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IL-17 is a cytokine that induces neutrophil-mediated inflammation, but its role in protective immunity against intracellular bacterial infection remains unclear. In the present study, we demonstrate that IL-17 is an important cytokine not only in the early neutrophil-mediated inflammatory response, but also in T cell-mediated IFN-γ production and granuloma formation in response to pulmonary infection by Mycobacterium bovis bacille Calmette-Guérin (BCG). IL-17 expression in the BCG-infected lung was detected from the first day after infection and the expression depended on IL-23. Our observations indicated that γδ T cells are a primary source of IL-17. Lung-infiltrating T cells of IL-17-deficient mice produced less IFN-γ in comparison to those from wild-type mice 4 wk after BCG infection. Impaired granuloma formation was also observed in the infected lungs of IL-17-deficient mice, which is consistent with the decreased delayed-type hypersensitivity response of the infected mice against mycobacterial Ag. These data suggest that IL-17 is an important cytokine in the induction of optimal Th1 response and protective immunity against mycobacterial infection.


Although neutrophils are not considered to be effective antimycobacterial effector cells, it has been reported that neutrophils participate in the immune response against mycobacterial infection (4, 5). Depletion of neutrophils at the early stage of mycobacterial infection resulted in an increase of bacterial burden in several reports (5, 6) and reduced pulmonary granuloma formation in another report (7). The depletion of neutrophils roughly 2 wk after mycobacterial infection has been reported to induce an increase in the bacterial count in the lung (8), although the bacterial burden did not increase under similar conditions in another report (5). In contrast, the induction of neutrophils by LPS or rG-CSF resulted in enhanced protection against infection. Because mycobacteria induce cytokine production by neutrophils (5), cytokines are likely candidates in the mechanism of neutrophil-mediated enhancement of protection and granuloma formation. These reports suggest that neutrophils participate in the immune response to mycobacterial infection.

IL-17 is a proinflammatory cytokine secreted by T lymphocytes (9–13) that enhances the generation, activation, and migration of neutrophils through the induction of CXC chemokines, IL-6, IL-8, G-CSF, and TNF (14–16). Studies have shown the importance of IL-17 in various physiological and pathophysiological processes, including the induction of granulopoiesis (17, 18), host defense against Klebsiella or Candida infections (19, 20), rheumatoid arthritis (21, 22), allograft rejection (23, 24), and asthma (25, 26). IL-17 also triggers neutrophil migration to the lung (15). It has recently been reported that IL-17 is required for the optimal induction of Th1-type and Th2-type immune response, although the mechanism has not yet been clarified (27).

IL-23 was recently identified as a cytokine which induces IL-17 expression (28). IL-23 is a disulfide-bonded heterodimeric cytokine composed of an IL-23-specific p19 subunit and a p40 subunit common to IL-12 and IL-23. It is produced by macrophages and dendritic cells (29). The IL-23R also consists of two components a unique IL-23R subunit (IL-23R) and a subunit shared by IL-12...
and IL-23 receptors (IL-12Rβ1) (30). The biological functions of IL-12 and IL-23 are similar but not identical. IL-23 is not as potent as IL-12 in the induction of IFN-γ production (29). Instead, IL-23 (but not IL-12) induces the proliferation of memory CD4+ T cells and the IL-17 production of CD4+ T cells (29, 28, 31). IL-23-transgenic mice consistently develop multiorgan inflammation associated with neutrophilia (32). Furthermore, mice deficient in IL-23 but not IL-12 are highly resistant to experimental autoimmune encephalomyelitis, whereas mice deficient in IL-12 but not IL-23 display defective development of Th1-type immune responses (33). A recent study reported that IL-23 enhances protective immunity to Cryptococcus neoformans infection and a deficiency of IL-23 is linked to a decrease in IL-17 production (34). All of these results indicate that IL-23 is an important regulator of the inflammatory immune response mediated by IL-17.

The role of IL-23 in mycobacterial infection has also been investigated. Mice lacking both IL-12 and IL-23 (IL-12/23p40-deficient mice) had a higher bacterial burden in infected lungs (37). It is therefore controversial whether the IL-23/IL-17 axis is important in protective immunity against mycobacterial infections.

In the present study, we hypothesized that IL-17 participates in the immune response against mycobacterial infection through neutrophil induction and Th1 enhancement. We analyzed IL-17 expression in wild-type mice as well as the immune responses of IL-17-deficient mice (35, 36). These results suggest an involvement of IL-23 in host defenses against mycobacterial infection. In contrast, an analysis of IL-23p19-deficient mice showed that IL-23 is superfluous in the response to Mycobacterium tuberculosis infection (37). It is therefore controversial whether the IL-23/IL-17 axis is important in protective immunity against mycobacterial infections.

Materials and Methods

Animals

IL-17 gene-KO mice were generated as described previously (27). IL-17 KO mice of 129/Sv × C57BL/6 mixed background were backcrossed to the C57BL/6 for more than eight generations. The genotyping of IL-17 KO mice was conducted using the following PCR primers: primer 1, 5'-GAG TCA TTA AGG TAC ACC TAT-3'; primer 2, 5'-GGT CAC TCT GGC ACA C-3'. Primers 1 and 2 were used to detect the wild-type allele and primers 1 and 3 were used to detect the mutant allele. The lung was perfused with PBS through the right ventricle before excision of the thoracic cavity. The excised lung tissue was minced and incubated for 1 h at 37°C in 5 ml of PBS containing 10% heat-inactivated FBS, 125 U/ml collagenase I (Sigma-Aldrich), 60 U/ml hyaluronidase (Sigma-Aldrich), and DNase I (Sigma-Aldrich), and 60 U/ml hyaluronidase (Sigma-Aldrich). Single-cell suspensions (pulmonary infiltrated (PIF) cells) were prepared by passing through a 30-mm stainless steel mesh. The cells were collected and resuspended with 1.0% FBS, 125 U/ml collagenase I (Sigma-Aldrich), 60 U/ml hyaluronidase (Sigma-Aldrich), and 60 U/ml hyaluronidase (Sigma-Aldrich). Single-cell suspensions (pulmonary infiltrated (PIF) cells) were prepared by passing through a 30-mm stainless steel mesh. The cells were collected and used for in vitro culture or flow cytometric analysis. Single-cell suspensions from the mediastinal LNs and spleens were also prepared by passing through a 30-mm stainless steel mesh.

In the bronchoalveolar lavage fluid (BAL) recovered at the indicated times after infection with M. bovis BCG, the BAL was collected with 1 ml of RPMI 1640 medium containing 10% FBS and 68 mM EDTA. Cells in the BAL were collected and resuspended with 50% FBS-RPMI 1640 medium. Cytospin slides were prepared using a Cytospin model IV (Shandon). Fifty microliters of a 5 × 106 cells/ml cell suspension was placed into the chamber which was attached to a Cytospin slide, and centrifuged at 2000 rpm for 2 min at 20°C. The cells at the interface were collected and used for in vitro culture or flow cytometric analysis. Single-cell suspensions from the mediastinal LNs and spleens were also prepared by passing through a 30-mm stainless steel mesh.

Microorganisms and bacterial infection

M. bovis BCG (Japan BCG Association) was grown in 7H9 medium (Difco) supplemented with albumin-dextrose-catalase enrichment (Difco). Small aliquots of M. bovis BCG suspended in 7H9 medium containing 10% glycerol were stored at -80°C until use. The viable bacterial numbers were determined by 7H10 (Difco) plate supplemented with oleic acid-albumin-dextrose-catalase enrichment (Difco). The concentration of bacteria was quantified by colony counting. The bacteria were resuspended in PBS before use. Mice were inoculated intratracheally (i.t.) with 5 × 106 CFU of M. bovis BCG in 50 μl of PBS. M. bovis BCG-infected mice were sacrificed on days 0, 1, 3, 5, 7, 10, 14, 21, and 28. For the in vitro mycobacterial infection of splenocytes, 5 × 106 splenocytes were infected with 5 × 103 CFU of M. bovis BCG in 0.5 ml of antibiotic-free RPMI 1640 medium (Sigma-Aldrich) containing 10% heat-inactivated FBS for 90 min and an equal volume of RPMI 1640 containing 10% FBS, 100 U/ml gentamicin was subsequently added. The splenocytes were incubated for either 24 or 48 h.

Delayed-type hypersensitivity (DTH) responses

M. bovis BCG-infected mice were tested for a DTH response to the purified protein derivative (PPD; Japan BCG Association) derived from M. tuberculosis by the following formula: (footpad thickness of the PPD-injected right footpad - footpad thickness of the un injected left footpad) (millimeter).

IL-17

The stable transfectant producing IL-17 was described previously (41). Briefly, FBL-3 erythroleukemia cells were transfected with pEF-BOS mammalian expression vector (42) carrying the cDNA for full-length mouse IL-17 and pBL-hygB carrying the hygromycin B-resistance gene. Hygromycin B-resistant clones producing IL-17 were selected. The culture supernatant was collected and the IL-17 concentration was determined and used as rIL-17.

Cell preparation

The lung was perfused with PBS through the right ventricle before excision from the mice. The excised lung tissue was minced and incubated for 1 h at 37°C in 5 ml of PBS containing 10% FBS, 125 U/ml collagenase I (Sigma-Aldrich), 60 U/ml DNase I (Sigma-Aldrich), and 60 U/ml hyaluronidase (Sigma-Aldrich). Single-cell suspensions (pulmonary infiltrated (PIF) cells) were prepared by passing through a 30-mm stainless steel mesh. To enrich the pulmonary lymphocytes, PIF cells were resuspended in 8 ml of 45% Percoll solution (Amersham Biosciences), overlaid on 5 ml of 67.5% Percoll solution, and centrifuged at 2200 rpm for 20 min at 20°C. The cells at the interface were collected and used for in vitro culture or flow cytometric analysis. Single-cell suspensions from the mediastinal LNs and spleens were also prepared by passing through a 30-mm stainless steel mesh.

In the bronchoalveolar lavage fluid (BAL) recovered at the indicated times after infection with M. bovis BCG, the BAL was collected with 1 ml of RPMI 1640 medium containing 10% FBS and 68 mM EDTA. Cells in the BAL were collected and resuspended with 50% FBS-RPMI 1640 medium. Cytospin slides were prepared using a Cytospin model IV (Shandon). Fifty microliters of a 5 × 106 cells/ml cell suspension was placed into the chamber which was attached to a Cytospin slide, and centrifuged at 800 rpm for 3 min. The cells were morphologically examined and stained with May-Grünewald and Giemsa solutions (Wako Pure Chemical).
Magnetic separation
To enrich T cells, spleen cell suspension was passed through a nylon wool column. The T cell subsets were further fractionated by high-gradient MACS (Miltenyi Biotec). The cells were incubated with FITC-conjugated anti-CD4 (BD Biosciences), PE-conjugated anti-CD4, and allophycocyanin-conjugated anti-CD3e mAbs (BD Biosciences) for 15 min at 4°C. After washing, cells were resuspended and incubated with anti-FITC microbeads (Miltenyi Biotec) for 15 min at 4°C. After another washing step, CD8+ cells were isolated using an AutoMACS (isolation mode: depletion). Negative fractions containing CD4+ and CD8+ cells were incubated with anti-PE microbeads (Miltenyi Biotec) for 15 min at 4°C. After washing, CD4+ cells were isolated using an AutoMACS (isolation mode: depletion). Negative fractions containing CD4+ and CD8+ cells were incubated with anti-allophycocyanin microbeads (Miltenyi Biotec) for 15 min at 4°C. CD3-positive or -negative CD4+ CD8+ cells were isolated using an AutoMACS (isolation mode: depletion). Aliquots of the unsorted (whole splenocytes) and sorted cell fractions (CD4+, CD8+, CD3+ CD4+ CD8+ , and CD3+ CD4+ CD8− cells) were analyzed by flow cytometry (FCM) as described below. The CD4+, CD8+, CD3+ CD4+ CD8−, and CD3− CD4+ CD8− cell populations were sorted to a purity of >98, >98, >95, or >93%, respectively.

Expression of cytokine/chemokine genes
Total RNA was extracted from various organs, such as the lung, medias- linal LN, or the spleen, using TRIzol reagent (Invitrogen Life Technolo- gies). First-strand cDNA was synthesized from 2 μg of RNA using reverse transcriptase (SuperScript II; Invitrogen Life Technologies) and 20 μM of random primer in 20 μl of reaction buffer. The synthesized first-strand cDNA were amplified by quantitative real-time PCR using 20 μM of each primer pair with 2.5 U of the Taq polymerase (Takara Shuzo) in a total volume of 20 μl of the reaction buffer consisting of 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 0.1% gelatin, 0.2 mM dNTP, and SYBR Green I (Cambrex Bio Science). Thermal cycling was initiated with a no denaturation step of 5 min at 95°C, followed by 40 cycles of 95°C for 10 s and 60°C for 30 s. The fluorescence emitted from amplified DNA was read at 60°C at the end of each cycle. The data of the real-time PCR amplification were analyzed using the iCycler iQ and the Real-Time PCR Optical System Software version 3.0 (Bio-Rad). The cycle number at which the various transcripts were detectable, referred to as the threshold cycle (Ct), was compared with that of β-actin and referred to as ΔCt. The relative gene level was expressed as 2−ΔΔCt in which ΔΔCt equals ΔCt of the experimental sample minus ΔCt of the control sample. The specific primers were as follows: IL-7 sense (5′-AAG CGA GCA GCA ATC ATC C-3′), IL-7 antisense (5′-GGG ACT TGT GAT GTA GTC G-3′); IL-23p19 sense (5′-CCT GCT GTC TCA CAT CCT C-3′); IL-23p19 antisense (5′-TGG GCA TCT GGT GGT CTC C-3′); IL-12p35 sense (5′-AGT TGG GCA GGT GAC ATC C-3′); IL-12p35 antisense (5′-CCA CCC TTC TTC TAC AA-3′); IL-12p35 antisense (5′-GGG AGC CCT TCC TTG TTG-3′); IL-15 sense (5′-AAA CCC ATG GCA GGT ATG AA-3′); IL-15 antisense (5′-AAG TAC CAG GAC ATG GT-3′); KC sense (5′-TCG CAA GGC CCA GCC-3′); KC antisense (5′-GCT TCA GGG TCA AGG CAA GCC-3′); MIP-2 sense (5′-GAG CTG GGT GAC GGC CAC ACG-3′); MIP-2 antisense (5′-GTT AGC CAT GCC GGC CCC AGG-3′); β-actin sense (5′-CAT GTC TCA GCT CAC T-3′); β-actin antisense (5′-CGT TTA TTA ACG ACG TAT CTC TG-3′). The cells were pretreated with culture supernatant from 2.4G2 hybridoma producing mAb specific for FeγR IIb (Fc blocker), and then were surface stained with allophycocyanin-conjugated anti-CD3, NK1.1, CD8α, or CD4 (Mac-1), FITC-conjugated anti-TCR Cβ or Gr-1, and biotin- conjugated anti-TCR C8, CD4, or CD45R/B220 (BD Biosciences) mAbs plus TriColor-streptavidin. Surface-stained cells were subjected to intracellular IL-17 staining. For intracellular cytokine staining, we used PE-conjugated anti-IL-17 mAb after permeabilization of the cells using Cytofix/Cytoperm kits (BD Biosciences).

To examine the Ag-specific Th1 immune-response in the in vivo infection system, pulmonary lymphocytes at 7, 14, and 28 days after M. bovis BCG infection were incubated with or without 5 μg/ml PPD in the presence of mitomycin C-treated spleen cells (1 × 106 cells) from naive mice for 24 h at 37°C and 5% CO2, with the addition of GolgiPlug for the last 6 h. Cells were pretreated with Fc blocker, and subsequently surface stained with allophycocyanin-conjugated anti-CD3 mAb. To detect Th1 cells, we used PE-conjugated anti-IFN-γ mAb.

For both intracellular IL-17 and IFN-γ staining, cells were detected by a flow cytometer, FACSCalibur (BD Biosciences). The data were analyzed with CellQuest software (BD Biosciences).

Bacterial counts in organs
Seven, 14, and 28 days after infection with M. bovis BCG, 1 × 105 cells were sacrificed and their lungs, spleens, and livers were removed. The organs were homogenized in saline containing 0.05% Tween 80. Ten-fold serial dilu- tions of the homogenates were placed onto Middlebrook 7H10 agar (Difco). The plates were incubated at 37°C for 3 wk. After incubation, the colonies were counted and the bacterial counts in organs were calculated as log10 CFU per organ.

Histopathology
The mice were sacrificed at 3, 7, 14, and 28 days after infection with M. bovis BCG. Approximately one-third of the lung and the spleen were fixed in buffered formalin and embedded in paraffin for histopathological exam- ination. Thin sections with 4-mm thickness were prepared and stained with H&E.

To quantify the granuloma area, histological data were acquired using a charge-coupled device camera (Olympus). The digital data were analyzed using the Image J program distributed by the National Institutes of Health.

The threshold was set to discriminate between granuloma tissue and nor- mal tissue and the percentage granuloma area was calculated by the Ana- lyze Particle command. Ten to 15 sections were analyzed and the mean and SD of the percentage granuloma area were calculated.

Statistical analysis
The statistical significance of the data was determined by Student’s t test. A p value of <0.05 was considered to indicate a significant difference.

Results
Expression of IL-17 and IL-17-inducing cytokine IL-23 in the lungs of mice inoculated with M. bovis BCG
To assess the involvement of IL-17 in the immune response against mycobacteria, we initially analyzed the expression of IL-17 in the lungs of mycobacteria-infected mice. IL-17 mRNA in the lung of M. bovis BCG-infected C57BL/6 mice was detected on day 1 postinfection, peaked on day 5, and then returned to the baseline by day 7 (Fig. 1). We also investigated the factors which induced the IL-17 expression. Other studies have reported that IL-15 and IL-23 are potential inducers of IL-17 production (28, 43). We de- tected no correlation between IL-15 and IL-17 expression (Fig. 1). In contrast, IL-23p19 expression increased on day 1 and stayed at the same level for up to 3 days (Fig. 1). IL-23p19 expression increased again on day 7 and maintained high expression level up to day 21. IL-12p35 expression also increased on days 1–3, but returned to baseline by day 5. IL-23 is a heterodimeric cytokine consisting of a unique p19 subunit and a p40 subunit shared by IL-12 and IL-23, and IL-12 consists of unique p35 and common
p40. The expression of p40 was not induced at the early stage of infection although its expression was enhanced at a later stage. This finding suggests that constitutively expressed IL-12/23p40 is used to form IL-12 or IL-23. We subsequently tested the involvement of both IL-23 and IL-12 in the induction of IL-17. rIL-23 but not rIL-12 induced IL-17 production in resident pulmonary lymphocytes in a dose-dependent manner, thus suggesting that IL-23 but not IL-12 plays an important role in IL-17 production (Fig. 2A). Furthermore, CD3+ T cells in the resident pulmonary lymphocytes were the major IL-17-producing cells significantly induced by rIL-23; however, they were only slightly induced by rIL-12 (Fig. 2B). We also found that IL-23 but not IL-12 induced IL-17 production in resident peritoneal exudate cells (44). To further confirm the importance of IL-23 in the induction of IL-17 after *M. bovis* BCG i.t. infection, we infected wild-type and IL-12/23p40 KO mice with *M. bovis* BCG. The expression of IL-17 in the lungs of the IL-12/23p40 KO mice was markedly diminished in comparison to that in the wild-type mice on days 3 and 28 after the *M. bovis* BCG infection (Fig. 2C). However, it is still possible that IL-12 contributes to IL-17 induction because IL-12/23p40 KO mice lack both IL-12 and IL-23. To confirm the role of IL-23 in the induction of IL-17 production in the resident pulmonary lymphocytes, the lymphocytes of wild-type mice were infected in vitro with *M. bovis* BCG in the presence or absence of neutralizing Ab to IL-23p19 or control Ig. As shown in Fig. 2D, IL-17 expression increased significantly after the mycobacterial infection, while the
addition of neutralizing Ab to IL-23p19 to the culture suppressed the induction of IL-17 expression. Furthermore, we detected comparable levels of IL-23p19 mRNA (Fig. 2E) and IL-23 protein (data not shown) in the lungs of the wild-type and IL-17KO mice after M. bovis BCG infection, indicating that IL-23 production is independent of IL-17. These data indicate that IL-23 is required for IL-17 production by M. bovis BCG-infected mice.

Impaired neutrophil induction in infected lungs after inoculation with M. bovis BCG

It has been reported that IL-17 induces the production of neutrophil CXC chemokines such as human IL-8, murine KC/CXCL1, and murine MIP-2/CXCL2 (45–47). IL-17 also induces the production of cytokines important in the induction, activation, or survival of neutrophils, such as G-CSF, IL-6, and TNF (15, 48). Therefore, we investigated whether IL-17 is responsible for the chemokine/cytokine expression induced by M. bovis BCG infection. As shown in Fig. 3, the expression of KC and MIP-2 were severely impaired on days 1–3 and on day 1, respectively, in the lungs of infected IL-17KO mice in comparison to that in wild-type mice. The expressions of G-CSF, IL-6, and TNF were also diminished in the IL-17KO mice in comparison to those in the wild-type mice. In findings consistent with the decrease of chemokine/cytokine expression in the IL-17KO mice, the BAL cells of the IL-17 KO mice contained significantly lower number of neutrophils on days 1–5 after infection (Table I). These results indicate that IL-17 participates in the induction of acute neutrophil-mediated inflammation in the lungs of M. bovis BCG-infected mice. In addition, the numbers of monocytes and lymphocytes in the BAL cells of the IL-17 KO mice were also lower in comparison to the wild-type mice after M. bovis BCG infection (Table I).

Identification of IL-17-expressing cells in response to M. bovis BCG infection in vitro

We established an in vitro infection system to determine the phenotype of IL-17-producing cells at the early stage of mycobacterial infection. Splenocytes from naive wild-type mice were infected with M. bovis BCG in vitro and were separated into several fractions after 24 and 48 h of culturing. The expression of IL-17 was subsequently analyzed by RT-PCR. Unexpectedly, strong IL-17 mRNA expression was detected in the CD4+ CD8− cells (Fig. 4A). Because spleen TCR γδT cells are CD4− CD8− CD3+ T cells, we theorized that CD4+ CD8− CD3+ TCR γδ+ T cells produce IL-17 in the culture system. Therefore, CD4−CD8− cells were separated into CD3-positive or -negative populations and analyzed. The expression of IL-17 induced by M. bovis BCG infection was detected not only in CD4+ CD8− CD3+ T cells but also in CD4− CD8− CD3− (non-T) cells (Fig. 4B, left panels), thus indicating that IL-17 is expressed by both TCR γδ+ T cells and non-T cells. To confirm the expression of IL-17 by TCR γδ+ T cells, an in vitro infection analysis was conducted using spleen cells from TCR C5 KO mice. IL-17 expression of CD4− CD8− CD3+ T cells was markedly reduced in the TCR C5 KO mice (Fig. 4B, right panels). Interestingly, IL-17 production by CD4− CD8− CD3−

Table I. Subsets of the BAL from wild-type mice or IL-17KO mice after BCG infection

<table>
<thead>
<tr>
<th>Days</th>
<th>Total Cells</th>
<th>Neutrophils</th>
<th>Monocytes</th>
<th>Lymphocytes (×10^7 cells)</th>
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<tr>
<td></td>
<td>Wild type</td>
<td>IL-17KO</td>
<td></td>
<td></td>
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<tr>
<td>0</td>
<td>4.51 ± 0.37</td>
<td>4.53 ± 0.38</td>
<td>nd</td>
<td>4.29 ± 0.18</td>
</tr>
<tr>
<td>1</td>
<td>15.50 ± 4.99</td>
<td>3.93 ± 0.56**</td>
<td>0.26 ± 0.16**</td>
<td>3.55 ± 0.25</td>
</tr>
<tr>
<td>3</td>
<td>50.04 ± 14.39</td>
<td>25.97 ± 5.84**</td>
<td>12.64 ± 2.05**</td>
<td>10.89 ± 2.55**</td>
</tr>
<tr>
<td>5</td>
<td>33.46 ± 8.80</td>
<td>9.66 ± 2.92**</td>
<td>3.23 ± 2.24</td>
<td>15.29 ± 2.94</td>
</tr>
<tr>
<td></td>
<td>12.86 ± 2.61</td>
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</tbody>
</table>

*The BAL fluid was recovered at the indicated times after infection with M. bovis BCG. Cells were morphologically examined after staining with May-Grünwald and Giemsa solutions and then counted by microscopy. The values from the IL-17KO mice were significantly different from the values for wild-type mice infected with M. bovis BCG (*, p < 0.005; **, p < 0.001; nd, not detectable).
(non-T) cells was also decreased in the TCR Cγ KO mice after infection.

To confirm IL-17 is expressed by CD4+ CD8− cells at the protein level, in vitro M. bovis BCG-infected or uninfected splenocytes were stained for cell surface markers and cytoplasmic IL-17 and analyzed by FCM. Consistent with the RT-PCR results shown in Fig. 4B, both the CD3+ T cells and CD3− non-T cells from M. bovis BCG-infected splenocytes produced IL-17 (Fig. 4C). Among the CD3+ cells, the TCR Cδ+ and TCR Cβ+ cells produced IL-17, although Cδ+ T cells represented >80% of the IL-17-producing T cells (Fig. 4D). The ratio of IL-17-producing cells in TCR Cδ+ cells (~45%) was higher than that in TCR Cβ+ cells (~3%).

To confirm IL-17 production by TCR γδ+ T cells and non-T cells in vivo in the mycobacteria-infected lung, we i.t. infected the wild-type mice with M. bovis BCG, and analyzed the IL-17 expression by PIF cells and pulmonary lymphocytes. CD3+ T cells were the major IL-17-producing cells in the infected lungs, although CD3− non-T cells produced IL-17 as well (Fig. 5A, left panel). To determine which T cells produce IL-17 upon M. bovis BCG-infection, pulmonary lymphocytes were stained with mAb against CD4 or CD8 and IL-17, and analyzed by FCM. Approximately 20% of the IL-17+ CD3+ T cells were CD4+, but the remaining IL-17-producing T cells were the CD4− CD8− phenotype (Fig. 5A, right panels). Among the CD3+ T cells, both the TCR Cβ+ and TCR Cδ+ T cells produced IL-17 (Fig. 5B). The ratio of IL-17-producing cells in TCR Cδ+ T cells (~45%) was higher than that in TCR Cβ+ T cells (~3.5%). In addition, the mean fluorescence intensity of IL-17 staining of the TCR Cδ+ T cells (2150.6) was higher than that of the TCR Cβ+ T cells (425.1). These results indicate that TCR γδ+ T cells produce IL-17 at a higher frequency and intensity than TCR αβ+ T cells. The data indicate that TCR γδ+ T cells are the major

FIGURE 4. Identification of CD4+CD8− TCR γδ+ T cells as the major IL-17-expressing cells in response to M. bovis BCG infection in vitro. A and B, Spleen cells of wild-type C57BL/6 (A) or TCR Cγ KO mice (A and B) were infected with M. bovis BCG in vitro. Cells were collected and separated by magnetic cell sorting after 48 h of the culture and the expression of IL-17 was analyzed by RT-PCR. C and D, Spleen cells of C57BL/6 mice were infected with M. bovis BCG in vitro. After 40 h of culture, GolgiPlug was added and the cells were incubated for another 8 h. The cells were then surface-stained with allophycocyanin-anti-CD3, FITC-anti-TCR Cβ, and biotin-anti-TCR Cδ mAbs plus TriColor-streptavidin. Surface-stained cells were subjected to intercellular cytokine staining with PE-anti-IL-17 mAb. Samples were analyzed by FCM. The IL-17 expression of total spleen cells (C) or CD3+ cells (D) is shown. Data representative of three independent experiments are demonstrated in all panels.

FIGURE 5. Identification of TCR γδ+ T cells as IL-17-producing cells after M. bovis BCG infection of the lung in vivo. Wild-type C57BL/6 mice were infected i.t. with M. bovis BCG. The PIF cells were collected 3 days after infection and cultured with or without 1 μg/ml calcium ionophore A-23187 and 25 ng/ml PMA for 4 h at 37°C and 5% CO2 in the presence of GolgiPlug. The cells were surface-stained with allophycocyanin-conjugated anti-CD3, FITC-conjugated anti-TCR Cβ, and biotin-conjugated anti-TCR Cδ, CD4, or CD45R/ B220 mAbs plus TriColor-streptavidin. Surface-stained cells were subjected to intracellular cytokine staining. For intracellular cytokine staining, we used PE-conjugated anti-IL-17 mAb after the permeabilization of the cells. The IL-17 expression in lung lymphocytes in the PIF cells (A–C, upper panels) or whole PIF cells (C, lower panels) are shown. Lymphocytes in the PIF cells were gated on CD3+ cells (A, right panels; B). Data representative of three independent experiments are demonstrated in all the panels.
IL-17-producing cells in vivo. We further investigated the markers of IL-17-producing non-T cells. However, we could not detect IL-17 production in NK1.1<sup>-</sup>/H<sup>11001</sup>, CD45R/B220<sup>-</sup>/H<sup>11001</sup>, CD11b<sup>-</sup>/H<sup>11001</sup>, or Gr-1<sup>-</sup>/H<sup>11001</sup> cells (Fig. 5 C).

Impaired granuloma formation in the lungs of IL-17 KO mice infected with M. bovis BCG

Because the participation of neutrophils in granuloma formation was suggested, and IL-17 itself was reported to enhance the Th1 response, we hypothesized that a lack of IL-17 during mycobacterial infection influences the establishment of the acquired immune response and granuloma formation. A histological examination was conducted on the lungs of M. bovis BCG-infected IL-17 KO mice on day 28 of infection, when the acquired immune response and granulomas were established in the lungs of the wild-type mice (Fig. 6). The size and number of granulomas in the lungs of the IL-17 KO mice were reduced in comparison to the granulomas in the wild-type mice on day 28 of infection (Fig. 6, A and B). The granulomas in the lungs of the IL-17 KO mice were less densely packed with mononuclear cells in comparison to those in the wild-type mice (Fig. 6A, e and f). This result indicates that IL-17 is an important factor in the establishment of granulomas.

To further compare the cellular composition in the granulomas of the wild-type and the IL-17 KO mice, FCM analysis of monocyte and granulocyte lineage markers was conducted on PIF cells on day 28 after i.t. M. bovis BCG infection. As shown in Fig. 6C, the ratios of CD11b<sup>+</sup>Gr-1<sup>low</sup> macrophages (R1 of the Fig. 6C) and CD11b<sup>+</sup>Gr-1<sup>high</sup> neutrophils (R2) were lower in the lungs of the IL-17KO mice than those in the wild-type mice. In contrast, the ratio of macrophages to granulocytes in the infected lungs of IL-17 KO mice (~1:0.6) was nearly identical with that in the wild-type mice. These results suggest that the accumulation of both monocytes and granulocytes was reduced in the granulomas in the IL-17 KO mice.

Impaired IFN-γ production by mycobacterial Ag-specific T cells and DTH in the IL-17 KO mice after infection with M. bovis BCG

We investigated whether the absence of IL-17 affected the Ag-specific Th1 immune response to mycobacterial Ags after i.t. infection with M. bovis BCG. On day 14 after infection, there was no statistically significant difference in IFN-γ production by pulmonary lymphocytes in the wild-type and IL-17 KO mice, although IL-17KO mice tended to show slightly lower levels of production (Fig. 7A). On day 28 after infection, the lung lymphocytes from the IL-17 KO mice showed a significantly lower level of IFN-γ production than those from the wild-type mice (p < 0.01). In contrast, IL-4 and IL-13 were not produced in the pulmonary lymphocytes of either the wild-type or the IL-17 KO mice at any stage of M. bovis BCG infection (data not shown), indicating that the diminished IFN-γ production in the IL-17KO mice was not due to a deviation to the Th2-type immune response. To determine the population of IFN-γ-producing cells, we analyzed the CD3<sup>+</sup> pulmonary lymphocytes by intracellular IFN-γ staining and FCM analysis (Fig. 7B). T cells from the IL-17 KO mice showed a slightly
lower percentage of IL-17-producing cells (1.7% of CD3+ T cells) in comparison to that of the wild-type mice (2.8% of CD3+ T cells) (Fig. 7B, upper panels). The ratio of IFN-γ-producing cells was significantly lower in the T cells from the IL-17 KO mice than that from the wild-type mice (5.1 ± 0.7% in the IL-17 KO mice vs 11.7 ± 1.4% in the wild-type mice) on day 28 after infection (p < 0.01). These results indicated that the generation of Th1 cells was impaired in IL-17 KO mice infected with M. bovis at 4 wk.

It is possible that IL-17 directly induced IFN-γ production of T cells. To examine the possibilities, we stimulated the pulmonary lymphocytes on day 28 after infection with graded concentrations of rIL-17. rIL-17 did not affect the IFN-γ production of the pulmonary lymphocytes from either the wild-type or the IL-17 KO mice (Fig. 7C). These results suggest that IL-17 is not a direct inducer of IFN-γ production in T cells.

To further investigate the cell-mediated immune response in the IL-17 KO mice, we evaluated the ability of the IL-17 KO mice to mount DTH responses. We sensitized the wild-type and IL-17 KO mice by M. bovis BCG infection, elicited DTH responses 4 wk later by injection of PPD into the right hind footpads, and measured specific footpad swelling 24 and 48 h after the challenge. We found that the DTH to mycobacterial Ag was inhibited in the IL-17 KO mice in comparison to that of wild-type mice (Fig. 7D). Therefore, IL-17 is indispensable to the optimal induction of DTH responses and a lack of IL-17 leads to an inefficient cell-mediated immune response.

Bacterial loads of various organs in IL-17 KO mice after infection with M. bovis BCG

To analyze the role of IL-17 in protective immunity against mycobacterial infection, we examined the bacterial growth in various
organs of the wild-type and IL-17 KO mice after i.t. infection with *M. bovis* BCG. As shown in Fig. 8, the bacterial numbers in the lungs, livers, and spleens of the IL-17 KO mice were similar to those in the wild-type mice on days 7, 14, and 28 after infection. The data suggest that IL-17 is superfluous during the early protective immune response that suppresses bacterial expansion in the infected organs.

**Discussion**

In this study, we demonstrate that IL-17 plays a key role in neutrophil induction after pulmonary mycobacterial infection. The recruitment of neutrophils to the lungs has been described in patients in the acute phase of tuberculosis (49, 50) and in experimental animals infected with mycobacteria (51, 52), but the molecular mechanism was not clarified. To determine the involvement of IL-17 in the induction of neutrophils in response to mycobacterial infection, we analyzed the migration of neutrophils to the lungs in the *Mycobacterium*-infected IL-17KO mice. We demonstrated that neutrophil mobilization in the *M. bovis* BCG-infected lungs was significantly suppressed in the IL-17-deficient mice. Furthermore, IL-17 was induced in the lung from an early stage of *M. bovis* BCG pulmonary infection in the wild-type mice. These results demonstrate the importance of IL-17 in the induction of neutrophils after *M. bovis* BCG infection. The macrophage/DC-derived cytokines IL-23 and IL-15 have been reported to induce IL-17 production (28, 43). We identified IL-23 as an IL-17 inducer in pulmonary mycobacterial infection because IL-17 production was significantly suppressed in the IL-12/23p40-deficient mice and IL-12 failed to induce IL-17 (Ref. 53 and this report). Because the expression of neutrophil-inducing chemokines KC/CXCL1 and MIP-2/CXCL2 and neutrophil-inducing/activating cytokines G-CSF and IL-6 was also decreased in the absence of IL-17, IL-17-mediated neutrophil induction may depend on these chemokines and cytokines. IL-17 has been reported to be an important mediator of neutrophil induction, and its role depends on these chemokines and cytokines. IL-17 has been shown to be a pro-inflammatory cytokine that boosts the Th1 response and promotes the differentiation of Th1 cells (54). Therefore, we speculated that IL-17-mediated neutrophil induction may depend on these chemokines and cytokines. IL-17 has been reported to be an important mediator of neutrophil migration and host defense against pneumonia by *Klebsiella pneumoniae* (19, 53). In the experimental model of *Klebsiella* infection, it was suggested that bacterial products induce a subset of T cells to secrete IL-17. A similar mechanism may be involved in mycobacteria-induced neutrophil migration.

In this study, we found that TCR γδ⁺ T cells and unidentified non-T cells were the major IL-17-producing cells in the mycobacterial infection. The macrophage/DC-derived cytokines IL-23 and IL-15 have been reported to induce IL-17 production (28, 43). We identified IL-23 as an IL-17 inducer in pulmonary mycobacterial infection because IL-17 production was significantly suppressed in the IL-12/23p40-deficient mice and IL-12 failed to induce IL-17 (Ref. 53 and this report). Because the expression of neutrophil-inducing chemokines KC/CXCL1 and MIP-2/CXCL2 and neutrophil-inducing/activating cytokines G-CSF and IL-6 was also decreased in the absence of IL-17, IL-17-mediated neutrophil induction may depend on these chemokines and cytokines. IL-17 has been reported to be an important mediator of neutrophil migration and host defense against pneumonia by *Klebsiella pneumoniae* (19, 53). In the experimental model of *Klebsiella* infection, it was suggested that bacterial products induce a subset of T cells to secrete IL-17. A similar mechanism may be involved in mycobacteria-induced neutrophil migration.

Among the T cell subset identified by CD4/CD8 expression, CD4⁺CD8⁻ thymocytes (54) as well as by activated CD4⁺ and CD4⁺CD45RO⁺ memory T cells (9). Activated CD8⁺ and CD8⁺CD45RO⁺ memory T cells are also produced IL-17 in humans (55). However, we previously reported that both TCR αβ⁺ T cells and TCR γδ⁺ T cells produced IL-17 after stimulation with the Fas ligand (41). The ratio of IL-17-producing cells in the TCR γδ⁺ T cells was higher than that in the TCR αβ⁺ T cells. Among the T cell subset identified by CD4/CD8 expression, CD4⁺CD8⁻ cells were the major producers of Fas ligand-induced IL-17, although some CD4⁺CD8⁻ cells produced it as well. This distribution of IL-17-producing T cell subsets in *M. bovis* BCG-infected spleen cells is similar to that observed in Fas ligand-induced IL-17 production. We confirmed that both TCR αβ⁺ T cells and TCR γδ⁺ T cells produce IL-17 in splenocytes and pulmonary lymphocytes after *M. bovis* BCG infection (Figs. 4D and 5B). The percentage of IL-17-producing TCR γδ⁺ T cells among the IL-17-producing T cells (~60–70%) was higher than that of IL-17-producing TCR αβ⁺ T cells. It is noteworthy that the proportion of IL-17-producing cells among the TCR γδ⁺ T cells (~45%) was higher than that in the TCR αβ⁺ T cells (~3.5%) after *M. bovis* BCG infection, and that the mean fluorescence intensity of IL-17 staining is higher in TCR γδ⁺ T cells than TCR αβ⁺ T cells. These observations suggest that TCR γδ⁺ T cells are the major IL-17-producing cells in our system. Furthermore, a portion of IL-17-producing cells were non-T cells without CD3 expression (Figs. 4C and 5A). Recently, it was reported that neutrophils produce IL-17 in a model of LPS-induced lung inflammation (56); however, no IL-17 production by neutrophils was detected in our system. The identification of IL-17-producing non-T cells is ongoing.

The role of neutrophils at the early stage of mycobacterial infection is controversial. Several in vitro studies suggested that human neutrophils are able to kill virulent *M. tuberculosis* (57, 58) but other reports failed to reproduce the result (59). A recent report demonstrated the increased bacterial burden in infected organs from the early stage of mycobacterial infection when neutrophils are depleted from mice before and/or during this stage (60). However, another report failed to detect any difference in the bacterial counts in the neutrophil-depleted mice (60). We demonstrated that the absence of IL-17 resulted in a significant reduction of neutrophil accumulation in the lung (Table I and Fig. 6C) without compromising the control of bacterial growth on days 7–28 after infection (Fig. 8). The data indicate that IL-17-induced neutrophils themselves are not effective effector cells in the elimination of mycobacteria at the early stage of infection before the establishment of acquired immunity. Although neutrophils may not act as direct effector cells against mycobacteria, they may serve as important immunoregulators. Cytokines and chemokines are produced by mycobacteria-activated neutrophils (7, 61). Furthermore, Ab-mediated neutrophil depletion resulted in the formation of disorganized granulomas in the mycobacteria-infected lung (7). This observation is similar to that seen in the IL-17 KO mice. We hypothesize that the IL-17-mediated migration of neutrophils into the lung may have an important role in the formation of organized granulomas.

Our data further demonstrate that IL-17 is an important cytokine not only in the early neutrophil recruitment but also in the induction of Th1-type acquired immunity after pulmonary mycobacterial infection. The IL-17 KO mice showed a decreased level of mycobacterial Ag-specific Th1 response on day 28 after *M. bovis* BCG infection. It has been reported that IL-17 is required to induce optimum Th1 and DTH responses against hapten (27), which is consistent with our observation that the Th1-type response is decreased in the absence of IL-17. However, IL-17 failed to directly enhance mycobacterial Ag-specific IFN-γ production because the level of IFN-γ production was not altered when the T cells from the *M. bovis* BCG-infected IL-17 KO mice were cultured with rIL-17 (Fig. 7C). This finding is consistent with a report showing that exogenously added IL-17 affected neither Th1/Th2 phenotype differentiation nor IL-17 production (62). Yao et al. (63) reported that IL-17 stimulates the activity of the transcriptional factor NF-κB, which is known to up-regulate gene products involved in cell activation and growth control. Therefore, we speculate that IL-17-mediated NF-κB activation of APCs or other immunoregulatory cells may be important in the induction of an optimum level of Th1 response. The mechanism of IL-17-mediated enhancement of the Th1 response is now under investigation.

In summary, we investigated IL-17 production and IL-17-mediated immune regulation in mycobacterial infection. We found that IL-23-induced IL-17 production in *M. bovis* BCG-infected mice in vivo and infected spleen cells in vitro. The major IL-17 producers were TCR γδ⁺ T cells. IL-17 induced by *M. bovis* BCG infection affected not only early pulmonary neutrophil induction,
but also the development of IFN-γ-producing T cells and granulo-
oma formation. Our data suggest that IL-17 exerts benefit effects on the development of protective cell-mediated immunity against mycobacteria, it is likely to be used as an im-
munoadjuvant to enhance the efficacy of vaccination inducing the protective Th1 response.

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