Neutralization or Absence of the Interleukin-23 Pathway Does Not Compromise Immunity to Mycobacterial Infection[∇]

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Interleukin-23 (IL-23), a member of the IL-12 family, is a heterodimeric cytokine that is composed of the p40 subunit of IL-12 plus a unique p19 subunit. IL-23 is critical for autoimmune inflammation, in part due to its stimulation of the proinflammatory cytokine IL-17A. It is less clear, however, if IL-23 is required during the immune response to pathogens. We examined the role of IL-23 during *Mycobacterium bovis* BCG infection. We found that IL-23 reduces the bacterial burden and promotes granuloma formation when IL-12 is absent. However, IL-23 does not contribute substantially to host resistance when IL-12 is present, as the ability to control bacterial growth and form granulomata is not affected in IL-23p19-deficient mice and mice treated with a specific anti-IL-23p19 antibody. IL-23p19-deficient mice are also able to mount an effective memory response to secondary infection with BCG. While IL-23p19-deficient mice do not produce IL-17A, this cytokine is not necessary for effective control of infection, and antibody blocking of IL-17A in both wild-type and IL-12-deficient mice also has little effect on the bacterial burden. These data suggest that IL-23 by itself does not play an essential role in the protective immune response to BCG infection; however, the presence of IL-23 can partially compensate for the absence of IL-12. Furthermore, neutralization of IL-23 or IL-17A does not increase susceptibility to mycobacterial BCG infection.

Interleukin-12 (IL-12) and IL-23 are heterodimeric cytokines that are closely related. Both of these cytokines contain a p40 chain, and IL-12 and IL-23 contain the unique subunits p35 and p19, respectively. The heterodimeric receptors for both cytokines contain the IL-12Rβ1 chain. Despite the presence of common cytokine and receptor subunits, the roles of these cytokines are different. IL-12 is essential for the generation of immunity to intracellular pathogens and is characterized by its ability to stimulate gamma interferon (IFN-γ) production from T and NK cells (10). In contrast, IL-23 contributes to the development of IL-17A-producing T cells that promote autoimmune inflammation (17). IL-23, but not IL-12, is important for the pathogenesis of experimental autoimmune encephalitis and collagen-induced arthritis, and IL-23p19 transgenic mice develop severe multiorgan inflammation (5, 21, 26). The immune system, however, has evolved to protect us from infections and tumors rather than to promote autoimmunity; thus, a key question is whether IL-23 can also play a protective role in addition to its characterized pathogenic role.

IL-12 plays a crucial role in the development of cell-mediated immune responses necessary for host resistance to infections. However, in several mouse models of infection, p40-deficient mice have a more severe phenotype than IL-12p35-deficient mice (3, 6–8, 13, 18, 19). These observations suggest that either IL-23 or free p40 by itself adds a level of protection in addition to

that provided by IL-12. With the development of more specific reagents, including p19-deficient animals and recombinant IL-23, it has been possible to assess the role of IL-23 during infection directly. It has been shown recently that IL-23 plays a prominent role in host resistance against acute *Klebsiella pneumoniae* infection through its induction of IL-17A (11). Pulmonary administration of adenovirus vectors expressing IL-23 can improve host resistance to *Mycobacterium tuberculosis* in wild-type mice (12). However, studies using IL-23-deficient mice have shown that the absence of IL-23 has little or no effect on host resistance to *Toxoplasma gondii*, *Cryptococcus neoformans*, and *M. tuberculosis* infection, unless IL-12 is also absent (14, 16, 19). These studies suggest that, compared to the dominant role of IL-12, the role of IL-23 in chronic infections is more subtle.

Bacillus Calmette-Guérin (*Mycobacterium bovis* BCG) is a live attenuated strain of *M. bovis* that is used as an antituberculosis vaccine in many countries. Interestingly, BCG infection is one of the very few infections which affects humans who carry genetic mutations in the common p40 subunit or IL-12Rβ1 and therefore cannot produce or respond to both IL-12 and IL-23 (9). In contrast, mutations in the human p19 and p35 subunits or their specific receptors (IL-23R and IL-12Rβ2, respectively) have not been described, indicating that the absence of IL-12 and IL-23 together may lead to greater susceptibility to infection than the absence of either interleukin alone.

We evaluated the role of IL-23 during BCG infection by comparing wild-type (WT), IL-23-deficient (p19KO), IL-12-deficient (p35KO), and IL-12- and IL-23-deficient (p40KO and p19/35DKO) mice. We found that IL-23 reduces the severity of infection and promotes granuloma formation only when IL-12

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is absent. Although IL-17A production is augmented in IL-12-deficient mice, IL-23-mediated protection in these animals is probably not due to IL-17A, as treatment of p35KO mice with anti-IL-17A antibody did not increase the bacterial burden. In contrast, IL-23 deficiency does not affect the ability of otherwise immunocompetent mice to control infection, form granulomata, generate a memory immune response, or produce key cytokines. While treatment of infected animals with blocking monoclonal antibody to p40 significantly increases the bacterial burden, treatment with anti-p19 antibody does not have an appreciable effect. These data suggest that IL-23 plays a minimal role in the immune response to BCG infection in immunocompetent animals and has a noticeable antimycobacterial effect only when the potent IL-12-mediated Th1 response is absent.

MATERIALS AND METHODS

Bacteria. For in vivo infections, Theracys-BCG Live (Aventis Pasteur Inc., Swiftwater, PA), a freeze-dried preparation of the Connaught strain of BCG, was reconstituted as recommended by the manufacturer, and the concentration was adjusted to approximately 6×10^7 CFU/ml in a 10% glycerol-saline solution. For in vitro infections, BCG-TICE (Organon, Roseland, NJ) was reconstituted in saline and grown for 10 days in Middlebrook 7H9 broth with oleic acid-albumin-dextrose-catalase enrichment (Becton Dickinson, Sparks, MD). Aliquots were stored at -80°C . Prior to injection into mice or in vitro infection, aliquots were thawed, sonicated three times for 10 s, triturated through an insulin syringe, and diluted to obtain an appropriate concentration in 0.02% Tween 80–0.09% saline. Heat-killed BCG was prepared by incubating Theracys-BCG at 80°C for 30 min.

Mice and infections. Six- to 12-week-old female C57BL/6 mice (Jackson Laboratories, Bar Harbor, ME) and C57BL/6 mice genetically deficient in IL-23p19 (B6.129-IL23p19^{tm1Dnax}) (p19KO), IL-12p40 (B6.129S1-IL12b^{tm1Jm/J}) (p40KO), IL-12p35 (B6.129S1-IL12a^{tm1Jm/J}) (p35KO), and both IL-23p19 and IL-12p35 (B.129-IL23p19^{tm1Dnax} IL12a^{tm1Jm/J}) (p19/35DKO) were infected intravenously via the lateral tail vein with 2.3×10^5 to 5×10^6 CFU of BCG as indicated below. Mice were sacrificed at specific times after infection by CO_2 narcosis. All knockout animals were bred and maintained at Schering-Plough Biopharma (formerly DNAX Research, Inc.), and p19/35DKO animals were generated by crossing p19KO mice with p35KO mice. All animals were housed in a specific-pathogen-free environment and were negative for pathogens in routine screening. All study protocols were reviewed and approved by the DNAX Institutional Animal Care and Use Committee.

Antibodies. Monoclonal antibodies specific for tumor necrosis factor alpha (TNF- α) (clone MP6.XT22, rat immunoglobulin G1 [IgG1]), IL-12p40 (clone C17.8, rat IgG2a), IL-17A (clone JL7.1D10, rat IgG1), and IL-23p19 (clone MB490, mouse IgG1) (2) and isotype controls specific for hexon (clone 27F11, mouse IgG1), *Escherichia coli* β -galactosidase (clone GL117, rat IgG2a), and human IL-4 (clone 25D2, rat IgG1) were produced at Schering-Plough Biopharma. Mice were given 1 mg of antibody subcutaneously 1 day prior to infection and again at 1, 2, and 3 weeks postinfection. Serum antibody titers indicated that monoclonal antibodies MB490, C17.8, and JL7.1D10 were present in the circulation at expected concentrations at the time of necropsy.

CFU determination. Blood was perfused out of a lung with RPMI 1640 through the right ventricle after the inferior vena cava was severed. The left lobe of the lung, one half of the spleen, and the left lateral liver lobe were placed in 1 ml of 0.02% Tween 80–0.9% saline and homogenized for 1 min using a Mini-Bead Beater 8 with 2.5-mm zirconia/silica beads (Biospec Products Inc., Bartlesville, OK). Dilutions of the homogenates were plated on 7H10 Middlebrook and Cohn agar plates (Becton Dickinson, Cockeysville, MD), and colonies were counted 2 weeks later.

In vitro infections. Peritoneal exudates were collected by injecting and withdrawing 8 to 10 ml cold RPMI 1640 from the peritoneal cavity of naïve mice. Cells were allowed to adhere to plastic for 2 h at 37°C . Nonadherent cells were aspirated off, and adherent cells were washed four times with warm complete medium (RPMI 1640 with L-glutamate, 10% fetal bovine serum, 20 mM HEPES, 100 $\mu\text{g}/\text{ml}$ penicillin/streptomycin, $1 \times$ nonessential amino acids, and 1 mM sodium pyruvate). Bacteria were added to the cell cultures at a multiplicity of infection of approximately 1:1 and cultured for 24 h. The medium was aspirated off, and adherent cells were prepared for gene expression analysis.

RNA extraction and real-time quantitative PCR gene expression. Real-time PCR analysis of cellular and tissue samples was performed as described previously (16). Gene expression levels were normalized relative to the expression of ubiquitin in each sample.

Histopathology and morphometric analysis. Sections (5 μm) of formalin-fixed, paraffin-embedded livers and lungs were stained for acid-fast bacteria (AFB) using a modified Kinyoun method and with hematoxylin and eosin for morphometric analysis. Briefly, an entire section stained with hematoxylin and eosin (a minimum of five consecutive microscopic fields) was examined, and the mean number of granulomata per $10\times$ field was determined as described previously (16). Individual granulomata were further evaluated to determine the cellular composition and the presence of AFB using a $20\times$ oil immersion objective. The proportion of granulomata harboring AFB (number of AFB-positive granulomata/total number of granulomata) was determined for each histologic section, and a mean percentage was derived for each murine genotype at each time postinfection.

In vitro restimulation assays. Perfused lung tissue was finely minced with a razor blade and incubated for 1.5 h with 150 U/ml collagenase type IV (Sigma Chemical Co., St Louis, MO) in RPMI. Spleens, lung draining lymph nodes, or digested lungs were pressed through 100- μm nylon cell strainers, and cells were incubated with red-blood-cell-lysing buffer (Sigma Chemical Co.), washed, and resuspended in complete medium. Cells were cultured at a concentration of 2×10^6 cells/ml for 72 h with various concentrations of tuberculin (Colorado Serum Co., Denver, CO) or heat-killed BCG at a multiplicity of infection of 1:1. Organs from five mice per group were pooled and assayed in duplicate. Supernatants were analyzed to determine cytokine levels using OptEIA enzyme-linked immunosorbent assay sets (BD Biosciences, San Diego, CA).

RESULTS

BCG-induced expression of IL-23 and IL-12 and their receptors. We first asked if BCG can stimulate IL-23 and IL-23R expression. p19, p40, and p35 expression was measured in resident peritoneal adherent cells isolated from naïve WT, p19KO, p35KO, and p40KO mice which were cultured in vitro with live BCG for 24 h. BCG induced upregulation of IL-23p19 mRNA in WT, p35KO, and p40KO cells but not in p19KO cells (Fig. 1a). An IL-12p40 message was induced in all cells, while an IL-12p35 message was induced in all but p35KO cells (Fig. 1a). It should be noted that p40KO mice produce a mutated nonfunctional p40 mRNA species which the PCR primers can detect (20). WT adherent cells isolated from bronchoalveolar lavage also upregulated the message for IL-12 and IL-23 subunits upon incubation with BCG (not shown). We also looked for BCG-induced expression of the receptor subunits for IL-12 and IL-23 on whole splenocytes. For all strains of mice, BCG induced modest upregulation of the specific IL-23 receptor subunit IL-23R and substantial upregulation of the shared IL-12 and IL-23 receptor subunit IL-12R β 1 and the specific IL-12 receptor subunit IL-12R β 2 (Fig. 1b).

Blockade or absence of IL-23 and IL-12. We compared the abilities of mice genetically deficient in p19, p35, and p40 to control intravenous BCG infection. In WT mice, the numbers of bacterial CFU in the spleen and lung peaked at 2 weeks postinfection, and this was followed by gradual clearance (Fig. 2a). The course of infection in p19KO mice was indistinguishable from that in WT mice. The numbers of CFU in WT and p19KO mice were similar as much as 15 weeks postinfection (not shown). Both p40KO and p35KO mice failed to control the growth of BCG; however, p35KO mice had significantly lower bacterial burdens than p40KO mice (Fig. 2a). Thus, a deficiency in IL-23 alone does not alter the ability of an organism to control BCG infection; however, a deficiency of IL-12 and IL-23 together is more conducive to bacterial growth than a deficiency of IL-12 alone is.

FIG. 1. IL-23 message is upregulated after BCG infection. RNA was obtained from adherent unelicited peritoneal exudate cells (a) and splenocytes (b) from WT and KO mice after 24 h in culture with or without live BCG organisms. The levels of p19, p35, p40, IL-23R, IL-12Rβ1, and IL-12Rβ2 mRNA were determined by reverse transcription-PCR, and the levels of expression are relative to the amounts of ubiquitin mRNA in the samples. The data are representative of the results of the two experiments performed.

Similar differences in numbers of CFU between mice lacking p35 and mice lacking p40 have been reported previously (13). Both p40KO and p35KO mice are unable to make bioactive IL-12, while the p40KO mice also lack IL-23 and the ability to generate bioactive p40 monomers and homodimers. Mice deficient in both p35 and p19 were generated in order to determine whether the greater susceptibility of p40KO mice was due to a lack of p40 by itself or to a lack of IL-23. Interestingly, the bacterial burdens in p19/35DKO and p40KO mice were equivalent (Fig. 2a). Thus, the difference between p35KO and p40KO mice is due to a lack of IL-23 in the p40KO mice and not due to a lack of p40 by itself, and IL-23 can substantially influence the bacterial burden only when IL-12 is absent.

Targeting cytokines that play a role in autoimmune inflammation, such as TNF, is a strategy that is currently used clinically to treat conditions such as rheumatoid arthritis and Crohn's disease; however, the risk involved with the use of these drugs is that infections can develop (25). To determine the risk of BCG infection associated with targeting IL-23 compared with targeting other cytokines, WT mice were treated with neutralizing antibodies to IL-23p19, IL-12p40, TNF, or isotype controls 1 day before and 1 and 2 weeks after infection. Mice treated with anti-p40 or anti-TNF antibodies had significantly higher numbers of CFU in their spleens, livers, and

FIG. 2. Blockade or absence of IL-23 during BCG infection. (a) Mean numbers of CFU in the spleen and lung after intravenous infection with approximately 4.7×10^5 CFU BCG per mouse. The numbers of CFU for WT and p19KO mice differed significantly from the numbers of CFU for p35KO, p40KO, and p19/35DKO mice at weeks 2, 4, and 8 in the spleen and at weeks 4 and 8 in the lung. The numbers of CFU in p35KO mice differed significantly from the numbers of CFU for p40KO and p19/35DKO mice at weeks 4 and 8 in the spleen and at week 8 in the lung. There were no significant differences between the numbers of CFU for WT and p19KO mice in either organ. (b) WT mice were treated with neutralizing antibodies to p40, TNF, or p19 or with appropriate isotype control antibodies 1 day before and 1 and 2 weeks after intravenous infection with approximately 2.5×10^5 CFU BCG per mouse. Organ bacterial burdens were determined at 20 days postinfection. The values are means $\pm$ standard deviations. One asterisk indicates that the *P* value is <0.05 and two asterisks indicate that the *P* value is <0.001 , as determined by one-way analysis of variance after log transformation (Bonferroni posttest; five mice/group). The knock-out data are representative of the results of three experiments, and the antibody treatment data are representative of the results of two experiments.

lungs than isotype control-treated mice had (Fig. 2b), as observed previously (15, 24). In contrast, the numbers of CFU in anti-p19-treated mice did not differ from the numbers of CFU in isotype control-treated mice. Thus, IL-23 blockade alone does not significantly affect the ability of mice to control BCG infection; however, blockade of TNF or blockade of IL-12 and IL-23 together enhances bacterial growth.

IL-23, IL-12, and granuloma formation. The formation of granulomata provides control and containment of mycobacterial infections. It has previously been reported that mice deficient in both IL-12 and IL-23 have defective granuloma formation compared with p35KO mice (13); therefore, we

FIG. 3. Granuloma formation. (a) Mean number of granulomata per 100 $\times$ field. (b) Percent of granulomata containing AFB in the liver and lung. There were no significant differences in granuloma counts and AFB percentages between WT and p19KO mice. The liver and lung granuloma counts and AFB percentages for WT and p19KO mice differed significantly from those for p40KO and p19/35DKO mice at weeks 2 and 8. At week 8, the lung and liver AFB percentages for p35KO mice differed significantly from those for p40KO and p19/35DKO mice. The values are means $\pm$ standard errors of the means ($P < 0.001$ for significant differences, as determined by a one-way analysis of variance [Bonferroni posttest; three to five mice/group]). Similar results were obtained in a separate experiment.

examined the role of IL-23 in granuloma formation and resolution. For WT and p19KO mice there was no significant difference in the number of granulomata or the percentage of intralesional AFB when the animals were compared at sequential times postinfection (Fig. 3). Relatively high numbers of granulomata were present in the liver and lung at 2 weeks postinfection (Fig. 3a). By 8 weeks there were few or no granulomata, and the infection had resolved in most animals, as indicated by the low frequency or absence of AFB (Fig. 3b) and the low numbers of CFU (Fig. 2). No appreciable differences in the cellular composition of granulomata or the distribution of AFB in WT and p19KO mice were noted (not shown).

In contrast, in p35KO, p40KO, and p19/35DKO mice there was delayed granuloma formation compared to the formation in WT and p19KO mice (Fig. 3a). At 2 weeks postinfection, WT and p19KO mice had significantly more granulomata in the liver than p35KO, p40KO, and p19/35DKO mice had. Although IL-12-deficient mice and IL-12- and IL-23-deficient mice eventually formed granulomata in the liver and lung, these lesions did not limit growth of the organisms, as the percentages of granulomata containing AFB in these mice increased over time, whereas the granulomata in WT and p19KO mice significantly reduced and appeared to clear the infection (Fig. 3b). Interestingly, granulomatous inflammation was more severe in the lungs of

FIG. 4. Gene expression during BCG infection. (a) mRNA expression determined by reverse transcription-PCR 24 h after in vitro incubation of splenocytes with and without BCG. (b) mRNA expression in spleens of WT and knockout mice at 0, 2, 4, and 8 weeks postinfection. The dotted lines indicate the limit of detection (three mice/group). (c) Splenocytes and lung draining lymph node cells from mice infected for 3 weeks with approximately 5×10^5 CFU BCG were restimulated in vitro for 72 h with medium alone, a 1:100 dilution of tuberculin, or heat-killed BCG (hkBGG) at a multiplicity of infection of 1:1. Supernatant IFN- γ and IL-17A levels were determined by an enzyme-linked immunosorbent assay. The data are means $\pm$ standard deviations. All data are representative of the results of at least two experiments.

p40KO and p19/35DKO mice than in the lungs of p35KO mice at 8 weeks postinfection (Fig. 3a), suggesting that IL-23 may influence the inflammatory response in the lungs of IL-12-deficient mice.

Effects of IL-23 and IL-12 on gene expression. Expression of genes encoding important factors that mediate the antimycobacterial host response was assessed after BCG infection in vitro, in vivo, and during restimulation of cells ex vivo. Following incubation with BCG, the levels of IFN- γ and nitric oxide synthase 2 (NOS2) mRNA increased sharply in cultured splenocytes from both WT and p19KO mice, whereas minimal increases were observed in splenocytes from p35KO and p40KO mice (Fig. 4a). BCG-induced expression of the proinflammatory cytokines TNF- α , IL-1 α , IL-1 β , and IL-6 was similar in splenocytes from all four strains of mice (Fig. 4a and data not shown). Finally, BCG upregulated IL-17A mRNA expression in splenocytes from WT and p35KO mice but not in splenocytes from mice that lacked IL-23.

The temporal expression of a variety of genes after in vivo infection was also monitored. WT and p19KO mice similarly upregulated messages for IFN- γ , NOS2, IL-27p28, and IL-12R β 1 in the spleen, with expression peaking at 2 weeks postinfection (Fig. 4b). Similar expression patterns were seen in the lung (data not shown). Maximal expression of these genes in the spleen coincided with the peak of infection in these mice (Fig. 2a). In contrast, the gene expression in IL-12-deficient mice and in IL-12- and IL-23-deficient mice differed greatly from the gene expression in WT mice. Notably, IFN- γ , NOS2, IL-12R β 1, and IL-27p28 were expressed at lower levels or with different kinetics (Fig. 4b). Mice deficient in both IL-12 and IL-23 had a different pattern of gene expression than mice deficient in IL-12 alone, as p35KO mice exhibited slightly higher expression of these genes at later times. Interestingly, p35KO mice had enhanced IL-17A mRNA expression compared to WT mice. While the IL-23p19 mRNA levels in vivo were low at the times examined, we observed greater expression in p35KO and p40KO mice than in mice having other genotypes.

We also compared cytokine production after in vitro restimulation of splenocytes and lung draining lymph node cells from infected WT and knockout mice. Both WT and p19KO cells made nanogram quantities of IFN- γ in response to tuberculin or heat-killed BCG, while p35KO and p40KO cells made minimal IFN- γ (Fig. 4c). IL-17A production was absent in p19KO and p40KO cells, while p35KO cells made more IL-17A than WT cells made.

IL-23 and the memory response. The experiments described above showed that IL-23 plays a minimal role in the primary response to BCG infection in immunocompetent mice. However, the effects of IL-23 could be manifested during a memory response rather than during a primary immune response. To test this possibility, mice were infected with a low dose of BCG, and the numbers of CFU in organs were determined after 15 weeks (Fig. 5a). These mice and age-matched naïve mice were then infected with a high dose of BCG. Three weeks after the high-dose infection, the CFU burdens in naïve and previously infected (memory-immune) mice were compared. Naïve WT and p19KO mice had indistinguishable responses to a high-dose infection (Fig. 5a). WT and p19KO memory-immune mice controlled the high-dose infection better than naïve mice, and there were no significant differences in the number of CFU between the two groups (Fig. 5a). After 15 weeks, p35KO and p40KO mice failed to control the initial low-dose infection

FIG. 5. Memory response. (a) Mice were infected with approximately 2.25×10^5 CFU/mouse, and the numbers of CFU were determined at 15 weeks postinfection (open bars). The memory-immune mice (solid bars) and age-matched naïve mice (cross-hatched bars) were then infected with 5×10^6 CFU/mouse, and the numbers of CFU in organs were determined 3 weeks later (five mice/group). One asterisk indicates that the P value is <0.05 , two asterisks indicate that the P value is <0.01 , and three asterisks indicate that the P value is <0.001 , as determined by one-way analysis of variance. The dotted line indicates the limit of detection. n.s., not significant. (b) Cells were isolated from the lungs and spleens of infected naïve and memory-immune mice and restimulated in vitro with tuberculin (diluted 1:1,000 for the IFN- γ assay and 1:100 for the IL-17A assay) for 72 h. Supernatants were collected, and cytokines were analyzed by an enzyme-linked immunosorbent assay. The IL-17A levels in splenocyte cultures were low or undetectable except in p35KO mice (not shown). The values are means $\pm$ standard deviations. The experiment was performed once.

FIG. 6. Effect of blocking IL-17A. WT, p35KO, and p40KO mice were treated with anti-IL-17A or isotype control antibody 1 day before and 1, 2, and 3 weeks after intravenous infection with 5.5×10^5 CFU BCG. At 4 weeks postinfection, organs were harvested, and the numbers of bacterial CFU were determined. The differences in the numbers of CFU in the lung between the anti-IL-17A and isotype control treatments were not significantly significant. The values are means $\pm$ standard deviations (five mice/group).

(Fig. 5a); thus, their ability to generate a memory response is difficult to interpret.

Lung and spleen cells derived from memory-immune WT and p19KO mice produced more IFN- γ than cells from infected naïve mice produced when they were restimulated in vitro with tuberculin (Fig. 5b). The amounts of IFN- γ mRNA in spleens and lungs isolated from memory-immune WT and p19KO mice were also similar (not shown). Both p35KO and p40KO cells produced little IFN- γ in this assay, and the levels were equivalent in the memory-immune and infected naïve groups. IL-17A production in WT and p35KO mice was modestly increased in lung cells from memory-immune mice compared to lung cells from infected naïve mice (Fig. 5b). IL-23 drives the production of IL-17A from T cells (1), and accordingly, a minimal amount of IL-17A was detected in restimulated lung cells from p19KO mice or p40KO mice.

Blockade of IL-17A. Our data suggest that the absence of IL-23 has little effect on the course of BCG infection, unless IL-12 is also absent. A key question is how IL-23 protects IL-12-deficient mice. IL-17A is a proinflammatory cytokine linked to IL-23, and high levels of IL-17A are produced in p35KO mice compared to the levels produced in p40KO mice (Fig. 4c and 5b), although the IL-17A message was detected in p40KO mice at a late time (Fig. 4b). We administered anti-IL-17A or control antibody to wild-type, p35KO, and p40KO mice 1 day prior to infection and weekly thereafter to determine if IL-17A production in p35KO mice influenced BCG growth. Anti-IL-17A had no statistically significant effect on the bacterial burden in the lung and spleen after 4 weeks of treatment (Fig. 6a and b).

However, there was a trend toward a reduced bacterial burden in WT and p35KO mice and an increased burden in p40KO mice with anti-IL-17A treatment. In a second experiment, treatment of WT mice resulted in a statistically significant reduction in the number of splenic CFU (not shown). These experiments suggest that the robust IL-17A expression in p35KO mice is not likely to be the dominant IL-23-induced factor that makes these mice more resistant to BCG infection than p40KO mice.

DISCUSSION

This work resolved several questions concerning the role of IL-23 during mycobacterial BCG infection. First, we found that IL-23 is dispensable for the control of infections in mice which have intact IL-12. Although there is strong upregulation of the p19 message upon incubation of adherent peritoneal exudate cells with BCG, we found no significant differences between WT and p19KO animals with regard to bacterial burden, granuloma formation, or the memory immune response. Expression of numerous genes, including the IFN- γ and NOS2 genes, appears to be normal in infected p19KO mice, but IL-17A production is absent. However, the absence of IL-17A appears to have little effect on the ability of IL-23-deficient animals to contain an infection. We also examined the discrepancy in the responses to infection seen in p40KO and p35KO mice. p35KO mice have lower numbers of CFU in the organs than p40KO mice and altered granuloma formation. Although free p40 has been proposed to mediate this difference (13), we show here that IL-23, not p40, is responsible, as p19/35DKO mice had responses to infection identical to those of p40KO mice. Therefore, IL-23 has the ability to reduce the bacterial burden and alter disease pathology when IL-12 is absent.

Given that p19KO mice showed no diminished protection, the increased susceptibility of p19/35DKO mice compared to p35KO mice appears to be counterintuitive. However, IL-12 is a potent inducer of IFN- γ . When the IL-12/IFN- γ response is intact, as it is in the p19KO mice, BCG organisms are killed efficiently, and the contribution of IL-23 is insignificant. However, when the IL-12/IFN- γ response is missing, the host cannot efficiently kill BCG, and only then does IL-23 have a moderate effect on reducing the bacterial burden. IL-23 also affects the function and formation of granulomata in IL-12-deficient mice, as p35KO mice have a lower percentage of AFB-containing granulomata and fewer lung granulomata than p40KO and p19/35DKO mice have. It is possible that IL-23-driven IFN- γ is responsible for the difference in disease outcome in p35KO mice and IL-12- and IL-23-deficient mice, as IL-23 was originally described as a cytokine that was able to induce IFN- γ production from human T cells (22). In the context of some infections, there is evidence that IL-23 might drive IFN- γ production, as more IFN- γ was produced from restimulated p35KO cells than from IL-12- and IL-23-deficient cells (13, 14, 18). However, in many infections, including infections with *Mycobacterium avium*, *C. neoformans*, *T. gondii*, *K. pneumoniae*, and *Francisella tularensis*, no increase in the amount of IFN- γ was observed in p35KO mice compared with IL-12- and IL-23-deficient mice, indicating that IL-23 does not induce significant IFN- γ production (6–8, 11, 19). We also have no conclusive evidence that IL-23-driven IFN- γ pro-

fects p35KO mice during BCG infection. The IFN- γ response in p35KO mice is minor compared to that in WT and p19KO mice, and, in a variety of assays, we did not consistently observe more IFN- γ in p35KO mice than in p40KO and p19/35DKO mice (Fig. 4 and 5b).

We did consistently observe more IL-17A in p35KO mice than in p40KO mice, and we specifically were interested in whether the IL-17A production was responsible for the differences between IL-12-deficient mice and IL-12- and IL-23-deficient mice. Certainly during *K. pneumoniae* infection, IL-23-induced IL-17A plays a protective role (11). Our results indicate that this does not appear to be the case for BCG infection. Instead, blocking IL-17A for 4 weeks had no major effect on the bacterial burden in both immunocompetent and IL-12-deficient animals. If anything, anti-IL-17A treatment tended to reduce the number of CFU in the lungs of WT and p35KO mice, although the difference was significant only in WT spleens in one experiment (not shown). IL-23-driven IL-17A-producing T cells have been shown to cause significant pathology in experimental autoimmune models (5, 17, 21). In addition, it has been reported that IL-17A-producing T cells contribute to the liver pathology associated with *Schistosoma mansoni* infection (23), and it has recently been suggested that IFN- γ regulates a population of inflammatory IL-17A-producing T cells during BCG infection (4). While it is possible that IL-17A-producing T cells contribute to an inflammatory environment that is conducive to BCG growth, we saw no gross histological differences between mice treated with isotype and mice treated with anti-IL-17A (data not shown). In addition, we found that p19KO mice have reduced levels of IL-17A compared to the levels in wild-type mice, yet the responses to infection are identical, as assessed by multiple parameters. Conversely, p35KO mice have increased levels of IL-17A message and IL-17A-producing T cells, but they have reduced histopathology compared with p40KO and p19/35KO mice that do not produce IL-17A. Therefore, we have no clear evidence that IL-17A is responsible for mycobacterium-associated immunopathology. Extending the time of antibody treatment may clarify whether IL-17A contributes to the long-term inflammatory response; however, the anti-rat antibody response limits the length of time that this cytokine can be neutralized in vivo.

We observed no effect of the loss of IL-23 on the ability of mice to mount a memory immune response. Although IL-23 was initially shown to stimulate the proliferation of memory CD4⁺ cells but not the proliferation of naïve CD4⁺ cells (22), this result is subject to reinterpretation in light of recent findings. In the original description, the CD4⁺CD45RB^{low} memory cells that responded to IL-23 were isolated from IL-10 knockout mice. IL-10 knockout mice get spontaneous colitis when they are 3 months old, which has recently been shown to be dependent on IL-23p19 (27). Yen et al. showed that in IL-10 knockout mice, IL-23 actually stimulates a unique subset of activated/memory T cells that produce IL-17A (Th17 cells), whereas IL-23 has no effect on memory cells from wild-type mice. Our data suggest that IL-23-induced IL-17A production does not play a major role in the host defense against BCG infection; thus, the effect of IL-23 on Th17 cells with a memory phenotype may not be relevant.

The roles of IL-23 and IL-17A during the pathogenesis of

autoimmune inflammation make these cytokines attractive targets for anti-inflammatory drugs. It is important to assess the potential risks involved with blocking any immune mediator, particularly with regard to infection. Indeed, the use of TNF antagonists as immunotherapy for certain inflammatory diseases carries the risk that mycobacterial and other infections may develop (25). We show that blocking IL-23 or IL-17A during BCG infection with monoclonal antibodies does not appear to affect the bacterial burden in immunocompetent mice. In contrast, blocking TNF- α or both IL-23 and IL-12 with anti-p40 dramatically enhances bacterial growth. From a safety perspective, antibody blockade of IL-23 or IL-17A rather than IL-12p40 or TNF- α in the context of inflammation might be preferable in patients who have been or may be exposed to mycobacterial infection. Furthermore, these potential therapies may not affect a patient's ability to respond to vaccinations, since IL-23p19KO mice can generate a functional memory response to immunization. However, it is important to bear in mind that our studies, performed with an attenuated form of mycobacteria in a mouse model of infection, may not mimic the immune response that occurs during virulent *M. tuberculosis* infection in humans. Further investigations using aerosolized *M. tuberculosis* infection of mice or primates should be carried out to better assess the infection risk of these potential therapeutics.

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