IL-23 Is Required for Long-Term Control of Mycobacterium tuberculosis and B Cell Follicle Formation in the Infected Lung

Shabaana A. Khader, Lokesh Guglani, Javier Rangel-Moreno, Radha Gopal, Beth A. Fallert Junecko, Jeffrey J. Fountain, Cynthia Martino, John E. Pearl, Michael Tighe, Yin-yao Lin, Samantha Slight, Jay K. Kolls, Todd A. Reinhart, Troy D. Randall and Andrea M. Cooper

J Immunol 2011; 187:5402-5407; Prepublished online 14 October 2011; doi: 10.4049/jimmunol.1101377
http://www.jimmunol.org/content/187/10/5402

References
This article cites 31 articles, 16 of which you can access for free at:
http://www.jimmunol.org/content/187/10/5402.full#ref-list-1

Subscription
Information about subscribing to The Journal of Immunology is online at:
http://jimmunol.org/subscription

Permissions
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
IL-23 Is Required for Long-Term Control of *Mycobacterium tuberculosis* and B Cell Follicle Formation in the Infected Lung

Shabaana A. Khader,* Lokesh Guglani,* Javier Rangel-Moreno,† Radha Gopal,*
Beth A. Fallert Junecko,‡ Jeffrey J. Fountain,§ Cynthia Martino,§ John E. Pearl,§
Michael Tighe,§ Yin-yao Lin,* Samantha Slight,* Jay K. Kolls,¶ Todd A. Reinhart,¶
Troy D. Randall,‡ and Andrea M. Cooper§

IL-23 is required for the IL-17 response to infection with *Mycobacterium tuberculosis*, but is not required for the early control of bacterial growth. However, mice deficient for the p19 component of IL-23 (Il23a−/−) exhibit increased bacterial growth late in infection that is temporally associated with smaller B cell follicles in the lungs. Cxcl13 is required for B cell follicle formation and immunity during tuberculosis. The absence of IL-23 results in decreased expression of Cxcl13 within *M. tuberculosis* infection that is temporally associated with smaller B cell follicles in the lungs. Cxcl13 is required for B cell follicle formation in this regard. The Journal of Immunology, 2011, 187: 5402–5407.

Tuberculosis remains a worldwide threat to world health that resists ongoing attempts at control. The increasing threat from drug-resistant strains requires that we understand the host immune response as this will allow us to promote protective responses by vaccination and to limit pathologic and regulatory responses. B cell follicles are formed in *Mycobacterium tuberculosis*-infected lungs in both mouse and human (1, 2); however, the induction and maintenance of these follicles and the role that they play in protection has not been extensively studied. B cells and Ig

---

**Materials and Methods**

**Mice and infection**

Mice were bred at the Trudeau Institute (Saranac Lake, NY) or the University of Pittsburgh (Pittsburgh, PA). IL-23p19−/−mice (Il23a−/−) were provided by Dr. Nico Ghiardi and Dr. Fred deSauvage (Genentech, South San Francisco, CA) (9). IL-17A−/−mice were from Angen (Thousand Oaks, CA) (10). IL-22−/−mice (Il22−/−) were from Dr. Wenjun Ouyang (Genentech) (11). C57BL6/J mice (B6) were used as wild-type controls. Age- and sex-matched mice were used between the ages of 7 and 12 wk. Mice received either isotype control Ab (clone 50104) or 100 μg anti–IL-17A (clone 50104; both from R&D...
or the University of Pittsburgh. Institutional Animal Care and Use Committees at either Trudeau Institute
CFUs in homogenized tissue (12). All experiments were approved by the
matically, as described (12). Bacterial numbers were counted by viable
in 10% FBS (Invitrogen) in DMEM (14).

sections were fixed with acetone/ethanol (75:25) for 10 min at room
and 30 min. The digest was then passed through a 70-μm filter and released cells cultured
in 10% FBS (Invitrogen) in DMEM (14).

Detection of IFN-γ-producing cells
Lung and draining lymph node tissue was prepared as described (12). Ag-specific IFN-γ-producing 1A-restricted T cells from infected lungs or lung draining lymph nodes were enumerated using peptide-driven ELISPOT (7). Single-cell suspensions were analyzed for CD4 (clone GK1.5), CD44 (clone IM7), and IFN-γ (clone XMGL2) (13). Cells were gated based on their forward and side-scatter characteristics and the frequency of specific cell types determined using FlowJo (Tree Star).

Generation of primary lung fibroblast cultures
Lung fibroblast cultures were prepared by digesting lung sections in 0.2% trypsin, 0.1% collagenase type IV (both from Invitrogen, Carlsbad, CA), and 400 μg/ml DNAse (Walthington, Lakewood, NJ) for 30 min. The digest was then passed through a 70-μm filter and released cells cultured in 10% FBS (Invitrogen) (14).

Immunohistochemistry
The formalin-fixed caudal lobe of the lung from infected mice was pro-
cessed for immunohistological analysis as described (15). Sections were probed with biotinylated rat anti-mouse B220 (BD Pharmingen) and goat anti-mouse CD3 (Santa Cruz Biotechnology). Secondary Abs or streptavidin were labeled with Alexa Fluor 594-conjugated or Alexa Fluor 488 (both from Invitrogen). A Zeiss Axioscop 2 microscope and a Zeiss Axio-Cam digital camera (Carl Zeiss) were used to generate images. B220+ B cell follicles were counted in four to five different mice and results expressed as the average number of B cell follicles/lobe. All B220+ B cell follicles were outlined with the automated tool of the Zeiss Axioskop 2 microscope (Carl Zeiss) and average size in squared microns calculated. A Zeiss Axioscop 2 microscope and a Zeiss Axio- Cam digital camera (Carl Zeiss), and the average ± SD was calculated. Samples were analyzed in a blinded fashion.

CXCL13-specific staining was performed on 6-μm cryosections of M. tuberculosis-infected mouse lung instilled with Tissue-Tek CRYO-OCT Compound (Fisher Scientific, Pittsburgh, PA) and adhered to coated slides. Sections were fixed with acetone/ethanol (75:25) for 10 min at room temperature and then blocked with 5% normal mouse serum in PBS (nms-PBS) for 30 min. The primary Ab was 17 μg/ml goat anti-mouse CXCL13 (AF470; R&D Systems) in 5% nms-PBS for 2 h followed by the secondary donkey anti-goat Alexa Fluor 647 (A21447; Invitrogen) at 1:250 in nms-PBS. PE-labeled rat anti-mouse B220 (553090; BD Pharmingen) was used at 1:100 in nms-PBS to detect B cells in the same sections.

In situ hybridization
Mouse Cxcl13 cDNA was RT-PCR amplified with primers BFJ.mCxc13_F1 (5’-GAACTCCACCTCGAGCAGA-3’) and BFJ.mCxc13_R1 (5’-CTTT-
TGAGATGAGTGGGCT-3’). Rhesus macaque Cxcl13 cDNA was RT-
PCR amplified with primers 5’-AGACAGAAGTTGTCATCT-3’ and 5’-GTGGAATATACCGACAGGG-3’. PCR products were ligated to the pGEM-T vector (Promega) and DNA sequenced. The pGEMT-Cxcl13 plasmid was linearized by restriction digest. Gene-specific riboprobes were synthesized by in vitro transcription using a MaxiScript SP6/T7 kit (Ambion), and unincorporated nucleotides were removed using RNA Mini Quick Spin Columns (Roche). Paraffin-embedded tissue specimens were pretreated as described (16), following deparaffinization in xylene and rinsing in ethanol. In situ hybridization with 35S-labeled riboprobes was performed at 50°C overnight as described (17), with 0.1 M DTT included in the hybridization mix. Tissue sections were coated with NTB-2 emulsion (Kodak) and exposed at 10°C for 10 d. The sections were counterstained with hematoxylin (Vector Laboratories) and mounted with Permount (Fisher). Images were visualized using an Olympus BX41 microscope (Olympus) and captured using a SPOT RT3 digital camera (Diagnostic Instruments).

Real-time PCR
RNA was extracted from lung tissue and analyzed by real-time PCR as previously described (7).

Statistical analysis
Differences between the means of experimental groups were analyzed using the two-tailed Student t test or ANOVA as appropriate. Differences were considered significant when p ≤ 0.05. Inherently logarithmic data from bacterial growth and RT-PCR were transformed for statistical analysis.

Results
IL-23 is required for the long-term containment of bacterial growth in M. tuberculosis-infected mice
We showed that IL-23 does not impact the early control of bacterial growth in M. tuberculosis-infected mice but that it augments the IFN-γ response in the absence of IL-12 (7). To determine whether IL-23 plays a role during long-term infection, we compared bacterial burden in B6 and Il23a−/− mice over time. We found that whereas early bacterial burden did not differ, by day 150, bacteria had significantly increased in the lungs of Il23a−/− mice, a difference that was maintained through day 250 (Fig. 1). Upon histo-
logical evaluation of the lungs, we noted that Il23a−/− mice had reduced numbers of B cell follicles surrounding the lesions (Fig. 1B). In addition, the B cell follicles appeared disorganized in the lungs of Il23a−/− mice at day 200 following infection (Fig. 1C). Thus, the development of inducible BALT (18) was compromised in the absence of IL-23.

Absence of IL-23 does not compromise the expression of type 1 immune responses in the lung
We previously showed that IL-23 compensates for IL-12 in the response of mice to M. tuberculosis infection (7). Therefore, to

![FIGURE 1. Mice lacking IL-23 are less able to control bacterial growth when chronically infected with M. tuberculosis.](http://www.jimmunol.org/)

A

B

C

B6

Il23a−/−

 paw length (mm) by treatment

Average number of B cell follicles/lobe

Days post infection

Log10 CFU bacterial burden

B6

Il23a−/−

C

Il23a−/−

Graph shows the combined data of three experiments, all of which showed a significant difference between B6 and Il23a−/− in bacterial burden during day 120. Data points are the mean ± SD for an n of 12–15 mice per group. B. The number of B cell follicles in the lungs of B6 (closed circles) and Il23a−/− (open circles) mice infected for 200 d was determined. Data points are from n = 4 mice/group and represent one experiment of two total. C. B6 mice and Il23a−/− mice were infected as in A for 200 d, and representative sections stained with H&E are shown (top panels). Original magnification ×100. Sections were probed with Abs to the B cell marker B220 (green), the T cell marker CD3 (red), and macrophage marker inducible NO synthase (also red) (bottom panels). Original magnification ×200. The significance was determined by the Student t test with *p ≤ 0.05, **p ≤ 0.0001.
examine the expression of protective immunity, we measured the induction of mRNA for molecules associated with protection in B6 and Il23a−/− *M. tuberculosis*-infected mice. Although mRNA expression of IFN-γ and the markers of macrophage antimicrobial activity Lrg-47 and inducible NO synthase (not shown) were equivalent, the expression of IL-22 and IL-17A was reduced in Il23a−/− lungs (Fig. 2A). In contrast, the expression of IL-21, another Th17-associated cytokine, was not significantly affected (data not shown). Although induction of IL-17A and IL-22 was dramatically reduced, there was some early expression of IL-17A and some late expression of IL-22 mRNA. Using flow cytometry and ELISPOT assays, we saw a reduction in both total numbers of IFN-γ-producing cells (Fig. 2B, left panel) and Ag-specific IFN-γ-producing (Fig. 2B, right panel) cells in the lungs of the Il23a−/− mice at the latest time point. These data suggest that although type 17 responses are reduced in the absence of IL-23, the accumulation of Th1 cells in the lung tissue is only modestly impacted.

Neither the absence of IL-17A nor IL-22 is responsible for the increased bacterial burden in IL-23–deficient mice

To determine whether the increased bacterial growth in the IL-23–deficient mice was a result of the loss of either IL-17A or IL-22, we infected IL-22–deficient or IL-17RA–deficient mice with *M. tuberculosis*. We found that both Il22−/− and Il17ra−/− mice controlled bacteria as well as B6 mice for up to 200 d (Fig. 3A, 3B). We also blocked IL-22 signaling by administering anti–IL-22 Ab to B6 mice every other day for 30 d prior to harvest at days 50, 90, 150, or 200 with similar results (Fig. 3C). To investigate whether combined deficiency resulted in increased bacterial growth, we treated IL-17RA–deficient mice with anti–IL-22 and Il22−/− mice with anti–IL-17A and observed no increase in bacterial growth (Fig. 3D, E). These data suggest that neither IL-17A nor IL-22 (either alone or in combination) can directly impact the ability of *M. tuberculosis*-infected mice to control bacterial growth.

**IL-23, IL-17R, and IL-22 impact the size of B cell follicles in the lungs of *M. tuberculosis*-infected mice at different times during infection**

We investigated whether absence of IL-17R or IL-22 impacted the generation of B cell follicles. Using morphometric analysis of lesions in the lung, we found that the absence of IL-17RA resulted in reduced size of B cell follicles early postinfection but not after day 50 (Fig. 4A, 4Ei). In contrast, the absence of IL-22 had an impact on B cell follicle size at day 80 as shown both by the gene-deficient mice and the anti–IL-22–treated animals (Fig. 4B, 4Eii). By day 150 (Fig. 4C) and including day 200 (Fig. 4D), only the absence of IL-23a had an impact on B cell follicle size.

**IL-23 is required for CXCL13 expression in the B cell follicles and to promote migration of T cells away from the blood vessels**

B cell follicles are dependent upon the chemokine CXCL13 (19), and we have previously shown that there is reduced expression of immunity in *M. tuberculosis*-infected Cxcl13−/− mice and that this is associated with inappropriate T cell accumulation around vessels in the lung (15). We therefore investigated the availability...
of CXCL13 and the preponderance of T cell cuffing around vessels in the lungs of the IL-23–deficient mice using microscopy. We found that expression of CXCL13 protein was abundant in the B cell follicles in lungs of M. tuberculosis-infected B6 mice (Fig. 5Ai), but that this was reduced in the infected lungs of Il23a−/− mice (Fig. 5Aii). We also performed in situ hybridization for the Cxcl13 mRNA on the infected lungs and found that the lymphoid aggregates contained Cxcl13 mRNA (Fig. 5Bi) and that this expression was lost in infected lungs of Il23a−/− mice (Fig. 5Bii). These data demonstrate that IL-23 is required for the expression of CXCL13 within the lymphoid aggregates that develop in the lungs of M. tuberculosis-infected mice.

We also analyzed the distribution of T cells within the lesions and found that in the absence of IL-23a, there was a significant increase in the amount of T cell cuffing around the blood vessels close to the lesional site (Fig. 5C). An increased level of T cell cuffing was also seen in the absence of IL-17RA or IL-22, but this was significantly less for Il17ra−/− mice and trended toward less for the anti–IL-22–treated mice when compared with the Il23a−/− mice (Fig. 5C). The similarity of the T cell distribution in this model to that seen in the M. tuberculosis-infected Cxcl13−/− mice caused us to investigate the ability of IL-17A and IL-22 to induce CXCL13. To do this, we exposed mouse fibroblasts to IL-17A or IL-22 and found that lung stromal cells produced the chemokine CXCL13 (Fig. 5D) as well as IL-6, GM-CSF, and keratinocyte chemoattractant (data not shown). In contrast, lymphotoxin α, CXCL12, and CCL21, which can play a role in lymphoid tissue formation, were not induced in response to IL-17 or IL-22 (data not shown). These data suggest that IL-23 is required for CXCL13-dependent generation of B cell follicles as well as the development of a T cell-containing granuloma during M. tuberculosis infection. Further, IL-23–dependent IL-17 and IL-22 may contribute to the development of the granuloma via induction of CXCL13 in the stromal cells of the lung.

Discussion

We show that IL-23 is required for the long-term containment of M. tuberculosis growth in mouse lungs and that it is critical for the CXCL13-dependent development of B cell follicles in infected lung tissue. We have previously reported that the lack of CXCL13 compromises immunity to tuberculosis and that this is linked to poor lymphoid follicle formation and increased accumulation of T cells around the vessels (15). In the model reported in this study, we show that IL-23 is critical for CXCL13 expression in lymphoid follicles and that there is increased T cell cuffing in the absence of IL-23 as the disease becomes chronic. We hypothesize therefore that the increased susceptibility to bacterial growth that occurs later in disease in the absence of IL-23 is a result of poor CXCL13 expression and the subsequent reduced ability of T cells to migrate from the vessels to the infected phagocytes and activate them efficiently.

We screened a large number of genes for differences in expression between the M. tuberculosis-infected B6 and IL-23a−/− mice infected for 50 d (i) and the lungs of B6 (left panel), Il23a−/− (upper right panel), and anti–IL-22–treated (lower right panel) mice infected for 80 d (ii). The lymphocyte accumulations were characterized as in Fig. 1C. Original magnification ×200.

![Figure 4](http://www.jimmunol.org/)
not, however, recapitulate the deficiency in bacterial control seen in the absence of IL-23. Deficiency of IL-17 signaling or IL-22 did, however, modestly impact the development of the B cell follicles, but this was not a sustained effect. Our observation that these cytokines were able to induce production of CXCL13 from lung stromal cells suggests that although IL-23 is critical for the CXCL13 expression in the lymphoid follicles, IL-23 may act through induction of IL-17 and IL-22 to mediate its effect. The fact that absence of either cytokine only modestly affects the development of follicles suggests that they compensate for each other in vivo.

We know that IL-23 can compensate for the absence of IL-12 in the IFN-γ response during mouse tuberculosis (7), and we see in this study a minor decrease in the IFN-γ-producing cells in the chronically infected Il23a−/− mice. Important recent data have shown that during chronic inflammatory conditions such as examined in this paper, the development of Ag-specific cells from an IL-17–producing phenotype to an IFN-γ–producing phenotype is dependent upon the IL-23a subunit (20). Together with our data, this suggests that in the absence of IL-23, there may be a chronic, if small, inability to develop an IFN-γ–producing phenotype over time, and this may also contribute to the increase in bacterial growth over the long term.

IL-17 has been implicated in germinal center formation (21) and promotion of IgG2a and IgG3 isotypes (22) as well as being associated with lymphoid neogenesis in graft rejection (23). In a model of neonatal pulmonary inflammation in which LPS treatment results in tertiary lymphoid tissues in the lung, a clear dependence on IL-17–producing CXCR5-expressing T follicular helper cells was demonstrated (24). We propose that as we see an early, albeit low, level of IL-23a-independent IL-17A and delayed initiation of B cell follicle formation in the IL-17RA−/− mice, that a similar process of IL-17 induction of CXCL13 production by lung stromal cells occurs during the early response to M. tuberculosis. In contrast to the data reported in this study, however, the size of tertiary lymphoid tissues in the neonatal inflammation model was not impacted by the absence of IL-23a, although there were fewer lymphoid accumulations seen. Together, these two different models suggest that although IL-17 and IL-23 play significant roles in the development of tertiary lymphoid structures, their relative roles may vary depending upon the nature of the stimulus. A critical issue may be the length of time and nature of the stimulus and the actual nature of the induced lymphoid follicle. We further show that IL-22 can augment the induction of CXCL13 in lung stromal cells, and as infection progresses, it may be that there is a requirement for IL-22 in driving and maintaining CXCL13 production, and this is why we see a requirement for IL-22 in B cell follicle formation in M. tuberculosis-infected lungs by day 80 of our study. As disease progresses, however, B cell follicle growth in the Il17ra−/− and Il22−/− mice becomes equivalent with the B6 mice, and it is the absence of IL-23 that has the greatest effect on the maintenance of the B cell follicle. Recent data show that IL-23 is critical for the expansion of infection-associated lymphoid tissue-inducer cells (25) and that IL-22 is a critical product of these cells (26). Although these cells are associated with the development of lymphoid tissues, they are also able to mediate protection against bacterial infection in the gut (25, 26); however, preliminary studies do not show any appreciable differences in the lymphoid tissue-inducer populations between infected control and Il23a−/− mice (not shown).

Although we propose that IL-23 is required to drive CXCL13 production and focus T cells to the granuloma, the possibility that IL-17 and IL-22 may be mediating direct effects should be considered. In more acute models of bacterial infection (27) and specifically in high-dose intratracheal bacillus Calmette-Guérin infection, IL-17 activity has been shown to be required for the early inflammatory response as well as protection; these activities are dependent upon IL-17 derived from γδ T cells (28, 29). We do not see a requirement for IL-17RA in protection in the low-dose aerosol model, suggesting that rapid expression of IL-17 activity within the lung is a requirement that depends upon dose and acuteness of challenge. Others have also reported a small role for IL-22 in protection against M. tuberculosis infection (30).
have not directly investigated the impact of the loss of IL-23 on the B cell function in this study, but others have shown that blockade of CXCL13 does not impact B cell activation in tertiary lymphoid follicles (31).

Our data support the need to investigate the role of IL-23 in the containment of tuberculosis. Humans lacking the IL-12p40 subunit that contributes to both the IL-12p70 and IL-23 cytokine (32) are particularly susceptible to mycobacterial diseases, and this deficiency may reflect a role of both IL-12 and IL-23 in control of infection. In contrast to a protective role for IL-23, we recently published that excess IL-23 is associated with increased pathologic consequences (8), and this highlights that, for a chronic disease such as tuberculosis, a balance in the level of this cytokine is essential. Too much and there is pathology; too little and there is a loss of protection.

Disclosures

The authors have no financial conflicts of interest.

References


