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*J Immunol* published online 12 September 2012
http://www.jimmunol.org/content/early/2012/09/12/jimmunol.1201061
Blockade of IL-10 Signaling during Bacillus Calmette-Guérin Vaccination Enhances and Sustains Th1, Th17, and Innate Lymphoid IFN-γ and IL-17 Responses and Increases Protection to Mycobacterium tuberculosis Infection

Jonathan M. Pitt,* Evangelos Stavropoulos,* Paul S. Redford,* Amy M. Beebe,† Gregory J. Bancroft,‡ Douglas B. Young,§ and Anne O’Garra*  

Vaccination with Mycobacterium bovis bacillus Calmette-Guérin (BCG) remains the only prophylactic vaccine against tuberculosis, caused by Mycobacterium tuberculosis, but gives variable protection against pulmonary disease. The generation of host Th1 responses following BCG vaccination is accepted as the major mechanism of protection against M. tuberculosis infection. Early production of IL-17 in the lungs following M. tuberculosis challenge of mice previously vaccinated with M. tuberculosis peptides in adjuvant has been shown to be required for efficient Th1 cell recruitment. IL-10 regulates various processes involved in generation of Th1 and Th17 responses. Previous studies have shown IL-10 as a negative regulator of the immune response to pulmonary M. tuberculosis infection, with 10^10^ mice having reduced lung bacterial loads. In this study we show that inhibition of IL-10 signaling during BCG vaccination enhances host-generated Ag-specific IFN-γ and IL-17A responses, and that this regimen gives significantly greater protection against aerogenic M. tuberculosis challenge in both susceptible and relatively resistant strains of mice. In M. tuberculosis-susceptible CBA/J mice, Ab blockade of IL-10R specifically during BCG vaccination resulted in additional protection against M. tuberculosis challenge of >1-log_10^ compared with equivalent isotype-treated controls. The protection observed following BCG vaccination concurrent with anti–IL-10R mAb treatment was sustained through chronic M. tuberculosis infection and correlated with enhanced lung Th1 and Th17 responses and increased IFN-γ and IL-17A production by γδ T cells and an innate-like Thy1.2^CD^3^ lymphoid population. We show that IL-10 inhibits optimal BCG-elicted protection, therefore suggesting that antagonists of IL-10 may be of great benefit as adjuvants in preventive vaccination against tuberculosis. The Journal of Immunology, 2012, 189: 000–000.
role that IL-17 plays in vaccination against _M. tuberculosis_ infection (26, 27).

IL-10 regulates the immune response induced by various pathogens and their products, thereby preventing damage to host tissues (28). However, with some infections IL-10 impedes the ability of the host immune response to eliminate the pathogen, contributing to chronic infection (29–32). We and others have shown IL-10 to be a negative regulator of the immune response to primary _M. tuberculosis_ infection in vivo without overt evidence of immunopathology in lethally infected mice (33–37). In our previously published study, in the absence of IL-10 signaling, mice showed greater control of bacterial load in lungs and spleens as compared with wild-type (WT) control animals, corresponding with earlier and enhanced cytokine levels in these organs and in the serum (36).

The potential for IL-10 to similarly regulate the response to immunization has been demonstrated by the observation that neutralization of IL-10 during priming with soluble OVA protein in the presence of LPS leads to an enhanced Th1 response upon tralization of IL-10 during priming with soluble OVA protein in greater control of bacterial load in lungs and spleens as compared with BCG vaccination alone. We also showed that C57BL/6 mice display decreased lung and spleen bacterial burdens compared with BCG vaccination alone (38–42). In established lymphocytic choriomeningitis virus infection, blockade of IL-10R signaling during an otherwise ineffective therapeutic DNA vaccination resulted in enhanced clearance of infection by increasing numbers of multifunctional virus-specific T cells (43). In mycobacterial infection, anti–IL-10R mAb administered before vaccination with _M. tuberculosis_ culture filtrate protein enhanced the immunomodulatory functions of culture filtrate protein without the requirement for additional adjuvant, and gave the vaccine the ability to protect against i.v. challenge with _Mycobacterium avium_ (44). Another study has shown that systemic BCG infection of C57BL/6 Il10−/− mice resulted in lower bacterial burdens than those in WT controls (45). It has been suggested that blockade of IL-10 signaling might enhance BCG-elicted protection, because at 30 d after _M. tuberculosis_ challenge, BCG-vaccinated C57BL/6 Il10−/− mice have decreased bacterial burdens compared with WT BCG-vaccinated animals (24). However, given that C57BL/6 Il10−/− mice display decreased lung and spleen bacterial burdens beyond 28 d after aerogenic _M. tuberculosis_ infection in the absence of vaccination (36), it is unclear using C57BL/6 Il10−/− mice whether IL-10 has regulatory effects both during _M. tuberculosis_ challenge and vaccination, or whether IL-10 has a regulatory role specifically at the level of initial vaccination as has been shown in other models of infectious disease (38–42).

In the present study we have found that inhibition of IL-10 signaling during BCG vaccination enhances Th1 and Th17 responses, as well as IFN-γ and IL-17A production by CD8+ T cells, γδ T cells, and an innate-like Th1.2·CD3+ population in vivo. This resulted in significantly increased protection against aerogenic challenge with _M. tuberculosis_, compared with BCG vaccination alone. Furthermore, this protective response is sustained during the course of chronic _M. tuberculosis_ infection, in both _M. tuberculosis_-susceptible CBA/J mice as well as in the relatively resistant C57BL/6 mouse strain, as compared with BCG vaccination alone. We also highlight the key lymphoid sources of the cytokines IFN-γ and IL-17A that correlate with the strongest level of protection against _M. tuberculosis_ challenge in BCG-vaccinated/anti–IL-10R-treated mice.

**Materials and Methods**

**Animals**

Female C57BL/6 and C57BL/6 Il10−/− mice (8–12 wk of age) were bred and housed in the specific pathogen-free facilities at the Medical Research Council National Institute for Medical Research (London, U.K.). Female CBA/J mice (8–10 wk of age) were obtained from The Jackson Laboratory (Bar Harbor, ME). Experiments were reviewed by and in accordance with the Home Office regulations (United Kingdom).

**BCG vaccination and _M. tuberculosis_ infection**

Experiments were performed under containment level 3 conditions. BCG vaccine Danish 1331 (Statens Serum Institut, Copenhagen, Denmark) and _M. tuberculosis_ H37Rv were grown in Middlebrook 7H9 broth supplemented with 10% oleic acid albumin dextrose complex (Difco), 0.05% Tween 80, and 0.5% glycerol to mid-log phase before freezing at −80°C. For vaccination, mice received 5 × 105 CFU of _M. tuberculosis_ infections, 2 × 107 CFU _M. tuberculosis_ H37Rv in PBS was aerosolized using a three-jet Collision nebulizer unit (BGI, Waltham, MA) during a period of 15 min with ∼30 CFU delivered to the lungs as confirmed by enumeration of bacteria on day 1 postinfection.

**Anti–IL-10R mAb treatment**

One day prior to BCG vaccination, mice were injected i.p. with 1 mg either anti–IL-10R mAb (1B1.3A; a gift from DNAX, now Merck, Palo Alto, CA) that specifically binds the ligand-binding domain of IL-10R (46), or with IgG1 isotype control mAb (GL133; Merck) (46). Following vaccination, mice received 0.35 mg respective mAb i.p. weekly for 6 wk.

**Processing of organs for cell culture and determination of bacterial burden**

Lungs and spleens were aseptically removed from mice after sacrifice, placed into RPMI 1640 medium (BioWhittaker), and homogenized through a 70-μm nylon sieve. To determine bacterial burden (CFU), cell suspensions were serially diluted onto 7H11 agar plates supplemented with oleic acid albumin dextrose complex. After 18 d at 37°C, visible colonies were counted and the bacterial load was calculated per organ. For cell culture, erythrocytes were lysed using 0.83% ammonium chloride in water, before lung cells (1 × 108/ml) and splenocytes (5 × 107/ml) were restimulated with 20 μg/ml purified protein derivative (PPD) (Serum Statens Institut) and 2 μg/ml anti-CD28 (37.51; National Institute for Medical Research) for 16 h in 24-well tissue culture plates (final well volume of 1 ml). In the initial experiments using C57BL6 and C57BL6 Il10−/− mice, 10 μg/ml PPD alone was used for cell restimulations for 48 h. Supernatants were collected at these time points and assessed by ELISA for IFN-γ or IL-6 concentrations using matched Ab pairs (National Institute for Medical Research) (limits of detection 70 and 50 pg/ml, respectively), and TNF-α or IL-17A using ELISA kits (Ebioscience; limit of detection 30 pg/ml for each). Following the manufacturer’s instructions, Brefeldin A (10 μg/ml; Sigma-Aldrich) was added to the cultures for the last 4 h before the cells were stained for extracellular markers and intracellular cytokines for analysis by flow cytometry.

**Flow cytometry**

Harvested cells were washed in Dulbecco’s PBS (Life Technologies), treated with 5 μg/ml anti-FcγRIIa/RIIIa (anti-CD16/CD32; 2.4G2; National Institute for Medical Research), stained to exclude dead cells using 7-aminoactinomycin D (Invitrogen) before analysis by flow cytometry. Stained cells were analyzed using FlowJo version 8.8.6 software (Tree Star) and Flow cytometric data was presented as a frequency dot plot, where 20,000 events were acquired per sample. Statistical analysis was performed using Graphpad Prism software. Differences were considered significant when p < 0.05.
Results
BCG vaccination in the absence of IL-10 enhances IFN-γ and IL-17 responses and increases protection against M. tuberculosis challenge

Infection or vaccination with BCG induces IL-10 production in both humans (47, 48) and mice (45, 49), and we have observed that T cells are a major source of IL-10 following BCG vaccination (data not shown). Previous studies by others have shown that IL-10 reduces the cytokine response to systemic BCG infection in vivo, with Il10−/− mice displaying significantly lower BCG bacterial burden and no signs of host-mediated immunopathology (45, 50). In accord with this, recent studies in our laboratory have shown an enhanced and earlier cytokine response in the lung, spleen, and serum of Il10−/− mice following aerogenic M. tuberculosis infection compared with WT control mice (36). In light of this work and the work of others (24, 45), we questioned whether BCG vaccination of Il10−/− mice could broadly enhance cytokine responses, leading to greater protection against aerogenic M. tuberculosis challenge.

C57BL/6 or C57BL/6 Il10−/− mice were vaccinated with BCG and, after 16 wk, mice were killed and lung and spleen cell suspensions were restimulated ex vivo with M. tuberculosis PPD. Levels of IFN-γ, TNF-α, and IL-17A from splenocytes were significantly increased in BCG-vaccinated C57BL/6 Il10−/− mice when compared with vaccinated WT animals (Fig. 1). The same significant increase in the levels of these cytokines from PPD-restimulated splenocytes was also observed 6 wk following BCG vaccination of C57BL/6 mice in the presence of anti-IL-10R mAbs, as compared with BCG vaccination in the presence of isotype control mAbs (Supplemental Fig. 1). These cytokines could not be detected from PPD restimulations of lung cells (data not shown), likely due to the lack of recruitment of T cells to the lung following i.d. BCG vaccination.

The increased levels of PPD-induced IFN-γ and TNF-α from BCG-vaccinated Il10−/− mice, both well-characterized protective factors in the immune response to M. tuberculosis (6, 9, 51), led us to investigate whether these mice would show any increased protection to subsequent M. tuberculosis challenge. Six weeks following BCG (or PBS control) vaccination, C57BL/6 or C57BL/6 Il10−/− mice were challenged with low-dose M. tuberculosis by aerosol, and at 28 and 56 d after challenge mice were killed and the bacterial loads in the lungs and spleen were determined (Fig. 2). At day 28 after M. tuberculosis challenge, BCG-vaccinated Il10−/− mice showed lower bacterial burdens in the lung compared with BCG-vaccinated WT animals (Fig. 2), in line with a previous report (24). There was no difference in M. tuberculosis bacterial load between PBS-administered WT and Il10−/− groups at this time point. No differences in CFU were observed at day 28 between any of the groups in the spleen, possibly due to low and variable levels of dissemination at this time point (Fig. 2). At day 56 after challenge, BCG-vaccinated Il10−/− mice continued to show enhanced bacterial control in the lungs as compared with BCG-vaccinated WT counterparts. As previously reported, the bacterial load was lower in the Il10−/− mice at this time point after M. tuberculosis challenge independent of BCG vaccination (36). Furthermore, the M. tuberculosis bacterial load in the spleen was also significantly lower in BCG-vaccinated Il10−/− mice at day 56 (observed in two separate identical experiments; Supplemental Fig. 2), and it showed only a minimal increase from day 28, suggesting greater control of M. tuberculosis dissemination in these animals (Fig. 2, Supplemental Fig. 2).

To determine the immune responses underlying the enhanced protection observed, we carried out ex vivo PPD restimulations on lung and spleen cell suspensions from these mice (Fig. 3). Concentrations of IFN-γ, TNF-α, IL-6, and IL-17A from lung restimulations were significantly enhanced at day 28 after M. tuberculosis challenge in WT mice that had been BCG vaccinated compared with PBS-treated WT animals. No significant differences were observed between BCG-vaccinated WT and BCG-vaccinated Il10−/− mice at this time point. By day 56 following M. tuberculosis challenge, the concentrations of these cytokines, however, showed no significant differences between BCG-vaccinated WT and PBS-treated WT mice. Production of TNF-α, IL-6, and IL-17A remained more strongly sustained in the lungs of BCG-vaccinated Il10−/− mice compared with BCG-vaccinated WT animals at day 56 after challenge. Cytokine responses in the spleen were generally greatest in Il10−/− mice after M. tuberculosis challenge, regardless of BCG vaccination, and they were elevated in comparison with WT animals at both time points investigated.

Taken together, these data confirm that BCG vaccination in the absence of IL-10 increases protection in the lung (24), but the data extend upon these findings by showing that decreased M. tuber-

FIGURE 1. C57BL/6 Il10−/− mice have enhanced IFN-γ and IL-17 responses in the spleen after i.d. BCG vaccination. C57BL/6 or C57BL/6 Il10−/− were vaccinated with either 5 × 10^5 CFU BCG i.d. or PBS i.d. as control. After 16 wk, splenocytes were isolated and restimulated with PPD as described in Materials and Methods. The levels of production of IFN-γ, TNF-α, and IL-17A were measured from the supernatants of these restimulations by ELISA. ND, below the level of detection. Data shown are means ± SEM from two combined identical experiments, totaling six mice per group. *p < 0.05, **p < 0.001 by one-way ANOVA.

FIGURE 2. BCG-vaccinated C57BL/6 Il10−/− mice show greater protection to M. tuberculosis challenge. C57BL/6 or C57BL/6 Il10−/− mice were vaccinated with either 5 × 10^5 CFU BCG i.d. or PBS i.d. as control. Mice were challenged by M. tuberculosis aerosol 6 wk after vaccination, and bacterial load per lungs and spleen was determined at 28 and 56 d after challenge. Data shown are means ± SEM from two combined identical independent experiments (n = 5–8/group/experiment) totaling 12–16 per group. ***p < 0.001 by one-way ANOVA; *p < 0.05, **p < 0.01, ***p < 0.001 by unpaired Student t test. Mtb, M. tuberculosis.
culosis bacterial burdens in the lungs, as well as in the spleens, are also observed at later time points after M. tuberculosis challenge of BCG-vaccinated Il102/2 mice. Furthermore, the enhanced protection seen in these mice is associated with increased PPD-specific cytokine responses in both the lungs and spleen after M. tuberculosis challenge. However, from these experiments it is unclear whether these effects are at the level of infection, vaccination, or both.

IL-10R blockade during BCG vaccination alone enhances protection against M. tuberculosis in resistant C57BL/6 mice

As Il102/2 mice have previously been shown to have an earlier and enhanced immune response to aerogenic M. tuberculosis infection, resulting in significantly reduced bacterial burdens (33, 36, 37), we determined whether abrogation of IL-10 signaling during BCG vaccination only would lead to enhanced protection against M. tuberculosis challenge. This would show whether IL-10 has a regulatory role specifically at the level of initial BCG vaccination, as has been shown in other immunization models (38–42). To do this we administered anti–IL-10R mAb, or isotype control mAb, to C57BL/6 mice during BCG vaccination only for 6 wk, stopping mAb administration for 2 wk before mice were challenged with M. tuberculosis (Fig. 4). M. tuberculosis bacterial load was measured at 28 and 63 d after M. tuberculosis challenge in lungs and spleens. At day 28 after M. tuberculosis challenge, although BCG-vaccinated/isotype-treated mice had significantly greater protection following M. tuberculosis challenge compared with unvaccinated/isotype-treated animals, there was no additional protection observed in BCG-vaccinated/anti–IL-10R-treated mice. However, by day 63 after M. tuberculosis challenge, BCG-vaccinated/anti–IL-10R-treated mice showed a decrease in lung bacterial load over BCG-vaccinated/isotype-treated animals (Fig. 4). This difference was also observed in the spleen at day 63 (although this did not reach statistical significance; p = 0.173). These data suggest that blockade of IL-10R signaling at the time of administering BCG enhances the vaccine-driven protective host response against M. tuberculosis infection.

Anti–IL-10R mAb treatment during BCG vaccination of M. tuberculosis-susceptible CBA/J mice significantly enhances protection against aerogenic M. tuberculosis challenge

Strains of mice that are more susceptible to M. tuberculosis have been argued to better reflect the pathology of human TB than do the more commonly used C57BL/6 and BALB/c M. tuberculosis-resistant strains (52, 53). The CBA/J mouse strain, alongside DBA/2 and C3H strains, is more susceptible to M. tuberculosis infection, with escalating bacterial burden in the lungs until death at around 150 d after aerosol infection (34, 35, 52, 54, 55). To determine whether blockade of IL-10 signaling during BCG vaccination could also induce a greater cytokine response in this susceptible strain, CBA/J mice were vaccinated as before with concomitant administration of anti–IL-10R mAb or isotype control mAb. Seven weeks following the final mAb administration (13 wk after BCG vaccination) we determined the PPD-specific cytokine response (Fig. 5). BCG vaccination with concomitant blockade of IL-10 signaling induced significantly greater levels of
M. tuberculosis isotype control mAb i.p. weekly following vaccination for 6 wk. Two PBS i.d. as control. Mice then received 0.35 mg anti–IL-10R mAb or

Materials and Methods as described in section), splenocytes were isolated and restimulated with PPD and anti-CD28 3 weeks following the last mAb administration (13 wk after BCG vaccination alone appeared to wane at 112 d after challenge (Fig. 6). Whereas the effect of BCG vaccination in CBA/J mice, we examined how the immune response was altered during the M. tuberculosis challenge of these animals. We were unable to find any significant differences in total numbers of CD4+ T cells, CD8+ T cells, or γδ T cells in the lungs between any of the groups at 28, 63, and 112 d after challenge (data not shown). This suggests the increased protection observed by blockade of IL-10R signaling during BCG vaccination in CBA/J mice. We were unable to find any significant differences in total numbers of CD4+ T cells, CD8+ T cells, or γδ T cells in the lungs between any of the groups at 28, 63, and 112 d after challenge (Fig. 6).

To explore potential mechanisms to account for the considerable protection observed by blockade of IL-10R signaling during BCG vaccination in CBA/J mice, we examined how the immune response was altered during the M. tuberculosis challenge of these animals. We were unable to find any significant differences in total numbers of CD4+ T cells, CD8+ T cells, or γδ T cells in the lungs between any of the groups at 28, 63, and 112 d after challenge (data not shown). This suggests the increased protection observed by blockade of IL-10R signaling during BCG vaccination in CBA/J mice.

FIGURE 4. Anti–IL-10R mAb treatment during BCG vaccination increases protection against M. tuberculosis in resistant C57BL/6 mice. C57BL/6 mice were treated with 1 mg anti–IL-10R mAb or isotype control mAb i.p. and the next day vaccinated with either 5 × 10^7 CFU BCG i.d. or PBS i.d. as control. Mice then received 0.35 mg anti–IL-10R mAb or isotype control mAb i.p. weekly following vaccination for 6 wk. Two weeks after the final weekly mAb administration, mice were challenged by M. tuberculosis aerosol, and bacterial load per lungs and per spleen was determined at 28 and 63 d after challenge. Data shown depict the means ± SEM from one experiment (n = 5/group). ***p < 0.001 by one-way ANOVA; **p < 0.01 by unpaired Student t test. Mtb, M. tuberculosis.

IFN-γ and TNF-α from splenocytes compared with BCG vaccination alone (Fig. 5). Low levels of PPD-specific IL-17A were also observed in these mice, but not in any other group. PPD-specific cytokine production was not detected in lung cell supernatants from these mice (data not shown).

We next investigated whether anti–IL-10R mAb treatment in combination with BCG vaccination would increase protection against M. tuberculosis challenge in M. tuberculosis-susceptible CBA/J mice (Fig. 6). Strikingly, at day 63 after M. tuberculosis challenge, BCG-vaccinated CBA/J mice that had received anti–IL-10R mAb during vaccination had a 10-fold lower bacterial burden in the lungs compared with isotype control mAb-treated BCG-vaccinated animals, extending to a 13-fold difference by day 112 after challenge (Fig. 6). Whereas the effect of BCG vaccination alone appeared to wane at 112 d after M. tuberculosis challenge, this was to a much greater extent than in mice that had been vaccinated with BCG in the presence of anti–IL-10R mAbs. These data suggest that blockade of IL-10R during BCG vaccination results in a sustained protective control of bacterial load and provides significantly greater protection than BCG alone. The bacterial load in the spleen was similarly reduced at day 112 in BCG-vaccinated/anti–IL10R-treated mice and showed the smallest increase from the spleen bacterial burdens at day 63 (Fig. 6). At both time points investigated it was observed that PBS-vaccinated/anti–IL-10R-treated control mice had a small reduction in bacterial load compared with PBS-vaccinated/isotype-treated animals. This was more marked at the earlier time point, suggesting a low remaining concentration of the anti–IL-10R mAb early during M. tuberculosis infection, despite ceasing administration before M. tuberculosis challenge (Fig. 6).

FIGURE 5. Blockade of IL-10R signaling during BCG vaccination enhances Th1- and Th17-type responses in M. tuberculosis-susceptible CBA/J mice. CBA/J mice were treated with 1 mg anti–IL-10R mAb or isotype control mAb i.p. and the next day vaccinated with either 5 × 10^7 CFU BCG i.d. or PBS i.d. as control. Mice then received 0.35 mg anti–IL-10R mAb or isotype control mAb i.p. weekly following vaccination for 6 wk. Seven weeks following the last mAb administration (13 wk after BCG vaccination), splenocytes were isolated and restimulated with PPD and anti-CD28 as described in Materials and Methods. The levels of production of IFN-γ, TNF-α, and IL-17A were measured from the supernatants of these restimulations by ELISA. ND, below the level of detection. Data shown are means ± SEM from two combined identical experiments, totaling six mice per group. *p < 0.05 by one-way ANOVA.

FIGURE 6. Blockade of IL-10R signaling during BCG vaccination significantly increases protection to M. tuberculosis challenge in the susceptible CBA/J strain. CBA/J mice were treated with 1 mg anti–IL-10R mAb or isotype control mAb i.p. and the next day vaccinated with either 5 × 10^7 CFU BCG i.d. or PBS i.d. as control. Mice then received 0.35 mg anti–IL-10R mAb or isotype control mAb i.p. weekly following vaccination for 6 wk. One week after the final weekly mAb administration, mice were challenged by M. tuberculosis aerosol, and bacterial load per lungs and per spleen was determined at 63 and 112 d after challenge. Data shown are from one of two independent experiments (n = 5/group) and depict the means ± SEM. ***p < 0.001 by one-way ANOVA; **p < 0.01 by unpaired Student t test. Mtb, M. tuberculosis.
mAb only during BCG vaccination, compared with BCG vaccination alone. We did not observe cytokine-producing CD8+ T cells or innate lymphocytes at day 20 after challenge, nor any cells positive for intracellular IL-17A at this time point (Supplemental Fig. 3 and data not shown).

PPD restimulation of lung cell homogenates from *M. tuberculosis*-challenged BCG-vaccinated/anti–IL-10R-treated CBA/J mice revealed elevated production of IFN-γ at day 112 after challenge, compared with BCG-vaccinated/isotype-treated counterparts (Fig. 7). Levels of IFN-γ at day 112 after challenge in the other groups of mice were, however, seen to wane to lower levels as compared with day 63, whereas IFN-γ concentrations in BCG/anti–IL-10R-treated mice remained high. Levels of PPD-specific TNF-α in restimulated lung cell suspensions were similar between all the *M. tuberculosis*-challenged groups at both time points investigated. In BCG-vaccinated/anti–IL-10R-treated mice, IL-17A production was enhanced in the lungs at 63 d after challenge, although by day 112, PPD-elicited production of IL-17A and IL-6 was strongly enhanced compared with all other control groups (Fig. 7).

**Lung cellular sources of cytokine production in *M. tuberculosis*-challenged CBA/J mice after vaccination with BCG in the presence of anti–IL-10R mAb**

To identify the cellular sources of PPD-specific IFN-γ and IL-17A during *M. tuberculosis* challenge, intracellular cytokine staining was carried out on the PPD-restimulated lung cell cultures for flow cytometric analysis. Live T cell populations were gated as shown (Supplemental Fig. 4A). Analysis of IFN-γ and IL-17A production by lung cells taken from mice at days 63 (data not shown) and 112 after *M. tuberculosis* challenge revealed that the percentages of IFN-γ-producing CD4+ Th1 and also IL-17A–producing CD4+ Th17 T cells were increased in BCG-vaccinated/anti–IL-10R-treated animals compared with all other groups (Fig. 8A, Supplemental Fig. 4). These findings also translated to total numbers of cytokine-producing cells (Fig. 8B). Similarly, percentages and numbers of PPD-specific IFN-γ– and IL-17–producing CD8+ T cells were significantly increased in this group compared with BCG-vaccinated/isotype-treated mice (Fig. 8, Supplemental Fig. 4). Most IFN-γ+ cells in the lungs of BCG-vaccinated/anti–IL-10R-treated CBA/J mice were CD4+ T cells or CD8+ T cells at day 112 after *M. tuberculosis* challenge. A major source of IL-17A was from γδ T cells during *M. tuberculosis* challenge, regardless of vaccination, as has previously been shown during either *M. tuberculosis* or BCG infections in the lungs of mice (56, 57), although this was not significantly higher in BCG-vaccinated/anti–IL-10R-treated mice as compared with other groups (Fig. 8). However, a high percentage of IL-17A+ cells was seen in a Thy1.2+CD3+CD4+CD8−γδTCR+ innate-like lymphoid population in BCG-vaccinated/anti–IL-10R-treated mice that was greatly enhanced over BCG vaccination alone, where these IL-17A–producing cells were barely detectable (Fig. 8A, Supplemental Fig. 4). This innate lymphoid population also made IFN-γ after *M. tuberculosis* challenge of BCG-vaccinated mice in the absence of IL-10R signaling. Blockade of IL-10 signaling during BCG vaccination resulted in small increases in numbers of IFN-γ/IL-17A double-producing CD4+, CD8+, γδ T cells and innate-like Thy1.2+CD3+ cells in the lung on *M. tuberculosis* challenge (Fig. 8B).

**Discussion**

Despite its efficacy in protecting infants from disseminated forms of TB, the only current TB vaccine, BCG, has proved variable for protection against pulmonary disease (3–5). The global burden of TB, as well as the variability in the current BCG vaccine, highlights the need to gain greater understanding of the complex balance of immune mediators following vaccination and their correlation with the best levels of protection (3, 5). In this study we asked whether IL-10 action following BCG vaccination limited vaccine efficacy in terms of the T cell responses generated, and most importantly whether this would limit protection against *M. tuberculosis* challenge. We found that blockade of IL-10 signaling during BCG vaccination increased PPD-specific Th1, Th17, and innate lymphoid IFN-γ and IL-17 responses, and that this regimen enhanced the protection provided by BCG against *M. tuberculosis* challenge in two mouse strains with different *M. tuberculosis* susceptibility.

Our findings are in line with the role of IL-10 in suppressing vaccination against other pathogens. Blockade of IL-10 signaling during vaccination against *L. major* infection has similarly been shown to boost Th1 responses and enhance parasitic control following challenge with *L. major* promastigotes (39–42). Also,
administration of anti–IL-10R mAb during chemotherapy of *Schistosoma mansoni* infection increases anti-worm Th1 and Th17 responses, correlating with significantly enhanced resistance to reinfection (58). The increased Th1 responses in these studies, as well as in our own observations following BCG vaccination in the absence of IL-10 signaling, are likely to be due to removal of the regulation of IL-10 on APCs, including inhibition of IL-12 production and of cell surface molecules involved in Ag presentation (28, 37). In our study, the highest PPD-specific IFN-γ responses were seen to occur in BCG-vaccinated C57BL/6 *Il10^−/−* mice and BCG-vaccinated/anti–IL-10R-treated CBA/J mice; however, despite the necessity of IFN-γ production in control of *M. tuberculosis* infection, several studies have demonstrated that the level of IFN-γ alone does not correlate with protection (59–61). Therefore, the high level of protection in these animals is unlikely to be solely explained by increased levels of this cytokine.

The increased Th17 responses we observed may also be partly due to released inhibition of APC function following anti–IL-10R mAb treatment. However, in light of recent data showing that IL-10 can act directly on Th17 cells to regulate their responses (62), and can promote regulation of Th17 cells via effects on regulatory T cells (63), the blockade of direct IL-10R signaling on these cell types may also explain the increased proportion of Th17 cells in BCG-vaccinated/anti–IL-10R-treated mice. In line with this we also observe increased mRNA levels of IL-17A, IL-17F, and IL-22 in the lungs of BCG-vaccinated/anti–IL-10R-treated CBA/J mice during *M. tuberculosis* challenge (data not shown). The presence of IL-17-producing CD4+ T cells in the lung following peptide/adjuvant vaccination has previously been shown to be necessary for the early recruitment of Th1 memory cells to the lung on *M. tuberculosis* challenge (23). Although throughout our study we see a strong correlation between protection and lung IL-17A levels in BCG-vaccinated mice with blockade or absence of IL-10 signaling, a direct contribution of IL-17A to efficient recruitment of Th1 cells and/or the increased protection in this setting remains to be determined.

In addition to increased CD4+ Th1 and Th17 cell responses, we also observed increased numbers of IFN-γ- and IL-17A–producing CD8+ T cells, γδ T cells, and an innate-like Thy1.2^CD3+^CD8− population of cells produce IL-17 following primary *M. tuberculosis* infection in vivo (56), although whether the latter IL-17–producing population is similar to the Thy1.2^CD3+^ CD8−–producing cells we observe is not known. The Thy1.2^CD3+^ population we observed may include NK cells or it could include IFN-γ– and IL-17A–producing members of the recently described family of innate lymphoid cells (64, 65). The increased numbers of CD8+ T cells observed to produce IFN-γ and IL-17A may be of advantage to the host given the important ancillary role of these cells in TB (66). Irrespective of its source, a major role of IL-17 in many infectious diseases is the promotion of granulocyte accumulation in tissues, including neutrophils (67). The adverse and beneficial roles of this latter cell type in TB are under much scrutiny (68). A recent study has shown an association between IL-17–dependent accumulation of neutrophils and severe lung immunopathology in *M. tuberculosis*-infected mice given subsequent repeated BCG vaccination (25). Although we observed a significantly elevated level of IL-17A in the lungs of *M. tuberculosis*-challenged BCG-vaccinated/anti–IL-10R-treated CBA/J mice, we saw no matching increase in total numbers of lung neutrophils at any of the time points investigated (data not shown). In light of recent data showing that the presence of IFN-γ regulates neutrophils during *M. tuberculosis* infection (69), we postulate that the high levels of IFN-γ in these mice control the emergence of adverse IL-17–driven neutrophil-associated lung inflammation.

Perhaps the most striking finding of this study was the significantly enhanced protection seen in CBA/J mice that had received anti–IL-10R blocking mAb in parallel with BCG vaccination. The
greater protective effect of this regimen in the CBA/J strain as compared with C57BL/6 animals at 63 d after M. tuberculosis infection may reflect their inherent differences in susceptibility to M. tuberculosis infection (54). Importantly, BCG-vaccinated/anti-IL-10R-treated susceptible CBA/J mice had sustained control of M. tuberculosis infection, as at 112 d after M. tuberculosis challenge a 13-fold additional increase in lung M. tuberculosis bacterial burden was observed, compared with BCG-vaccinated/isotype-treated counterparts. We presume the effect of anti-IL-10R mAb in enhancing BCG-mediated protection occurs predominantly during the initial period of vaccination, as the last administration of Ab was at least 1 wk before M. tuberculosis challenge, and the protective effects we have observed occur several weeks after challenge. We cannot, however, exclude the possibility of remaining anti-IL-10R mAb in the system during M. tuberculosis challenge, although this may be beneficial for the host given the negative regulatory effect IL-10 plays during primary M. tuberculosis infection (33, 34, 36). The use of M. tuberculosis-susceptible mouse strains such as the CBA/J alongside the commonly used M. tuberculosis-resistant C57BL/6 and BALB/c strains is important in the development of new TB vaccines, given that susceptible mouse strains may more accurately depict humans that progress to active TB (52).

An important obstacle in the development of efficient protection in the lung following M. tuberculosis infection is the time it takes for T cells to migrate to infected areas where they can help direct macrophage-mediated killing of internalized bacilli via the production of IFN-γ (6, 53). Indeed, it has been demonstrated that the earlier control of a secondary aerogenic M. tuberculosis infection in antibiotic-cured “M. tuberculosis-immunized” mice correlates with the earlier migration of Th1 cells to the lung (19). Our previous data show that the enhanced protection of IL10−/− mice following aerogenic M. tuberculosis infection also occurs with earlier and enhanced lung cytokine responses (36). In the present study, we observed that BCG-vaccinated/anti-IL-10R-treated C57BL/6 mice have a small but significant increase in IFN-γ+ CD4+ T cells at day 20 after M. tuberculosis challenge, when compared with control groups (Supplemental Fig. 3). This may be the result of earlier migration of M. tuberculosis-specific CD4+ T cells to the lungs, and may play some part in the increased level of protection to M. tuberculosis challenge observed following BCG vaccination in the absence of IL-10 signaling. More detailed early kinetics experiments of T cell migration during M. tuberculosis challenge of BCG-vaccinated/anti-IL-10R-treated mice will shed more light on these dynamics.

In summary, our data show that inhibition of IL-10 signaling during BCG vaccination increases and balances IFN-γ and IL-17A responses in favor of the host, providing significantly enhanced and sustained protection to aerogenic M. tuberculosis challenge over BCG vaccination alone, without inducing overt host pathology. The enhanced protection to M. tuberculosis challenge is associated with increased Th1, Th17, and innate lymphoid IFN-γ and IL-17 responses, is sustained during M. tuberculosis challenge, and increases protection in both M. tuberculosis-susceptible and M. tuberculosis-resistant mouse strains. This study gives added insight into the negative regulatory role of IL-10 during vaccination, and it suggests that the use of antagonists of IL-10 signaling may be of great benefit as adjuvants in preventive vaccination against TB.

Acknowledgments

We thank the Medical Research Council National Institute for Medical Research Biological and Procedure Services, Safety Services, and the Flow Cytometry Facility for technical help and assistance. We also thank DNA

Disclosures

The authors have no financial conflicts of interest.

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