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Maintenance of Pulmonary Th1 Effector Function in Chronic Tuberculosis Requires Persistent IL-12 Production

Carl G. Feng,† Dragana Jankovic,* Marika Kullberg,* Allen Cheever, † Charles A. Scanga,* Sara Hieny,* Patricia Caspar,* George S. Yap, ‡ and Alan Sher*

The mechanisms that prevent reactivation of latent *Mycobacterium tuberculosis* infection in asymptomatic individuals are poorly understood. Although IL-12 is critical for the induction of IFN-γ-dependent host control of *M. tuberculosis*, the requirement for the cytokine in the maintenance of host resistance and pulmonary Th1 effector function has not yet been formally examined. In this study, we reconstituted IL-12p40-deficient mice with IL-12 during the first 4 wk of infection and then assessed the effects of cytokine withdrawal. Although IL-12 administration initially resulted in restricted mycobacterial growth and prolonged survival, the reconstituted animals eventually succumbed to infection. This breakdown in bacterial control was accompanied by a marked reduction in the numbers of IFN-γ-producing CD4+ T cells in lungs. Moreover, whereas CD4+ T cells isolated from chronically infected wild-type mice expanded and transferred long-term protection to *M. tuberculosis*-challenged RAG−/− mice, they failed to do so in IL-12p40-deficient RAG−/− recipients and were clearly reduced in frequency within pulmonary granulomas in the latter animals. These studies establish that continuous IL-12 production is necessary for maintenance of the pulmonary Th1 cells required for host control of persistent *M. tuberculosis* infection and suggest that breakdown of this mechanism could be a contributing factor in reactivated disease. The Journal of Immunology, 2005, 174: 4185–4192.

Although an estimated one-third of the world’s population is infected with *Mycobacterium tuberculosis*, only 5–10% of this group develop active disease in later life. The immunologic explanation for the breakdown of host resistance in these individuals is unclear. A major hypothesis based largely on studies in experimental models is that the reactivation of latent infection stems from a failure of tissue granulomas to contain the organism (1), particularly in lungs, its major target organ. Mycobacterial granulomas are typically composed of lymphocytes and infected macrophages (2, 3). The former cell population, in addition to walling off infected cells, is thought to provide cytokine mediators necessary for macrophage activation and restriction of intracellular growth of mycobacteria (1). Therefore, the development of defects in lymphocyte recruitment, retention, or effector function within granulomas during chronic infection could explain the breakdown of latency and induction of disease.

Host control of *M. tuberculosis* infection in both humans and mouse models has been shown to be associated with the production of IFN-γ by CD4+ T cells (4). IL-12 is known to be a crucial cytokine in the differentiation of IFN-γ-producing Th1 cells (5), and patients with genetic defects in IL-12 signaling have been reported to display increased susceptibility to environmental mycobacteria and bacillus Calmette-Guérin (6). Although only a few of these individuals have been diagnosed with tuberculosis per se (7), a role for IL-12 in resistance to *M. tuberculosis* was suggested by the improved clinical outcome observed when the cytokine was combined with drug therapy in a case study (8).

Early administration of IL-12 to *M. tuberculosis*-infected BALB/c mice has been shown previously to result in significantly decreased bacterial burdens and increased mean host survival time (9) and in other settings to reduce pathology (10). Moreover, neutralization of IL-12 at the initiation of *M. tuberculosis* infection led to increased bacterial loads and reduced granuloma integrity (11). In parallel studies, IL-12p40−/− mice were found to be highly susceptible to *M. tuberculosis* infection, a defect associated with decreased IFN-γ production by these animals (12–14).

Although the above experiments established an essential role for IL-12 in the induction of Th1-dependent control of mycobacterial growth, they did not address whether continuous production of the cytokine is required to maintain IFN-γ-producing effector CD4+ T cells and host control of persistent infection once the Th1 response has been initiated. This issue is of particular importance in tuberculosis because a breakdown in IL-12 production and/or responsiveness could provide an explanation for disease reactivation. Indeed, while IL-12 was initially thought to be important only in the initiation of antimicrobial immunity (15, 16), more recent reports have demonstrated a major requirement for the cytokine in the maintenance of effector Th1 responses during parasitic infections (17–19). The latter studies, however, did not examine whether continuous IL-12 production is necessary for persistent Th1 reactivity in nonlymphoid tissue sites, such as the lungs, and if so whether this requirement is explained by effects on regional Th1 cell dynamics or cytokine production.

In the present study, we investigate the requirement for persistent IL-12 production in the maintenance of Th1 responsiveness and bacterial control in *M. tuberculosis*-infected mice. Our data indicate that in IL-12-reconstituted IL-12p40−/− mice, continuous treatment with the cytokine is necessary for maintenance of pulmonary Th1 cell numbers, granuloma formation, and mycobacterial immunity, and that adoptively transferred CD4+ T cells from

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chronically infected donors require IL-12p40 for both their expansion and effector function. Together, these findings suggest that defects leading to an interruption in IL-12p40 signaling could contribute to the breakdown of latency that manifests as clinical tuberculosis.

Materials and Methods

**Experimental animals**

Wild-type (WT) C57Bl/6 mice were obtained from the Division of Cancer Treatment, National Cancer Institute. Mice deficient in IL-12p40 or RAG2 were provided by Taconic Farms from the National Institute of Allergy and Infectious Diseases Animal Supply Contract. IL-12p40 × RAG2 double knockout (KO) mice were generated by crossing single gene-deficient animals. All mice were maintained in specific pathogen-free conditions at an American Association of Laboratory Animal Care-accredited, BioSafety Level 3 physical containment animal facility at the National Institute of Allergy and Infectious Diseases, National Institutes of Health. Female 8- to 12-wk-old mice were used in all experiments.

**M. tuberculosis infection and measurement of bacterial loads**

*M. tuberculosis* H37Rv harvested from infected mouse lungs were expanded in Middlebrook 7H9 broth supplemented with oleic acid-albumin-dextrose-catalase (Difco) for 14 days at 37°C and stored in aliquots at −80°C. Mice were exposed to *M. tuberculosis* via aerosol route by using a nose-only exposure unit (CH Technologies). Each mouse received ~100 CFU measured in lung at 24 h after exposure. Bacterial loads in infected organs were quantitated by culture on 7H11 agar (Difco).

**In vivo reconstitution of IL-12p40−/− mice with rIL-12**

Beginning on the day of bacterial exposure, IL-12p40−/− animals were injected i.p. twice weekly with 0.25 μg of murine IL-12p70 protein (a gift from Wyeth Research) until day 28 of infection.

**Adoptive transfer of CD4+ T cells**

Pooled splenocytes and mediastinal lymph node cells from 12- to 18-wk infected WT mice were passed through T cell enrichment columns (R&D Systems), and the resulting cell populations were incubated with anti-CD4 MACS beads (Miltenyi Biotec). CD4+ T cells were then positively selected using magnetic cell sorting. The resulting population contained greater than 90% CD4+ T cells, as determined by flow cytometry (data not shown). For adoptive transfer experiments, RAG2−/− or IL-12p40−/− animals were injected i.v. with the purified CD4+ T cells (10⁶/mouse) 1 day after *M. tuberculosis* infection.

**Purification of pulmonary leukocytes**

Lungs were perfused with PBS through the heart before removal from mice. Single cell suspensions were prepared by passing lung tissue through 40-μm nylon cell strainers. To enrich pulmonary leukocytes, lung cells were centrifuged in 35% Percoll (Pharmacia Biotech) for 15 min at 700 × g. The cell pellets were then collected, and erythrocytes were lysed.

**ELISPOT assay**

Ag-specific IFN-γ-producing cells in lungs were enumerated using an ELISPOT assay. Ninety-six-well nitrocellulose plates (MultiScreen IP; Millipore) were coated with 15 μg/ml anti-IFN-γ mAb (clone AN18; Mabtech) in PBS overnight at 4°C. After blocking with complete RPMI 1640 medium (RPMI 1640 medium supplemented with 10% heat-inactivated FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 μM glutamine, 10 mM HEPES, and 50 μM 2-ME) for 1 h at 37°C, serial dilutions of pulmonary leukocytes (starting at 8 × 10⁵/well) in complete RPMI 1640 medium were added to wells containing irradiated syngeneic WT splenocytes (5 × 10⁵/well) in the presence or absence of purified protein derivative from *M. tuberculosis* (PPD; 10 μg/ml; Statens Seruminstitut). The plates were then incubated for 16 h at 37°C and washed extensively with PBS. After incubation with 1 μg/ml second biotinylated anti-IFN-γ mAb (clone R4-6A2; Mabtech) for 2 h at room temperature, the plates were washed and incubated with alkaline phosphatase-conjugated streptavidin. The spots were visualized using an alkaline phosphatase substrate kit (Bio-Rad). Specific responses were quantitated by subtracting the number of spots detected in the absence of PPD. The frequencies of PPD-specific CD4+ T cells were determined by dividing the number of Ag-specific spots per well by the number of CD4+ T cells plated per well. The numbers of CD4+ T cells were calculated by multiplying the number of viable cells by the percentage of CD4+ T cells determined by flow cytometry.

**Flow cytometry**

Single cell suspensions from individual mice were stained, as previously described (20), with minor modifications. For intracellular detection of IFN-γ, total lung cells were stimulated with plate-bound anti-CD3 (clone 145-2C11; 10 μg/ml) at 37°C for 6 h, and brefeldin A was added during the last 2 h. Cells were then surface stained with mAb to CD4 (clone RM4-5) and CD8 (clone 53-6.7), fixed, and permeabilized. Intracellular IFN-γ was detected with anti-IFN-γ-mAb (clone XMG1.2). Data were collected using a FACS Calibur (BD Immunocytometry Systems) with CellQuest (BD Biosciences) and analyzed with FlowJo (Tree Star) program. All mAb were obtained from BD Pharmingen.

**Delayed-type hypersensitivity (DTH) measurements**

Four-week-infected mice were inoculated intradermally in one footpad with 10 μg of PPD and in the contralateral footpad with an equal volume of sterile PBS. Footpad thickness was measured 48 h later with dial calipers.

**Histopathology**

Tissues were fixed with Formalin, sectioned, and stained with H&E. The Ziehl-Neelsen method was used to stain acid-fast mycobacteria in tissue sections.

**Statistics**

Student’s t test was used to determine the significance of differences between groups. Values of p < 0.05 were considered significant.

**Results**

**IL-12 reconstitution results in transient control of mycobacterial infection in IL-12p40−/− mice**

It has been shown previously that high levels of IL-12p40 expression are maintained during chronic infection with *M. tuberculosis* (21, 22). As a first step in evaluating the role of IL-12 in the maintenance of host resistance and the Th1 response to persistent *M. tuberculosis* infection, we tested the effects of Ab-mediated neutralization of the cytokine on bacterial burdens in lung and spleen. In these experiments, in contrast to those in previous studies, we deliberately initiated anti-IL-12 mAb injection during chronic infection at 12 wk following aerosol exposure. Administration of anti-IL-12p40 mAb in vivo resulted in only minimal elevations in tissue CFU in chronically infected mice, and these differences were statistically significant in the case of the splenic bacterial counts (data not shown).

Because it could be argued that the minor effect of Ab treatment observed reflects incomplete neutralization of the cytokine, we adopted two additional strategies for evaluating the role of IL-12 in the maintenance of host resistance to *M. tuberculosis*. In the first of these approaches, rIL-12 was administered to IL-12 p40-deficient mice thrice weekly beginning on the day of aerosol infection and for a total 28 days, and mice were assessed for both survival and bacterial burdens. As previously described (13, 14), IL-12 p40−/− mice were highly susceptible to aerosol *M. tuberculosis* infection and succumbed by ~60 days postinfection (p.i.) (Fig. 1A). In contrast, all IL-12-reconstituted IL-12 p40−/− animals remained alive during the same time period. The increase in survival resulting from cytokine reconstitution was associated with significantly reduced pulmonary and splenic CFU measured at 28 days p.i. (Fig.

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2 Abbreviations used in this paper: WT, wild type; DTH, delayed-type hypersensitivity; KO, knockout; p.i., postinfection; PPD, purified protein derivative from *M. tuberculosis*. 
In contrast, the equivalent tissues from IL-12p40-infected WT mice displayed well-formed granulomas as well as a reversion to a normal histological appearance in which the lymphocytes were retained (Fig. 2F). Acid-fast staining of the same sections at day 84 revealed that pulmonary granulomas of WT mice contained only very small numbers of bacilli (Fig. 2G), whereas lesions in the IL-12-reconstituted KO animals displayed abundant mycobacteria (Fig. 2H). This loss in bacterial control was evident even at the level of individual macrophages.

Cytokine withdrawal in infected IL-12-reconstituted IL-12p40−/− mice leads to reduced Th1 responses

Before examining the effects of IL-12 withdrawal on mycobacterium-induced Th1 responses, we first asked whether CD4+ T cells from IL-12p40−/− mice reconstituted with IL-12 do indeed produce high levels of IFN-γ. When isolated at day 28 and stimulated with anti-CD3 for 6 h ex vivo, greater than 33% of pulmonary CD4+ T cells in both WT and IL-12-reconstituted KO mice stained positively for intracellular IFN-γ vs <9% in IL-12p40−/− animals (Fig. 3A). Comparable results were obtained when the frequency of M. tuberculosis-specific IFN-γ-producing cells in lungs was quantitated by ELISPOT assays (Fig. 3B). Although the frequency of total CD4+ T cells in the lungs of the reconstituted IL-12p40−/− mice (21%) was considerably lower than that observed in the equivalent organs from WT animals (40%), it was higher than their nonreconstituted IL-12p40−/− counterparts (12%) (Fig. 3A), suggesting that IL-12p40 is required for the expansion or maintenance of CD4+ T cells potentially able to synthesize IFN-γ in the infected lungs. Perhaps related to this observation, IL-12 treatment failed to restore DTH responsiveness to IL-12p40−/− mice when measured by skin testing with PPD at day 28 p.i. (Fig. 3C).

In addition to CD4+ T lymphocytes, pulmonary CD8+ T cells are also known to produce IFN-γ during M. tuberculosis infection (23, 24). Nevertheless, the same frequency of total and IFN-γ+CD8+ T cells was observed in lungs of infected WT, IL-12p40−/−, and IL-12-reconstituted IL-12p40−/− mice, arguing that CD8+ T cells IFN-γ responses are IL-12p40 independent (Fig. 3A).

Although IL-12 reconstitution clearly restored the ability of IL-12p40−/− mice to mount a protective Th1 response against M. tuberculosis, this capacity decayed following the cessation of IL-12 treatment at day 28. When analyzed at day 84 p.i., lungs from IL-12-reconstituted IL-12p40−/− mice displayed a marked reduction in the frequency of IFN-γ+CD4+ T cells compared with WT animals assayed at the same time point (Fig. 3D) or IL-12-reconstituted IL-12p40−/− mice measured at the time of cytokine withdrawal (Fig. 3A). This frequency was comparable to that observed in nonreconstituted IL-12p40−/− animals at day 28. A similar loss in PPD-specific IFN-γ-producing T cells was evident using ELISPOT assay to pulmonary effector cells (Fig. 3E).

Thus, within a period of 56 days or less, IL-12 withdrawal is associated with a reduction in the frequency of M. tuberculosis-primed Th1 cells to the levels found in IL-12p40−/− mice. This loss in Th1 responsiveness was also reflected in the decrease in the percentage of total CD4+ T cells (from 21 to 14%) in lung tissues (Fig. 3, A and D).

Primed CD4+ T cells adoptively transfer protection to M. tuberculosis-challenged RAG−/−, but not IL-12p40-deficient RAG−/− mice

To further examine the requirement for IL-12p40 in the maintenance of Th1 effector cells and host resistance in M. tuberculosis infection, we compared the protective effects of transferred CD4+ T cells from chronically infected donors into IL-12p40-sufficient vs deficient RAG−/− recipient animals (Fig. 4). As expected, both
RAG−/− and IL-12p40−/− × RAG−/− mice were unable to control *M. tuberculosis* infection, and all succumbed by day 45 p.i. (Fig. 4A). In contrast, when reconstituted with purified CD4+ T cells from 12- to 18-wk infected WT mice, the IL-12-sufficient RAG−/− recipients displayed prolonged survival (median survival time = 120 days). IL-12-deficient RAG−/− recipients, in contrast, exhibited transient control of infection (median survival time = 65 days). When examined at day 28 p.i., CD4+ T cell-reconstituted RAG−/− and IL-12p40−/− × RAG−/− animals displayed significant reductions in pulmonary bacterial loads compared with their nonreconstituted counterparts, these differences being more pronounced in spleen (Fig. 4B). However, by day 49 p.i., CD4+ T cell-reconstituted IL-12p40−/− × RAG−/− animals exhibited an approximate 2- to 3-log increase in CFU in both lungs and spleens compared with RAG−/− recipients (Fig. 4C).

Flow cytometric analysis of pulmonary cell populations was performed to determine whether the observed differences in host resistance in the IL-12-deficient vs sufficient recipients were reflected in the numbers of IFN-γ-producing CD4+ T cells present (Fig. 5). These experiments revealed that at day 49 the lungs of IL-12-sufficient recipients contained ~50% more IFN-γ+ CD4+ T cells than IL-12-deficient recipients, and that the cells in the former animals produced more cytokine as assessed by a ~2-fold increase in mean florescence intensity (Fig. 5A). Although the numbers of CD4+ T cells in lungs (Fig. 5B) and spleens (3.8 ± 0.19 vs. 3.4 ± 0.10 × 10^6) of reconstituted, but uninfected RAG−/− and IL-12p40−/− × RAG−/− animals were comparable at 28 days after transfer, a major difference was observed in the numbers of total CD4+ T cells recovered from the lungs of the two groups of recipients following infection. Thus, at day 28 p.i., lungs of the IL-12-sufficient reconstituted mice contained greater than 2-fold more CD4+ T cells than their IL-12-deficient counterparts, and this difference increased to over 7-fold by d49 (Fig. 5B). As a consequence of the reduction in the percentage of IFN-γ+ CD4+ T cells and the total CD4 numbers, significantly lower numbers of pulmonary IFN-γ+ CD4+ T cells were present in the lungs of reconstituted IL-12-deficient RAG−/− mice than those of IL-12-sufficient recipients (Fig. 5C). Consistent with these findings, pulmonary granulomas of T cell-reconstituted IL-12p40-deficient RAG−/− recipients showed far less lymphocyte infiltration than their counterparts in similarly reconstituted IL-12-sufficient RAG−/− animals when examined at the day 49 time point (Fig. 5D). Together, these observations indicated that IL-12p40 is important to expand Th1 effector cells in *M. tuberculosis*-infected lungs.

**Discussion**

Although IL-12-dependent IFN-γ production is clearly important in the induction of host resistance to *M. tuberculosis* (4, 13, 14), much less is known about the role of this pathway in containing chronic infection once initial control of the pathogen has been established. Because CD4+ T cells (25) as well as production of TNF (26, 27), IFN-γ (28, 29), and NO (30) have each been shown to be required for maintenance of latent *M. tuberculosis* in mice, it has been assumed that IL-12-dependent Th1 effector function underlies chronic as well as acute control of infection. Nevertheless, a requirement for IL-12 at this stage has never been formally demonstrated. Our initial attempt to approach this question by treatment with anti-IL-12p40 mAb resulted in only marginal effects on bacterial loads, leaving open the possibility of incomplete neutralization, a technical problem confronted previously in other studies using this strategy (31, 32). Therefore, we used two alternative methods to specifically block IL-12 function. First, we used an anti-IL-12p40 mAb that did not block other cytokines such as IL-12, IL-18, IL-35, or IL-39 (33). As expected, this treatment resulted in only marginal effects on bacterial loads (Fig. 1A). Second, we used a strategy designed to block IL-12 production at the transcriptional level by transfection with an anti-IL-12p40 siRNA to abrogate its expression (34). This approach also had only marginal effects on bacterial loads (Fig. 1B). Given these results, we conclude that IL-12 is not a critical driver of chronic *M. tuberculosis* infection in vivo.
approaches using cytokine or CD4+ T cell reconstitution of IL-12p40−/− animals. The results from both types of experiments establish a major role for IL-12 in the maintenance of pulmonary Th1 cell numbers as well as their host-protective effector function in chronic M. tuberculosis infection.

IL-12 is thought to serve a number of distinct functions in the regulation of CD4+ T cell responses. IL-12 is known to drive Th1 effector choice, although recent studies have indicated that its role in this pathway is not obligatory (33–35). Although originally thought to function primarily during the initiation of Th1 responses (15, 16), a more up-to-date view is that IL-12 by maintaining both the strength of IFN-γ production and Th1 cell survival/expansion also plays a critical role in determining Th1 response persistence (5). That IL-12 is critical for the maintenance of IFN-γ production by CD4+ T cells in vivo was first revealed in three studies in which continuous IFN-γ-dependent resistance to Leishmania major (17, 18) and Toxoplasma gondii (19) infections. Later studies in the Leishmania model demonstrated that part of the requirement for IL-12 in these experiments reflects a role for IL-12 in maintaining Th1 cell numbers in draining lymph nodes (36), a conclusion supported by subsequent work analyzing the function of the cytokine in Th1 expansion/survival in an in vitro system (34).

The results presented in this work in which Th1 function was measured in M. tuberculosis-infected lung provide the first evidence demonstrating that IL-12 plays a major role in the maintenance of Th1 effector cells in a nonlymphoid tissue. In both of the approaches used, the absence of IL-12p40 led to a decrease in pulmonary CD4+ T cell IFN-γ production not only at the level of the total population, but also in terms of individual cells, as evidenced by mean fluorescence intensity. At the same time, IL-12p40 clearly affected both the frequency and numbers of CD4+ T

FIGURE 3. Transient IL-12 treatment is sufficient to establish, but not sustain, Th1 responses in the lungs of infected IL-12p40−/− mice. Lung cells isolated from day 28 (A) or 84 (D) infected mice were stimulated with plate-bound anti-CD3 mAb for 6 h, and intracellular IFN-γ production was determined by flow cytometry after gating on lymphocyte populations as described. The FACS profiles shown are representative of two performed.

FIGURE 4. Primed CD4+ T cells adoptively transfer prolonged protection to infected RAG−/−, but not IL-12p40-deficient RAG−/− mice. CD4+ T cells purified from mediastinal lymph nodes and spleens of 12- to 18-wk infected WT animals were adoptively transferred by i.v. injection into RAG−/− or IL-12p40−/− x RAG−/− mice aerogenically infected with M. tuberculosis 1 day previously. Survival of infected animals was monitored thereafter (A). Pulmonary and splenic CFU determined in nonreconstituted (□) and reconstituted (□) animals at day 28 p.i. (B), as well as at day 49 p.i. (C) in CD4+ T cell-reconstituted IL-12p40-sufficient (□) and IL-12p40-deficient RAG−/− (□) mice are shown (mean ± SD, n = 3–4, *, p < 0.05. All experiments shown are representative of two performed.
cells maintained in lung tissue in each of the two reconstitution scenarios. Whether this outcome reflects IL-12-dependent effects on Th1 cell proliferation (37–39) or survival (40–42) is unclear from the present data. Moreover, because these analyses were performed in a defined organ site, a possible function of IL-12p40 in lymphocyte recruitment/migration cannot be excluded. Indeed, a previous study has suggested that IL-12 may play a role in promoting the exit of CD4+ T cells from lymphoid organs (34). Pulmonary CD4+ T cell numbers in M. tuberculosis-infected mice have been shown to persist with a low proliferation rate for a prolonged period following adult thymectomy (43). This observation suggests that the effect of IL-12 withdrawal in our experiments reflects a major function for IL-12 in regulating CD4+ T cell survival within these lesions, or, alternatively, a requirement for the cytokine in determining the Th1 phenotype of de novo primed CD4+ T cells during the chronic infection.

The p40 subunit of IL-12 is also shared by IL-23, in which it dimerizes with p19 rather than p35 (44). Therefore, IL-12p40−/− mice are both IL-12 and IL-23 deficient, and it could be argued that IL-23 may partially explain the effects on host resistance and Th1 effector response observed in the present study. Indeed, a role for IL-23 in immunity to M. tuberculosis was suggested by the observation that while both p35−/− and p40−/− mice are susceptible to infection, the former animals are clearly more resistant (13, 14). However, more recent work has indicated that p19−/− mice are highly resistant to aerosol M. tuberculosis infection and develop normal Th1 responses to the pathogen (45), arguing that it is unlikely that the IL-12p40−/−dependent effects on host resistance and CD4+ T cell IFN-γ production described in this study reflect the contribution of IL-23.

Although not required for the development of IFN-γ-producing cells, IL-23 has been shown to contribute to the induction and maintenance of inflammatory responses (46, 47). We found that granuloma formation in the lungs of IL-12-reconstituted IL-12p40 KO mice never reached the extent of that observed in infected WT animals. Moreover, the same IL-12-reconstituted IL-12p40 KO mice displayed impaired DTH responses to PPD. Although the possibility of incomplete reconstitution due to the limited period of IL-12 administration or the failure of the cytokine to reach the appropriate tissue site cannot be formally ruled out, one possible explanation is that the partial restoration observed in these experiments stems from the lack of IL-23 in the cytokine-treated animals. Indeed, previous studies have demonstrated that in contrast to IL-12p40−/− mice, M. tuberculosis-infected p35−/− animals are able to generate normal delayed skin test reactions to PPD (13, 14), indicating that IL-12 is not required for DTH responses to this pathogen. Moreover, IL-23 has been shown to be required for DTH responses (48). Based on these observations, a contribution of IL-23 to the inflammatory response to M. tuberculosis would be expected. Nevertheless, the data reported in this work and elsewhere (45, 49) argue that in terms of both host resistance to intracellular pathogens and regulation of CD4+ T cell IFN-γ production, IL-12, rather than IL-23, is the critical cytokine involved. The possible contribution of other as yet to be described p40-containing members of the IL-12 family has not yet been formally ruled out in the current study, particularly in the CD4+ T cell reconstitution experiments. However, because rIL-12p70 was used in the cytokine restoration approach described in this study, it is clear that IL-12 itself is sufficient to mediate the effects on Th1 response observed. Studies involving IL-12p35−/− as opposed to IL-12p40−/− RAG−/− recipients have been initiated to directly address this point.
Our observation of a requirement for continuous IL-12 production in the control of *M. tuberculosis* infection raises the question as to whether IL-12 impairment underlies the breakdown of latency that leads to reactivated tuberculosis. If, as described in this study, IL-12 is necessary for both the persistence of effector CD4+ T cells in infected lung and their continued production of IFN-γ, then immunoregulatory changes negatively influencing IL-12 synthesis or function would be expected to lead to a loss in Th1-dependent control of bacterial growth within granulomas and reactivation of infection. Indeed, it is clear from previous work in experimental models (25) and the re-emergence of tuberculosis seen in AIDS patients (50, 51) that CD4 depletion can lead directly to breakdown of granulomas and reactivated disease. Similarly, depletion of IFN-γ has been shown to reactivate *M. tuberculosis* infection in the Cornell latency model (28, 29), and based on the increased susceptibility of patients with IFN-γ receptor signaling defects (6), one would predict a similar causal relationship in infected humans. Nevertheless, an effect of IL-12 deficiency/impairment on IFN-γ synthesis by CD4+ T cells need not necessarily lead to reactivated disease, because both CD4+ T cell-dependent, IFN-γ-independent (25, 52), as well as CD8+ T cell-dependent, IFN-γ-dependent (29, 53) control of *M. tuberculosis* has been described. Thus, IL-12 is more likely to regulate latency based on its pronounced effects on the maintenance of *M. tuberculosis*-specific Th1 cell numbers in lung.

Regardless of the final impairment mechanism, a number of different immunoregulatory alterations occurring during chronic *M. tuberculosis* infection could lead to a loss in IL-12 activity. For example, an age-influenced development of regulatory T cells producing down-modulating cytokines, such as IL-10 and/or TGF-β, could contribute to a damping in IL-12 synthesis and/or function. Comparable effects could result from changes in IL-12R expression due to factors such as increased Th2 cytokine (e.g., IL-4) production (54–57). Correlative studies on IL-12 or IL-12R gene expression in *M. tuberculosis*-infected populations analogous to those previously reported in patients with tuberculosis vs lepromatous leprosy (58) could be used as initial approach to address the involvement of IL-12 in the maintenance of latency and its breakdown.

In addition to its relevance to mechanisms of latency, the finding that persistent IL-12 production is necessary for the maintenance of Th1-dependent host resistance to *M. tuberculosis* is of direct importance in vaccine design. Previous studies in an L. major experimental model showed that when used as an adjuvant with a DNA vaccine, IL-12 administered in a form of a recombinant plasmid stimulates long-term Th1-mediated immunity against parasite challenge, whereas soluble IL-12 induces only transient protection (59). This work argued that, in addition to persistent Ag, continuous production of IL-12 is necessary to sustain protective Th1 responses elicited by vaccines. Plasmid IL-12 DNA has also been shown to enhance DNA vaccination against aerosol *M. tuberculosis* (60) and to have therapeutic value as a treatment for latent infection (61). Although not directly evaluated, it is possible that its efficacy in the latter studies was due to the persistent cytokine production resulting from this form of delivery. A requirement for continuous IL-12 production may also explain the superior protection induced by inoculation of live bacillus Calmette-Guerin as opposed to replication-deficient avutzrophic mycobacteria (62, 63). One important implication of this hypothesis is that in vaccine approaches using nonlivering Ag, boosting strategies that stimulate IL-12 production should be used to ensure the maintenance of the Th1 effector/memory cells required for long-term protection against *M. tuberculosis*.

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