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IL-23-dependent IL-17 drives Th1-cell responses following *Mycobacterium bovis* BCG vaccination

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The generation of effective type 1 T helper (Th1)-cell responses is required for immunity against intracellular bacteria. However, some intracellular bacteria require interleukin (IL)-17 to drive Th1-cell immunity and subsequent protective host immunity. Here, in a model of *Mycobacterium bovis* Bacille Calmette–Guerin (BCG) vaccination in mice, we demonstrate that the dependence on IL-17 to drive Th1-cell responses is a host mechanism to overcome bacteria-induced IL-10 inhibitory effects. We show that BCG-induced prostaglandin-E2 (PGE2) promotes the production of IL-10 which limits Th1-cell responses, while simultaneously inducing IL-23 and Th17-cell differentiation. The ability of IL-17 to downregulate IL-10 and induce IL-12 production allows the generation of subsequent Th1-cell responses. Accordingly, BCG-induced Th17-cell responses precede the generation of Th1-cell responses in vivo, whereas the absence of the IL-23 pathway decreases BCG vaccine-induced Th17 and Th1-cell immunity and subsequent vaccine-induced protection upon *M. tuberculosis* challenge. Importantly, in the absence of IL-10, BCG-induced Th1-cell responses occur in an IL-17-independent manner. These novel data demonstrate a role for the IL-23/IL-17 pathway in driving Th1-cell responses, specifically to overcome IL-10-mediated inhibition and, furthermore, show that in the absence of IL-10, the generation of BCG-induced Th1-cell immunity is IL-17 independent.

Key words: Immune regulation · Immune responses · Intracellular bacteria · T helper cells


Supporting Information available online

Introduction

Tuberculosis (TB), caused by the intracellular pathogen *Mycobacterium tuberculosis*, remains a crucial worldwide health problem. Approximately one-third of the world's population is latently infected with *M. tuberculosis*, with 9 million new cases of active TB and 2 million deaths occurring annually. The recent emergence of Extensively Drug Resistant (XDR) strains of *M. tuberculosis*, along with HIV-associated TB, has further compounded the problem. *M. bovis* Bacille Calmette–Guerin (BCG) is still the most widely used vaccine, but exhibits variable efficacy [1]. In order to improve upon the current efficacy of BCG vaccination, it is critical to understand the requirements for effective vaccine-induced immune responses following BCG vaccination. The interleukin (IL)-12 type 1 T helper (Th1) pathway is critical for host immunity against *M. tuberculosis* in humans [2], and in experimental models [3]. Consistent with

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these findings, BCG vaccine-induced protection against TB is also dependent on the accumulation of Th1-cell memory cells that produce the cytokine IFN-γ that activates macrophages for mycobacterial control [4]. However, factors required for effective generation of Th1-cell responses following BCG vaccination are not completely understood. The identification of factors required for BCG vaccine-induced Th1-cell responses will result in a major improvement in our ability to vaccinate effectively against TB and contribute to better control of global TB burdens.

The cytokine IL-12, made up of IL-12p35 and IL-12p40 subunits, is critical for the induction of IFN-γ from T and NK T cells [5]. IL-23, composed of the p40 and p19 subunit [6], is required for maintenance of Th type 17 (Th17) cells [7, 8]. Th17 cells produce the cytokines IL-17A (IL-17), IL-17F, IL-21, and IL-22 [9] and are involved in the induction of inflammation associated with models of autoimmune diseases [10]. In contrast, IL-23-dependent IL-17 responses are important for protective immunity against extracellular bacterial infections via induction of chemokines required for neutrophilic recruitment and bacterial killing [11]. However, more recently we and others have shown that IL-17 is also required for protective immunity against some intracellular pathogens such as *Francisella tularensis* LVS [12] and *Chlamydia muriduram* [13]. IL-17-induced protective immunity against these intracellular pathogens occurs via IL-17-dependent induction of IL-12 in DCs [12, 13] and the resulting generation of Th1-cell responses [12]. Accordingly, the absence of the IL-23/IL-17 pathways results in decreased induction of Th1-cell immune responses and increased susceptibility to infection [12, 13]. Interestingly, pulmonary acute infection with *M. bovis* BCG also requires IL-17 to drive Th1-cell immune responses, without playing a role in protection [14]. These studies project the important question why some intracellular bacteria such as *F. tularensis*, *C. muridarum*, and *M. bovis* BCG [12–14] require IL-17 to induce Th1-cell immunity.

In light of these recent findings and since the BCG is the most widely used vaccine worldwide, the goal of this study was to determine if the generation of BCG vaccine-induced Th1-cell immune responses and subsequent protection against *M. tuberculosis* challenge is IL-23/IL-17 dependent. Furthermore, we wanted to delineate the mechanism behind the basis for IL-17 dependence for the generation of Th1-cell immunity. Accordingly, we show here that IL-23-dependent IL-17 is required for effective generation of Th1-cell BCG vaccine-induced immune responses and protection following *M. tuberculosis* challenge. We show for the first time that the requirement for IL-17 in driving Th1-cell immunity is a host response to overcome bacteria-induced IL-10 and its inhibitory effects on Th1-cell generation. Prostaglandin E2 (PGE2) is a common inflammatory mediator that can directly suppress the production of IL-12 in DCs [15, 16], instead enhancing the production of IL-12 antagonists, IL-10 and IL-12p40 [16, 17]. Furthermore, recent studies have shown that PGE2 acts on DCs through its receptors EP2 and EP4 to drive IL-23 responses and mediate Th17-cell differentiation in vitro [18, 19]. Here, for the first time we show the existence of a dual function for pathogen-induced PGE2 since it can direct both BCG-induced IL-10 and IL-23, thereby simultaneously limiting Th1-cell responses and driving Th17-cell responses. Importantly, we show that IL-17 can downregulate IL-10 and induce IL-12 production in DCs, thereby allowing the generation of Th1-cell responses; in the absence of IL-10, BCG-induced Th1-cell responses occurs in an IL-17-independent manner. These data therefore project a critical role for IL-23/IL-17 pathway in overcoming BCG-induced IL-10-mediated inhibitory effects.

Results

Vaccine-induced IL-17 is required to generate Th1-cell responses following BCG vaccination in mice

IL-17 is required for the generation of Th1-cell responses and host immunity against *F. tularensis LVS* and *C. muridarum* [12, 13]. Therefore, we determined if IL-17 was involved in the generation of Th1-cell responses following vaccination with BCG. We subcutaneously vaccinated WT C57BL/6J (B6) mice or IL-17 receptor A-deficient mice (*il17ra*$^{-/-}$) with live BCG and evaluated IFN-γ responses in the draining LNs (DLNs) of vaccinated mice. BCG-vaccinated B6 mice induced both $CD4^+$ and $CD8^+$ IFN-γ-producing cells in DLNs, with higher numbers of $CD8^+$ IFN-γ-producing cells, when compared with unvaccinated mice (Fig. 1A and B). Interestingly, *il17ra*$^{-/-}$ mice had significantly decreased $CD4^+$ and $CD8^+$ IFN-γ-producing cells compared with B6 BCG-vaccinated mice (Fig. 1A and B; Supporting Information Fig. 1A and B). In order to detect Ag-specific responses in $CD4^+$ T cells, the $Ag85B_{240–254}$ peptide containing the motif that is conserved for class II $I\text{-}A^b$ and requires processing by APCs to prime Ag85B-specific $CD4^+$ T cells was used to stimulate LN cells [20]. Ag85B-specific Th1 cells were significantly lower in *il17ra*$^{-/-}$ BCG-vaccinated DLNs (Fig. 1C) and correlated with a decreased expression of IFN-γ mRNA in *il17ra*$^{-/-}$ DLN cells when compared with B6-vaccinated mice (Fig. 1D). However, no Ag85B-specific cytokine responses were detected in the lungs of BCG-vaccinated mice at any of the time points tested (data not shown).

IL-17 induces the production of IL-12 in DCs [12, 13] and subsequent Th1-cell differentiation [12]. Therefore, we hypothesized that reduced Th1-cell responses in the *il17ra*$^{-/-}$ BCG-vaccinated mice was due to decreased induction of IL-17-dependent IL-12 production. Consistent with this hypothesis, significantly reduced IL-12p35 and IL-12p40 mRNA levels were detected in DLN cells of BCG-vaccinated *il17ra*$^{-/-}$ mice (Fig. 1D) and correlated with decreased mRNA expression of the Th1-cell transcription factor, Tbet [21]. As expected [12], there was increased induction of IL-17 mRNA in the *il17ra*$^{-/-}$ BCG-vaccinated DLN cells compared with DLN cells isolated from B6 BCG-vaccinated mice (Fig. 1D). Also, the increased levels of IL-17 mRNA correlated with increased expression of the Th17-cell transcription factor, RORγt [22] in DLN cells from *il17ra*$^{-/-}$ BCG-vaccinated mice (Fig. 1D). These data suggest that IL-17 is

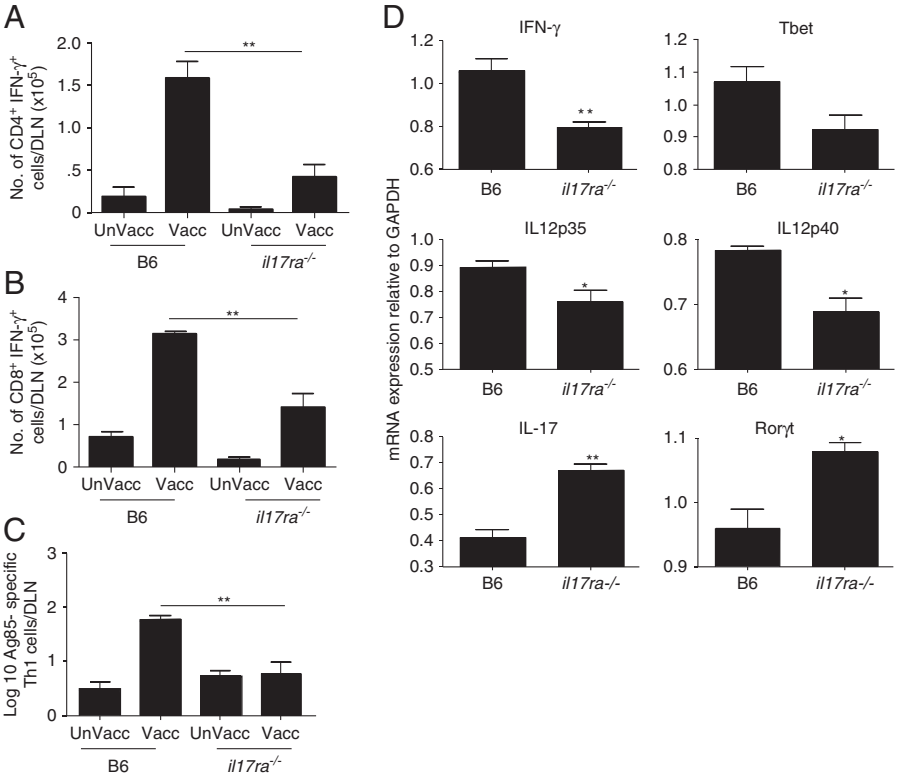

Figure 1. Vaccine-induced IL-17 responses are required for Th1-cell responses following BCG vaccination. B6 and *il17ra*$^{-/-}$ mice were either subcutaneously vaccinated or left unvaccinated with 1×10^6 BCG. On day 14, the number of IFN-γ-producing (A) CD4$^+$ T cells or (B) CD8$^+$ T cells in DLNs were detected by intracellular staining and flow cytometry. (C) Ag85B-specific Th1-cell responses were detected in BCG-vaccinated or unvaccinated B6 and *il17ra*$^{-/-}$ DLNs on day 14 postvaccination using an Ag-driven ELISpot assay. (D) The mRNA levels of specific genes relative to GAPDH expression were determined in DLN cells of B6 and *il17ra*$^{-/-}$ mice using RT-PCR. The data are shown as the mean+SD of values from four to six mice. *$p \leq 0.05$, **$p \leq 0.05$, two-tailed Student's *t*-test. Data representative of (A, B, D) two and (C) three experiments.

required for the induction of vaccine-induced Th1-cell responses following BCG vaccination.

IL-23-dependent Th17 response is required for BCG induced immunity following *M. tuberculosis* challenge

IL-23 is critical for Th17-cell responses in vivo following mycobacterial exposure [23–25] and therefore, we vaccinated B6 mice and IL-23 gene-deficient mice (*il23p19*$^{-/-}$) and evaluated the generation of Ag85B-specific Th17- and Th1-cell responses in the DLNs. The generation of Ag85B-specific Th17-cell responses (Fig. 2A) and Th1-cell responses (Fig. 2B) were significantly decreased in *il23p19*$^{-/-}$ mice when compared with B6 BCG-vaccinated mice. Induction of an effective Th1-cell vaccine response is crucial for vaccine-induced protection against *M. tuberculosis* challenge [25]. Therefore, we tested whether reduced Th17- and Th1-cell vaccine-induced responses resulted in decreased protection in the *M. tuberculosis*-challenged *il23p19*$^{-/-}$ BCG-vaccinated mice. *il23p19*$^{-/-}$ mice were vaccinated with BCG, rested for 30 days, following which they were challenged with aerosolized *M. tuberculosis* and the lung bacterial burdens determined in BCG-vaccinated and unvaccinated mice. As previously described, no differences in bacterial burden in the lungs of B6 and *il23p19*$^{-/-}$ unvaccinated mice were detected [23] (Fig. 2C). However, we found significantly higher lung bacterial burden in *il23p19*$^{-/-}$ *M. tuberculosis* challenged BCG-vaccinated mice when compared with B6 BCG-vaccinated mice (Fig. 2C). These data demonstrate the importance of the IL-23/Th17 pathway in mediating Th1-cell responses and protective BCG vaccine-induced immunity in response to pulmonary *M. tuberculosis* challenge.

Since IL-17 appeared to be a prerequisite for effective generation of BCG-induced Th1-cell responses (Fig. 1), we determined the kinetics of Ag85B-specific Th1- and Th17-cell responses in B6 and *il17ra*$^{-/-}$ BCG-vaccinated mice. We found that significant Ag85B-specific Th17-cell responses occurred between days 4 and 8 in the DLNs of BCG-vaccinated B6 mice, which was prior to the detection of Ag85B-specific Th1-cell responses on day 14 postvaccination (Fig. 2D). Furthermore, IL-17 signaling is crucial for the generation of Ag85B-specific Th1-cell responses, since *il17ra*$^{-/-}$ BCG-vaccinated mice, although able to induce an early Ag85B-specific Th17-cell response, are unable to induce an effective Ag85B-specific Th1-cell response on day 14 (Fig. 2E). To further confirm these findings, we purified CD4$^+$ T cells from B6 BCG-vaccinated and unvaccinated DLNs at different time points postvaccination and measured cytokine mRNA induction in these cells. Consistent

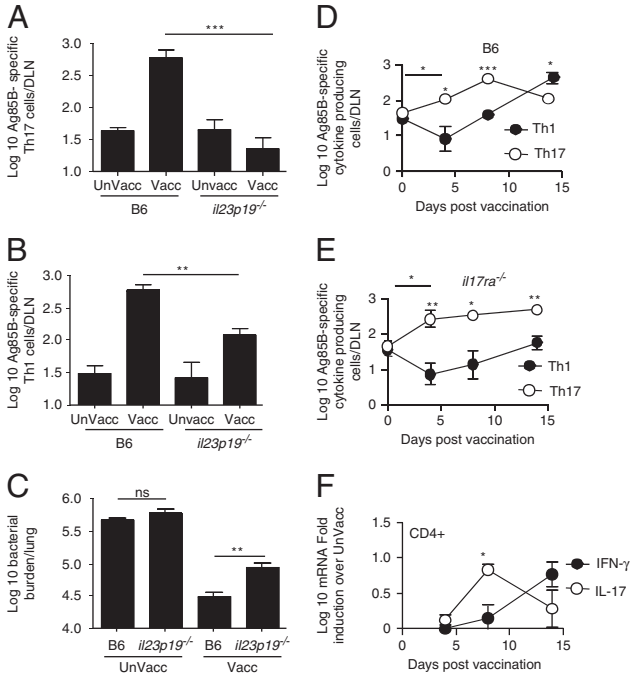

Figure 2. IL-23-dependent IL-17 is required for BCG vaccine-induced Th1-cell immunity and protection following *M. tuberculosis challenge*. (A, B) B6 and *il23p19*$^{-/-}$ mice were vaccinated with BCG or left unvaccinated and the number of (A) Ag85B-specific Th17 cells and (B) Ag85B-specific Th1 cells in DLNs was determined on day 14 by an Ag-driven ELISpot assay. (C) B6 and *il23p19*$^{-/-}$ mice were vaccinated and rested for 30 days, then challenged with ~100 CFU *M. tuberculosis* by the aerosol route. Thirty days later, bacterial CFUs were determined in the lungs of unvaccinated and vaccinated mice. The number of Ag85B-specific CD4$^+$ Th1 and Th17 cells was determined in DLNs of BCG-vaccinated or unvaccinated (D) B6 mice or (E) *il17ra*$^{-/-}$ mice by Ag-driven ELISpot assay. (F) The $\log_{10}$ fold induction of IL-17 and IFN-γ mRNA in CD4$^+$ sorted cells in B6 BCG-vaccinated DLN cells compared with unvaccinated B6 control cells was determined by RT-PCR. The data are shown as the mean ± SD of values from four to six mice. $^*p \leq 0.05$, $^{**}p \leq 0.005$, $^{***}p \leq 0.0005$, two-tailed Student's *t*-test. One of the two independent experiments is shown. ns; not significant.

with data shown in Fig. 2D, IL-17 mRNA induction occurred in CD4$^+$ T cells earlier than the induction of IFN-γ mRNA, which was detected on day 14 postvaccination (Fig. 2F). Together, our data show that BCG vaccination induces an early IL-23-dependent Th17-cell response that precedes the Th1-cell response, and is required for the induction of an effective BCG vaccine-induced Th1-cell response.

BCG-induced PGE 2 drives IL-10 and inhibits IFN-γ production in T cells

Th17 cells are induced early in vivo following BCG vaccination and are important for subsequent generation of vaccine-induced Th1 cells at later time points (Figs. 1 and 2). Therefore, we then addressed whether the Th1- and Th17-cell polarizing cytokines namely IL-12 or IL-23 are induced in DCs in response to BCG exposure. We found that following BCG exposure, DCs produced both IL-23 and IL-12 cytokines (Fig. 3A and B). Interestingly, BCG also induced high levels of the anti-inflammatory cytokine, IL-10 in BCG-exposed DCs (Fig. 3C). IL-10 is an anti-inflammatory cytokine that inhibits IL-12 production and Th1-cell differentiation [26]. Accordingly, IL-10 also inhibits IL-12 production in BCG-infected DCs and the generation of IFN-γ-producing cells [27]. Based on these data, we hypothesized that the absence of early Th1-cell responses in vivo following BCG vaccination was due to high BCG-induced IL-10 levels (Fig. 3C) and that IL-17 dependence to induce Th1-cell responses (Fig. 1) was a host strategy to overcome the IL-10-mediated inhibition. To address this hypothesis, we first treated BCG-stimulated DCs with IL-10-neutralizing antibody and measured IL-12 production in supernatants. As expected [27], neutralization of BCG-induced IL-10 resulted in significantly increased production of IL-12 (Fig. 3D). We also determined the effect of IL-10 neutralization on Th1 cell generation by coculturing naïve OT-II TCR Tg T cells with BCG/OVA$_{323–339}$-treated DCs in the presence of IL-10-neutralizing antibody. Consistent with our hypothesis, we report that T-cell-derived IFN-γ production was inhibited in the presence of BCG and neutralization of IL-10 reversed BCG-mediated inhibition of IFN-γ production in T-cell supernatants (Fig. 3E). These data suggest that despite induction of some IL-12 in BCG-exposed DCs, coincident induction of IL-10 inhibits Th1-cell responses. Importantly, Ag85B-specific Th1-cell responses detected in vivo were also increased in BCG-vaccinated *il10*$^{-/-}$ mice when compared with B6 BCG-vaccinated mice (Fig. 3F). Consistent with a recent published study [28], we did not find any differences between lung bacterial burden in B6 and *il-10*$^{-/-}$ unvaccinated mice at early time points following infection with *M. tuberculosis* (Fig. 3G). However, we found that *il10*$^{-/-}$ BCG-vaccinated mice when challenged with aerosolized *M. tuberculosis* mediated significantly better bacterial control in the lungs when compared with challenged B6 BCG-vaccinated mice (Fig. 3G). These data suggest that IL-10 expression reduces the efficacy of BCG vaccine-induced immunity against *M. tuberculosis* challenge.

We then further determined the molecular mechanism by which BCG-induced IL-10 inhibits Th1-cell responses. PGE2 is known to induce IL-10 and inhibit IL-12 production in DCs [16]. However, it is not known if BCG can induce PGE2 production in DCs and whether it impacts the generation of BCG-induced T-cell responses. We report that BCG induced high levels of PGE2 in DC culture supernatants (Fig. 4A). PGE2 synthesis involves the release of endogenous arachidonic acid and conversion to PGE2 via the rate-limiting enzyme cyclooxygenase 2 (COX2). Accordingly, cotreatment of BCG-exposed DCs with a COX2 inhibitor (Celecoxib) abrogated PGE2 production (Fig. 4A). Consistent with a role for PGE2 in IL-10 production, addition of COX2 inhibitor significantly reduced BCG-induced IL-10 levels (Fig. 4B) and increased IL-12 production (Fig. 4C). Furthermore, treatment with COX2 inhibitor was also able to reverse BCG-mediated inhibition of IFN-γ production in T cells cultured with BCG-exposed DCs (Fig. 4D) in DC–T-cell cocultures. These data show that BCG exposure induces PGE2 and downstream induction of IL-10; however, this pathway also limits early IL-12 production and T-cell-

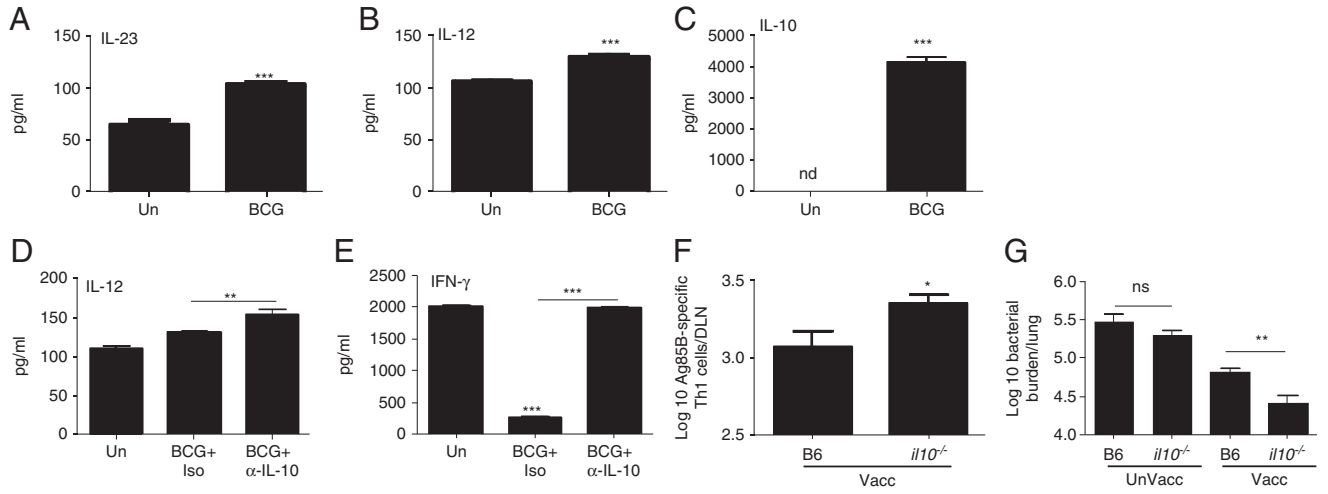

Figure 3. BCG-induced IL-10 inhibits the generation of Th1-cell responses. (A–C) B6 DCs were either left untreated (Un) or stimulated with BCG (MOI-5) for 24 h and supernatants assayed for (A) IL-23, (B) IL-12, and (C) IL-10 production. (D) B6 DCs were left untreated (Un), stimulated with BCG and isotype antibody control (BCG+Iso) or BCG and IL-10-neutralizing antibody (BCG+α-IL-10) and the levels of IL-12 were determined. (E) In some experiments, B6 DCs were left untreated (Un), stimulated with BCG and isotype antibody control (BCG+Iso) or BCG and IL-10-neutralizing antibody (BCG+α-IL-10) and cultured with naïve OT-II TCR-Tg $CD4^+$ T cells and $OVA_{323–339}$. On day 5, IFN-γ protein levels were determined in culture supernatants. (A–E) Data are shown as the mean + SD of triplicate samples. (F) B6 and *il-10*$^{-/-}$ mice were vaccinated with BCG as described in Fig. 1 and Ag85B-specific Th1-cell responses were detected in DLNs on day 14 by ELISpot assay. (G) B6 and *il10*$^{-/-}$ BCG-vaccinated mice were rested for 30 days, following which they were challenged with ~100 CFU *M. tuberculosis* by the aerosol route. Thirty days later, lung CFUs were determined in unvaccinated and vaccinated mice. (F, G) Data are shown as the mean+SD from four to five mice. nd, not detectable; ns, not significant; *$p \leq 0.05$, **$p \leq 0.005$, ***$p \leq 0.0005$, two-tailed Student's *t*-test. One of the two independent experiments is shown.

derived IFN-γ responses. These data together show that the presence of BCG-induced IL-10 is detrimental to the generation of effective Th1-cell responses and vaccine-induced protection against *M. tuberculosis* challenge.

BCG-induced PGE2 drives IL-23 and Ag85B-specific Th17-cell responses

Addition of exogenous PGE2 is a potent inducer of IL-23 in DCs and drives the production of IL-17 in T cells in vitro [18, 19]. Since PGE2 drives IL-10 in BCG-exposed DCs (Fig. 4B), we then examined whether PGE 2 had dual functions following mycobacterial exposure and can also drive IL-23 production in DCs. Accordingly, we treated BCG-exposed DCs with COX2 inhibitor and determined IL-23 levels in culture supernatants. Our data show that BCG-induced PGE 2 is critical for the induction of IL-23 since we detected decreased IL-23 production in response to BCG stimulation in COX2-treated samples (Fig. 4E). To further determine if PGE2-induced IL-23 production is required for the generation of BCG-induced Th17-cell responses, we cocultured naïve $CD4^+$ OT-II TCR Tg T cells with BCG/$OVA_{323–339}$-treated DCs in the presence or absence of COX2 inhibitor. We found BCG/$OVA_{323–339}$-treated DCs primed T cells produced IL-17, whereas the addition of COX2 inhibitor significantly reduced the production of IL-17 in T-cell cultures (Fig. 4F). These data show for the first time that BCG-induced PGE2 production in DCs serves dual functions not only does it mediate IL-10 production and limit IFN-γ production (Fig. 3), but also mediates IL-23 production and IL-17 production in T cells (Fig. 4). In support of this, we found that the treatment of BCG-vaccinated mice with COX2 inhibitor in vivo significantly reduced Ag85B-specific Th17-cell responses (Fig. 4G), but not Ag85B-specific Th1-cell responses (Fig. 4H) or vaccine-induced protection in the lung following *M. tuberculosis* challenge (Fig. 4I). These data suggest that both in vivo and in vitro, blocking PGE2 results in reduced Th17-cell responses. Importantly, despite reduced Th17-cell responses, a competent Th1-cell response is generated, likely due to coincident loss of IL-10 production that can confer vaccine-induced protection. These data suggest a role for IL-17 specifically to overcome IL-10 inhibitory effects.

IL-17 is not required to drive Th1-cell responses in the absence of IL-10

Consistent with a role for IL-17 in the induction of IL-12 in DCs [12, 13], we found that IL-17 treatment of BCG-exposed DCs enhanced IL-12 (Fig. 5A). Importantly, the treatment of IL-17 significantly decreased BCG-induced IL-10 production in DCs (Fig. 5B). These data suggest that BCG exposure results in the induction of PGE2 and high levels of IL-10 that initially inhibits IL-12 production and Th1-cell responses in vivo (Fig. 2). Accordingly, the peak of PGE2 induction in vivo following BCG vaccination was at day 4, with significantly lower levels of PGE2 at later time points (Fig. 5C). However, BCG-induced PGE2 also mediates IL-23 induction and drives subsequent Th17-cell responses. IL-17 then induces IL-12 production and inhibits IL-10 production and mediates IFN-γ responses at later time points. Therefore, IFN-γ protein expression was not detected early,

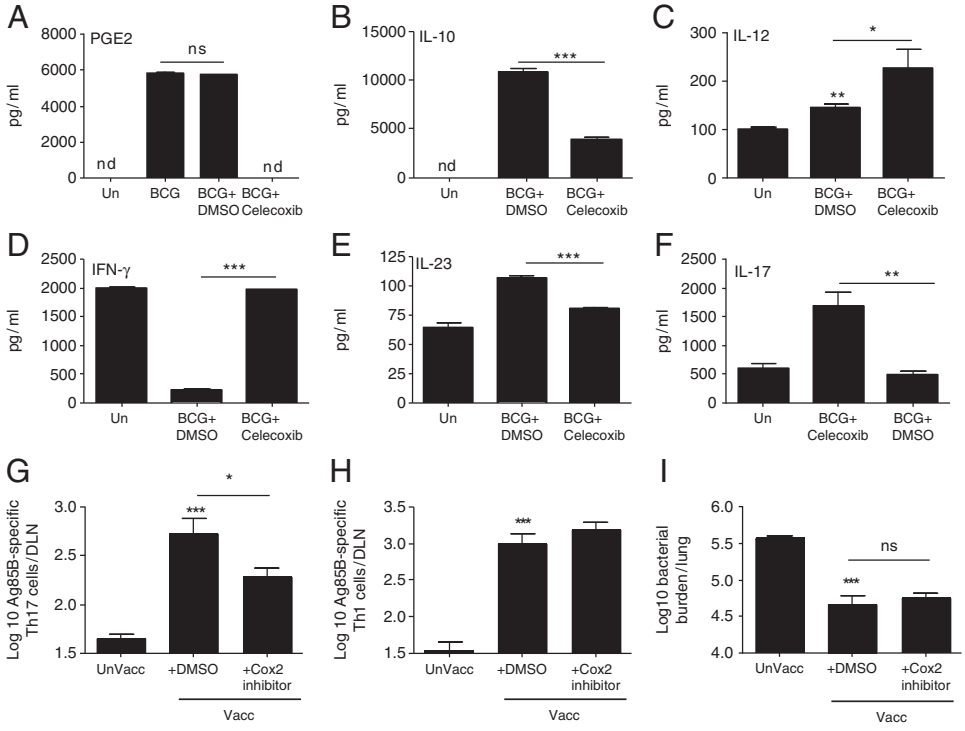

Figure 4. BCG-induced PGE2 drives IL-23 and Ag85B-specific Th17-cell responses. (A–C) DCs were untreated (Un), stimulated with BCG (MOI = 5), or BCG and COX2 inhibitor (BCG+Celecoxib; 10 μM). Since Celecoxib was dissolved in DMSO, a similar volume of DMSO was added into BCG-treated wells (BCG+DMSO). Supernatants were assayed for the levels of (A) PGE2, (B) IL-10, and (C) IL-12. (D) B6 DCs were stimulated with BCG+DMSO or BCG and COX2 inhibitor (BCG+Celecoxib) and cultured with naïve OT-II TCR-Tg $CD4^+$ T cells and $OVA_{323–339}$. Protein levels of IFN-γ were determined in supernatants on day 5. Untreated samples shown in Figs 3E and 4D are the same. (E) B6 DCs were either left untreated (Un), stimulated with BCG (MOI-5) or BCG and COX2 inhibitor (BCG+Celecoxib; 10 μM) and the levels of IL-23 were determined. (F) B6 DCs were stimulated with BCG+DMSO or BCG and COX2 inhibitor (BCG+Celecoxib) and cultured with naïve OT-II TCR-Tg $CD4^+$ T cells and $OVA_{323–339}$ and protein levels of IL-17 determined in day 5 supernatants. (A–F) Data are shown as the mean+SD of triplicate samples. (G, H) B6 mice were BCG vaccinated or BCG vaccinated and treated with COX2 inhibitor (NS-398) and the Ag85B-specific (G) Th17-cell responses or (H) Th1-cell responses were determined in DLNs on day 14 by ELISpot assay. (I) B6 BCG-vaccinated mice were treated with DMSO or with COX2 inhibitor between days 1 and 15 postvaccination following which they were rested for 30 days and challenged with ~100 CFU *M. tuberculosis* by the aerosol route. Thirty days later, lung CFUs were determined in unvaccinated and vaccinated mice. (G–I) Data are shown as the mean+SD from four to five mice. nd, not detectable; ns, not significant; $^*p \leq 0.05$, $^{**}p \leq 0.005$, $^{***}p \leq 0.0005$, two-tailed Student's *t*-test. One of the two independent experiments is shown.

but at later time points following BCG vaccination in vivo (Fig. 5D). In order to confirm that PGE-dependent IL-17 mediates Th1-cell responses to overcome BCG-mediated IL-10 inhibition, we depleted IL-17 in $il10^{-/-}$ BCG-vaccinated mice and measured Ag85B-specific Th1-cell responses in DLNs. Our data show that the depletion of IL-17 in B6 mice resulted in decreased Ag85B-specific Th1-cell response (Fig. 5E). However, depletion of IL-17 in $il10^{-/-}$ mice did not result in decreased Ag85B-specific Th1-cell responses when compared with $il\text{-}10^{-/-}$ BCG-vaccinated mice treated with isotype control antibody (Fig. 5E). These data suggest that IL-17 responses are required to drive Th1-cell responses, specifically to overcome IL-10-dependent Th1-cell inhibitory effects.

Discussion

PGE2 is critical for the induction of IL-23 responses and Th17-cell responses [18, 19], while inhibiting IL-12 responses through the production of IL-10 [16]. However, since PGE2-matured DCs can effectively induce IFN-γ-producing T cells [29, 30], we show that the immune system has developed means of counteracting the PGE2-mediated suppression of Th1-cell immunity. In this article, we show that the role for mycobacteria-induced PGE 2 is bifunctional since it not only induces IL-10 and limits early Th1-cell response, but also simultaneously induces IL-23 and subsequent IL-17 responses. PGE2-dependent IL-17 production is then required to overcome PGE2- and IL-10-mediated inhibition of IL-12 and for the generation of Th1-cell responses. Accordingly, IL-23 is important for inducing vaccine-induced Th17 and Th1-cell immunity following vaccination with an attenuated intracellular live bacteria, BCG, and vaccine-induced protection following *M. tuberculosis* challenge.

Following BCG vaccination, both Th1- and Th17-cell responses are detected in the DLNs on day 14 postvaccination. However, by tracking kinetics of Th1- and Th17-cell responses, we show that the Th17 responses occur early, coincide with high induction of PGE2 production in vivo, and precede the induction of Th1-cell responses. The induction of Th1-cell responses is IL-17 dependent since the $il17ra^{-/-}$ mice and depletion of IL-17 results in reduced Th1-cell

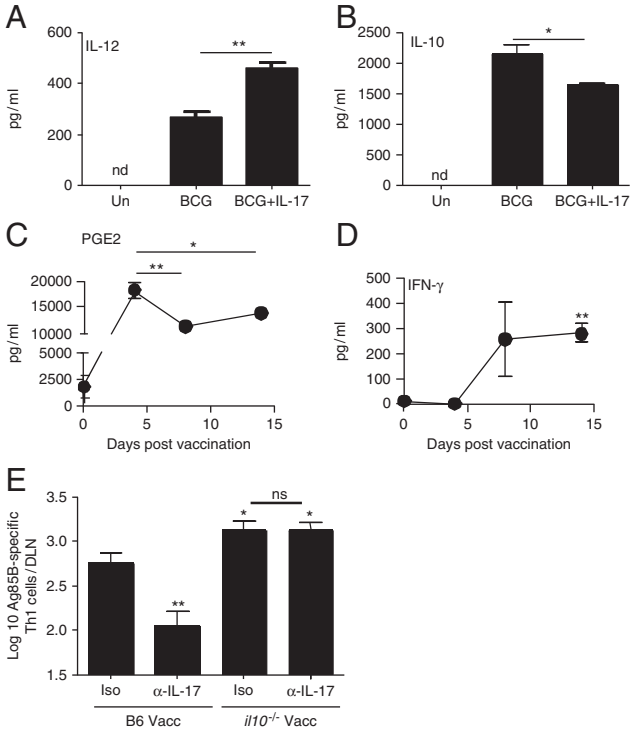

Figure 5. IL-17 is not required to drive Th1-cell responses in the absence of IL-10. (A, B) DCs from B6 mice were left untreated (Un), stimulated with BCG (BCG), or BCG and IL-17 (100 ng/mL; BCG+IL-17) and supernatants assayed for the levels of (A) IL-12 and (B) IL-10. Data are shown as mean+SD of triplicate samples. B6 mice were BCG vaccinated and (C) the serum was analyzed for PGE2 protein levels postvaccination, whereas (D) DLNs were cultured in media for 24 h and supernatants were assayed for IFN-γ protein. (E) $il10^{-/-}$ BCG-vaccinated mice were treated with isotype control antibody or IL-17-neutralizing antibody and the Ag85B-specific Th1-cell responses were determined in DLNs on day 14. B6 BCG-vaccinated mice treated with IL-17-neutralizing antibody or control isotype antibody were also included. (C–E) The data are shown as the mean+SD of values for four to five mice. nd, not detectable. *$p \leq 0.05$, **$p \leq 0.005$, ***$p \leq 0.0005$, ns – not significant, two-tailed Student's *t*-test. One of the two independent experiments is shown.

responses. Until recently, nonimmune cells such as fibroblasts and epithelial cells were considered primary responders to IL-17 (reviewed in [31]). However, recently, myeloid cells such as macrophages [12, 32, 33] and DCs [12] have been shown to express IL-17 receptors, respond to IL-17 [12, 32] and mediate host immune responses. IL-17 can act on macrophages for direct bacterial killing [12, 34], whereas IL-17-dependent responses in DCs results in the induction of IL-12 [12, 13] and Th1-cell differentiation [12]. Collectively, these studies suggest that the IL-17 pathway when required provides critical "help" in the generation of Th1-cell responses. This is evident from the reduced IL-12p40 and IL-12p35 mRNA levels and the decreased IFN-γ responses in vivo in DLNs of BCG-vaccinated $il17ra^{-/-}$ mice when compared with B6 BCG-vaccinated mice. We also show that dependence on IL-17 to drive Th1-cell responses is a host strategy to overcome Th1-cell inhibitory effects of IL-10, which is also induced by BCG. Accordingly, neutralization of IL-10 results in IL-12 production in DCs and increased IFN-γ responses in T cells. However, it cannot be eliminated that factors other than IL-12 are also modulated by inhibition of IL-10 and mediate the increased Th1-cell responses. Importantly, in contrast to B6 mice, $il10^{-/-}$ BCG-vaccinated mice were able to induce effective Th1-cell responses in the absence of IL-17, suggesting that IL-17 is required to drive Th1-cell responses in order to overcome Th1-cell inhibitory effects of IL-10.

IL-23 is critical for in vivo generation of Th17 cells following mycobacterial exposure [23–25] and not surprisingly, $il23p19^{-/-}$ BCG-vaccinated mice had reduced Th17- and Th1-cell responses, which correlated with lower protection upon challenge with *M. tuberculosis*. However, since vaccine-induced protection is reduced and not completely lost in the absence of IL-23, it is likely that factors other than IL-23 can also mediate vaccine-induced protection. These studies imply that IL-23-dependent IL-17 is a critical factor in deciding efficacy outcomes of BCG vaccine-induced immunity against TB. Th17 cells have also been implicated as key players in models of autoimmune diseases [10]. Accordingly, a rare IL-23R polymorphism in humans protects against the development of Crohn's disease [35], likely due to reduced Th17-cell responses. In contrast, our data predict that humans with IL-23R variants, although protected against autoimmune diseases, may not generate effective BCG vaccine-induced Th1-cell immunity, potentially resulting in poor protection outcomes upon *M. tuberculosis* challenge. Furthermore, since recombinant BCG strains are a likely choice for priming or boosters in future TB vaccine strategies against TB [36], the findings presented here suggest that including IL-23-promoting factors into recombinant BCG vaccines may be one approach to promote Th17-cell responses and improve upon current levels of Th1-cell-induced protection against TB. In contrast, identifying and eliminating IL-10-inducing factors in BCG may directly increase Th1-cell responses and generate better efficacy against *M. tuberculosis* challenge as seen in the $il10^{-/-}$ BCG-vaccinated mice. Our data also suggest that eliminating PGE2-inducing factors in BCG may eliminate IL-10 production and directly induce Th1-cell responses without dependence on IL-17. Therefore, our study defines several molecular mechanisms that can be exploited to improve upon current vaccine strategies against TB.

In summary, we propose that some intracellular bacteria such as BCG avoid direct induction of Th1-cell responses by producing PGE2 and IL-10. The fact that BCG-induced IL-10 inhibits IL-12 production and limits IFN-γ production has been demonstrated previously [27]. However, our study extends these findings and shows that $il\text{-}10^{-/-}$ BCG-vaccinated mice have better vaccine-induced protection outcomes. Moreover inhibitory effects of IL-10 are not limited to attenuated strains of mycobacteria, since even in models of virulent *M. tuberculosis* infection, $il10^{-/-}$ mice exhibit enhanced IFN-γ production and reduced lung bacterial loads during chronic stages of infection [28]. Furthermore, novel data presented here show that pathogen-induced PGE2 has dual functions to play in host immunity, apart from its role in driving IL-10 production, PGE2 is also required to drive IL-23 responses in DCs and subsequent IL-17 production in T cells. IL-17 then overcomes IL-10-mediated inhibition of Th1-cell induction by downregulating IL-10 and upregulating IL-12 production in DCs, thereby allowing for the generation of an effective IFN-γ response. The broader understanding of the specific host factors

required to induce an optimal Th1-cell immune response against intracellular bacteria will allow us to exploit this knowledge in design of better vaccine strategies against infections.

Materials and methods

Animals

C57BL/6 (B6), OT-II αβ TCR Transgenic (Tg) mice (OT-II) which are MHC class II I-A^b restricted and specific for $OVA_{323–339}$ and *il-10*$^{-/-}$ mice were purchased from The Jackson Laboratory (Bar Harbor, ME). *il23p19*$^{-/-}$ [37], *il17ra*$^{-/-}$ [11] on the B6 background were maintained at the University of Pittsburgh animal facility. All experimental mice were age and sex matched and were used between the ages of 6 and 8 weeks according to University of Pittsburgh IACUC guidelines.

Vaccinations

BCG Pasteur was grown in Proskauer Beck (PB) medium containing 0.05% Tween-80 to mid-log phase and then frozen in 1-mL aliquots at −80°C. Bacterial stocks were plated on 7H11 agar plates to calculate colony forming units (CFUs). Mice were vaccinated subcutaneously with 1×10^6 CFU of BCG in PBS. BCG-vaccinated mice received COX2 inhibitor (NS-398; Sigma 10 mg/kg of body weight), isotype control antibody (Clone 54447, R&D Biosystems) and IL-17-neutralizing antibody (Clone 50104, R&D Biosystems) every 48 h following vaccination.

Experimental infection

The H37Rv strain of *M. tuberculosis* was grown as described previously [23]. For aerosol infections, mice were infected with 100 CFU of bacteria using a Glas-Col airborne infection system as described earlier [23]. Lung bacterial burden was estimated by plating the lung homogenates on 7H11 agar plates.

DLN cell preparation

DLNs were collected in ice-cold DMEM and dispersed through a 70-μM pore size nylon tissue strainer (Falcon; BD Biosciences). Cells suspensions were treated with Gey's solution, washed, and counted (Beckman Coulter). Single cells were used for ELISpot, flow cytometric analyses or for sorting purified populations.

Detection of cytokine-producing cells by ELISpot assay

Detection of Ag-specific IFN-γ- and IL-17-producing cells was carried out using an ELISpot assay as described earlier [25]. Cells from unvaccinated and vaccinated mice were seeded at an initial concentration of $2–5 \times 10^6$ cells/well and doubling dilutions made. Irradiated B6 splenocytes were used as APCs, whereas $Ag85B_{240–254}$ was used as Ag in assays from BCG-vaccinated mice to detect responding $CD4^+$ cells [20]; mouse rIL-2 (Sigma-Aldrich; 10 U/mL) was added to all wells. Spots were enumerated by using CTL-Immuno Spot analyzer and the frequency of responding cells was determined and applied to the number of cells per sample to generate the total number of responding cells per organ. Wells without Ag were included as controls and did not yield cytokine-producing spots.

Generation and stimulation of BMDCs

BMDCs (DCs) were generated by culturing BM cells in cDMEM-containing GM-CSF (PeproTech) [23]. On day 7, nonadherent cells were collected and stimulated with BCG at a multiplicity of infection (MOI) of 5. Culture supernatants were analyzed by Luminex assays.

Naïve $CD4^+$ T-cell isolation and in vitro effector generation

Naïve $CD4^+$ T cells were isolated from OT-II TCRαβ Tg mice using magnetic $CD4^+$ beads (L3T4) (Miltenyi Biotec). Naïve OT-II $CD4^+$ T cells (1×10^6 cells/mL) were cultured with BCG-stimulated DCs (MOI = 5) or unstimulated DCs (1×10^6 cells/mL) and $OVA_{323–339}$ peptide (5 μM) for 5 days. In some wells, DCs were treated with COX2 inhibitor (Celecoxib, 10 μM), anti-IL-10 (10 μg/mL; Clone JES 052A5, R&D Biosystems) [38]; isotype control (10 μg/mL; Clone 43414, R&D Biosystems), or IL-17A (100 ng/mL, R&D Biosystems) was added. Protein levels in the supernatants were assayed by ELISA.

Determination of protein amounts

IL-17, IFN-γ, and IL-10 protein amounts were measured in cell-culture supernatants using a mouse Luminex assay (Linco/Millipore). ELISA antibody pairs were used to detect IL-12p70 levels (R&D Biosystems) and IL-23 levels (R&D Biosystems) in supernatants. PGE2 quantitative ELISA was performed in DC culture supernatants according to the manufacturer's protocol (R&D Biosystems) and serum PGE2 levels determined using DetectX PGE2 kit validated for mouse serum (Arbor Assays).

Real-time PCR

RNA was extracted as described previously, reverse transcribed, and amplified using FAM-labeled probe and primers on the ABI Prism 7900 detection system [23]. Fold increase in signal over that derived from control samples was determined using the ΔΔct

calculation. In some cases, the levels of mRNA relative to housekeeping gene (GAPDH) were calculated. The primer and probes sequences have been published previously [23] or were commercially purchased (ABI Biosystems).

Flow cytometry and cell sorting

Single-cell suspensions were stained with fluorochrome-labeled antibodies specific for CD3 (17A2), CD4 (RM4-5), and CD8 (53–6.7). Intracellular staining was performed by using anti-IFN-γ (XMG1.2) on cells stimulated with PMA and ionomycin as per the method described [12]. To sort for a purified DLN cell population, stained cells were sorted on BD FACS Aria flow cytometer as $CD3^+$ $CD4^+$ (purity, $>94\%$). For analysis, FlowJo (Tree Star, CA) was used.

Statistical analysis

Differences between the means of groups were analyzed using the two-tailed Student's *t*-test in GraphPad Prism 5 (La Jolla, CA). Inherently, logarithmic data from bacterial growth and RT-PCR were transformed for statistical analyses.

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Abbreviations: **COX:** cyclooxygenase · **DLN:** draining LN · **PGE2:** prostaglandin-E2 · **RT-PCR:** real-time PCR · **TB:** tuberculosis

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