Increased Pulmonary Tumor Necrosis Factor Alpha, Interleukin-6 (IL-6), and IL-17A Responses Compensate for Decreased Gamma Interferon Production in Anti-IL-12 Autovaccine-Treated, 
*Mycobacterium bovis* BCG-Vaccinated Mice

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Interleukin-12 (IL-12) and IL-23 (which share a p40 subunit) are pivotal cytokines in the generation of protective Th1/Th17-type immune responses upon infection with the intracellular pathogen *Mycobacterium tuberculosis*. The role of IL-12 and IL-23 in protection conferred by the tuberculosis vaccine *Mycobacterium bovis* bacillus Calmette-Guérin (BCG) is, however, less well documented. By using an autovaccine approach, i.e., IL-12p70 cross-linked with ovalbumin and PADRE peptide formulated with the GSK proprietary adjuvant system AS02, we could specifically neutralize IL-12 while leaving the IL-23 axis intact. Neutralization of IL-12 before *M. tuberculosis* challenge rendered C57BL/6 mice highly susceptible, resulting in 30-fold-higher CFU in spleen and lungs and accelerated mortality. In contrast, neutralization of IL-12 in BCG-vaccinated mice prior to *M. tuberculosis* challenge only marginally affected vaccine-mediated protection. Analysis of cytokine production in spleen and lungs 3 weeks post-TB challenge by enzyme-linked immunosorbent assay and functional and flow cytometric assays showed significantly reduced mycobacterium-specific gamma interferon (IFN-γ) responses in *M. tuberculosis*-infected and BCG-vaccinated mice that had been treated with the autovaccine. Purified protein derivative-induced tumor necrosis factor alpha (TNF-α), IL-6, and IL-17A levels, however, were highest in lungs from BCG-vaccinated/IL-12-neutralized animals, and even unstimulated lung cells from these mice produced significant levels of the three cytokines. Mycobacterium-specific IL-4 and IL-5 production levels were overall very low, but IL-12 neutralization resulted in increased concanavalin A-triggered polyclonal secretion of these Th2-type cytokines. These results suggest that TNF-α, IL-6, and IL-17A may be more important pulmonary effector molecules of BCG-mediated protection than IFN-γ in a context of IL-12 deficiency.
tant effects of both cytokines. Moreover, these studies have examined the effect of IL-12/IL-23 on the initiation of the immune response by the BCG vaccine but not on the role of IL-12 in the protective effector phase conferred by the vaccine against *M. tuberculosis* infection.

As compensation phenomena may occur when working with genetically inactivated knockout mice, we used an alternative approach of IL-12 neutralization, i.e., autovaccination, to revisit the role of this cytokine in protection against TB infection and in protection conferred by prior *M. bovis* BCG vaccination. In the anti-IL-12 autovaccine, murine IL-12p70 is cross-linked with ovalbumin (OVA) and pan-HLA-DR epitope (PADRE peptide, which binds to murine I-A<sup>β</sup> molecules and is immunogenic in C57BL/6 mice) (2) through glutaraldehyde cross-linking. This IL-12-(OVA)-PADRE vaccine induces anti-IL-12 antibodies which specifically neutralize the biological activity of IL-12 but, as reported here, not that of IL-23. We have previously shown that immunization of mice with anti-IL-12 autovaccine can protect against experimental autoimmune encephalitis (EAE), but at the expense of increased sensitivity to infection by *Leishmania major* (35). Treatment with this anti-IL-12 autovaccine was also capable of blocking the development of atherosclerosis in low-density lipoprotein receptor-deficient (LDL-R<sup>−/−</sup>) mice (18).

In order to analyze the role of IL-12 in resistance against TB infection and protection conferred by the BCG vaccine, C57BL/6 mice were immunized with anti-IL-12p70 autovaccine (formulated with the GSK proprietary adjuvant system; AS02<sub>a</sub>) 2 months prior to challenge with *M. tuberculosis* H37Rv and monitored for bacterial replication in the spleen and lungs. Alternatively, mice were first vaccinated with *M. bovis* BCG, immunized subsequently against IL-12p70, and finally challenged with *M. tuberculosis*. Our results confirmed that IL-12 is essential for protection against primary infection with TB but much less so for protection induced by the BCG vaccine. Analysis of mycobacterium-specific and concanavalin A (ConA)-induced cytokine responses in spleen and lungs by enzyme-linked immunosorbent assay (ELISA), functional assays, and flow cytometric cytokine assays indicates that TNF-α, IL-6, and IL-17A are important pulmonary effector molecules of BCG-mediated protection that can compensate for decreased IFN-γ production in these IL-12-neutralized mice.

**Materials and Methods**

Mice. Specific-pathogen-free female C57BL/6 mice, 6 to 8 weeks old at the start, were obtained from the breeding facilities of the Ludwig Institute. The experimental protocol and animal handling was approved by the ethical committees of the Faculty of Medicine, Université Catholique de Louvain, and of the WIV-ISP.

*M. bovis* BCG vaccination. Mice were vaccinated subcutaneously with 0.5 mg (2 × 10<sup>8</sup> CFU) of freshly prepared *M. bovis* BCG (strain GL2) grown as a surface pellicle on synthetic Sauton medium (20). Two months after BCG vaccination, half of the mice were treated with the anti-IL-12 autovaccine.

Immunization with the anti-IL-12 autovaccine. Naïve and BCG-immunized mice were vaccinated intramuscularly five (experiment 1) or four (experiments 3 and 4) times at 2-week intervals with 3 to 5 × 10<sup>6</sup> CFU of freshly prepared *M. bovis* BCG (strain GL2) grown as a surface pellicle on synthetic Sauton medium (20). Two months after BCG vaccination, half of the mice were treated with the anti-IL-12 autovaccine.

Analysis of IL-12-neutralizing activity. Sera were collected 1 month prior to *M. tuberculosis* challenge (experiment 4). IL-12 inhibitory activity present in the sera of vaccinated mice was assessed in a bioassay using a mouse B7a/F3 murine hematopoietic cell line transfected with the IL-12Rα and β chains as described before (18). Briefly, serial serum dilutions were incubated for 24 h in the presence of 10<sup>4</sup> Ba/F3mIL-12R cells and 1 ng/ml recombinant IL-12 (R&D Systems). One microcurie of 3<sup>125</sup>I-hI-lumidine (Perkin-Elmer) was then added for another 16 h. Incorporation was measured with a Topcount NXT counter (Perkin-Elmer).

Inhibitory titers were calculated as the serum dilution that inhibited 50% of the biological activity of 1 ng/ml IL-12.

**Analysis of IL-12-neutralizing activity.** Anti-IL-12 functional activity was measured in vitro by testing the inhibition of IL-12-induced proliferation of Ba/F3 cells expressing the IL-23 receptor complex. The murine IL-12Rβ1 and IL-23R coding sequences were amplified by reverse transcription-PCR (RT-PCR) from ConA-stimulated C57BL/6 T cells with the following primers: 5′-ACTCGGCT CTCATGCACT-3′ (IL-12Rβ1 sense primer), 5′-TCGCAACGACACGC CTTGTG-3′ (IL-12Rβ1 antisense primer), 5′-CAGGGGACGCGCCTGAT T-3′ (IL-23R sense primer), and 5′-GACTTGTAGCGATGCTACCT-3′ (IL-23R antisense primer). Both PCR products were cloned into the pEG/6V5-His-Topo vector (Invitrogen) and sequenced to exclude any PCR-induced mutations. The murine IL-12Rβ1 cDNA was further subcloned into the pEF-Bos, pro expression vector, which contains a purumycin resistance cassette (10). Five million Ba/F3 cells were co-electroporated with 25 μg of each expression vector (pEF-Bos, proto-IL-12 and pEF-Topo-IL-12Rβ1) in 800 μl Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal calf serum (FCS). Transfected Ba/F3 cells were cultured in DMEM with 10% FCS and IL-3 (100 U/ml). After 48 h, cells were cultured alternatively in the presence of purumycin (3 μg/ml) and bleomycin (16 μg/ml). Transfected cells surviving the selection procedure were then cloned by limiting dilution in the presence of IL-3 and selective agents. Clones were tested for IL-23-dependent proliferation.

IL-23-inhibitory activity present in the sera of anti-IL-12-vaccinated mice was tested by incubating serial serum dilutions for 3 days in the presence of 3 × 10<sup>7</sup> Ba/F3mIL-23R cells and 1 ng/ml recombinant IL-23 (R&D). Proliferation was measured by a hexosaminidase colorimetric assay (25). Inhibitory titers were calculated as the serum dilution that inhibited 50% of the biological activity of 1 ng/ml IL-23.

**Results**

*I. tuberculosis* challenge. Mice were challenged 2 months after the last administration of the anti-IL-12 autovaccine (6 months after BCG vaccination) with luminescent *M. tuberculosis* H37Rv, grown for 2 weeks as a surface pellicle on synthetic Sauton medium as described before (33). Bacterial replication in the spleen and lungs was monitored by luminescence. Briefly, luminescence was measured in a Turner Design TD 20/20 lumimeter as flash emission (15-s integration time) using 1% n-decanal (Sigma) in ethanol as substrate. In this assay, only live bacteria are enumerated, as light emission is dependent on the presence of reduced flavin mononucleotide (FMNH<sub>2</sub>), a cofactor only found in living cells. Mice were infected in a lateral tail vein with 2 × 10<sup>7</sup> CFU of *M. tuberculosis* and were sacrificed at the various indicated time points postinfection. Four to six mice were analyzed per group at each time point. For statistical analysis, results obtained in milli-relative light units (mRLU) were used for each group, mRLU/oriental analysis using a one-way analysis of variance with Tukey’s multiple comparison test.

**Cytokine production.** Mice were sacrificed 3 weeks after *M. tuberculosis* challenge. The spleen and lungs from three to four mice per group were removed aseptically and homogenized by gentle disruption in a Dounce homogenizer, and cells were adjusted to 4 × 10<sup>7</sup> white blood cells/ml in RPMI 1640 medium (Invitrogen) supplemented with 10% FCS (Greiner), 5 × 10<sup>−3</sup> M 2-mercaptoethanol, penicillin, streptomycin, amphotericin B (Fungizone; Invitrogen), and the prostaglandin E<sub>2</sub> inhibitor indomethacin (1 μg/ml). Spleen cell cytokine responses were tested in individual mice, and lung cell responses were evaluated on pooled cells for each group. Cells were stimulated with purified protein derivative of old tuberculin (PPD; 10 μg/ml; prepared at the former Pasteur Institute of Brussels, now WIV-ISP), recombinant Escherichia coli-derived mycolyl transferase Ag85B (Rv1886; 5 μg/ml), or polyclonal T cell mitogen ConA (4 μg/ml; Sigma) and incubated at 37°C in round-bottom, 96-well microplates in a humidified CO<sub>2</sub> incubator. Supernatants from three wells were pooled after 24 h and 72 h and stored at −20°C.

**Cytokine assays.** IL-9, IL-6, and TNF-α were detected as described previously using bioassays on TS1.C3 (36), 7TD1 (38), and WEHI 164 clone 13 (12) cells, respectively. IL-17A was detected using a home-made ELISA with MM17A-SOG as capture and MM17F3 as detection antibody. IFN-γ, IL-5, and IL-10 were detected using commercial ELISAs (R&D). These analyses were performed on 72-h culture supernatants.

**Flow cytometric cytokine assays.** Spleen or lung cell supernatants from unstimulated controls (three microwells) were harvested after 24 h and analyzed using the mouse Th1/Th2 cytokine flow Cytomix BMS720FF kit from Bender Med.
**RESULTS**

**IL-12- and IL-23-neutralizing activities in sera from anti-IL-12 autovaccine-treated mice.** C57BL/6 mice were immunized with mL-12p70-(OVA)-PADRE complexes emulsified in AS02a adjuvant. A number of the anti-IL-12-vaccinated mice were immunized with *M. bovis* BCG 2 months previously. Anti-IL-12-neutralizing antibody titers were determined using a Ba/F3 cell line transfected with murine IL-12Rα/IL-12Rβ1 chains and murine IL-12Rβ1/IL-23R chains, respectively. Inhibitory titers were calculated as the serum dilution that inhibited 50% of the biological activity of 1 ng/ml IL-12 or 1 ng/ml IL-23 (as measured in a proliferation assay using tritiated thymidine and a hexosaminidase colorimetric assay, respectively). Anti-IL-12-neutralizing titers were determined as described in Materials and Methods, with bioassays using Ba/F3 cells transfected with murine IL-12Rβ1/IL-23R chains, respectively. Inhibitory titers were calculated as the serum dilution that inhibited 50% of the biological activity of 1 ng/ml IL-12 or 1 ng/ml IL-23 (as measured in a proliferation assay using tritiated thymidine and a hexosaminidase colorimetric assay, respectively).

**Effect of in vivo IL-12 neutralization on susceptibility to intravenous *M. tuberculosis*.** Bacterial replication is shown for spleen (B) and lungs (C) of untreated (open squares) or IL-12-neutralized (filled triangles) C57BL/6 mice at day 1 (*n* = 2), day 30 (*n* = 4), and day 50 (*n* = 4 for untreated and *n* = 2 for IL-12-neutralized mice) post-TB challenge. Data are expressed as means ± the SD of the log10 mRLU/organ. NS, not statistically significant; ***, *P* < 0.001. (Results are from experiment 1A.)

Sera were also checked for their IL-23-neutralizing activity. Although IL-12 and IL-23 share the p40 subunit, anti-IL-12 autovaccine did not neutralize or only very weakly neutralized the IL-23-dependent proliferation of Ba/F3/mIL-23R (Fig 1A, open symbols). In the group of mice that received prior BCG vaccination, 5/12 animals had anti-IL-23 antibody titers below the detection level, but 1 animal had an anti-IL-23 antibody titer of >1:10,000 (1.32,612; mean antibody titer, 1/3,587 ± 2,656). In the group of mice that were infected with *M. tuberculosis*, 7/11 animals had antibody titers below the detection level, and none had a titer of >1:10,000 (mean antibody titer, 1/1,305 ± 812). These anti-IL-23-neutralizing titers were not statistically different between the two groups (*P* = 0.4378). No IL-12- or IL-23-neutralizing antibodies were detected in sera from mice vaccinated with ASO2a adjuvant only (Fig 1A).

**Effect of in vivo IL-12 neutralization on susceptibility to intravenous *M. tuberculosis*.** Treatment with the anti-IL-12p70 autovaccine increased the susceptibility of C57BL/6 mice to infection with luminescent *M. tuberculosis* H37Rv. In the spleen (Fig 1B), bacterial numbers (expressed in log10 mRLU, as determined by luminometry) increased between day 1 and day 28 postinfection (p.i.) in both groups and then stabilized between day 28 and day 50. Prior neutralization of IL-12 resulted in 30-fold-higher bacterial numbers, at both day 28 and day 50 postinfection. In lungs (Fig 1C), bacterial counts gradually increased between day 0 and day 50. At day 28 p.i., bacterial numbers were not significantly different between the two groups, but at day 50, mean bacterial counts in IL-12-neutralized mice were 25 times higher than in untreated mice. Furthermore, at day 50 postinfection 50% of the anti-IL-12-vaccinated mice had already succumbed to the infection, whereas control C57BL/6 mice infected intravenously with this infectious TB dose (2 × 105 CFU) survive for more than 140 days (14; K. Huygen et al., unpublished data).

**Effect of in vivo IL-12 neutralization on *M. bovis* BCG-mediated protection against *M. tuberculosis*.** In a second set of experiments, we analyzed the effect of the anti-IL-12p70 autovaccine on the protection conferred against *M. tuberculosis* by previous *M. bovis* BCG vaccination. As shown in Fig 2, C57BL/6 mice that had been vaccinated with BCG prior to TB challenge demonstrated a significantly reduced bacterial replication in spleen and in lungs compared to unvaccinated mice (*P* < 0.001) both at week 3 (Fig 2, upper panel) and at week 6 (Fig 2, lower panel) postinfection. Whereas IL-12 neutralization resulted in a dramatic increase in bacterial replication in the spleen and lungs from unvaccinated animals, IL-12 neutralization did not affect BCG-mediated immune protection in
the lungs at either week 3 or week 6 and affected BCG-mediated protection against *M. tuberculosis*. Interestingly, at week 6 p.i. one of the BCG-vaccinated/IL-12-neutralized mice showed 10-fold-higher bacterial numbers in the spleen and lungs than the other five mice of that group, and looking at this animal’s initial anti-IL-12 and anti-IL-23 titers prior to infection (Fig. 1A), it was precisely this mouse that showed the highest anti-IL-23 titer.

To further analyze the possible effect of IL-12 neutralization on BCG-mediated immune protection in the spleen and lungs, we also monitored mice in another experiment for a time period of 9 weeks (Fig. 3). As observed in the two previous experiments, the anti-IL-12 autovaccine markedly increased susceptibility against *M. tuberculosis* with more than 100-fold-higher bacterial loads in the spleen and lungs and an accelerated mortality (at day 63 postinfection, all the animals in the anti-IL-12 vaccine group had succumbed to the infection). C57BL/6 mice that had been vaccinated with BCG prior to TB challenge demonstrated a significantly reduced bacterial replication in the spleen and in lungs compared to unvaccinated mice. In the spleen (Fig. 3, upper panel), BCG offered a protection of 0.77 and 0.64 log_{10} mRLU (compared to the mean log_{10} values in naive TB-infected mice) at days 21 and 42, respectively. At day 63 postinfection, bacterial numbers in spleens from BCG-vaccinated animals were of the same magnitude as at day 42, but these numbers were no longer statistically different from bacterial numbers in spleens of naive infected animals. This was attributed to the fact that *M. tuberculosis* replication is progressively controlled to some extent in the spleens of C57BL/6 mice, resulting in slightly decreased bacterial numbers at later time points. In this experiment, BCG-vaccinated/IL-12-neutralized mice were protected to the same extent as BCG-vaccinated mice that had not received the anti-IL-12 autovaccine at the three time points tested (Fig. 3, upper panel).

As shown in the lower panel of Fig. 3, IL-12 neutralization did not affect BCG-mediated protection in the lungs, either. Thus, mice vaccinated with BCG showed 0.95-, 1.06-, and 1.19-log_{10} reductions at days 21, 42, and 63, respectively, and BCG-vaccinated/IL-12-neutralized mice showed similar reductions of 1.2, 0.94, and 0.81 log_{10} mRLU (*P* < 0.01). However, it must be noted that at the last time point, 2/4 BCG/anti-IL-12-vaccinated mice had bacterial numbers comparable to TB-infected mice, indicating that BCG vaccination was affected to some extent at this last time point by the IL-12 inhibition (TB only, 4.38 ± 0.21 log_{10}; BCG, 3.19 ± 0.11 log_{10} [not significant compared to BCG]).

**Effect of anti-IL-12p70 vaccine on Th1/Th2 cytokine production in *M. tuberculosis*-infected and in *M. bovis* BCG-vaccinated mice.** As IL-12 is an important cytokine for the induction of IFN-γ-producing T helper 1 cells, we analyzed the production of this cytokine at week 3 post-TB infection in spleen and lung cells stimulated with PPD of tuberculin or the polyclonal T cell
mitogen ConA. Spleen cells from animals treated with the anti-IL-12 vaccine prior to TB infection showed 10-fold-reduced mycobacterium-specific IFN-\(\gamma\) responses compared to IFN-\(\gamma\) responses in TB-infected animals that had not received the anti-IL-12 autovaccine. The polyclonal ConA-stimulated IFN-\(\gamma\) response was affected by the IL-12 neutralization to the same extent as the PPD-specific response and was also reduced about 10-fold, but not totally abolished (Fig. 4). In contrast, spleen cells from BCG-vaccinated mice produced comparable mycobacterium-specific and ConA-induced IFN-\(\gamma\) levels, irrespective of IL-12 depletion (Fig. 4).

In lungs, IFN-\(\gamma\) production was more strongly affected by IL-12 neutralization than in the spleen, and PPD-specific IFN-\(\gamma\) responses were below the detection level in TB-infected mice treated with the anti-IL-12 autovaccine. In lungs from BCG-vaccinated animals, PPD-specific IFN-\(\gamma\) levels were about 5-fold higher than in TB-infected mice, reflecting the recruitment of effector memory T cells to the lungs following vaccination. IL-12 neutralization in BCG-vaccinated animals resulted in a reduction of these pulmonary PPD-specific IFN-\(\gamma\) levels to about 20%, comparable to the values detected in lungs from animals that had not received a prior BCG vaccine.

FIG. 4. Effect of anti-IL-12 autovaccine on IFN-\(\gamma\) production in \(M. tuberculosis\)-infected and in \(M. bovis\) BCG-vaccinated mice. Spleen cells from four individual mice and lung cells (pooled per group) were collected at 3 weeks post-TB challenge (experiment 4) and cultured without stimulation (white bars) or in the presence of PPD (gray bars) or ConA (black bars). The 72-h culture supernatants were assessed for IFN-\(\gamma\) activity by ELISA. Data are reported as mean titers in pg/ml (± SD, for spleen only).

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FIG. 5. Effects of anti-IL-12 autovaccine on IL-5 production in \(M. tuberculosis\)-infected and in \(M. bovis\) BCG-vaccinated mice. Spleen cells from four individual mice and lung cells (pooled per group) were collected at 6 weeks post-TB challenge and cultured without stimulation (white bars) or in the presence of PPD (gray bars) or ConA (black bars). The 72-h culture supernatants were assessed for IL-5 activity by ELISA. Data are reported as mean titers in pg/ml (± SD, for spleen only).
Pulmonary IFN-γ production following stimulation with recombinant Ag85B (a culture filtrate antigen from *M. tuberculosis* with well-documented vaccine potential [3]) was also abolished by the anti-IL-12 autovaccine treatment in TB-infected mice and reduced to about 30% in BCG-vaccinated mice (data not shown). Pulmonary IFN-γ responses following ConA stimulation were not affected by the IL-12 neutralization (Fig. 4).

We expected the drop in IFN-γ levels in anti-IL-12-vaccinated B6 mice to be accompanied by increased Th2 cytokine production. To verify this point, we measured IL-5 in the various culture supernatants. No significant mycobacterium-specific IL-5 production could be detected in response to PPD in any of the groups. However, a sharp increase in ConA-induced IL-5 was seen in spleen and particularly in lung cell cultures from anti-IL-12 autovaccine-treated mice, confirming that the anti-IL-12 autovaccine had prompted a shift to a more-Th2-dominated environment (Fig. 5). This increased ConA-stimulated IL-5 response was observed both in TB-infected and in BCG-immunized mice vaccinated with the IL-12 autovaccine. IL-10 levels were overall very low but showed a weak tendency to increase in spleen from BCG-vaccinated/IL-12-neutralized mice in response to ConA (data not shown).

TABLE 1. Cytokine levels in unstimulated lung cell culture supernatant at week 3 post-TB infection

| Cytokine | Mean cytokine level (pg/ml) from lungs of mice treated with: |
|----------|----------------------------------------------------------|
|          | TB | TB/anti-IL-12 | BCG | BCG/anti-IL-12 |
| TNF-γ    | 163 | 248 | 61 | 2,266 |
| IL-6     | 2,288 | 1,737 | 330 | 14,486 |
| IL-17A   | 1,252 | 984 | <9.2 | 2,496 |
| IL-1α    | 563 | 313 | 41 | 1230 |
| IL-4     | <7.9 | <7.9 | <7.9 | <7.9 |

* Cytokine levels were determined in culture supernatants of lung cells from mice sacrificed 3 weeks after TB challenge and cultured for 24 h without additional stimulation. Results are reported as cytokine levels in supernatants from lung cells pooled from three mice/group as determined by flow cytometry. Intra-assay variation was in the range of 10%. (Results shown are from experiment 4.)
contrast, bacterial replication of virulent *M. tuberculosis* is less controlled in IL-17A KO mice (29). γδ T cells are the primary source of IL-17A in the BCG model (34), and granuloma formation is deficient in IL-17A KO mice (29), confirming the pivotal role of γδ cells in mycobacterial granuloma formation (11). It was therefore interesting to evaluate IL-17A production in our different experimental groups. Significant IL-17A levels were detected in lung cells from BCG-vaccinated/TB-infected mice cultured in the presence of PPD, and these levels were highest in anti-IL-12-treated mice (Fig. 7). Elevated IL-17A levels were also measured in unstimulated lung cell cultures from BCG-vaccinated/anti-IL-12-treated and from TB-infected and TB/anti-IL-12 autovaccine-treated animals (Table 1). Upon stimulation of lung cells with ConA, the highest IL-17A levels (about 4,000 pg/ml) were detected in both groups of anti-IL-12-treated mice (Fig. 7). In spleen cell cultures, very low IL-17A levels were found in response to PPD. ConA stimulated IL-17A production in all groups, and the response was highest in the BCG-immunized/anti-IL-12 autovaccine-treated group (Fig. 7). Spontaneous IL-17A production was very weak in unstimulated spleen cell cultures (Table 2).

IL-6 is an important cytokine for Th17 cell development in mice (39). Given the enhancement of IL-17A production observed in anti-IL-12-vaccinated mice, we finally measured IL-6 production in lung and spleen cell cultures. As for IL-17A, the highest pulmonary IL-6 levels were detected in BCG-vaccinated/anti-IL-12 treated mice in response to PPD and ConA (Fig. 8). Spontaneous pulmonary IL-6 production was also highest in the latter group (Table 1). IL-1α levels were also increased in culture supernatants from unstimulated lung cells of BCG-vaccinated mice that had been treated with the anti-IL-12 autovaccine (Table 1).

In spleen cell cultures, PPD and ConA stimulated the highest IL-6 levels in TB-infected/IL-12-neutralized mice (Fig. 8). Spontaneous spleen cell IL-6 production was also significantly higher in the latter group (Table 2).

The general picture is, thus, that besides increased pulmonary TNF-α production, IL-12 neutralization in BCG-vaccinated mice also results in increased pulmonary IL-17A and IL-6 responses, two essential components of Th17 biology.

### DISCUSSION

The regulatory cytokines IL-12 and IL-23 are important for the differentiation of Th1 and Th17 CD4⁺ T cells, respectively. Both molecules have the IL-12p40 subunit in common and signal through a common IL-12Rβ1 chain in their receptor, and this chain is essential for biological responsiveness to IL-12 and IL-23 and regulation of optimal IFN-γ responses (30, 44). The IL-12 axis is essential for induction of protective immunity against *M. tuberculosis* infection (9), but the role IL-12 in the protection conferred by tuberculosis vaccines is less well doc-

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**TABLE 2. Cytokine levels in unstimulated spleen cell culture supernatant at week 3 post-TB infection**

| Cytokine | Mean ± SD cytokine level (pg/ml) from spleens of mice treated with: |
|----------|---------------------------------------------------------------|
|          | TB | TB/anti-IL-12 | BCG | BCG/anti-IL-12 |
| TNF-α    | 101 ± 24 | 158 ± 64 | 97 ± 96 | 68 ± 41 |
| IL-6     | 408 ± 126 | 1,968 ± 544** | 680 ± 761 | 472 ± 173 |
| IL-17A   | 44 ± 46 | 166 ± 253 | 82 ± 142 | 118 ± 73 |
| IL-1α    | 84 ± 81 | 212 ± 49 | 34 ± 58 | <6.1 |
| GM-CSF   | 37 ± 34 | 74 ± 87 | 17 ± 29 | 44 ± 75 |
| IL-4     | <7.9 | 33 ± 57 | <7.9 | 49 ± 46 |

* Cytokine levels were determined in culture supernatants of spleen cells from mice sacrificed 3 weeks after *M. tuberculosis* challenge and cultured for 24 h without additional stimulation. Results are reported as mean ± SD values of three mice tested individually by flow cytometry. **, *P* < 0.01. (Results are from experiment 4.)

**FIG. 7. Effects of anti-IL-12 autovaccine on IL-17A production in *M. tuberculosis*-infected and in *M. bovis* BCG-vaccinated mice. Spleen cells from four individual mice and lung cells (pooled per group) were collected at 3 weeks post-TB challenge and cultured without stimulation (white bars) or in the presence of PPD (gray bars) or ConA (black bars). The 72-h culture supernatants were assessed for IL-17A activity by ELISA. Data are reported as mean titers in pg/ml (± SD, for spleen only).**
Wozniak et al. showed that codelivery of plasmid DNA expressing IL-23 or IL-12 with plasmid DNA encoding Ag85B in IL-12p40-deficient mice induced strong proliferative and IFN-γ-secreting T cell responses equivalent to those observed in wild-type mice immunized with Ag85B DNA. This response resulted in partial protection against aerosol M. tuberculosis; however, the protective effect was less than that in wild-type mice (42).

As compensation mechanisms may occur in genetically deleted KO mice, we revisited this issue using mice in which the IL-12 axis was specifically neutralized by an anti-IL-12p70 autovaccine. Our results confirmed the effectiveness of the IL-12-(OVA)-PADRE-AS02V combination for successful induction of anti-IL-12 p70 antibodies, resulting in specific neutralization of IL-12 but not of IL-23 in vivo. In a previous report, we observed that mice vaccinated with complexes made by direct cross-linking of IL-12 and PADRE (not including OVA) produced mainly anti-p40 antibodies (as detected by ELISA) (35). In the present experiments, a much stronger inhibition of IL-12 than IL-23 was observed in functional inhibition assays. It is possible that use of a different vaccine formulation, in which IL-12 was cross-linked to both OVA and PADRE peptides, may have masked p40 epitopes while making p35 more accessible.

Our results provide further evidence for the critical role played by IL-12p70 in resistance to TB infection, even in C57BL/6 mice that were not deprived of this molecule during their development, unlike IL-12 p40-deficient animals (7). However, while anti-IL-12 immunization rendered C57BL/6 mice highly susceptible to acute M. tuberculosis infection, treatment with anti-IL-12 autovaccine had only a marginal effect on BCG-vaccinated animals, which were able to control TB growth for several months, although bacterial expansion eventually progressed.

Analysis of in vitro cytokine production by spleen and lung infiltrating cells stimulated with PPD or ConA showed changes in cytokine production, attesting to the ability of the anti-IL-12-(OVA)-PADRE-AS02V autovaccine to modify cytokine profiles, including a drop in mycobacterium-specific Th1 (IFN-γ) and an increase in ConA-stimulated Th2 (IL-5) and Th17 markers. IFN-γ is known to be a key player in resistance to TB (6), but our analysis identified several compensating factors putatively implicated in the resistance conferred by M. bovis BCG vaccine under IL-12p70-limiting conditions. The most impressive difference was observed in lungs at 3 weeks postinfection, when TNF-α was enhanced more than 10-fold in IL-12-(OVA)-PADRE-AS02V-vaccinated mice primed with BCG compared to BCG-primed mice that did not receive the anti-IL-12 autovaccine. This increase was noted even in unstimulated lung cell cultures, strongly supporting the in vivo relevance of the observed TNF-α increase. This observation fits well with the importance attributed to TNF-α in acquired resistance to TB (13) and particularly in the control of latent M. tuberculosis infection (17, 21). Increased TNF-α production was only observed in lungs, and not in spleens, from BCG-vaccinated/IL-12-neutralized mice. The reason for this discrepancy between the spleen and lungs is not clear at the moment, but differences in distributions of CD4+ and CD8+ T cells in both organs may be involved. CD8+ T cells have been reported to be more abundant in TB-infected lungs than in the spleen following BCG vaccination (15), and the production of TNF-α by CD8+ T cells is an essential component of their control of TB infection (5). Although we have no formal proof for this, our findings may indicate that IL-12 is less important for the expression of protective memory responses induced by the BCG vaccine at the level of CD8+ than of CD4+ T cells.

ConA-triggered IL-17A production was increased 2- to 3-fold after IL-12-(OVA)-PADRE-AS02V vaccination in spleen and lung cell cultures tested 3 weeks after TB infection. IL-23 is well known to promote Th17 responses (1) and to be
required for IL-17 production during tuberculosis (22). Moreover, alternative stimuli of Th17 development, like IL-2, IL-15, IL-18, and IL-21, have been described (19, 41). Finally, besides Th17 cells, γδ T cells are known to be potent producers of IL-17A in *M. tuberculosis*-infected (26) and BCG-vaccinated mice (34), and innate production of IL-17 by these cells can be induced not only by IL-23 but also by IL-1 (32). Whatever the mechanism, it is likely that the increased IL-17A production was an attempt of the IL-12-depleted host to control TB expansion. Delivery of IL-17 to the lungs of IL-23p19-deficient and TB-infected mice was shown to restore the early accumulation of IFN-γ-producing cells, in agreement with the cellular recruitment function of chemokines induced by Th17 cells (22). Furthermore, IL-17 is known to act synergistically with TNF-α (28), and this synergism may further explain the limited effect of the anti-IL-12 autovaccine on BCG-mediated protection, even in the presence of significantly reduced IFN-γ production.

The results presented here are reminiscent of findings reported by Wozniak et al. in IL-12p40-deficient mice, who showed that bacillus Calmette-Guérin immune T cells generated in the absence of IL-12 and IL-23 exhibit a strong IL-17 secretion associated with some degree of protection against pulmonary *M. tuberculosis* infection (41, 43). By using an alternative anti-IL-12 autovaccine approach, we have confirmed these findings, monitoring the protective effects of BCG over a period of 9 weeks. Moreover, we have demonstrated that the increased IL-17 response is accompanied by increased TNF-α and IL-6 secretion. An essential difference between our report and the one of Wozniak and colleagues is the fact that we analyzed the effect of IL-12 neutralization on established BCG-induced memory, whereas in Wozniak’s study IL-12p40−/− mice were used for the induction of BCG-induced memory. Another important difference is the fact that our auto-IL-12 vaccine neutralized specifically the IL-12 but not the IL-23 axis, whereas in IL-12p40−/− KO mice the production of both cytokines was affected.

The present IL-12-(OVA)-PADRE autovaccine procedure induces neutralizing anti-IL-12p70 autoantibodies. It confers resistance to EAE (35) and impairs atherosclerosis in LDL-R−/− mice (18), but it increases sensitivity to infection by *L. major* (35). The present results show that it also increases susceptibility to TB infection but that protection mediated by BCG vaccination is not (or only modestly) affected, probably because of combined TNF-α and IL-6/IL-17A-mediated compensation mechanisms. The question remains of whether IL-12 neutralization with an anti-IL-12 autovaccine would result in reactivation of latent tuberculosis infection, as has been reported for treatment with anti-TNF-α antibodies (21, 27), but our observation that TNF-α production is upregulated in IL-12-neutralized mice suggests that this risk of reactivation might be low.

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