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

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*J Immunol* published online 14 October 2011
http://www.jimmunol.org/content/early/2011/10/14/jimmunol.1101377
IL-23 Is Required for Long-Term Control of *Mycobacterium tuberculosis* and B Cell Follicle Formation in the Infected Lung

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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*-induced lymphocyte follicles in the lungs, and this deficiency was associated with increased cuffing of T cells around the vessels in the lungs of these mice. *Il23a*−/− mice also poorly expressed IL-17A and IL-22 mRNA. These cytokines were able to induce Cxcl13 in mouse primary lung fibroblasts, suggesting that these cytokines are likely involved in B cell follicle formation. Indeed, IL-17RA–deficient mice generated smaller B cell follicles early in the response, whereas IL-22–deficient mice had smaller B cell follicles at an intermediate time postinfection; however, only *Il23a*−/− mice had a sustained deficiency in B cell follicle formation and reduced immunity. We propose that in the absence of IL-23, expression of long-term immunity to tuberculosis is compromised due to reduced expression of Cxcl13 in B cell follicles and reduced ability of T cells to migrate from the vessels and into the lesion. Further, although IL-17 and IL-22 can both contribute to Cxcl13 production and B cell follicle formation, it is IL-23 that is critical in this regard. *The Journal of Immunology*, 2011, 187: 000–000.

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 IgG play a minor role in protection, although there is mounting evidence that B cells can contribute to immunity to *M. tuberculosis* (3–5). In addition, manipulation of the B cell compartment could modulate the immune response to this intractable disease (6). It is important that the activity of B cells during tuberculosis be defined so that methods to control disease can be developed.

The requirement for IL-23 in the control of tuberculosis in the mouse model is secondary to the role of IL-12p70 (7); however, its role in chronic infection has not been investigated. Although IL-23 can drive the protective IFN-γ response, its primary role is in the maintenance of the IL-17A response (7); however, excess IL-17A and IL-23 contribute to the pathologic response to tuberculosis in the mouse lung (8). In this report, we show that IL-23 is required for long-term containment of *M. tuberculosis* as well as expression of CXCL13 within, and the maintenance of, B cell follicles within the lung lesions. We also show that IL-17A and IL-22 are involved in B cell follicle development at distinct times during infection and that IL-23 is necessary for the expression of both these cytokines in the lung. IL-17A and IL-22 are also shown to induce CXCL13 from lung stromal cells.

**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–deficient mice (*Il23a*−/−) were provided by Dr. Nico Ghilardi and Dr. Fred de Sauvage (Genentech, South San Francisco, CA) (9). IL-17A–deficient (*Il17ra*−/−) mice were from Amgen (Thousand Oaks, CA) (10). IL-22–deficient mice (*Il22*−/−) were from Dr. Wenjun Ouyang (Genentech) (11). C57BL6J 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
Systems) or 150 μg anti–IL-22 (a kind gift of Dr. Wenjun Ouyang) (11) i.p. The H37Rv strain of *M. tuberculosis* was used to infect mice aero-
genically, as described (12). Bacterial numbers were counted by viable CFUs in homogenized tissue (12). All experiments were approved by the Institutional Animal Care and Use Committees at either Trudeau Institute or the University of Pittsburgh.

**Detection of IFN-γ-producing cells**

Lung and draining lymph node tissue was prepared as described (12). Ag-specific IFN-γ-producing 1A-1-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 XMG1.2) (13). Cells were gated based on their forward and side-scatter characteristics and the frequency of CD44 (clone IM7), and IFN-γ (clone XMG1.2) (13). Cells were gated 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 (Worthington, Lakewood, NJ) for 30 min. The digest was then passed through a 70-μM filter and released cells cultured in 10% FBS (Invitrogen) in DMEM (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 strep-
tavidin were labeled with Alexa Fluor 594-conjugated or Alexa Fluor 488 (both from Invitrogen). A Zeiss Axioplan 2 microscope and a Zeiss Axio-
Cam digital camera (Carl Zeiss) were used to generate images. B220+ B cells 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 histological 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*. A, B6 (closed circles) and IL23a−/− (open circles) were infected by the aerosol route with 100 *M. tuberculosis* H37Rv, and the number of bacteria in the lungs was determined. The graph shows the combined data of three experiments, all of which showed a significant difference between B6 and IL23a−/− in bacterial burden after 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 were stained with H&E and 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 antimycobacterial 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 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 on day 150, 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, 3E). 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 Ab-treated animals (Fig. 4B). IL-22−/− mice were infected and treated with anti–IL-22 Ab for 30 d prior to harvest on day 150. For both D and E, data represent one experiment total with n = 4. There were no significant differences.

**FIGURE 2.** Mice lacking IL-23 maintain expression of IFN-γ and LRG-47 but not IL-17 or IL-22. A, B6 (closed circles) and Il23a−/− (open circles) were infected as for Fig. 1 and induction of mRNA for IFN-γ, Lrg-47, IL-17, and IL-22 relative to uninfected control mice determined by RT-PCR. One of two representative experiments is shown. B, A single-cell suspension from infected mice was analyzed by flow cytometry (left panel) or ELISPOT (right panel, day 238) for IFN-γ production. Data points are the mean ± SD for n = 3 to 4 mice per group. Significance was determined by the Student t test with *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001.

**FIGURE 3.** Mice lacking IL-17R or IL-22 are unaffected in their ability to control M. tuberculosis. A, B6 (closed circles) and Il17ra−/− (open squares) mice were infected as in Fig. 1, and the number of bacteria in the lungs was determined. One of two representative experiments is shown. B, B6 (closed circles) and Il22−/− (open triangles) mice were infected. One of two representative experiments is shown. C, B6 mice were infected and treated with isotype control Ab (closed circles) or anti–IL-22 Ab (open diamonds) for 30 d prior to each day of harvest. One of two representative experiments is shown. For A–C, data points are the mean ± SD for an n = 4 to 5 mice per group. D, Il17ra−/− mice were infected and treated with anti–IL-22 Ab for 30 d prior to harvest on day 150. E, Il22−/− mice were infected and treated with anti–IL-17 Ab for 30 d prior to harvest on day 150. For both D and E, data represent one experiment total with n = 4. There were no significant differences.
of CXCL13 and the preponderance of T cell cuffing around ves-
sels 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 ex-
pression was lost in infected lungs of Il23a−/− mice (Fig. 5Bii).
These data demonstrate that IL-23 is required for the expression
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–deficient IL-17 and IL-22 may contribute to the development of the granuloma via induction of
CXCL13 in the stromal cells of the lung.

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 Il23a−/− mice and trended toward less
for the anti-IL-22–treated mice when compared with the Il23a−/−
(control) 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. tuber-
culosis infection. Further, IL-23–deficient IL-17 and IL-22 may
contribute to the development of the granuloma via induction of
CXCL13 in the stromal cells of the lung.

We screened a large number of genes for differences in ex-
pression between the M. tuberculosis-infected B6 and IL-23a–
deficient mice. The absence of these genes either individually or in
concert did
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-deficient 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 we see a requirement for IL-22 in CXCL13 production.

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 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). We...
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 pathology. In mice, that contributes to both the IL-12p70 and IL-23 cytokine (32) are essential. Too much and there is pathology; too little and there is 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


