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Authors
Van Dis, Erik
Sogi, Kimberly M
Rae, Chris S
et al.

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STING-Activating Adjuvants Elicit a Th17 Immune Response and Protect against *Mycobacterium tuberculosis* Infection

Erik Van Dis[1,7], **Kimberly M. Sogi**[2,7,9], **Chris S. Rae**[3,8], **Kelsey E. Sivick**[3,8], **Natalie H. Surh**[3], **Meredith L. Leong**[3], **David B. Kanne**[3], **Ken Metchette**[3], **Justin J. Leong**[3], **Jacob R. Bruml**[3], **Vivian Chen**[2], **Kartoosh Heydari**[4], **Nathalie Cadieux**[5], **Tom Evans**[6], **Sarah M. McWhirter**[3], **Thomas W. Dubensky Jr.**[3], **Daniel A. Portnoy**[1,2], and **Sarah A. Stanley**[1,2,10,*]

[1]Molecular and Cell Biology, Division of Immunology and Pathogenesis, University of California, Berkeley, Berkeley, CA 94720, USA

[2]School of Public Health, Division of Infectious Disease and Vaccinology, University of California, Berkeley, Berkeley, CA 94720, USA

[3]Aduro Biotech, Inc., 740 Heinz Avenue, Berkeley, CA 94710, USA

[4]LKS Flow Cytometry Core, Cancer Research Laboratory, University of California, Berkeley, Berkeley, CA 94720, USA

[5]Aeras, 1405 Research Blvd., Rockville, MD 20850, USA

[6]Vaccitech Limited, King Charles House, Park End Street, Oxford OX1 1JD, UK

SUMMARY

There are a limited number of adjuvants that elicit effective cell-based immunity required for protection against intracellular bacterial pathogens. Here, we report that STING-activating cyclic dinucleotides (CDNs) formulated in a protein subunit vaccine elicit long-lasting protective immunity to *Mycobacterium tuberculosis* in the mouse model. Subcutaneous administration of this vaccine provides equivalent protection to that of the live attenuated vaccine strain Bacille Calmette-Guérin (BCG). Protection is STING dependent but type I IFN independent and

*Correspondence: sastanley@berkeley.edu.

[7]These authors contributed equally

[8]These authors contributed equally

[9]Present address: Immunotherapeutics and Vaccine Research Initiative, University of California, Berkeley, Berkeley, CA 94720, USA

[10]Lead Contact

SUPPLEMENTAL INFORMATION

Supplemental Information includes five figures and can be found with this article online at https://doi.org/10.1016/j.celrep.2018.04.003.

AUTHOR CONTRIBUTIONS

Conceptualization and Methodology, E.V.D., K.M.S., C.S.R., K.E.S., N.C., S.M.M., T.W.D., D.A.P., and S.A.S.; Investigation, E.V.D., K.M.S., C.S.R., K.E.S., N.H.S., M.L.L., D.B.K., K.M., J.J.L., J.R.B., and V.C.; Resources, K.H., N.C., and T.E.; Writing – Original Draft, E.V.D., K.M.S., and S.A.S.; Writing – Review and Editing, C.S.R., K.E.S., N.C., T.E., S.M.M., and D.A.P.; Supervision, S.M.M., T.W.D., D.A.P., and S.A.S.; Funding Acquisition, D.A.P. and S.A.S.

DECLARATION OF INTERESTS

D.A.P. has a consulting relationship with and a financial interest in Aduro Biotech, and both he and the company stand to benefit from the commercialization of the results of this research. E.V.D., K.M.S., C.S.R., D.A.P., and S.A.S. declare that parts of this work are the subject of a United States provisional patent application titled “Intranasal delivery of a cyclic-di-nucleotide adjuvanted vaccine for tuberculosis.”

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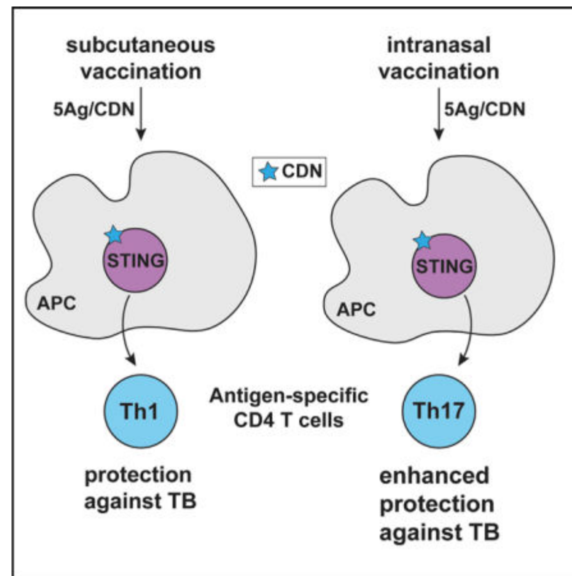
correlates with an increased frequency of a recently described subset of CXCR3-expressing T cells that localize to the lung parenchyma. Intranasal delivery results in superior protection compared with BCG, significantly boosts BCG-based immunity, and elicits both Th1 and Th17 immune responses, the latter of which correlates with enhanced protection. 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.

Graphical abstract

In Brief: Van Dis et al. demonstrate that STING-activating cyclic dinucleotides provide significant protection when used as adjuvants in a protein subunit vaccine against *Mycobacterium tuberculosis* and show that mucosal administration of this vaccine elicits a Th17 immune response that correlates with enhanced protection.



INTRODUCTION

Infection with *Mycobacterium tuberculosis* continues to be a leading cause of death worldwide, in part because of 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), but 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, which cannot always be given to immunocompromised individuals. However, subunit vaccines require an adjuvant to elicit a

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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 (McWhirter et al., 2009; Burdette et al., 2011). CDNs activate the cytosolic receptor stimulator of interferon genes (STING), leading to signaling through multiple immune pathways: TBK1/IRF3 leading to type I interferon (IFN), classical inflammation via nuclear factor κB (NF-κB), and STAT6-dependent gene expression (McWhirter et al., 2009; Chen et al., 2011; Burdette et al., 2011; Burdette and Vance, 2013). Treatment with CDNs stimulates innate immune cells to control *Klebsiella pneumoniae* and *Staphylococcus aureus* infection *in vivo* (Karaolis et al., 2007a, 2007b). 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* (Ebensen et al., 2007a, 2007b; Ogunniyi et al., 2008; Hu et al., 2009; Yan et al., 2009; Libanova et al., 2010; Madhun et al., 2011; Dubensky et. al., 2013). Finally, CDNs are under investigation as promising agents for cancer immunotherapy (Chandra et al., 2014; Woo et al., 2014; Hanson et al., 2015). 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 Toll-like receptor (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 because of 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. Therefore, 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 (s.c.) administration of a synthetic analog 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 parenchyma-homing T cells. Furthermore, intranasal (i.n.) delivery of 5Ag/RR-CDG resulted in 1.5–2 logs of protection 12 weeks after 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 what has been observed with any protein subunit vaccine for *M. tuberculosis* to date, and

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these results demonstrate that CDNs can elicit T cell responses that elicit protection against infection with an intracellular bacterial pathogen.

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 stereo-chemical configuration (RR-CDG) to prevent cleavage and inactivation by host cell phosphodiesterases (Corrales et al., 2015; Figure S1). 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 (Horwitz et al., 1995; Baldwin et al., 1998; Brandt et al., 2000; Weinrich Olsen et al., 2001; Olsen et al., 2004; Langermans et al., 2005). 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 1A).

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

To facilitate comparison with other vaccine adjuvants, RR-CDG was formulated with a fusion protein of ESAT-6 and Ag85B, antigens commonly used together in vaccine studies (Weinrich Olsen et al., 2001; Agger et al., 2008). 12 weeks after infection, the protection afforded by RR-CDG and the ESAT-6/Ag85B fusion protein was equivalent to 5Ag/RR-CDG (Figure S2). 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 an *M. tuberculosis* protein subunit vaccine to date (Skeiky et al.,

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2004; Bertholet et al., 2010; Aagaard et al., 2011; Baldwin et al., 2012; Billeskov et al., 2012; Ma et al., 2017).

5Ag/RR-CDG Vaccine Increases the Percentage of Parenchyma-Homing T Cells in the Lungs Relative to PBS- or BCG-Vaccinated Mice

At the peak of the immune response, 4 weeks after challenge, mice vaccinated with 5Ag/RR-CDG had a significantly higher percentage of CD4+ T cells in the lungs compared with mice vaccinated with PBS (Figure S3A) and a corresponding decrease in the percentage of CD8+ T cells (Figure S3B), 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. Because of 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 S4A). A robust ESAT-6-specific CD4+ IFN-γ+ T cell population was observed in 5Ag/RR-CDG immunized mice (Figure S4A), although it was lower than in PBS-immunized mice. A similar trend was observed for poly-functional T cells (Figure S4B). In total, although 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, which localize to the lung vasculature and produce abundant levels of IFN-γ, and CXCR3+ KLRG1–cells, which 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). 4 weeks after challenge, there was no significant difference in the percentage of CXCR3 KLRG1+ vascular CD4+ T cells among the groups (Figure 1F). 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 with PBS controls (Figure 1G). 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 with PBS immunized mice (Figure S4C). 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 (*Tmem173*$^{gt/gt}$) (Sauer et al., 2011) or IFNAR (*Ifnar1*$^{-/-}$) were immunized according to the schedule outlined in Figure 1A. Seven days after the second boost, both Ag85B- and ESAT-6-specific T cell responses were undetectable in PBMCs from *Tmem173*$^{gt/gt}$ mice, indicating that antigen-specific T cell responses promoted by 5Ag/RR-CDG are STING-dependent (Figures 2A and 2B). Interestingly, antigen-specific T cell responses were equivalent in wild-type and *Ifnar1*$^{-/-}$

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mouse PBMCs (Figures 2A and 2B), suggesting that 5Ag/RR-CDG responses are not dependent on IFNAR signaling.

Tmem173$^{gt/gt}$ mice immunized with 5Ag/RR-CDG had equivalent colony-forming units (CFUs) in the lungs 4 and 12 weeks after challenge with *M. tuberculosis* compared with *Tmem173*$^{gt/gt}$ mice immunized with PBS (Figures 2C and 2D), demonstrating that the protective efficacy of RR-CDG is dependent on STING. In contrast, *Ifnar1*$^{-/-}$ mice immunized with 5Ag/RR-CDG had equivalent protection as wild-type 5Ag/RR-CDG-vaccinated mice (Figures 2C and 2D). Thus, although 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.

i.n. but Not s.c. 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 3A, BCG primed mice received two boosts of 5Ag/RR-CDG or 5Ag alone via s.c. injection and were compared with mice that received three s.c. injections of 5Ag/RR-CDG as outlined in Figure 1A. After the second boost, IFN-γ ELISPOT using PBMCs showed that BCG-immunized mice boosted s.c. with 5Ag/RR-CDG had increased Ag85B- and ESAT-6-specific T cell responses compared with mice that were immunized only with BCG (Figure 3B). However, there was no difference in IFN-γ levels between mice immunized with BCG and boosted with s.c. 5Ag/RR-CDG compared with mice that received three s.c. administrations of 5Ag/RR-CDG alone (Figure 3B). Additionally, boosting BCG with s.c. 5Ag/RR-CDG did not result in enhanced protection against *M. tuberculosis* aerosol challenge (Figures 3C and 3D).

We next tested whether mucosal administration of 5Ag/RR-CDG via the i.n. route would enhance protection against *M. tuberculosis* infection using the vaccination schedule outlined in Figure 1A or as outlined in Figure 3A for i.n. boosting of BCG. Because AddaVax is not suitable for i.n. vaccination, 5Ag/RR-CDG was formulated in PBS. Seven days after the second boost, i.n. administration of 5Ag/RR-CDG resulted in an increase in IFN-γ-producing Ag85B-specific CD4+ T cells in PBMCs compared with PBS-vaccinated mice (Figure 4A). However, significantly fewer IFN-γ-producing cells were elicited by i.n. vaccination than by s.c. vaccination (Figure 4A). In contrast, i.n. administration of 5Ag/RR-CDG produced a robust interleukin-17 (IL-17) response from CD4+ T cells upon re-stimulation with Ag85B peptide pools (Figure 4B), a response that was not observed with s.c. administration of 5Ag/RR-CDG or with BCG vaccination.

Vaccinated mice were challenged with *M. tuberculosis* to determine the protective efficacy of i.n.-delivered CDN vaccines. As expected, ~1 log of pulmonary protection was seen in mice vaccinated with either BCG or s.c. 5Ag/RR-CDG (Figures 4C and 4D). However, i.n. administration of 5Ag/RR-CDG resulted in an additional 0.5 log of control 4 weeks after challenge (Figure 4C) and a trend toward increased control that was not statistically significant at 12 weeks (Figure 4D). Remarkably, BCG-vaccinated mice receiving i.n. boosts of 5Ag/RR-CDG had significantly lower CFUs in the lungs 12 weeks after challenge

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compared with BCG vaccination alone, resulting in 2 logs of protection against infection (Figure 4D). As with s.c. vaccination, the percentage of CD4+ IFN-γ+ T cells in the lungs of i.n.-vaccinated mice was not enhanced beyond infection-induced responses exhibited in PBS-immunized mice 4 weeks after challenge (Figure 4E). However, the pre-challenge increase in Th17 cells noted in the blood (Figure 4B) was reflected after challenge with a large fraction of CD4+ T cells in the lungs producing IL-17 (Figure 4F). i.n. immunization with 5Ag/RR-CDG resulted in significantly more IL-17+ CD4+ T cells than BCG vaccination or s.c. administration of 5Ag/RR-CDG, both alone and as a booster vaccine (Figure 4F; Figure S5). Thus, i.n. 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 i.n. route did not correlate with increases in Th1 cells but with increases in Th17 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 (Yi et al., 2013; Corrales et al., 2015). We therefore tested the adjuvant activity of ML-RR-cyclic guanosine monophosphate-adenosine monophosphate (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 common human STING alleles (Corrales et al., 2015). Mice were immunized via the i.n. or s.c. 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 first 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 i.n. (Figures 5A–5C). Administration of 5Ag/ML-RR-cGAMP s.c. did not elicit IL-17-producing CD4+ T cells (Figure 5C) but elicited more IFN-γ-producing CD4+ T cells than i.n. immunization (Figure 5B). This is similar to the trend seen with s.c. versus i.n. immunization of 5Ag/RR-CDG (Figures 4A and 4B).

Mice vaccinated with 5Ag/ML-RR-cGAMP were challenged with virulent *M. tuberculosis*, and protection was evaluated by CFUs in the lungs 4 weeks after challenge. Importantly, i.n. immunization with 5Ag/ML-RR-cGAMP provided 1.5 logs of protection when used as a sole vaccine (Figure 5D), equivalent to 5Ag/RR-CDG. These data demonstrate that ML-RR-cGAMP, a STING-activating compound with translational potential to human vaccines, behaves similarly as 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– parenchymahoming 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

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vaccine formulated with the Th1 adjuvant dimethyldioc-tadecylammonium liposomes with monophosphoryl lipid A (DDA/MPL) (Carpenter et al., 2017), the protection afforded by CDN-adjuvanted experimental vaccines was durable through 12 weeks after 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 (Skeiky et al., 2004; Bertholet et al., 2010; Aagaard et al., 2011; Baldwin et al., 2012; Billeskov et al., 2012) 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 s.c. Although we did not test whether the protection of mice immunized with CDNs delivered i.n. is dependent on type I IFN, others have shown that the immune response to mucosally delivered CDG does not require type I IFN (Blaauboer, Gabrielle, and Jin, 2014). STING also activates NF-κB, which induces classical pro-inflammatory cytokines, including tumor necrosis factor α (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 (Horwitz et al., 1995; Baldwin et al., 1998; Brandt et al., 2000; Skjøt et al., 2000; Weinrich Olsen et al., 2001; Olsen et al., 2004; Langermans et al., 2005). 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 as 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 (Kagina et al., 2010; Fletcher et al., 2016). 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/

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narrow or whether, in fact, a Th1 response is not sufficient to confer protective immunity (Kaufmann, 2014). Because 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 toward 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 s.c. and i.n. 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 i.n. 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 i.n.-vaccinated mice 4 weeks after 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 i.n. immunizations.

An ideal vaccine for *M. tuberculosis* would elicit memory T cells that traffic into the lung tissue because 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), 4 weeks after challenge. Despite inducing higher levels of parenchyma-homing T cells, vaccination with 5Ag/RR-CDG resulted in a lower percentage of these cells producing IFN-γ compared with 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 parenchyma-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

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CDN (ADU-S100) is currently in phase I clinical trials as a cancer therapeutic agent alone and in combination with checkpoint inhibition (ClinicalTrials.gov NCT02675439 and NCT03172936). Thus, there is an ongoing effort to develop CDN analogs with improved translational capacity, including a 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.

EXPERIMENTAL PROCEDURES

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 Guide for the Care and Use of Laboratory Animals, and the Public Health Service Policy on Humane Care and Use of Laboratory Animals.

Reagents

RR-CDG and ML-RR-cGAMP were synthesized at Aduro Biotech as described previously (Gaffney et al., 2010; Corrales et al., 2015). 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 the formulation of antigen and adjuvant as directed by the manufacturer. 5Ag fusion protein and peptide pools were provided by Aeras.

Mice

CB6F1 (Figure 1) and C57BL/6 mice (Figures 2, 3, 4, and 5) were obtained from The Jackson Laboratory (Bar Harbor, ME). *Ifnar1*$^{-/-}$ mice were obtained from the Vance lab (University of California, Berkeley) and were bred in-house. *Tmem173*$^{gt/gt}$ mice were a gift from the Raulet lab (University of California, Berkeley) and were bred in-house. Both *Tmem173*$^{gt/gt}$ and *Ifnar1*$^{-/-}$ mice were 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 min to inactivate the SVPD. 1×10^5 DC2.4 murine cells were incubated with 100 μM of CDG, RR-CDG, or

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saline pretreated with or without SVPD in triplicate for 30 min at 37°C. After 30 min, cells were washed and incubated with RPMI medium containing 10% fetal bovine serum (FBS) at 37°C with 5% CO_2. Supernatants were collected after 4 hr and added to L929 cells expressing luciferase under the control of an IFN-stimulated response element (ISRE). After 4 hr 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 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 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 i.n. delivery or in 2% AddaVax in PBS for s.c. delivery. Groups of sex-matched 6- to 10-week-old mice were vaccinated three times at 4-week intervals with 100 μL s.c. at the base of the tail (50 μL on each flank) or with 20 μL i.n. BCG-vaccinated mice were injected once with 2.5–5 × 10^5 CFUs/mouse in 100 μL of PBS s.c. in the scruff of the neck. At the indicated week after immunization, mice were bled retro-orbitally (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 CFUs in the lungs 1 day following infection (data not shown). Unless stated otherwise, groups of five mice were sacrificed 4 and 12 weeks after challenge to measure CFUs and immune responses in the lungs (4 weeks only). For bacterial enumeration, one lung lobe (the largest, 4 weeks after challenge) or all lung lobes (12 weeks after 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. The remaining lung lobes were used for ICS 4 weeks after challenge.

Pre-challenge ELISPOT and ICS Assays

Heparinized blood from five mice was analyzed separately (Figure 5) or pooled (Figures 1, 2, 3, and 4), and lymphocytes were isolated (Lympholyte-Mammal, Cedar Lane, catalog no. CL5115). For ELISPOTs, the lymphocytes (1 × 10^5 cells/well or 1 × 10^4 cells/well for Ag85B and ESAT-6) were put in plates pre-coated with IFN-γ capture antibody (BD Biosciences, 551881) containing splenocytes (1 × 10^5 cells/well) and peptide pools (2 μg/

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mL). Plates were incubated overnight and then washed and developed according to the BD Biosciences kit protocol. Spots were enumerated on a cytotoxic lymphocyte (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); carboxyfluorescein succinimidyl ester (CFSE)-labeled splenocyte feeder cells from an uninfected mouse (1×10^5 cells/well); and GolgiPlug and GolgiStop for 5 hr at 37°C. Cells were kept at 4°C overnight and then washed and stained with Live/Dead stain (Thermo Fisher Scientific, L34970), CD4 (BD Biosciences, 564933), CD8 (BD Biosciences, 563898), CD90.2 (BD Biosciences, 561616), major histocompatibility complex (MHC) class II (BioLegend, 107606), Ly6G (BD Biosciences, 551460), IFN-γ (eBioscience, 12-73111-81), TNF-α (BD Biosciences, 506324), and IL-17 (BioLegend, 506904). Data were collected using a BD LSR Fortessa flow cytometer with FACSDiva software (BD Biosciences) and analyzed using FlowJo Software (Tree Star, Ashland, OR).

Post-challenge Intracellular Cytokine Staining

Lungs (the four smallest lobes) were harvested 4 weeks after challenge into complete Roswell Part Memorial Institute medium (cRPMI) (RPMI 1640 medium, 10% FBS, 1% sodium pyruvate, 1% (4-(2-hydroxyethyl)-1-piperazinee-thanesulfonic acid) [HEPES], 1% L-glutamine, 1% non-essential amino acids, 1% penicillin/streptomycin [pen/strep], and 50 μM 2-mercaptoethanol [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 hr 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 room temperature (RT) for 20 min and removed from the BSL3. Data were collected and analyzed as outlined above.

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 7 (GraphPad, La Jolla, CA), and $p < 0.05$ was considered significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- Vaccines containing STING-activating adjuvants protect against TB infection
- Subcutaneous immunization elicits antigen-specific Th1 T cells
- Protection requires STING but not type I IFN signaling
- Mucosal immunization elicits Th17 T cells and enhances protective efficacy

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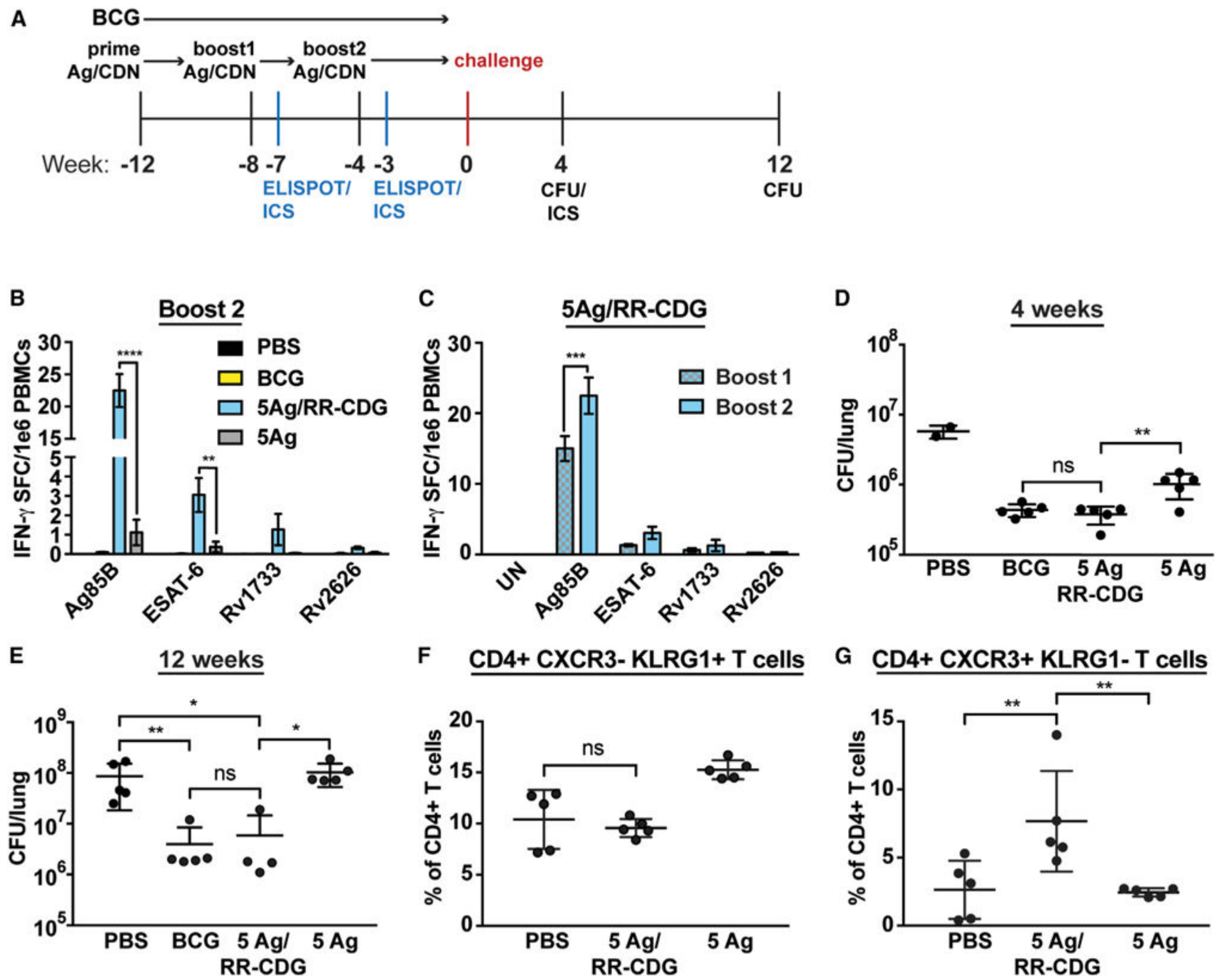

Figure 1. RR-CDG-Adjuvanted Vaccine Protects Equivalently to BCG Vaccination and Induces T Cell Populations Known to Protect against *M. tuberculosis* Infection

(A) Experimental timeline for vaccine experiments. Mice were vaccinated using the indicated schedule and challenged with ~100 CFUs of *M. tuberculosis* strain Erdman.

(B) IFN-γ ELISPOT from PBMCs harvested 7 days after the second 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 7 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).

(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 values; $**p < 0.002$, $***p < 0.001$, $****p < 0.0001$.

(D and E) CFU counts from lungs of vaccinated mice (D) 4 weeks and (E) 12 weeks after challenge. Mann-Whitney t test p values; $*p < 0.02$; $**p < 0.002$.

(F) ICS for percentage of CD4+ T cells in the lungs of mice that are CXCR3−KLRG1+ 4 weeks after challenge.

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(G) ICS for percentage of CD4+ T cells in the lungs of mice that are CXCR3+ KLRG1– 4 weeks after challenge. One-way ANOVA with Tukey's *post hoc* p value; $^{**}p < 0.002$.

(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. Error bars represent SD. See also Figures S1–S4.

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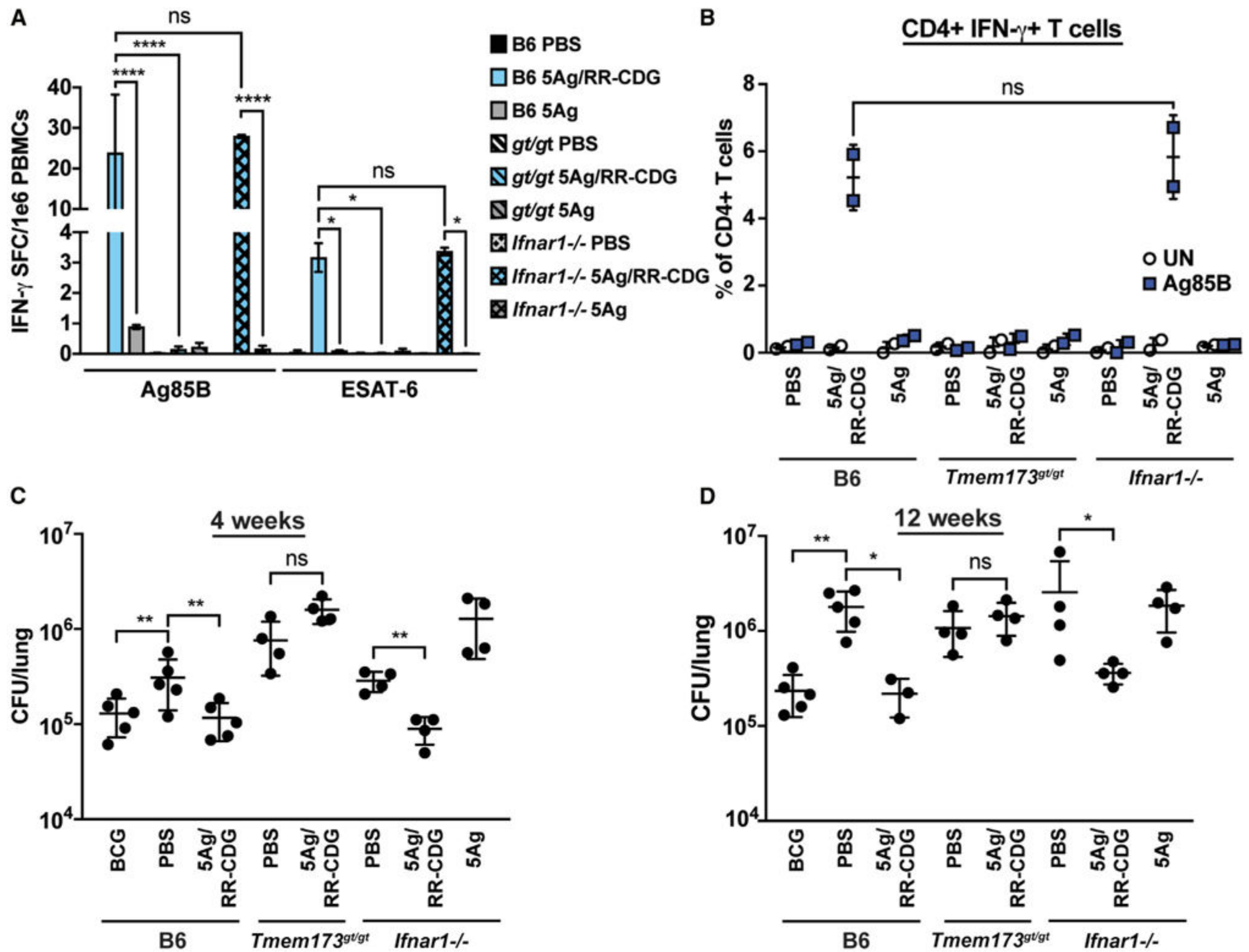

Figure 2. 5Ag/RR-CDG Vaccine-Induced Protection Requires STING but Not Type I IFN Signaling

(A) IFN-γ ELISPOT from PBMCs re-stimulated *ex vivo* with Ag85B and ESAT-6 peptide pools 7 days after the second 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 7 days after the second 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 values; *p < 0.05, ****p < 0.0001.

(C and D) CFU counts from lungs of vaccinated mice (C) 4 weeks and (D) 12 weeks after challenge. For CFU experiments, data are expressed as mean (± SD), and each symbol represents an individual animal. Mann-Whitney t test p values; *p < 0.05, **p < 0.002. Data are representative of experiments done in duplicate. Error bars represent SD.

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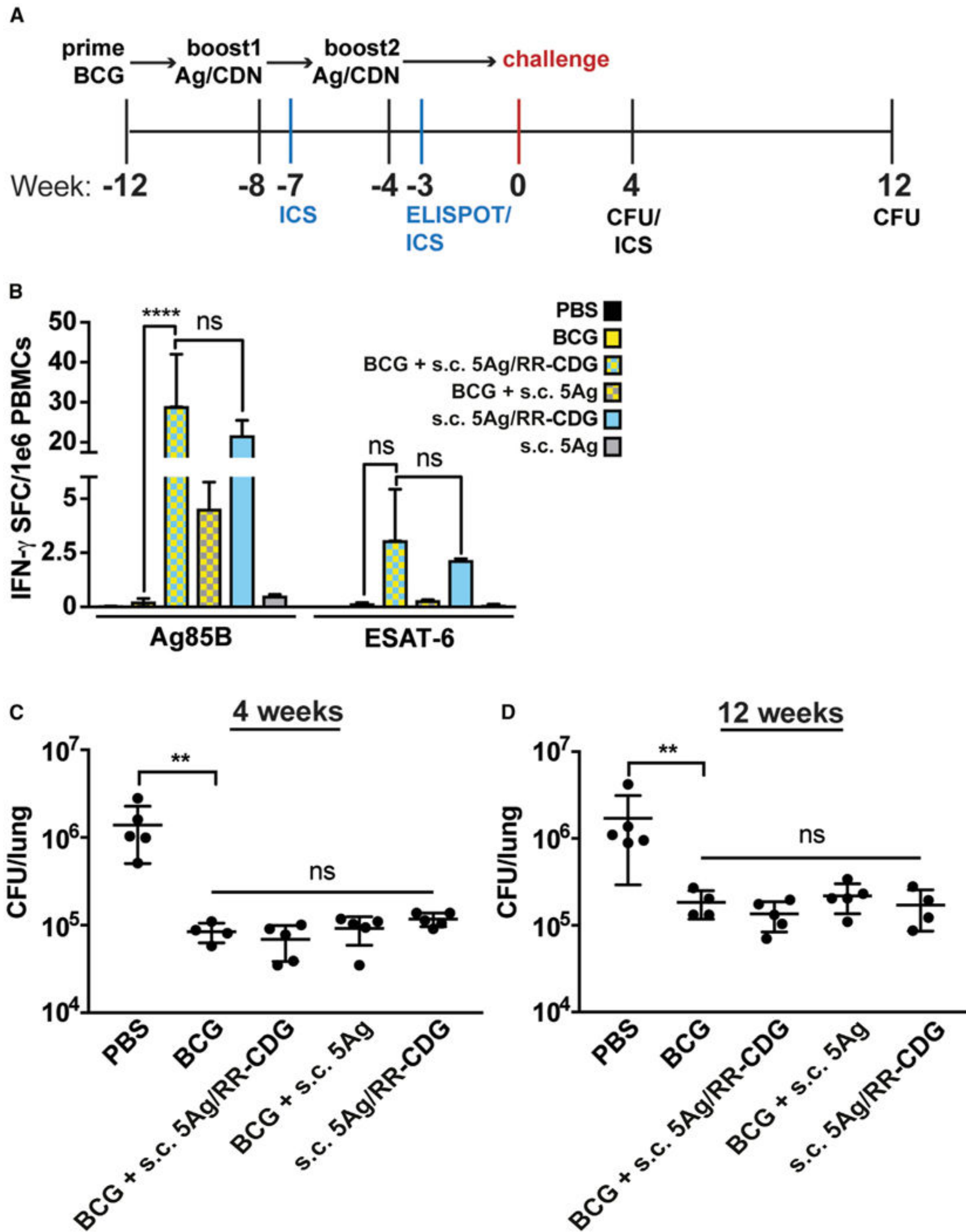

Figure 3. s.c. Boosting of BCG with 5Ag/RR-CDG Does Not Enhance Protection from *M. tuberculosis* Challenge

(A) Experimental timeline for BCG boosting experiments. Mice were vaccinated using the indicated schedule and challenged with 100 CFUs of *M. tuberculosis* strain Erdman.

(B) IFN-γ ELISPOT from PBMCs re-stimulated *ex vivo* with Ag85B and ESAT-6 peptide pools 7 days after the second 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 values; ****$p < 0.0001$.

(C and D) CFU counts from lungs of vaccinated mice (C) 4 weeks and (D) 12 weeks after challenge. For CFU experiments, data are expressed as mean (± SD), and each symbol represents an individual animal. Mann-Whitney t test p value; $^{**}p < 0.002$.

Data are representative of experiments done in duplicate. Error bars represent SD.

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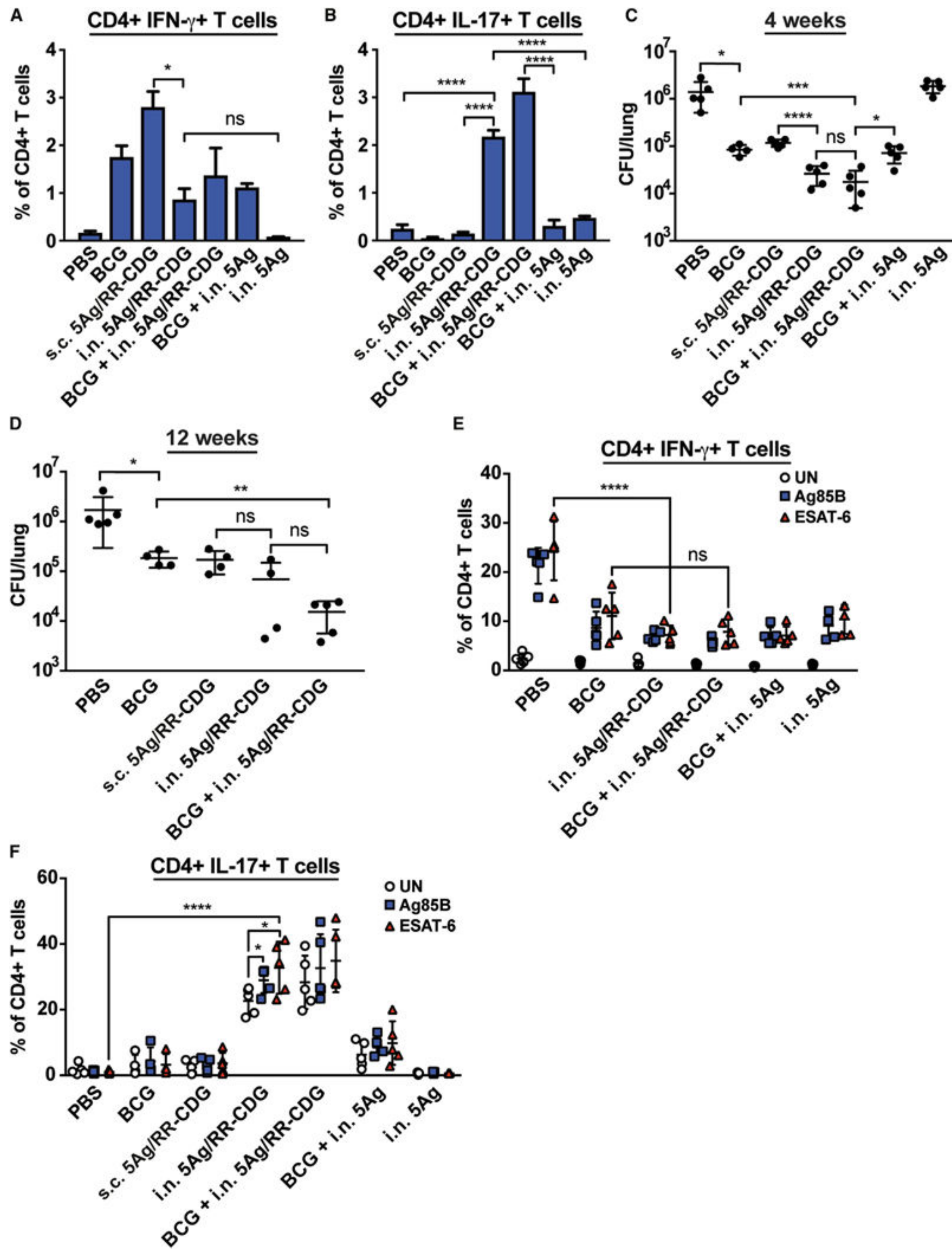

Figure 4. i.n. Administration of 5Ag/RR-CDG Induces Th17 Cells and Enhances Protection When Used as a Booster Vaccine for BCG

(A and B) ICS for percentage of Ag85B-specific CD4+ T cells that produce (A) IFN-γ or (B) IL-17 7 days after the second 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 values; *$p < 0.05$, ****$p < 0.0001$.

(C and D) CFU counts from lungs of vaccinated mice (C) 4 weeks and (D) 12 weeks after challenge. Mann-Whitney t test p values; *$p < 0.05$, **$p < 0.002$, ***$p < 0.0002$, ****$p < 0.0001$.

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(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 4 weeks after challenge. Two-way ANOVA with Tukey's *post hoc* p values; $*p < 0.05$, $****p < 0.0001$.

(C–F) Data are expressed as mean (± SD), and each symbol represents an individual animal. Data are representative of experiments done in duplicate. Error bars represent SD. See also Figure S5.

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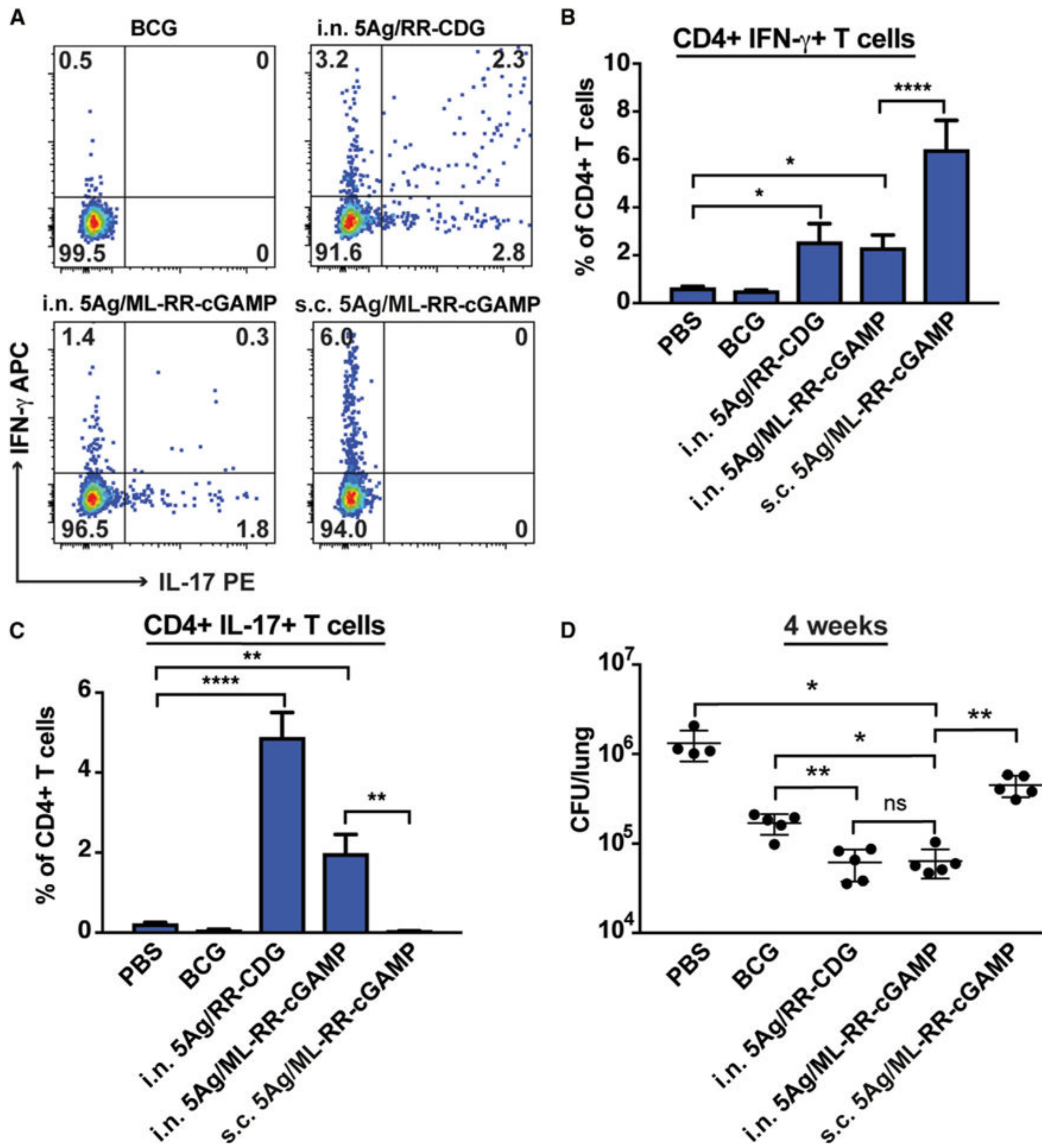

Figure 5. ML-RR-cGAMP-Adjuvanted Vaccine Elicits a Th17 Response and Protects against Challenge with *M. tuberculosis*

(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 first boost.

(B and C) ICS for percentage of Ag85B-specific CD4+ T cells that produce (B) IFN-γ or (C) IL-17 7 days after the first boost. Data are expressed as mean (± SD) of five mice per group. Two-way ANOVA with Tukey's *post hoc* p values; $*p < 0.05$, $**p < 0.002$, $****p < 0.0001$.

(D) CFU counts from lungs of vaccinated mice 4 weeks after challenge. Data are expressed as mean (± SD), and each symbol represents an individual animal. Mann-Whitney t test p values; $*p < 0.05$, $**p < 0.002$.

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Data are representative of experiments done in duplicate. Error bars represent SD.