL on each flank). BCG-vaccinated mice were injected once with 2.5 x 10^5 CFU/mouse in 100 µL of PBS plus 0.05% Tween 80 s.c. in the scruff of the neck.

Challenge Experiments with *M. tuberculosis*. Twelve weeks after the initial vaccine injection, mice were infected via the aerosol route with *M. tuberculosis* strain Erdman. Aerosol infection was done using a nebulizer and full-body inhalation exposure system (Glas-Col, Terre Haute, IN). A total of 9 mL of culture diluted in sterile water was loaded into the nebulizer calibrated to deliver 50 to 100 bacteria per mouse as measured by CFU in the lungs 1 day following infection (data not shown).

Pre-challenge ICS Assay. Heparinized blood lymphocytes were isolated 9 weeks post priming, processed as previously described (Van Dis et al., 2018), stained with Live/Dead stain (Thermo Fisher Scientific, L34970), Fc receptor block, CXCR3, major histocompatibility complex (MHC) class II, KLRG1, and TCRγ/δ (BioLegend, 101319,

126522, 107606, 107606, and 118124, respectively), and CD4, CD8, CD90.2 and Ly6G (BD Biosciences, 564933, 563898, 561616 and 551460), and fixed/permeabilized with BD Cytofix/Cytoperm Fixation/Permeabilization Solution Kit (Thermo Fisher, 554714) before staining with IFN-γ (eBioscience, 12-73111-81) and IL-17 (BioLegend, 506904). Data were collected using a BD LSR Fortessa flow cytometer and analyzed using FlowJo Software (Tree Star, Ashland, OR).

Post Challenge Cell Preparation. For bacterial enumeration, the largest lung lobe was homogenized in PBS plus 0.05% Tween 80, and serial dilutions were plated on 7H10 plates. CFU were counted 21 days after plating. The second largest lung lobe was fixed in 10% formalin for histological analysis or used for single cell RNA- sequencing. The remaining lung lobes were harvested into complete RPMI, dissociated, and strained through a 40 μm strainer. Cells were re-stimulated with no peptide or Ag85B peptide pools (2 μg/mL) with Protein Transport Inhibitor Cocktail (eBioscience, 00-4980-93) for 5 hours at 37C. Cells were washed and stained with antibodies used for pre-challenge ICS. Data were collected and analyzed as outlined above. Data presented are from Ag85B-stimulated samples for cytokine staining, and unstimulated samples for all other flow cytometric analyses.

Histological and immunohistochemical analysis. Lung lobes were fixed in 10% formalin at room temperature for at least 24 hours. Histology was performed by HistoWiz Inc. (histowiz.com) using a Standard Operating Procedure and fully automated workflow. Samples were processed, embedded in paraffin, and sectioned at 4μm. Antibodies used were rat monoclonal CD45R/B220 (Biolegend, 103229), rat monoclonal CD3 primary antibody (Abcam), and rabbit anti-rat secondary antibody (Vector). BOND polymer Refine Detection (Leica Biosystems) was used according to manufacturers' protocol. After staining, sections were dehydrated and film coverslipped using a TissueTek-Prisma and Coverslipper (Sakura). Whole slide scanning (40x) was performed on an Aperio AT2 (Leica Biosystems). Slide images are available online at https://app.histowiz.com/shared_orders/6d2c7816-b292-42c4-9ee2-71ffe032957e/slides/. Tissue sections were evaluated by a veterinary anatomic pathologist at the UC Davis Comparative Pathology Laboratory. Hemotoxylin and eosin (H&E) images were analyzed using Olympus cellSens software. Semiquantitative scoring was used to evaluate the degree of inflammation and the presence and severity of an array of inflammatory features. Here, lymphoid nodules are defined as discrete, nodular or ovoid, homogenous aggregates of lymphocytes comprising an area > 1000 um^2; lymphoid follicles exhibit germinal center development as well as the previously stated features; granulomas are defined as discrete, nodular aggregates of macrophages that efface pulmonary parenchyma, occasionally exhibit circumferential arrangement, and contain multinucleate giant cells. Immunohistochemical and immunoflourescent staining was analyzed using QuPath software(Bankhead et al., 2017). In brief, classifiers were trained to detect regions of inflammation, cytoplasmic CD3 immunoreactivity, and cytoplasmic B220 immunoreactivity by manual annotation of a subset of images. Classifiers were applied to either the total area of a lung section or to manually annotated regions of interest (ROIs) within a lung section. A single measurement classifier was generated for detection of cytoplasmic CD3

immunoreactivity within lesion. The classifier was applied to three equal-area ROI in each section of lung. For detection of cytoplasmic B220 immunoreactivity, classifiers were applied to the total sectional area of each lung section and the percentage of B220+ immune cells was determined. Lymphoid nodules were defined as discrete, nodular or ovoid, homogenous aggregates of lymphocytes with an area > 10000 um^2. Granulomas were defined as discrete, nodular aggregates of macrophages that efface pulmonary parenchyma, occasionally exhibit circumferential arrangement, and contain multinucleate giant cells.

Single Cell RNA Sequencing. The second largest lung lobes were pooled from three mock or i.n. H1/ML-RR-cGAMP vaccinated mice, tissue dissociated in RPMI containing liberase and DNase I in gentleMACS C tubes using the gentleMACS dissociator and strained through a 70μm filter for sorting on a Sony SH-800 sorter using a 100μm nozzle. Sorted CD4 T cells were washed in RPMI and counted. 5000-6000 CD4 T cells per sample were used for single cell RNA sequencing (scRNA-seq) according to the 10x Genomics protocol. Briefly, single cell suspensions were partitioned into Gel Beads in emulsion (GEMs) using the 3' Chromium Next GEM single Cell v3.1 system. 10x barcoded primers were used to reverse transcribe poly-adenylated mRNA to produce barcoded, full-length cDNA. Purified DNA was amplified, fragmented, and amplified again to attach Illumina adapter sequences by the Functional Genomics Laboratory at UC Berkeley. Libraries were sequenced on an Illumina NovaSeq SP 100SR and demultiplexed by the Vincent J. Coates Genomics Sequencing Laboratory at UC Berkeley. Reads were aligned to the mouse transcriptome mm10 with the 10x Cell Ranger analysis pipeline(Zheng et al., 2017), using the Savio computational cluster at UC Berkeley. After filtering, barcode counting, and UMI counting, the Scanpy toolkit (Wolf et al., 2018) was used to preprocess the data. The single cell variational inference (scVI)(Lopez et al., 2018) approach was utilized to project the data from both vaccinated and naïve mice into a shared lower-dimensional latent space. Uniform manifold approximation and projection (UMAP) and Leiden clustering were then applied on the *k*-Nearest-Neighbor graph of the latent space for visualization and clustering of the data. Leiden clusters that expressed high levels of myeloid, B cell, or CD8 T cell markers were excluded from the data before utilizing the scVI model a second time. The filtered data was annotated using signature genes from CD4 T cell subset in cellxgene (https://github.com/chanzuckerberg/cellxgene), a single-cell data exploration tool. All data has been deposited in GEO (GSE164590)**.** Scanpy was used to visualize data and test differential expression of genes between samples and CD4 T cell subsets. The single-cell ANnotation using Variational Inference (scANVI)(Xu et al.) variant of scVI was used to assign annotations to unassigned cells based on high-confidence seed labels annotated on cellxgene. High-confidence seed cells were identified using a high threshold of expression as cutoffs. In the scANVI analysis, *Tbx21* and *Rorc* were also used to define classical Th1 and Th17, respectively.

Statistical Analysis. Data are presented as mean values, and error bars represent SD. Symbols represent individual animals. The number of samples and statistical tests used are denoted in the legend of the corresponding figure for each experiment. Analysis of

statistical significance was performed using GraphPad Prism 8 (GraphPad, La Jolla, CA), and $p < 0.05$ was considered significant.

Results

We previously found that mucosal administration of a protein subunit vaccine with CDN adjuvants elicits significant protection against *M. tuberculosis* in the mouse model of infection (Van Dis et al., 2018). We therefore set out to define the mechanisms underlying the efficacy of CDN adjuvants for TB vaccines. For antigen we used either 5Ag—a five antigen fusion of *M. tuberculosis* proteins that includes the immunodominant proteins Ag85B and ESAT-6 and three proteins predicted to be expressed in bacterial latency (Van Dis et al., 2018)—or H1 antigen, a fusion of Ag85b-ESAT6 alone, as 5Ag and H1 were functionally equivalent for protection out to 4 weeks post challenge (data not shown) (Van Dis et al., 2018). Protein antigen was formulated with the CDN ML-RR-cGAMP (Corrales et al., 2015).

CDN-adjuvanted protein subunit vaccines elicit a rapid influx of T cells and control of infection upon *M. tuberculosis* challenge.

We first characterized the kinetics of protection and CD4 T cell infiltration elicited by H1/ML-RR-cGAMP in lungs after challenge. Mice were vaccinated 12 weeks prior to challenge with *M. tuberculosis*, either by the intranasal route (i.n.) with H1/ML-RR-cGAMP three times at 4 week intervals, or once with subcutaneous (s.c.) BCG, the standard route for BCG. Vaccination with H1/ML-RR-cGAMP resulted in increased protection when compared with BCG at 1 and at 2 weeks post challenge, demonstrating that mucosal CDN adjuvanted-vaccination induces immunity earlier than BCG vaccination (Figure 1A). Protection correlated with an influx of antigen-specific IL-17-producing T cells into the lungs at 1 week post challenge that increased steadily until 3 weeks post challenge (Figure 1B). Neither mock (PBS-treated) nor BCG-vaccinated mice showed appreciable Th17 responses (Figure 1B). Th1 frequency dramatically increased in mock vaccinated mice between 2-3 weeks post challenge (Figure 1C), eclipsing that of the vaccinated mice and coinciding with a larger bacterial burden (Figure 1A). A smaller population of CD4 T cells produced both IFN-γ and IL-17 (Figure 1D). While several sources have suggested that TCRγ/δ T cells are the main source of IL-17 in *M. tuberculosis*-infected mouse lungs during primary infection (Lockhart et al., 2006; Saitoh et al., 2012), we observed no difference in frequencies of TCRγ/δ^+ T cells or in the subset of IL-17^+ TCRγ/δ T cells in mock vs vaccinated mice after infection (Figure 2A, B). Thus, the evidence points to a role for vaccine-induced IL-17-producing conventional CD4 T cells in early and sustained mucosal vaccine efficacy.

IL-17 is required for full efficacy of CDN adjuvanted protein subunit vaccines.

To determine whether IL-17 is necessary for protective efficacy, we vaccinated wild type and *Il17*$^{-/-}$ mice and enumerated bacterial burdens in the lungs 4 weeks post challenge with *M. tuberculosis*. Bacterial burden was unaffected by the loss of IL-17 in mice that were mock vaccinated, vaccinated s.c. with 5Ag/ML-RR-cGAMP, or vaccinated s.c. with BCG (Figure 3A). However, we observed a significant loss of protection in *Il17*$^{-/-}$ mice that were vaccinated with i.n. 5Ag/ML-RR-cGAMP. While IL-17 has been reported to impact the development and recruitment of vaccine-induced Th1 cells (Gopal et al., 2012; Khader et al., 2007), Th1 cell production was unaffected in

Il17$^{-/-}$ mice at this timepoint (Figure3B). These data suggest that the efficacy of mucosal CDN vaccines is partially IL-17 dependent.

Type I interferon and IFN-γ contribute to CDN adjuvanted mucosal vaccine efficacy.

STING agonists strongly induce type I IFN expression through IRF3-dependent signaling (McWhirter et al., 2009). As type I IFN responses can negatively regulate the development of Th17 cells (Mourik et al., 2017) and type I IFN can play a detrimental role in primary *M. tuberculosis* infection immunity (Donovan et al., 2017; Ji et al., 2019; Stanley et al., 2007), it is somewhat surprising that STING agonists elicit robust Th17-dependent protective immunity. We tested the efficacy of i.n. 5Ag/ML-RR-cGAMP vaccination in IFNα/β receptor deficient mice (*Ifnar*$^{-/-}$). As expected, the Th17 response was more robust in the lungs of *Ifnar*$^{-/-}$ mice at 4 weeks post challenge (Figure 3C), while the Th1 response was unaffected (Figure 3D). However, despite an increased *M. tuberculosis*-specific Th17 response, we observed a small but statistically significant loss of control in vaccinated *Ifnar*$^{-/-}$ mice (Figure 3E). The increased Th17 response in *Ifnar*$^{-/-}$ mice thus cannot compensate for the loss of type I IFN in vaccine-elicited protective immunity.

STING also signals through both NF-κB and STAT6. STAT6 is a transcription factor that can be activated downstream of STING, leading to chemokine production that promotes control of viral infections (Chen et al., 2011). Loss of STAT6 did not affect Th17 responses in *Stat6*$^{-/-}$ vaccinated mice (Figure 3F). A slight decrease in Th1 frequency in *Stat6*$^{-/-}$ intranasal vaccinated mice (Figure 3G) did not translate to an impact on the efficacy of i.n. 5Ag/ML-RR-cGAMP (Figure 3H). Therefore, the efficacy of CDN adjuvanted TB vaccines is not dependent on STAT6 signaling.

We next sought to determine whether the classical Th1 response plays a significant role in protection. We vaccinated mice lacking IFN-γ (*Ifng*$^{-/-}$) with H1/ML-RR-cGAMP subcutaneously or intranasally and harvested lungs at 3 weeks post challenge to assess vaccine efficacy. Loss of IFN-γ did not affect the robust Th17 response in i.n. vaccinated mice prior to challenge (Figure 4A) or at the peak of the T cell response at 3 weeks post challenge (Figure 4B). While all *Ifng*$^{-/-}$ mice were more susceptible to *M. tuberculosis* infection, the subcutaneous and mucosal vaccines both provided significant protection compared to mock vaccination (Figure 4C, D). While this demonstrates that both routes of inoculation elicit IFN-γ-independent mechanisms of control, the magnitude of protection was significantly reduced in *Ifng*$^{-/-}$ mice (Figure 4D), indicating that IFN-γ does play some role in vaccine efficacy, in conjunction with IFN-γ-independent mechanisms.

Lymphocytes home to infected tissue in CDN vaccinated mice.

Proper localization of T cells in infected lungs is crucial for effective T cell-mediated immunity (Urdahl, 2014). CXCR3^{hi} CD4 T cells can localize to the infected mouse lung parenchyma and mediate superior bacterial resistance compared to KLRG1^{hi} T cells, which remain trapped in the vasculature (Lindenstrøm et al., 2018; Sakai et al., 2014). We found that i.n. vaccination with H1/ML-RR-cGAMP induced higher frequencies of CXCR3^{+} and lower frequencies of KLRG1^{+} CD4 T cells in infected mouse lungs than s.c. BCG vaccination or mock vaccination (Figure 5A, B). Conversely,

BCG vaccination induced a higher percentage of KLRG1$^+$ cells than i.n. H1/ML-RR-cGAMP vaccination (Figure 5B). In naïve infected mice, significant numbers of KLRG1$^+$ T cells appeared in the lungs at 3-4 weeks post *M. tuberculosis* challenge (Figure 5B). We next sought to determine whether i.n. vaccination with 5Ag/ML-RR-cGAMP changed T and B cell localization within infected lung tissue. Consistent with previous reports (Sakai et al., 2014), we found that in naïve infected mice, very few CD3$^+$ T cells were observed in lung lesions (Figure 5C). However, i.n. vaccination effectively enhanced infiltration of CD3$^+$ T cells into the lesion (Figure 5C, D) without increasing overall lung inflammation (Figure 6A). Although previous reports associated the efficacy of mucosal TB protein subunit vaccines with the formation of B cell follicles in vaccinated murine lungs (Gopal et al., 2013b; Monin et al., 2015), we did not observe B cell follicles with germinal center organization in any of the mice at 4 weeks post challenge (Figure 6B). However, i.n. vaccinated mouse lungs were more likely to contain lymphocyte aggregates without germinal centers (lymphoid nodules, Figure 6C) and B220$^+$ staining was increased in i.n. vaccinated mouse lungs (Figure 5E, F), suggesting that mucosal vaccination may boost the B cell response. Overall levels of inflammation did not correlate with protection (Figure 6A-G). Thus, mucosal vaccination leads to intralesional T cell homing and B cell infiltration into infected lungs.

Single cell analysis reveals shifts in naïve and vaccinated CD4 T cell compartment transcriptomes.

To gain a more detailed understanding of the characteristics of the CD4 T cell response during *M. tuberculosis* infection we performed single cell RNA-sequencing (scRNA-seq) (Papalexi and Satija, 2018) on CD4 T cells sorted from naïve and i.n. H1/ML-RR-cGAMP vaccinated infected mouse lungs at the peak of the T cell response, 3 weeks post infection. The filtered dataset, analyzed by single cell variational inference (scVI) (Lopez et al., 2018), consisted almost exclusively of CD4 T cells (Figure 7A). We first characterized the lung CD4 T cell response in naïve infected animals. Using signature transcription factor, cytokine, and cell surface marker expression, we were able to assign 56% of cells to classic CD4 T cell subsets while the rest of the cells could not be confidently assigned due to lack of marker expression. Our analysis identified clear populations of Th1 (*Ifng*), Treg (*Foxp3*), and naive (*Ccr7*, *Sell*; *Cd44^{lo}*) T cells (Figure 8A). The largest population of assigned cells expressed the Th1 gene marker *Ifng*, in agreement with our flow cytometry analysis (Figure1C). Very few T cells expressed the Th17 marker *Il17a*, and a small population of cells expressed *Foxp3* (Figure 8A, C). Few Th2 (*Gata3*, *Il4*) or Tfh (*Bcl6*, *Cxcr5*) cells were observed. Naive, Treg, and proliferating (*Mki67*) cells formed a clear cluster, while other cell subtypes—Th2, Th17, Tfh—varied more continuously in the transcriptional space. CD4 T cells were also isolated from i.n. H1/ML-RR-cGAMP vaccinated infected lungs, and 46% of cells were assigned to a CD4 T cell subset (Figure 8B). *Ifng*-expressing Th1 cells were the dominant CD4 subtype in vaccinated lungs, although fewer in frequency compared to the mock vaccinated sample (Figure 8A, B). As expected (Figure 1C, D), a significant population of CD4 T cells in mucosally vaccinated mice expressed *Il17a*. 20-30% of *Il17a*-expressing cells in vaccinated mice also expressed *Ifng* (Figure 8B), suggesting that these cells—denoted here as Th1-Th17—were not classical Th17 cells. Th17 signature transcription factor *Rorc* expression, but not Th1 signature transcription factor

Tbx21 expression, was detected in Th1-Th17 cells (Figure 7B). Thus, the CD4 T cell compartment in vaccinated infected mice is characterized by a new population of *Il17a*-expressing cells that is not apparent in naïve infected mice, and a portion of these Th17 cells co-express *Ifng*.

Signature gene expression was generally similar between mock and mucosally vaccinated CD4 T cell subtypes (Figure 7B). To determine whether functionality differed between the CD4 T cell compartments, we first identified genes upregulated in unvaccinated mice. *Ifng*, *Klrg1*, and *Cx3cr1* expression was increased in CD4 T cells from naïve infected lungs compared to vaccinated infected lungs (Figure 9B and Figure 7B), as expected (Figure 1C and Figure 5B, C). Interestingly, *Nkg7*, a regulator of cytotoxic granule exocytosis (Ng et al., 2020), was highly expressed in the bulk CD4 population of naïve infected lungs (Figure 9A, B), as were cytotoxic markers *Gzma*, *Gzmb*, but not *Prf1* (Figure 9B). These expression patterns were also evident in the *Ifng*-expressing subsets in both mock and mucosally vaccinated infected mice (Figure 9C). Mucosal vaccination led to upregulation of genes associated with the *Rorc*-expressing Th17 lineage (Figure 9D), such as *Tmem176a, Tmem176b* (Drujont et al., 2016), and *JunB* (Carr et al., 2017). The naïve and central memory T cell marker *Il7r* (Surh and Sprent, 2008) was highly expressed in the i.n. vaccinated CD4 T cell compartment (Figure 9D), but not naïve T cell markers *Ccr7* and *Sell* (Figure 7C, D). Increased *Rbpj* and *Cd44* expression in vaccinated mice (Figure 9D) may contribute to survival of effector and memory T cells (Helbig et al., 2012; Maekawa et al., 2015) and memory Th1 development (Baaten et al., 2010). Other activation marker genes such as *Il2ra*, *Cd69*, *Ctla4* (Figure 9D, E), but not *Pdcd1* (Figure 7C, D), also had increased expression within the bulk CD4 population and *Ifng*-expressing Th1 subset. Vaccination did not affect proliferation marker *Mki67* expression (Figure 7C, D). Within the Th1 subset, the top 10 differentially expressed genes identified—including *Klrg1, Cx3cr1, Nkg7, Il7r, Tmem176a, Tmem176b*—were also identified as differentially expressed in the bulk CD4 population (Figure 7E), suggesting that the bulk CD4 population differential expression is driven by transcriptional changes in the Th1 subset rather than changes in abundance of T cell subsets. The magnitude of the different expression patterns was either similar to the bulk CD4 population, or was amplified in the Th1 subset (Figure 9D, E and Figure 7D). Thus, single cell transcriptomics reveals that primary challenge with *M. tuberculosis* in unvaccinated mice induces lung CD4s that transcriptionally resemble terminally differentiated effector KLRG1$^+$ CX3CR1$^+$ Th1 cells that contribute little to control of *M. tuberculosis*. In contrast, mucosal vaccination elicits a more diverse CD4 T cell compartment with Th17, activation, and memory T cell markers.

Polyfunctional T cells expressing IFN-γ, TNF, and IL-2 have been implicated in vaccine-induced protection against TB (Lewinsohn et al., 2017). Neither *Ifng* (Figure 9B) nor *Il2* (Figure 7C, D) expression was significantly increased with mucosal vaccination, but *Tnf* expression was slightly elevated in the mucosal vaccinated T cell compartment (Figure 7C, D), particularly within the Th1-Th17 and Th17 subsets (Figure 9H). Besides *Ifng* expression, Th1-Th17 transcriptomes seemed much closer to transcriptomes from Th17s expressing *Il17a* alone than those of classical Th1s (Figure 7F). Intriguingly, mucosal vaccine-induced CD4 T cells expressed higher levels of the gene encoding CD153, *Tnfsf8* (Figure 9F, G), a marker for protective lung-homing T cells in mice

(Sallin et al., 2018) and humans (Du Bruyn et al.). Cells expressing *Il17a* but not *Ifng* expressed the highest levels of *Tnfsf8*, as well as *Il1r1*, a critical component of effector memory T cell function (Jain et al., 2018) (Figure 9H). CD153 and IL-1R1 may therefore promote the development or function of protective CD4 T cells in mice vaccinated with CDN-adjuvanted vaccines.

Many cells were not assigned to a particular helper T cell subset using signature gene expression (Figure 8A). This effect may be due to the limitations of scRNA-seq (Papalexi and Satija, 2018), which are often difficult to distinguish from inherent heterogeneity in the CD4 T cell compartment (Zemmour et al., 2020). We aimed to overcome these issues by using the single-cell ANnotation using Variational Inference (scANVI) deep generative model, which leverages high confidence seed labels based on signature marker genes to assign labels to unannotated cells (Xu et al.). We selected seed cells based on high expression of the gene signatures used in manual annotation (Figure 8A, B), and also added lineage-specific transcription factor signature genes (*Tbx21* for Th1 and *Rorc* for Th17) to increase confidence in denoting these classical Th subsets. No cells of the nonclassical Th1-Th17 subset co-expressed *Tbx21* and *Rorc*, thus, scANVI seed labels for Th1-Th17 only required *Ifng* and *Il17a* expression. scANVI successfully annotated all previously unassigned cells and delineated similar clusters as manual annotation with signature genes (Figure 10A), performing well at classifying cell types (Figure 10B). Differential expression analysis of the scANVI-determined Th1 subsets showed similar top hits between samples (Figure 10C) as manual annotation (Figure 7E). scANVI classified many more cells as Th1-Th17 (Figure 10D) compared to manual annotation (Figure 9F), even reclassifying many Th17 as Th1-Th17. While Th1-Th17 cells were not readily detectable in naïve mice with manual annotation, scANVI revealed a small population of these cells in the mock vaccinated as well as a large cluster in the mucosal vaccinated sample (Figure 10E). The pool of nonclassical CD4 T cells that do not fall into canonical *Tbx21*/*Ifng*-expressing Th1 or *Rorc*/*Il17a*-expressing Th17, therefore, is much larger than previously anticipated. These results show the promise and utility of recently developed tools in single cell transcriptomics and probabilistic models to study the heterogeneity of CD4 T cells in the context of vaccination and infection.

Discussion

The recent success of the phase IIb clinical trial of the M72/AS01$_E$ protein subunit vaccine has raised the possibility that improved adjuvants and protein antigens may result in a protein subunit vaccine that confers complete protection against TB infection. In addition, protein subunit vaccines have potential for safe use in HIV-infected or otherwise immunocompromised individuals. Our results here provide mechanistic insight into a highly efficacious CDN-adjuvanted TB protein subunit vaccine. We found that intranasal vaccine-induced protection is evident 1 week post *M. tuberculosis* challenge in mouse lungs, correlates with a robust Th17 response, and is dependent on IL-17 for full efficacy. In contrast, BCG-induced protection is Th1-dominated and is delayed until the peak of the CD4 T cell response at 2-3 weeks post infection. While the STING-activated transcription factor STAT6 is dispensable for vaccine efficacy, type I IFN signaling is necessary for full protection elicited by our CDN vaccine. Although this vaccine elicits IFN-γ$^+$ CD4 T cells when delivered through both the subcutaneous and intranasal routes, and IFN-γ plays an essential role in controlling *M. tuberculosis* infection, CDN vaccine efficacy requires a mixed T cell response requiring both IFN-γ and IL-17. Finally, single cell sequencing analysis of CD4 T cells at the peak of the T cell response reveal the emergence of a transcriptionally heterogeneous *Il17a*-expressing CD4 T cell cluster characterized by enhanced expression of functional markers is the most striking effect of mucosal vaccination. These results represent the first published single cell transcriptional analysis of naïve or vaccine-induced CD4 T cell responses during murine *M. tuberculosis* infection.

M. tuberculosis utilizes the ESX-1 secretion system to perforate host cell membranes to gain access to the cytosol and promote virulence, leading to dsDNA leak into the cytoplasm, activation of the cGAS/STING pathway, and subsequent induction of type I IFN and other pro-inflammatory cytokine responses (Collins et al., 2015; Wassermann et al., 2015; Watson et al., 2015). BCG, however, lacks the ESX-1 system and is unable to permeabilize the phagosome (Houben et al., 2012). Intriguingly, recombinant BCG vaccines that provide access to the cytosol provide superior protection (Grode et al., 2005; Gröschel et al., 2017; Saiga et al., 2015). Activation of cytosolic surveillance programs can therefore contribute to vaccine-induced immunity. To fully understand and improve upon the use of CDN adjuvants, it is essential to investigate the factors required for vaccine-induced protection downstream of STING activation. STING is primarily known for activating the transcription factor IRF3, resulting in type I IFN production, but transcription factors STAT6 and NF-κB can also be activated by STING (Cheng et al., 2020). We identified a partial role for type I IFN, but not STAT6, in mediating STING-dependent mucosal CDN vaccine efficacy. STING has also been shown to activate autophagy in a pathway independent of TBK1 and type I IFN induction (Collins et al., 2015; Watson et al., 2015). It is therefore of great interest to further investigate the roles of type I IFN, NFkB and autophagy in eliciting CDN-adjuvanted mucosal vaccine protection.

While it is clear that IFN-γ from CD4 T cells is essential for host defense against *M. tuberculosis*, our understanding of IFN-γ-independent mechanisms of control has been limited. Although *Ifng*$^{-/-}$ mice are extremely susceptible to *M. tuberculosis* infection in the presence or absence of the CDN-adjuvanted vaccine, both subcutaneous and

intranasal administration induced protection that was only partially dependent on IFN-γ. Therefore, mucosal vaccination presents an exciting opportunity to explore CD4 memory T cell responses distinct from the classical Th1 response. While Th1 frequency has not proven as an effective correlate of protective immunity, boosting the mucosal memory response of a TB vaccine could synergize with IFN-γ-mediated immunity.

Our results contribute to a growing body of evidence that mucosal vaccines can elicit robust Th17 responses that augment Th1 responses induced by subcutaneous vaccination. In concordance with our findings, it was reported that an intranasal LT-IIb adjuvanted TB vaccine leads to a robust Th17 memory response in mice, and that IL-17 itself is required for mucosal vaccine efficacy (Gopal et al., 2013b). However, the precise mechanism by which IL-17 specifically—or a Th17 T cell response more generally—protects against *M. tuberculosis* remains uncertain. We found that the efficacy of s.c. vaccination with either BCG or 5Ag/ML-RR-cGAMP is independent of IL-17. While it has been reported that subcutaneous administration of both a TB protein subunit vaccine and BCG results in IL-23-dependent polarization of inflammatory Th17 cells and protection (Gopal et al., 2013b; Khader et al., 2007), this mechanism of control may be distinct from that of mucosal vaccination, as IL-23 may also be required for an IL-17-independent function during infection (Chackerian et al., 2006). Further investigations are necessary to determine the role of IL-23 in CDN-adjuvanted vaccine efficacy.

One mechanism by which Th17 cells induce protection against TB may be the promotion of T cell localization to the lung parenchyma. In macaques, defective intralesional positioning limits the ability of T cells to interact with and promote *M. tuberculosis* killing in myeloid cells (Kauffman et al., 2018b). While CXCR3 has primarily been described as a marker of protective Th1 parenchymal homing cells during *M. tuberculosis* infection (Sakai et al., 2014), its role in tissue-homing of pathogen-specific T cells in the context of vaccination has not been well-studied. Early and sustained lung parenchymal localization of protective $CXCR3^+$ CD4 T cells may mediate our mucosal vaccine's protection. As IL-17 is thought to mainly recruit neutrophils to the site of infection, it is of great interest to determine if and how this cytokine might induce and maintain this protective memory T cell response.

Although a robust Th1 response was seen in mock vaccinated mouse lungs at 3 weeks post infection, CD4 T cells in these mice expressed markers of terminal differentiation—KLRG1 and CX3CR1—and failed to infiltrate into lesions. Cytotoxic genes such as *Nkg7*, *Gzma*, and *Gzmb* were also expressed in a subset of these cells. Terminally differentiated Th1s are not associated with protection against TB (Sakai et al., 2014; Sallin et al., 2017), and blood signature cytotoxic gene expression is anticorrelated with mouse susceptibility and progression from latent to active TB disease in humans (Moreira-Teixeira et al., 2020). Further investigation is necessary to determine if cytotoxic CD4s can mediate resistance to TB.

In contrast to the terminally differentiated Th1 marker expression seen in mock vaccinated mice, mucosal vaccination elicited higher expression of central memory T cell marker *Il7r* and activation/memory marker *Cd44* in the total CD4 and Th1 populations, and increased *Il1r1* expression specifically on IL-17-expressing T cells. Interestingly, i.n. vaccination also led to increased expression of *Tnfsf8*, which has been shown to be essential for IFN-γ-independent control of TB in a mouse model (Sallin et

al., 2018) and implicated as a mediator of protection against human TB (Du Bruyn et al.). We thus find potential markers of both IFN-γ-dependent and IFN-γ-independent CD4 T cell memory function. These markers may also suggest mechanisms for protection: signaling through these molecules may contribute to enhanced activation, survival, function and memory development of CD4 T cells during infection. It will be crucial to evaluate whether these transcriptional changes result in memory responses that can provide enduring protection, past the point at which BCG-mediated immunity wanes.

While we had previously found that Th17 cells were induced by i.n. vaccination (Van Dis et al., 2018), our latest findings reveal a surprisingly heterogeneous Th17 compartment, with 20-30% of *Il17a*-expressing cells in vaccinated mice also expressing *Ifng*. While CD4 T cell plasticity has been well documented in different contexts *in vivo*, it is uncertain whether this works to the host's advantage. In fact, Th1-Th17 presence correlates with TB disease severity in humans (Jurado et al., 2012), and numerous studies have identified pathogenic IFN-γ^+ Th17s as a driver of disease in mouse models of autoimmunity (Bystrom et al., 2019). Furthermore, a previous study using adoptively transferred Th17s reported that the ability to coproduce IFN-γ limits Th17 protective capacity during *M. tuberculosis* infection in mice (Monin et al., 2015). In our model, it is currently uncertain whether co-producing cells differ in protective capacity to classical Th1s or Th17s, and what cell types respond to IL-17 to mediate protection. Integrating single cell sequencing technology with spatial transcriptomics and cell surface proteomics provides exciting avenues for future investigations.

A recent mass cytometry study showed that the Th17 lineage maintains plasticity after *in vitro* differentiation (Tortola et al., 2020). Our scANVI analysis classified a surprisingly large proportion of both unassigned and *Il17a*-expressing "Th17" cells as Th1-Th17. This finding may indicate that Th1-Th17 cells are part of a larger pool of heterogeneous helper T cells, whose transcriptomes and effector functions may shift dynamically in response to inflammatory signals. Future fate-mapping studies could reveal whether CD4 T cell expression of IFN-γ and IL-17 changes over the course of vaccination, infection, and memory recall. While the plasticity in CD4 T cell phenotypes and function may be a hurdle in finding reliable correlates of vaccine correlates protection, true delineation of subtypes may be impossible, and it may be time to embrace heterogeneity in CD4 T cell-mediated protection for better TB vaccine design. Our studies add to the growing body of literature proving that mucosal vaccination leads to the development of Th17 and Th1-Th17 T cell compartments. This heterogeneity distinguishes mucosal vaccination from the terminally differentiated Th1-dominated response elicited by systemic delivery of a vaccine. While intranasal delivery of the BCG vaccine also induces Th17 responses and protection, there remains a need to find a vaccine with an increased safety profile. Our vaccine has proven effective when administered twice as a booster to BCG and when delivered three times on its own (Van Dis et al., 2018), inducing an IL-17-dependent response and protection that can outperform that of BCG. The studies presented here thus highlight the potentially profound impact of T cell diversity and lineage plasticity on antibacterial immune defense. Our findings suggest multiple avenues for improvement and optimism in designing improved vaccines against infectious respiratory diseases.

Acknowledgments

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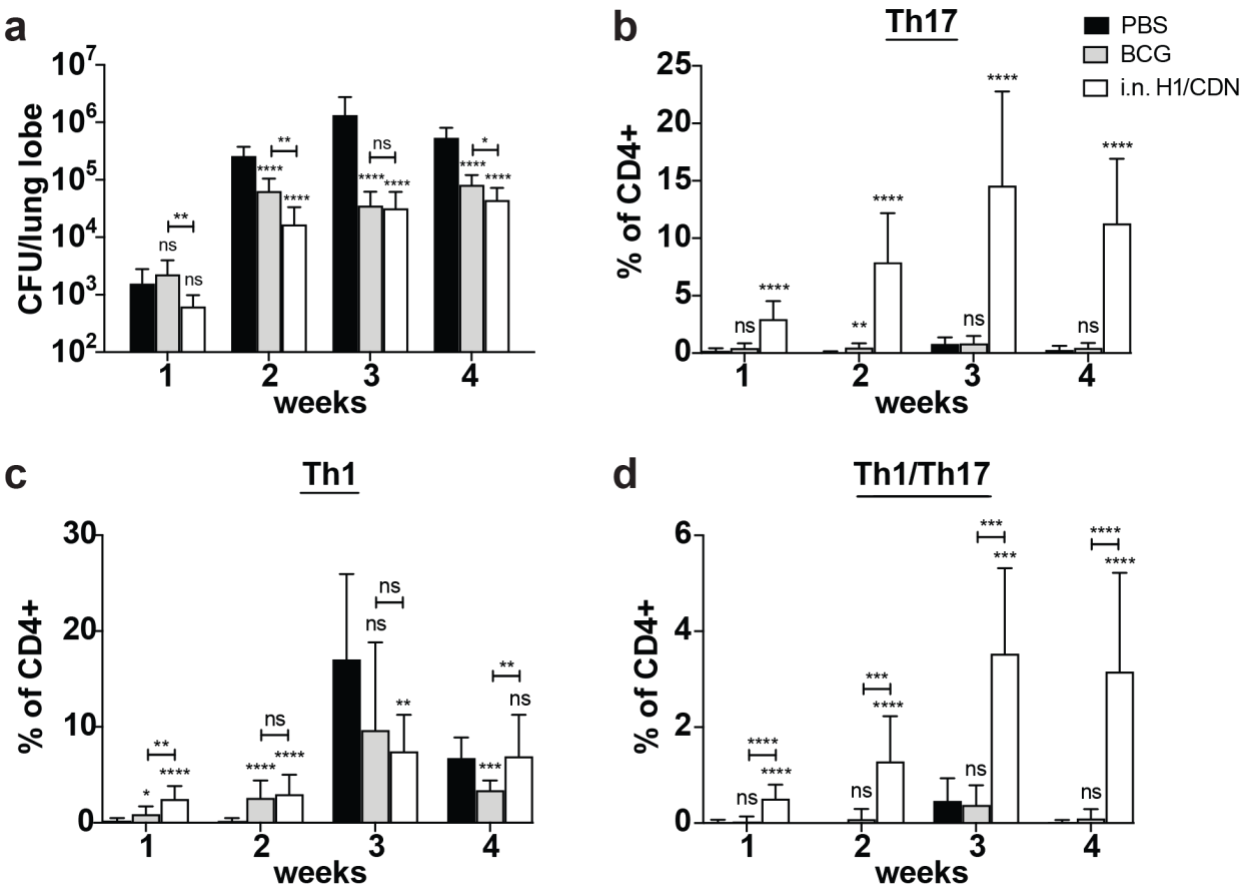


Figure 1: Intranasal immunization induces a Th17 response that precedes significant Th1 influx into the lungs of *M. tuberculosis*-challenged mice. Mice were vaccinated once with subcutaneous *M. bovis* BCG 12 weeks before challenge or primed with intranasal *M. tuberculosis* H1 antigen and ML-RR-cGAMP cyclic-di-nucleotide adjuvant (i.n. H1/CDN) 12 weeks before challenge, then boosted twice at 4 week intervals after priming, before *M. tuberculosis* aerosol challenge with 50-100 CFU. Mice were mock primed and boosted with PBS as a control. (**a**) Lung bacterial burden was enumerated at 1 – 4 weeks post *M. tuberculosis* challenge. (**b-d**) Intracellular cytokine staining (ICS) for percentage of lung CD4 T cells that produce (**b**) IL-17 (Th17), (**c**) IFN-γ (Th1), or (**d**) both IFN-γ and IL-17 (Th1/Th17) after restimulation *ex vivo* with recombinant Ag85b peptide pool. Data are expressed as mean (± SD) of eight to ten mice per group from two independent experiments. Mann-Whitney t test p values calculated in comparison to PBS vaccinated controls except where indicated; *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001. Significance is relative to PBS control unless otherwise indicated.

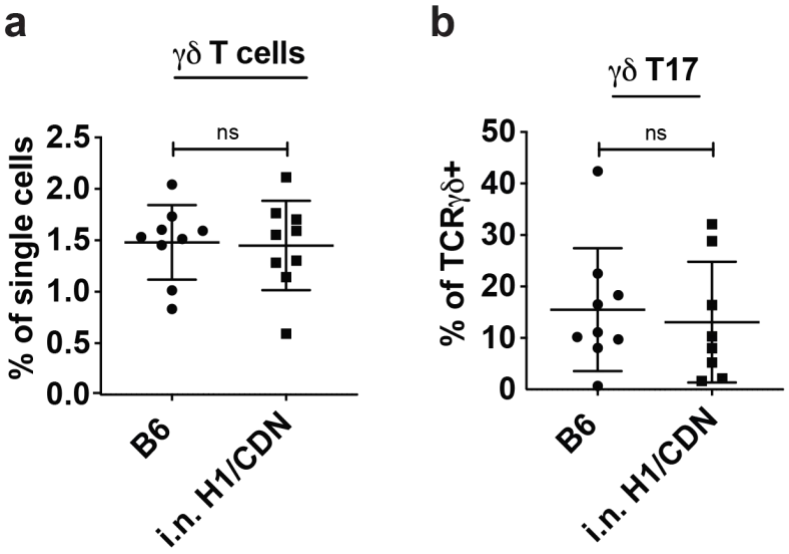
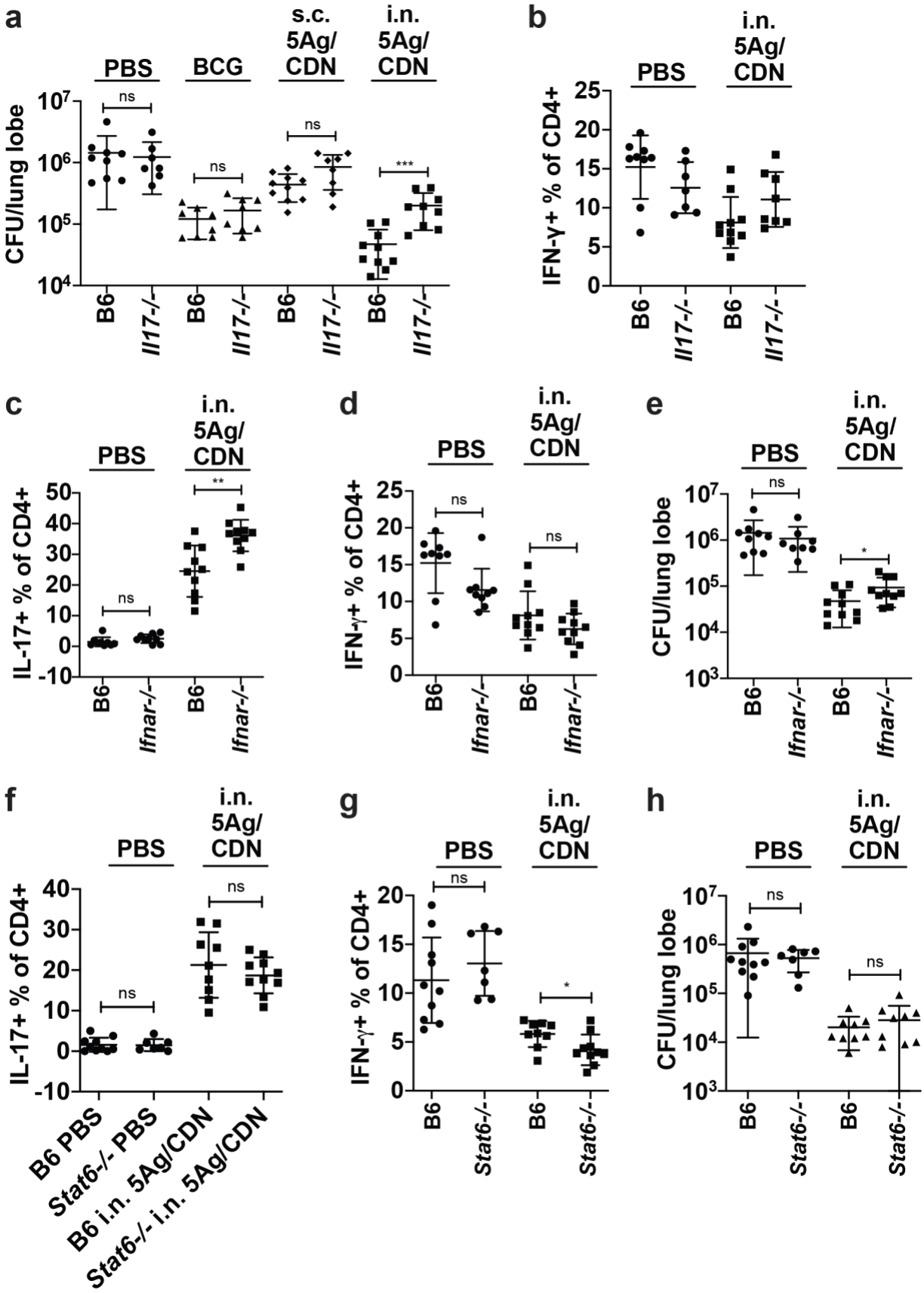


Figure 2: Intranasal immunization does not alter the frequency of TCRγ/δ or TCRγ/δ IL-17+ T cells. (a) Surface staining for percentage of live single cells that are TCRγ/δ+ in mock or i.n. H1/ML-RR-cGAMP vaccinated mouse lungs 4 weeks post M. tuberculosis challenge. **(b)** ICS for percentage of lung TCRγ/δ+ cells that produce IL-17 after restimulation ex vivo with recombinant Ag85b peptide pool. Data are expressed as mean (± SD) of eight to ten mice per group from two independent experiments; ICS data are representative of experiments with or without peptide restimulation. Mann-Whitney t test.


a
PBS
BCG
s.c. 5Ag/ CDN
i.n. 5Ag/ CDN
CFU/lung lobe
ns
ns
ns
***
B6
Il17-/-
b
i.n. 5Ag/ CDN
PBS
IFN-γ+ % of CD4+
c
i.n. 5Ag/ CDN
PBS
IL-17+ % of CD4+
**
ns
Ifnar-/-
d
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PBS
IFN-γ+ % of CD4+
e
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*
f
i.n. 5Ag/ CDN
PBS
IL-17+ % of CD4+
B6 PBS
Stat6-/- PBS
B6 i.n. 5Ag/CDN
Stat6-/- i.n. 5Ag/CDN
g
i.n. 5Ag/ CDN
PBS
IFN-γ+ % of CD4+
Stat6-/-
h
i.n. 5Ag/ CDN
PBS
CFU/lung lobe

Figure 3: IL-17 and Type I IFN, but not STAT6, are essential for full mucosal vaccine efficacy. Wild-type C57BL/6 (B6) and *Il17*$^{-/-}$ mice were vaccinated once with subcutaneous *M. bovis* BCG 12 weeks before *M. tuberculosis* aerosol challenge, or primed with either subcutaneous or intranasal *M. tuberculosis* 5Ag antigen and ML-RR-cGAMP cyclic-di-nucleotide adjuvant (i.n. 5Ag/CDN) 12 weeks before challenge, followed by two boosts. (**a, e, h**) At 4 weeks post challenge, mouse lungs were harvested and analyzed for bacterial burden. **(b-d, f, g)** ICS analysis of *ex vivo* restimulated lung leukocytes for **(c, f)** % IL-17^{+} and **(b, d, g)** % IFN-γ^{+} CD4 T cells. Data are expressed as mean (± SD) of eight to ten mice per group from two independent experiments. Mann-Whitney t test p values; *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001.

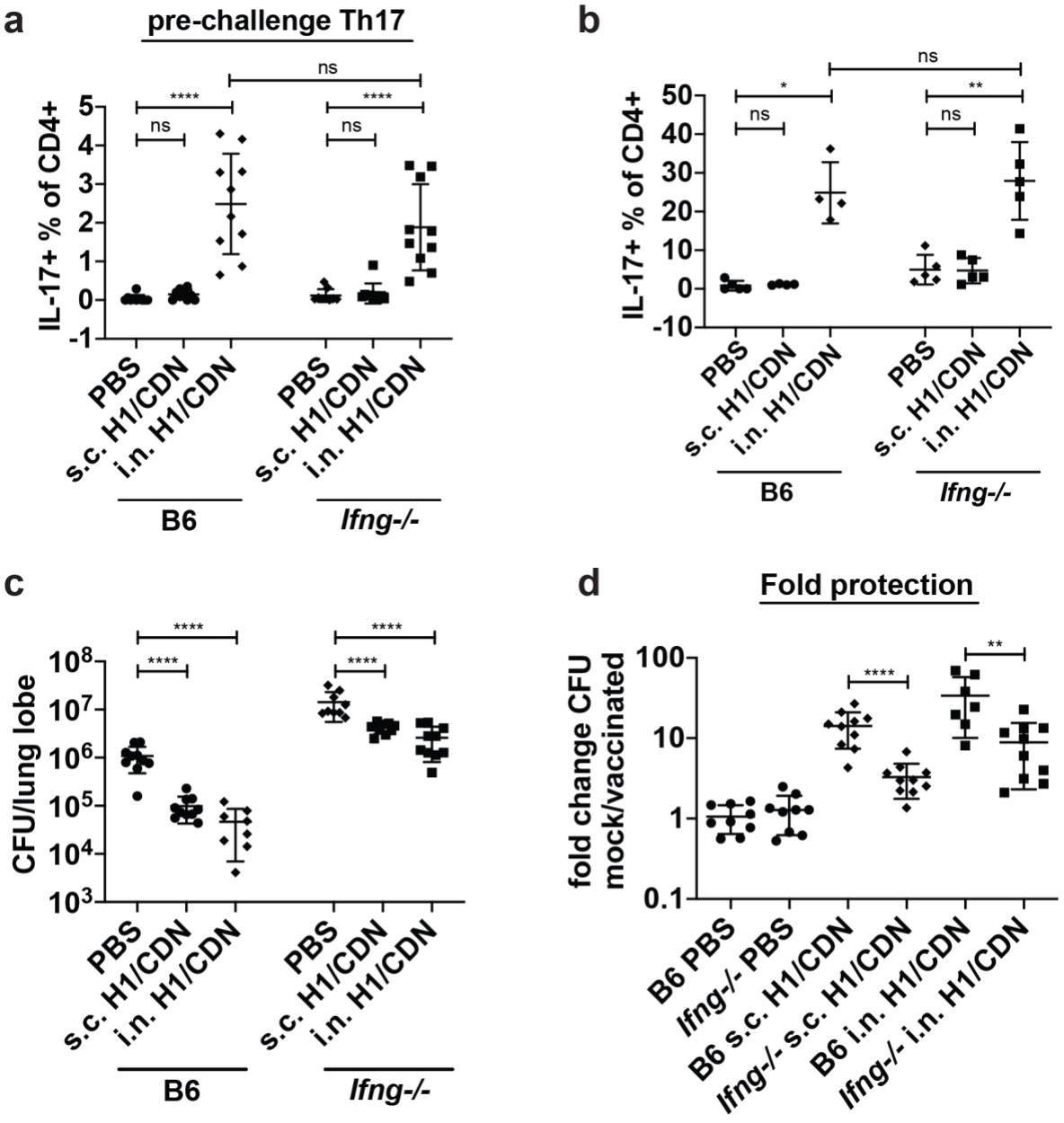

Figure 4: Vaccine efficacy is partially dependent on IFN-γ. (a) Pre-challenge ICS of ex vivo restimulated PBMCs from blood of vaccinated B6 or *Ifng*$^{-/-}$ mice for Th17 percentage of CD4 T cells one week after the second vaccine boost. **(b)** ICS analysis of *ex vivo* restimulated lung leukocytes from mock or i.n. H1/ML-RR-cGAMP (i.n. H1/CDN) vaccinated B6 or *Ifng*$^{-/-}$ mice 4 weeks post *M. tuberculosis* challenge for % IL-17^{+} CD4 T cells. **(c)** CFU counts from lungs of vaccinated mice 4 weeks post challenge. **(d)** Fold protection of vaccination, calculated as the fold-decrease in lung bacterial burden between vaccinated and mock vaccinated mice. Data are expressed as mean (± SD) of five to ten mice per group from (a) one representative experiment or (b, c) two independent experiments. Mann-Whitney t test p values; *p ≤ 0.05, **p ≤ 0.01.

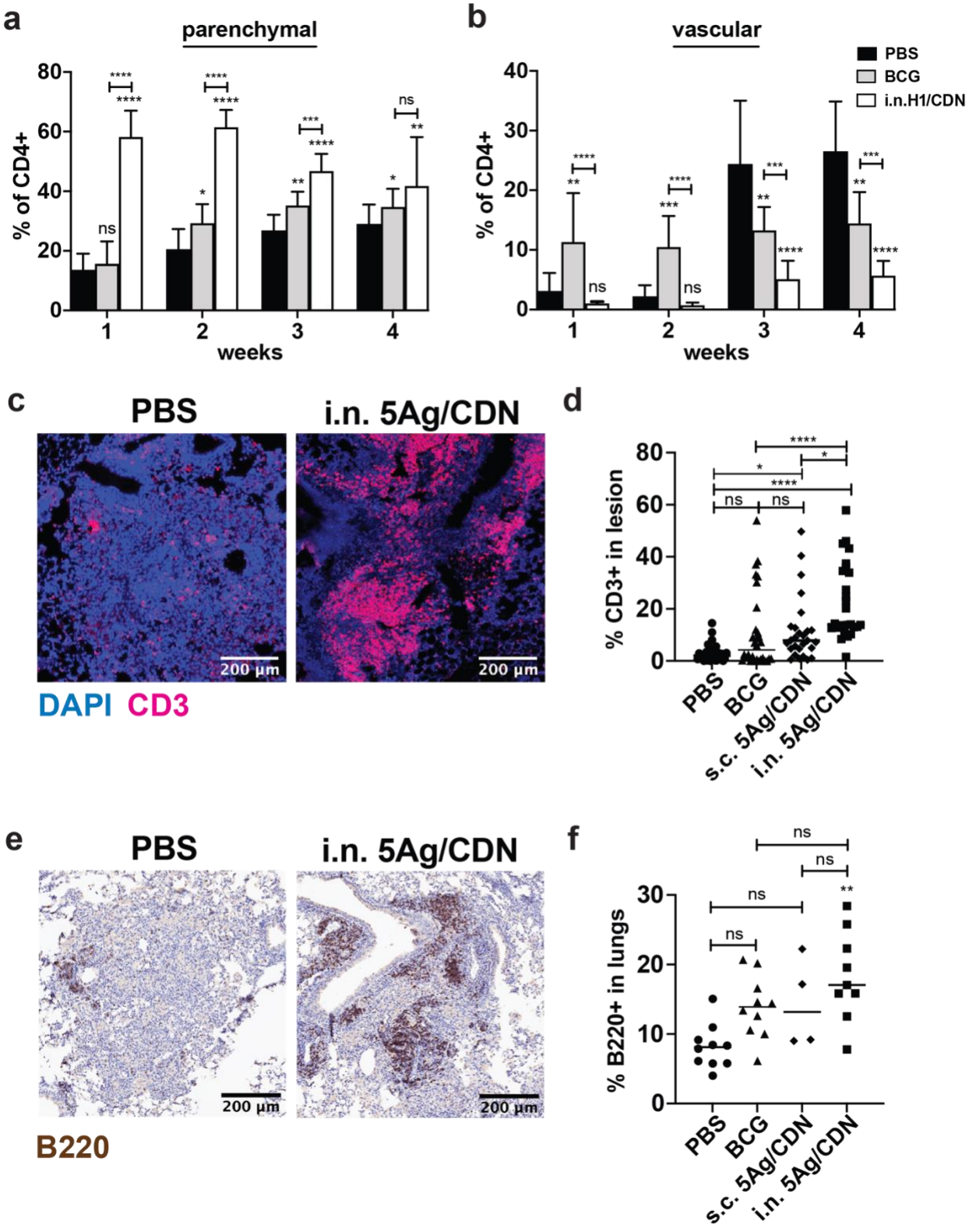

Figure 5: Lymphocytes home to lung lesions in mucosal vaccinated mice. (a) Surface staining for percentage of CXCR3^{+} KLRG1^{-} lung CD4 T cells from mock and i.n.

H1/ML-RR-cGAMP (i.n. H1/CDN) vaccinated mice. **(b)** Surface staining for percentage of $CXCR3^{-}$ $KLRG1^{+}$ lung CD4 T cells. **(c)** Representative immunofluorescent staining of formalin-fixed, paraffin-embedded lung sections from mock and 5Ag/ML-RR-cGAMP vaccinated mice for T cell marker CD3 and nuclear DAPI stain. **(d)** Quantification of $CD3^{+}$ immune cells out of total cells in lung lesions. **(e)** Representative immunohistochemical staining of lung sections for B cell marker B220. **(f)** Quantification of $B220^{+}$ lung area. Data are expressed as mean (± SD) of eight to ten mice per group from two independent experiments. (a, b) Mann-Whitney t test p values; (d, f) Kruskal Wallis test followed by Dunn multiple comparison posthoc p-values; *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$, ****$p \leq 0.0001$. Significance is relative to PBS control unless otherwise indicated.

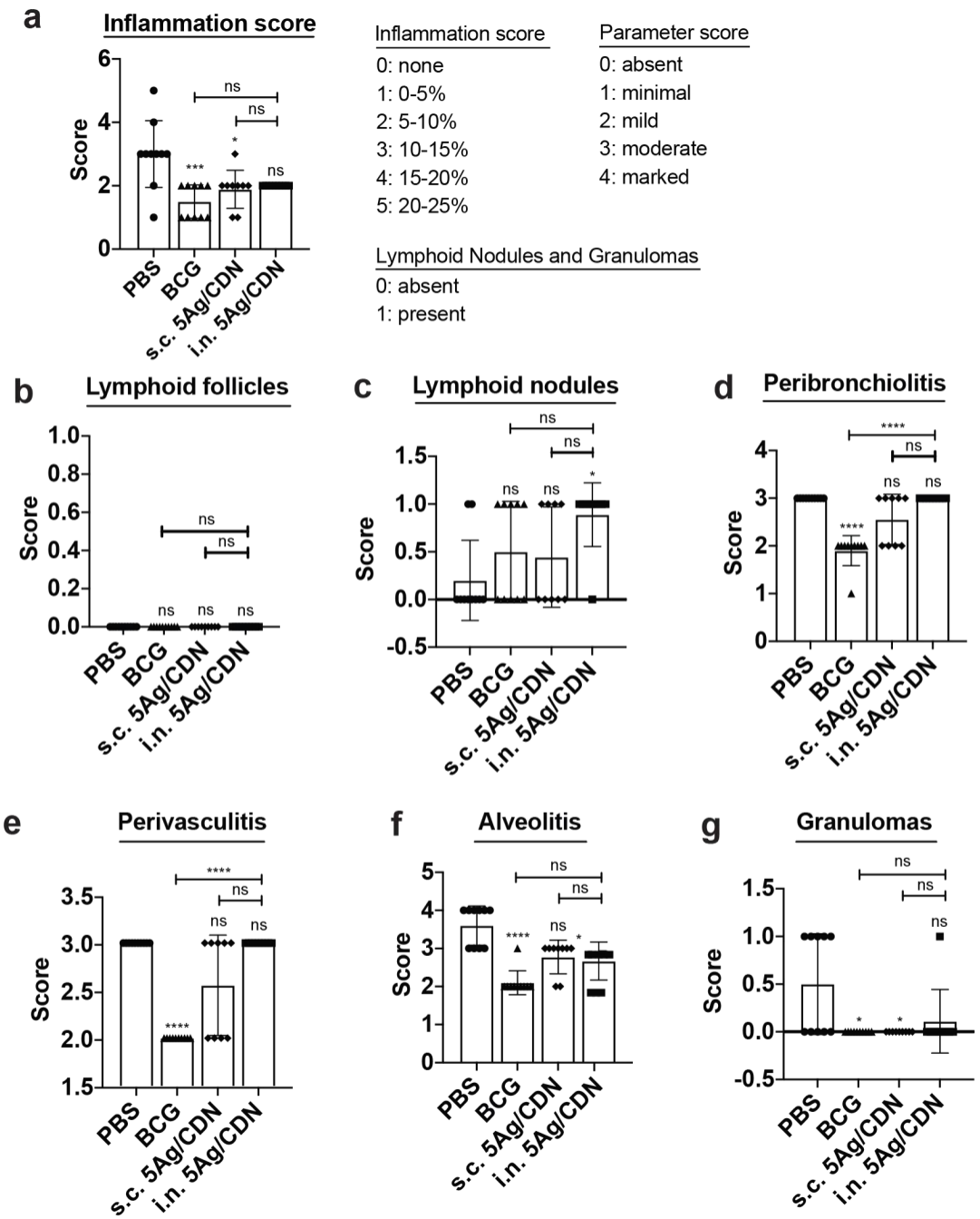

Figure 6: Semi-quantitative histopathological analysis of vaccinated mouse lungs. Formalin-fixed paraffin-embedded sections of mouse lungs 4 weeks post M. tuberculosis challenge were stained with H&E and assessed using a semi-quantitative scoring method for **(a)** inflammation area, **(b, g)** lymphoid follicle, lymphoid nodule, or granuloma presence, and **(d-f)** localized inflammation. Data are expressed as mean (± SD) of eight to ten mice per group from two independent experiments. Kruskal Wallis test followed by Dunn multiple comparison posthoc p-values; *p ≤ 0.05, ***p ≤ 0.001, ****p ≤ 0.0001.

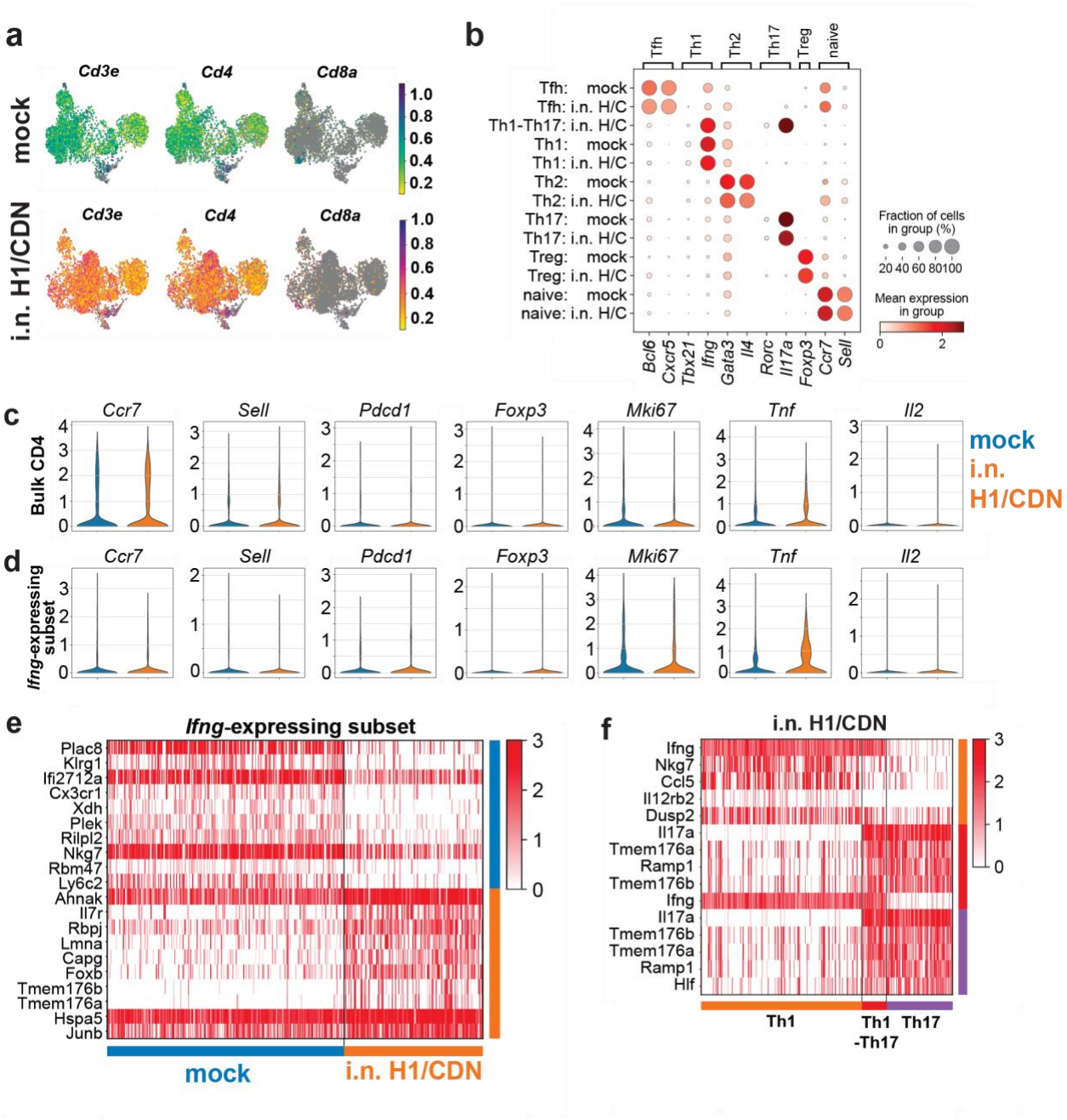

Figure 7: Single cell transcriptional analysis of mock and i.n. H1/ML-RR-cGAMP vaccinated mouse lung CD4 T cells. (a) UMAP plots of T cell marker expression in naïve (mock) or i.n. H1/ML-RR-cGAMP (i.n. H1/CDN) vaccinated mouse CD4 T cells. **(b)** CD4 subtype signature gene expression in naïve or i.n. H1/ML-RR-cGAMP (i.n. H/C) vaccinated mice. **(c)** Gene expression in bulk and **(d)** Ifng-expressing CD4 T cell populations. **(e)** Top differentially expressed genes in Ifng+ CD4 T cells. **(f)** Top differentially expressed genes in Ifng+ Il17a- (Th1), Ifng+ Il17a+ (Th1-Th17), and Ifng- Il17a+ (Th17) subsets from i.n. vaccinated mouse lungs. T test with overestimated variance.

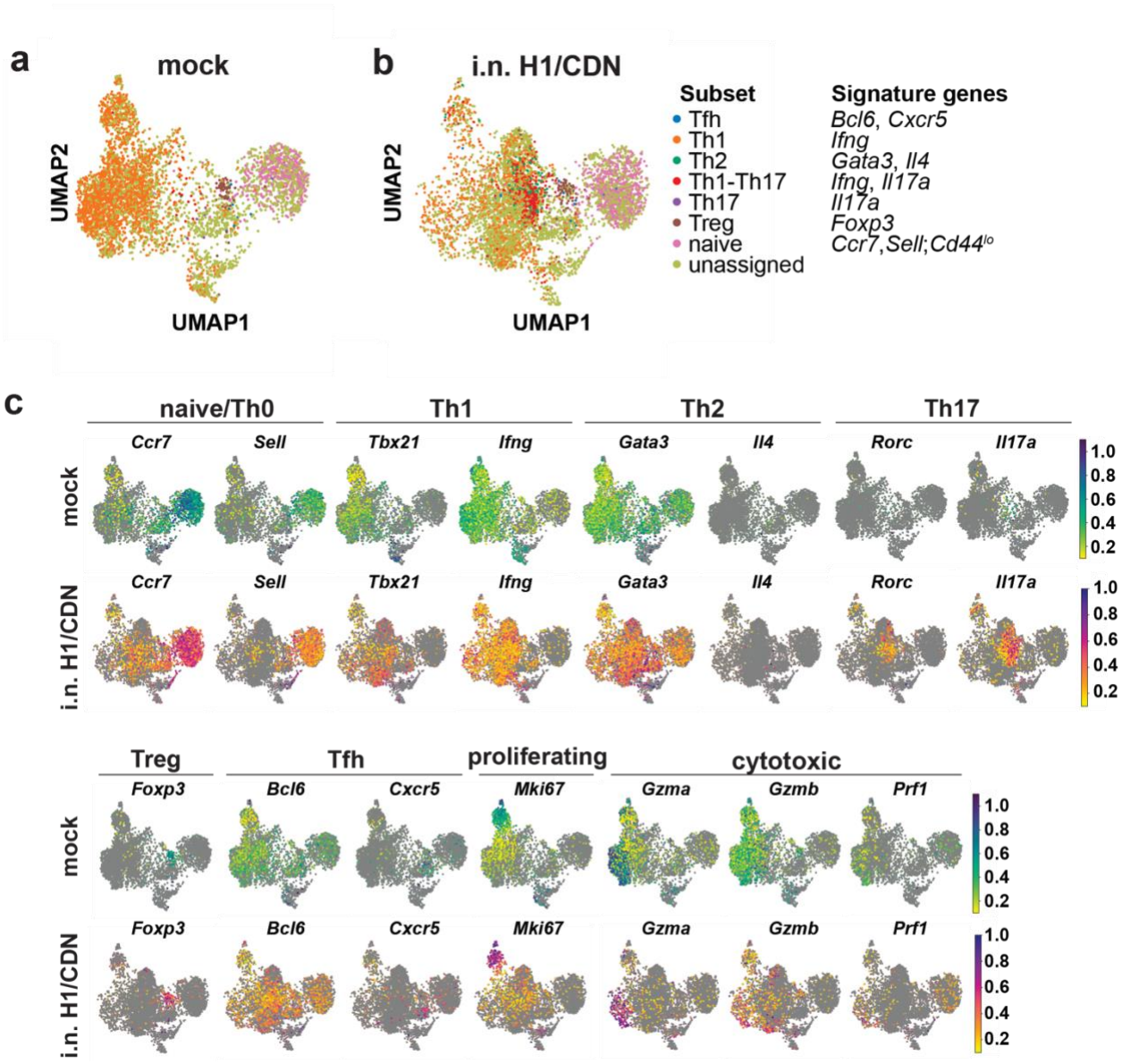

Figure 8: Single cell transcriptional analysis reveals the effects of mucosal vaccination on mouse lung CD4 T cell compartments. Subsets of CD4 T cells were manually annotated based on signature expression of lineage-specific transcription factor, cytokine, and cell surface markers. **(a)** UMAP plots of CD4 T cell subsets isolated from naïve (mock) or **(b)** i.n. H1/ML-RR-cGAMP (i.n. H1/CDN) vaccinated mice 3 weeks post infection. **(c)** Signature gene expression of CD4 T cell subtypes in naïve (mock) and i.n. H1/ML-RR-cGAMP vaccinated mice. The color scale indicates the percentile expression level for each cell that has non-zero expression.

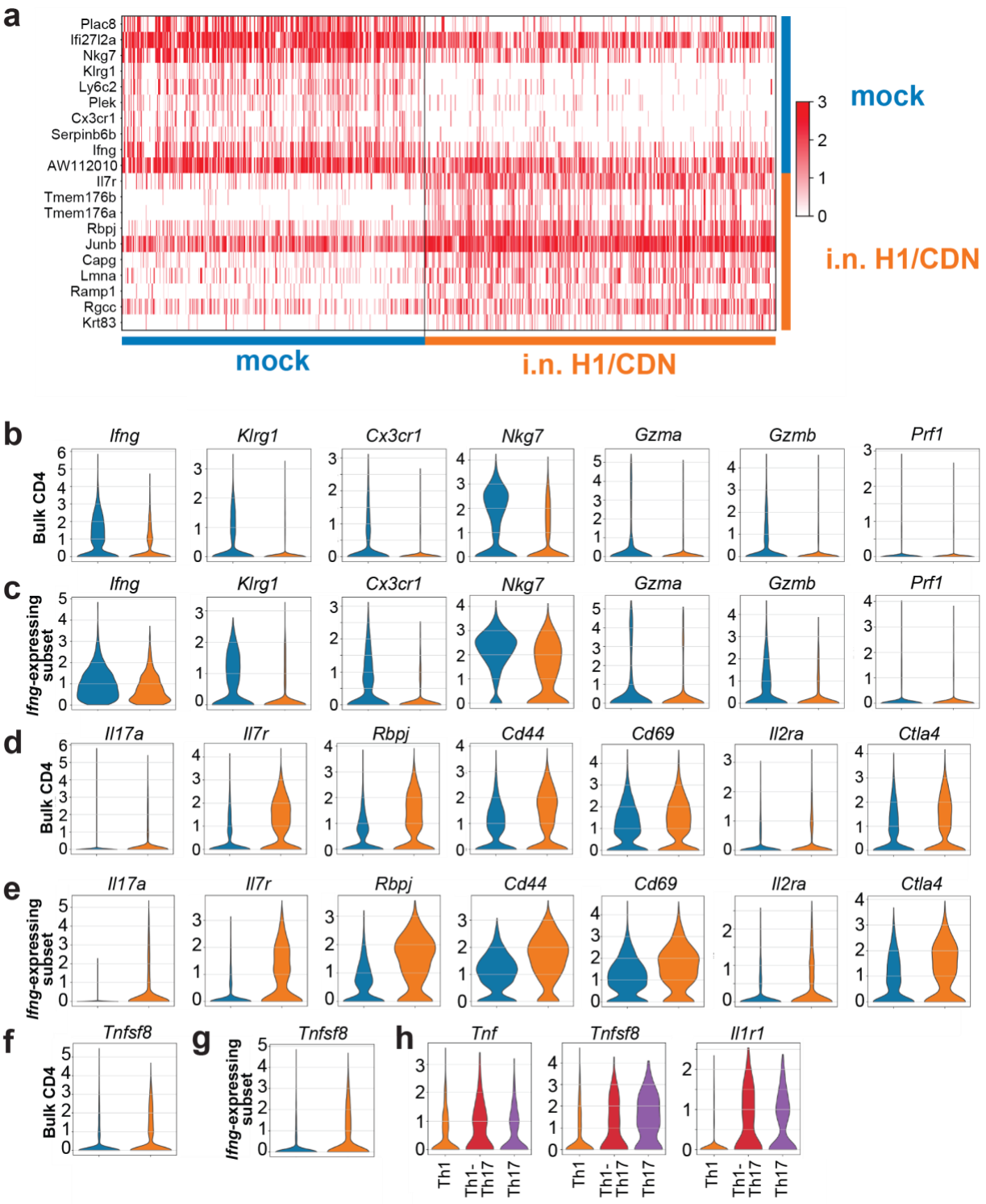

Figure 9: CD4 T cell transcriptomes from naïve infected mice show increased terminally differentiated Th1 marker expression, while mucosal vaccination leads to memory marker expression and the development of Th1-Th17 and Th17 cells. **(a)** Top differentially expressed genes in naïve (mock) and i.n. H1/ML-RR-cGAMP (i.n. H1/CDN) vaccinated mouse CD4 T cells obtained by Student's t-test with overestimated variance. **(b, c)** Markers of Th1 lineage, terminal differentiation, and cytotoxicity in **(b)**

bulk and **(c)** *Ifng*-expressing CD4 populations in naïve (blue) and i.n. H1/ML-RR-cGAMP vaccinated (orange) infected mouse lungs. **(d, e)** Markers of Th17 lineage, memory, and activation in **(d)** bulk and **(e)** *Ifng*-expressing CD4 populations. **(f, g)** *Tnfsf8* expression in **(f)** bulk and **(g)** *Ifng*-expressing CD4 populations. **(h)** Markers of polyfunctionality, protective capacity, and effector function in Th1, Th1-Th17, and Th17 subsets.

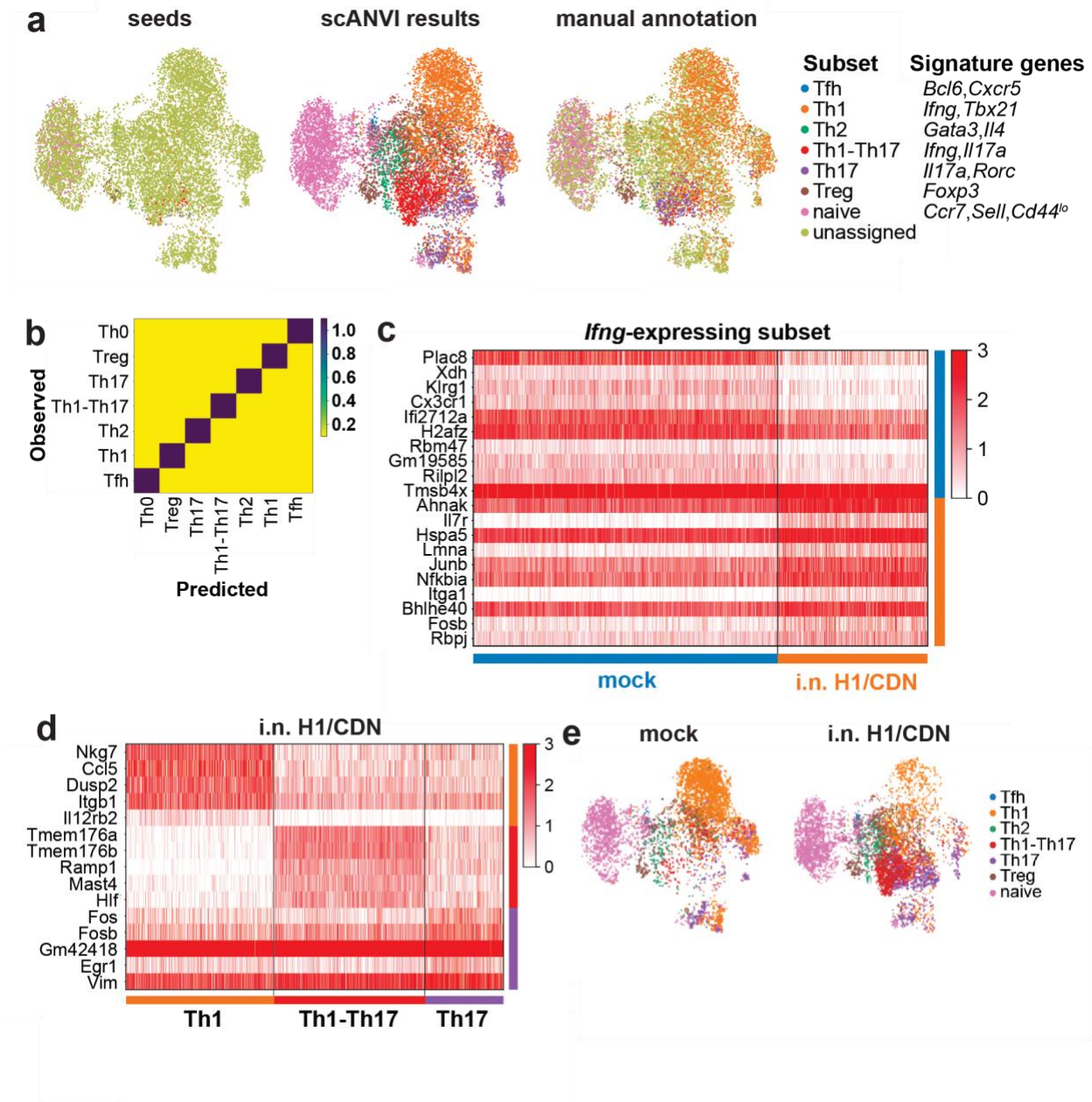

Figure 10: scANVI predicts T cell subset labels for unannotated cells. (a) UMAP plots of high confidence seed labels, scANVI annotations, and manual annotations used for analysis in Fig. 5 and 6 based on signature gene expression. **(b)** scANVI confusion matrix of observed and predicted labels to describe the model's performance. **(c)** Top differentially expressed genes in scANVI-predicted Th1 cells in naïve (mock) or i.n. H1/ML-RR-cGAMP (i.n. H1/CDN) vaccinated mouse lung CD4 T cells. **(d)** Top differentially expressed genes in scANVI-predicted Th1, Th1-Th17, and Th17 subsets from i.n. vaccinated mouse lungs. **(e)** UMAP plots of scANVI-predicted CD4 T cell subsets from naïve or i.n. H1/ML-RR-cGAMP vaccinated mouse lungs. T test with overestimated variance.

Chapter Four: Discussion and Future Directions

There are many approaches to vaccine design, but most experimental vaccines to *M. tuberculosis* that have been recently developed have been protein subunit-based vaccines since these tend to have been safety profiles than live attenuated vaccines, such as BCG. Of course, inducing an immune response to *M. tuberculosis* with a protein subunit vaccine requires formulation with an adjuvant in order to stimulate antigen presenting cells which then activate protective, antigen-specific T cells. A number of adjuvants for new vaccines for *M. tuberculosis* have been tested, including many that attempt to simulate the activation of cell surface pattern recognition receptors (PRRs) that recognize *M. tuberculosis* ligands present on the outside of the bacteria (Kaufmann et al., 2017). However, a critical aspect of *M. tuberculosis* virulence is the ability of the bacteria to perforate the phagosome using its Type VII Secretion System, ESX-1, which results in the delivery of virulence factors into the cytosol (Stanley et al., 2003, 2007). These virulence factors include *M. tuberculosis* DNA and bacteria-derived CDNs, which lead to activation of the PRRs cGAS and STING and stimulation of the cytosol surveillance pathway (Collins et al., 2015; Dey et al., 2015; Manzanillo et al., 2012; Wassermann et al., 2015; Watson et al., 2015). This is in contrast to the attenuated vaccine strain BCG, which is capable of activating many of the same cell surface PRRs as *M. tuberculosis* but lacks the ESX-1 secretion system and does not activate PRRs in the cytosol (Watson et al., 2015). This led to our main hypothesis, that formulating a protein subunit vaccine for *M. tuberculosis* using adjuvants that activate the cytosolic surveillance pathway, rather than cell surface PRRs, might stimulate a physiologically relevant immune response that more closely mimics that induced by virulent *M. tuberculosis*, and might provide superior protection against *M. tuberculosis* infection.

STING activation leads to multiple signaling pathways and protection against *M. tuberculosis* through unknown mechanisms

To stimulate the cytosol surveillance pathway, we formulated our protein subunit vaccine with CDNs, which activate the PRR STING. CDN activation of STING leads to, canonically, Type I IFN induction through activation of the transcription factor IRF3, as well as activation of the transcription factor STAT6 and production of inflammatory cytokines, such as TNFα, IL-1β and IL-12, through activation of the NF-kB signaling pathway (Burdette and Vance, 2013; Burdette et al., 2011; Chen et al., 2011; McWhirter et al., 2009). Finally, in the context of *M. tuberculosis*, STING is known to induce autophagy through activation of TBK1, independent of IRF3 (Watson et al., 2012, 2015). Thus, STING represents a particularly interesting innate immune signaling node with multiple downstream pathways that are relevant during *M. tuberculosis* infection. When designing this vaccine, we were of course interested in whether it would induce a protective immune response against *M. tuberculosis* infection, but we also hoped to learn something unique about the role of STING signaling during *M. tuberculosis* infection.

We found that vaccination with subcutaneous administration of protein antigen plus CDN adjuvant, followed by *M. tuberculosis* challenge, led to approximately 1 log fewer bacteria in the lungs at 4- and 12-weeks post-infection when compared to PBS-

immunized mice. This protection was entirely STING-dependent and was equivalent to that seen in mice vaccinated with subcutaneous BCG. This shows that CDN adjuvants are sufficient to induce a protective immune response against *M. tuberculosis* infection. Importantly, the protection mediate by subcutaneous immunization of our CDN-adjuvanted vaccine was completely independent of Type I IFN signaling. Since induction of Type I IFN is a prominent downstream effect of STING signaling, it may seem surprising that Type I IFN does not play a role in immunity elicited by a STING-activating vaccine. However, Type I IFN is generally detrimental to host control of *M. tuberculosis* infection as *Ifnar-/-* mice, which lack the Type I IFN receptor, have a reduced *M. tuberculosis* burden that wild type mice (Desvignes et al., 2012; Ji et al., 2019; Stanley et al., 2007). Conversely, *B6.Sst1* mice, which have aberrantly high Type I IFN, are extremely susceptible to TB and are rescued when crossed to *Ifnar-/-* mice (Ji et al., 2019). Since alternative STING signaling pathways lead to the production of inflammatory cytokines that are required for host control of *M. tuberculosis*, it is likely that Type IFN-independent pathways downstream of STING, such as NF-kB signaling, drive the protective efficacy against TB after subcutaneous administration of our CDN vaccine.

Mucosal vaccine administration can sometime more effectively protect against mucosal pathogens. To test whether mucosal immunization with our CDN vaccine would lead to enhanced protection against TB, we administered protein adjuvant plus CDN intranasally, following the same vaccine schedule as with subcutaneous administration. We found that intranasal administration induced superior protection to BCG, both as a sole vaccine and as a booster to BCG immunization, with approximately 2 logs of protection at 4 weeks post-challenge. To our knowledge, this is as good or better than any protein subunit vaccine tested against *M. tuberculosis* in mice to date. One interesting comparison is to the most promising new TB vaccine candidate, M72/AS01, which recently showed 50% efficacy in a human clinical trial at preventing progression from latent to active TB in adults (Van Der Meeren et al., 2018). Pre-clinical studies with this vaccine in mice during the early 2000s showed only ~0.5 log reduction in lung bacterial burden compared to PBS controls (Skeiky et al., 2004), significantly less protection than we observe with our CDN adjuvanted vaccine. Although comparing different experimental vaccines tested by different groups years apart is ill-advised, it is tempting to speculate that the generation of new, more effective adjuvants for use in *M. tuberculosis* vaccines, including perhaps CDNs, may put the eradication of TB within reach.

IL-17, IFN-γ and Type I IFN are required for mucosal vaccine efficacy

The protection afforded by our CDN-adjuvanted vaccine is likely mediated by a protective CD4 T cell population in the lungs that is either secreting the appropriate, protective milieu of cytokines, or is localizing to the correct location in the lungs, or both. To better understand the mechanism of protection, and to help define what a protective CD4 T cell response to TB entails, we performed single cell RNA sequencing on CD4+ T cells sorted from the lungs of mock and vaccinated mice after *M. tuberculosis* infection. The data presented here represent the first scRNAseq dataset on CD4+ T cells from the lungs of *M. tuberculosis*-infected mice. One striking difference between mock and CDN vaccinated mice was significant populations of Th17 cells and Th1-Th17

cells present only in the lungs of vaccinated mice. This finding is also reflected in flow cytometry from the lungs of mock and vaccinated mice after *M. tuberculosis* infection. Mock and vaccinated mice have relatively similar levels of IFN-γ-producing Th1 cells at 4 weeks post-infection, despite log differences in CFU burden. However, only mice immunized intranasally with our CDN vaccine have significant populations of IL-17-producing Th1 and IFN-γ- IL-17-double-producing Th1-Th17 cells. Thus, protection against *M. tuberculosis* infection correlates with the abundance of IL-17-producing T cells, and not with the abundance of the canonical protective subset during *M. tuberculosis* infection, Th1 T cells.

To explore whether the protective efficacy induced by our CDN vaccine simply correlates with IL-17, or whether protective efficacy actually requires IL-17, we mucosally immunized wild-type and IL-17-deficient mice with our CDN vaccine. While IL-17 is not required for primary immunity against *M. tuberculosis* challenge, or for BCG-induced immunity, IL-17-deficient mice have significantly less protection compared to wild-type mice after mucosal immunization with our CDN vaccine. So, the protection afforded by our vaccine depends significantly on IL-17, likely produced by Th17 and Th1-Th17 cells in the lungs. Given the known importance of IFN-γ in the immune response to *M. tuberculosis* infection (Flynn et al., 1993; Green et al., 2013), and the fact that immunized mice have *M. tuberculosis*-specific IFN-γ-producing Th1 and Th1-Th17 cells in the lungs, we next asked whether IFN-γ is required for vaccine-elicited protection. While IFN-γ-deficient mice have a significantly higher CFU burden in the lungs compared to wild-type mice, we see that our CDN vaccine is still capable of eliciting protection again *M. tuberculosis* in IFN-γ-deficient mice. Therefore, the protective immune response induced by our CDN vaccine is at least partially IFN-γ-independent. It will be interesting to test whether mice deficient in both IFN-γ and IL-17 have an additional loss of control, indicating distinct mechanisms of protection by these two CD4 T cell-derived cytokines.

Finally, we tested the contribution of Type I IFN after mucosal administration by immunizing mice with defects in Type I IFN signaling (*Ifnar-/-* mice) and comparing to wild type control mice. In contrast to subcutaneous administration, Type I IFN is required for full protective efficacy induced by mucosal administration of our CDN vaccine, despite the fact that *Ifnar-/-* mice actually have significantly more Th17 cells than control mice after vaccination. The enhanced efficacy and unique immune requirements of mucosal administration compared to subcutaneous administration suggests distinct mechanism of protection. The increase in Th17 cells is in accordance with studies showing Type I IFN suppresses Th17 differentiation (Mourik et al., 2017). However, this is the only condition in our studies where the abundance of Th17 cells does not correlate with the magnitude of protection. This suggests that either an excess of Th17 cells may actually be detrimental to control and Type I IFN plays an important role in regulating this response, that both wild type and *Ifnar-/-* mice have sufficient levels of Th17 cells after immunization and Type I IFN plays an as yet unknown role in mediating protection, or that the Th17 cells present in *Ifnar-/-* mice are somehow deficient in mediating protection compared to those present in wild type mice.

With the recent finding that *M tuberculosis*-specific CD4 T cells that express IL-17 are found in the lungs, but not blood, of human TB patients and correlate with better protection against infection (Ogongo et al., 2021), Th17 and Th1-Th17 cells may be key

CD4 T cell populations for protection against *M. tuberculosis.* Still, the exact role of IL-17 during TB infection is still being uncovered, and further research into the efficacy of our CDN vaccine will explore the protective role of IL-17-producing cells after vaccination. The canonical role for IL-17 is to recruit neutrophils but, as described in Chapter One, neutrophils tend to be detrimental to host control of infection. So, which cells require IL-17 signaling to help mount a protective immune response? Does IL-17 directly act on infected cells to inhibit bacteria replication, or is it immunomodulatory, acting on bystander cells to regulate and enhance the immune response to infection? While one study correlated Th17 cells with more rapid recruitment of protective Th1 cells in vaccinated mice after *M. tuberculosis* infection (Khader et al., 2007), the data presented here cannot support nor refute that hypothesis.

Interestingly, Th17 and Th1-Th17 cells are only present in the lungs of vaccinated mice which are mounting a secondary immune response to *M. tuberculosis* infection and are not present in the lungs of unvaccinated mice mounting a primary immune response. Furthermore, IL-17 is required for control of *M. tuberculosis* only in vaccinated mice. With IL-17 playing a clear role in suppressing bacteria replication in vaccinated mice, why is this cytokine not induced as part of a primary immune response? One possibility is that *M. tuberculosis* has mechanisms to prevent IL-17 production in unimmunized hosts, thereby supporting its growth.

How does vaccination with CDN adjuvants drive enhanced CD4 T cell localization?

For a CD4 T cell to effectively protect against *M. tuberculosis* infection, it must not only produce the appropriate combination of cytokines, it must also localize to the correct location within infected tissue. For CD4 T cells to be maximally protective during pulmonary *M. tuberculosis* infection, they must first become activated and polarize within lung-draining lymph nodes, then mobilize to the lung tissue, extravasate from the lung vasculature into the lung parenchyma, and localize to the site of the *M. tuberculosis* lesion and, finally, infiltrate into the core of the granulomatous lesion where the bacteria and infected cells are concentrated. The ability of CD4 T cells to penetrate the core of the *M. tuberculosis* granuloma is a key feature of effective vaccines for tuberculosis (Andersen and Urdahl, 2015; Hoang et al., 2013). To determine the effect of CDN adjuvants on CD4 T cell localization, we performed immunohistochemistry on lung sections from mock and vaccinated mice after *M. tuberculosis* infection and found that intranasal administration of our CDN vaccine induces significantly enhanced T cell homing to granulomatous lesions in the lungs compared to mock immunization, BCG immunization, or subcutaneous administration of our CDN vaccine.

This improved localization is likely due in part due to increased expression of the chemokine receptor CXCR3 on CD4 T cells in the lungs of CDN vaccinated mice (Sakai et al., 2014). We are interested in immunizing CXCR3 knockout mice to test whether a lack of CXCR3 results in less efficient CD4 T cell localization to the lung parenchyma and into the core of the *M. tuberculosis* lesion, and whether this deficiency in effective CD4 T cell homing would result in a loss of protection. These results would contribute to the growing literature on what makes these parenchymal-homing CD4 T cells so effective at controlling *M. tuberculosis* infection. We are also interested in which cells produce CXCR3 ligands, i.e. CXCL9, CXCL10 and CXCl11, and the role of STING

signaling pathways in driving expression of these chemokines after vaccination with CDN adjuvants. The ability to influence the localization of specific protective CD4 T cell subsets using specific vaccine adjuvants is an exciting prospect for future vaccine research.

Limitations, remaining questions, and future directions

There are several limitations to the data presented here, some of which have kindly been raised by audience members during past seminar presentations. One control group that we have not yet included in a vaccine cohort is mice immunized with CDN alone (without protein antigen). We expect that without priming and expanding CD4 T cells specific for *M. tuberculosis*, these mice would not be protected against infection. However, if these mice did have less bacterial growth in the lungs, or survive longer after infection than PBS immunized mice, that might indicate that CDNs are able to prime the immune system, perhaps through Type I IFN induction, in a way that creates a less hospitable environment for the bacteria. Another key limitation pertains to the studies on the role of IFN-γ in promoting vaccine efficacy. *Ifng-/-* mice have a very high lung burden compared to wild type mice, with or without vaccine-mediated protection. While our vaccine was able to protect in these mice, indicating some level of IFN-γ-independent control, it is possible that the high bacterial burden presented too large a challenge for the vaccine-elicited immune response and that with more physiological numbers of bacteria the vaccine would prove to be completely independent of IFN-γ. Finally, we have never compared intranasal administration of our vaccine to intranasal administration of BCG, which has been shown to be more effective that subcutaneous administration at controlling *M. tuberculosis* infection (Dijkman et al., 2019).

Numerous questions remain at the end of this dissertation. First, how exactly does STING signaling in antigen presenting cells drive the production of highly protective CD4 T cells? What is happening inside the innate immune cells that sense and respond to CDN stimulation? While we have defined here several CD4 T cell effector mechanisms that are required for protection and observe a small role for Type I IFN after mucosal immunization, the mechanism of CDN-mediated protection is still unclear, and we are interested in continuing to use genetic tools to dissect the molecular mechanism of CDN-mediated immunity. Nf-kB is downstream of STING and promotes TNFα production. TNFα expression was elevated in CD4 T cells in the lungs of CDN vaccinated has been shown to be required for immunity elicited by mucosal CDN adjuvants in vivo (Blaauboer et al., 2014). Is Nf-kB signaling required for protection? What is the role of TNFα in the context of a CDN vaccine for TB? Additionally, autophagy is induced after TBK1 activation downstream of STING during TB infection. We are interested in testing whether autophagy facilitates vaccine efficacy, perhaps by promoting antigen presentation, by immunizing mice deficient in key autophagy effectors such as ATG5. Finally, STING induces Type I IFN via the transcription factor IRF3. Would an IRF3 knockout mouse phenocopy an *Ifnar-/-* mouse in this context? If not, is this because of additional roles for IRF3 beyond Type IFN induction, or IRF3-independent Type I IFN induction after CDN stimulation?

We are also interested in further exploring the phenotype of the protective CD4 T cell populations in the lungs of infected mice. Why does a primary challenge with *M.*

tuberculosis lead to a Th1-dominant CD4 T cell population that is insufficient to control infection, while immunization with a CDN vaccine is capable of inducing a more heterogenous, and more protective, CD4 T cell population? Unocovering the similarties and differences in CD4 T cells between these two conditions may help inform studies on correlates of immunity during human clinical trials for new TB vaccines. Additionally, the intriguing result that protective efficacy is partially IFN-γ-independent contributes to growing literature that CD4 T cells can mediate IFN-γ-independent protection of TB infection (Cowley and Elkins, 2003; Gallegos et al., 2011). In particular, the TNFα superfamily member CD153 (*Tnfsf8*) has been implicated in IFN-γ-independent of TB (Sallin et al., 2018) and in promoting Th17 differentiation (Sun et al., 2010). We observe *Tnfsf8* mRNA expression in CD4 T cells from the lungs of mucosally immunized mice, especially on Th17 and Th1-Th17 cells, and are interested in exploring the role of CD153 in promoting CDN vaccine efficacy. We hope that by addressing the mechanism of efficacy of our CDN vaccine we can contribute to what is known about protective immunity to TB, help unravel the intricacies of the cGAS-STING pathway in the context of TB infection, and further define the most efficacious CD4 T cell population for protection against TB disease. Finally, we are excited about the idea that molecules that stimulate STING or other receptors in the cytosolic surveillance pathway may be effective adjuvants for other virulent intracellular pathogens.

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