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IL-17 Production Is Dominated by γδ T Cells rather than CD4 T Cells during *Mycobacterium tuberculosis* Infection[1]

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IL-17 is a cytokine produced by T cells in response to IL-23. Recent data support a new subset of CD4 Th cells distinct from Th1 or Th2 cells that produce IL-17 and may contribute to inflammation. In this study, we demonstrate that, in naive mice, as well as during *Mycobacterium tuberculosis* infection, IL-17 production is primarily from γδ T cells and other non-$CD4^+CD8^+$ cells, rather than CD4 T cells. The production of IL-17 by these cells is stimulated by IL-23 alone, and strongly induced by the cytokines, including IL-23, produced by *M. tuberculosis*-infected dendritic cells. IL-23 is present in the lungs early in infection and the IL-17-producing cells, such as γδ T cells, may represent a central innate protective response to pulmonary infection. ***The Journal of Immunology,*** **2006, 177: 4662–4669.**

Interleukin-23 is a member of the IL-12 family of cytokines and shares the IL-12p40 subunit with IL-12. IL-23 appears to be a driving force in chronic inflammatory disease. This cytokine promotes T cell production of IL-17A, IL-17F, TNF, and IL-6, which drive inflammation in experimental autoimmune encephalomyelitis (1). IL-23 also promotes IL-17-mediated chronic inflammation in collagen-induced arthritis (2). The profile of T cell genes induced by IL-23 is mostly distinct from that induced by IL-12, and IL-23 does not significantly prime for high IFN-γ production in mice (1, 3). A new subset of CD4 helper cells, distinct from that of Th1 and Th2 cells, termed Th17, has been described (4, 5). The development of this IL-17-producing subset is inhibited by IFN-γ or IL-4, and may be involved in autoimmune diseases. However, mature Th17 cells are resistant to the effects of IL-4 and IFN-γ, and are able to secrete IL-17 in Th1 or Th2 environments (4). Overexpression of IL-17 in lung epithelium results in lung pathology including epithelial hypertrophy and the presence of multinucleated macrophages in the parenchyma (5).

IL-23 induction of IL-17 in the lung leads to downstream events that mobilize cell infiltration. IL-17 induces chemokines, growth factors, and adhesion molecules, and augments neutrophil accumulation (6, 7). It has been demonstrated to play a role in control of pulmonary infection due to *Klebsiella*, an extracellular bacterial pathogen, in mouse models.

There are several lines of evidence that suggest γδ cells contribute to the immune response to *Mycobacterium tuberculosis*. In the macaque model of infection with *Mycobacterium bovis* bacillus Calmette-Guérin (BCG),[3] the $V\gamma2V\delta2^+$ T cell subset expand rapidly during primary exposure and also to secondary challenge with BCG or virulent *M. tuberculosis* (8). Similarly, intranasal infection of mice expanded resident Vγ2 T cells and caused an influx of other γδ subsets into the lung. These cells were capable of producing IFN-γ and were cytotoxic toward infected macrophages in vitro (9). In humans, γδ cells from BCG-vaccinated individuals expand upon restimulation with mycobacterial Ag, thereby displaying a memory-like phenotype (10). Human alveolar macrophages infected with *M. tuberculosis* release chemoattractants such as CXCL10 that cause chemotaxis of γδ cells and cytokine secretion (11). These chemoattractants are found in the lungs and axillary lymph nodes of patients with active disease. In the mouse model of *M. tuberculosis* infection, γδ knockout (KO) mice have a more pyogenic granulomatous response, implying a regulatory role in granuloma formation (12).

IL-12 has a central role in priming T cells to produce IFN-γ in response to *M. tuberculosis* (13, 14). IL-23 also contributes to resistance as suggested by the increased susceptibility of IL-$12p40^{-/-}$ compared with IL-$12p35^{-/-}$ mice during experimental infection (15). Somewhat surprisingly in light of that study, mice deficient in the p19 subunit of IL-23 did not display obviously increased susceptibility to *M. tuberculosis* (as measured by bacterial numbers). The relatively less susceptible phenotype of IL-$12p35^{-/-}$ mice may be due to the fact that IL-23 can also make a minor contribution to IFN-γ production in the absence of IL-12 (16). It was also demonstrated that IL-23 is an absolute requirement in $CD4^+$ T cell production of IL-17 during *M. tuberculosis* infection (16). The role of the IL-17-producing T cells in control of *M. tuberculosis* infection is not known.

In this study, we have used murine *M. tuberculosis* infection as a model to investigate the interaction between IL-23 and IL-17, with particular emphasis on the cells that respond to IL-23. By using a slow growing bacterium against which immune responses develop slowly, we have dissected the early IL-17 responses in the lungs, and the chronicity of cytokine production.

Recently, a homeostatic loop involving IL-23-induced IL-17 production by unconventional αβ T cells and γδ cells has been described (17). Whereas IL-23 and IL-17 promote granulopoiesis and influx of neutrophils into the lung, phagocytosis of apoptotic lung neutrophils reduces macrophage IL-23 secretion, and induction of IL-17, thereby limiting influx. The data presented here similarly demonstrate that, in naive mice, IL-17 production can be induced in γδ T cells. However, our study also demonstrates that

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[3] Abbreviations used in this paper: BCG, bacillus Calmette-Guérin; KO, knockout; DC, dendritic cell.

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M. tuberculosis infection of dendritic cells (DC) significantly increases production of IL-23 and other soluble factors. These factors drive the production of IL-17 from γδ T cells but surprisingly not from CD4 T cells. IL-23 is also present in the lungs early in infection. Production of IL-17 by γδ T cells occurs before Ag-specific αβ T cell priming or substantive IFN-γ production, consistent with a role in early lung remodeling and resistance to infection. Although CD4 Th17 cells appear in the lung as the infection progresses, the main contributors of IL-17 throughout the course of *M. tuberculosis* infection were non-CD4/CD8 cells, including γδ T cells. These results support a role for IL-23 and IL-17 involving γδ T cells as a first line of defense or inflammation before CD4 T cell responses are acquired, and suggest that the IL-23/IL17 homeostatic loop behaves as a proinflammatory network during infection and that non-$CD4^+$ T cells and γδ T cells can assume a dominant role in IL-17 production when $CD4^+$ T cells have been primed for IFN-γ production.

Materials and Methods

DC culture

Bone marrow cells from C57BL/6 mice were cultured in DMEM supplemented with 10% FBS, 1000 U/ml GM-CSF, and 1000 U/ml IL-4. Cytokines were replenished on day 3 of culture. On the sixth day of culture, the cells were plated at 1×10^6/ml and the medium was replenished without IL-4. DC were infected with *M. tuberculosis* strain Erdman at a multiplicity of infection of 3 for 18 h. Cells and supernatants were harvested by centrifugation.

Mice and bacteria

Female 6- to 8-wk-old C57BL/6 mice were infected via aerosol with ~50 CFU of *M. tuberculosis* strain Erdman as described previously (18). Mice deficient in γδ TCR expression (strain name, B6.129P2-*Tcrd*^{tm1Mom}/J) were purchased from The Jackson Laboratory. Breeding pairs of mice deficient in IL-12p40 or IL-12p35 were obtained from Dr. J. Kolls (University of Pittsburgh, Pittsburgh, PA), and bred as homozygotes in our breeding facility under specific pathogen-free conditions. For CFU determination, mice were euthanized at different time points and organ homogenates were plated on 7H10 plates and incubated for 3 wk at 37°C/5% CO_2. All animal procedures were approved by the University of Pittsburgh Institutional Animal Care Committee.

ELISPOT and protein analysis

Lung cells and spleens cells were obtained by crushing whole organs through cell strainers, washing with PBS, and lysing RBC as described previously (19). Cell yields were typically 1×10^6 live cells per naive lung, increasing 4- to 5-fold during the chronic phase of infection (4 wk). ELISPOT plates (Millipore 96-well MultiScreen HTS) were coated with 15 μg/ml anti-mouse IL-17A specific mAb (clone 50101; R&D Systems) or anti-mouse IFN-γ mAb (BD Biosciences) in PBS. IL-2 (PeproTech) was added to a final concentration of 30 U/ml, and cells were incubated for 48 h at 37°C/5% CO_2. Cells were usually plated at 2×10^5 cells/well, except for purified γδ cells, which were plated at lower concentrations due to low availability, and then normalized to 25,000 cells. Cytokines were detected with biotinylated anti-mouse IL-17 (R&D Systems) or anti-mouse IFN-γ Ab (BD Biosciences) and developed with avidin peroxidase and AEC substrate (Vectastain PK6100, PK4200). ELISPOT plates were read on an automatic ELISPOT reader (ImmunoSpot; Cellular Technology). Analysis of cytokine concentration from cell supernatants was done using a Bioplex Suspension Array (Bio-Rad Laboratories) and a Luminex reader according to the manufacturer's instructions.

Cell purification

In initial experiments, $CD90^+$, $CD4^+$, $CD8^+$, $B220^+$, and $CD11b^+$ cells were positively selected by direct labeling with MicroBeads, and γδ cells selected by indirect labeling with anti-γδ-TCR-biotin, and then anti-biotin MicroBeads (Miltenyi Biotec). Cell fractionation was confirmed by FACS. Spleen and lung cells that were depleted of both $CD4^+$ T cells and $CD8^+$ T cells contained cells that stained positively for B220, NK1.1, CD11b, γδ TCR, and CD3, and were negative for CD4 and CD8. In subsequent experiments, cells were purified by FACS sorting on a FACSAria (BD Biosciences).

Statistical analyses

All statistical analyses were performed using GraphPad Prism for Windows, version 4.03 (GraphPad Software). A one-way ANOVA followed by a Tukey posttest was used to determine significance. Values of $p < 0.05$ were considered significant.

Adenovirus

A lymphocytic choriomeningitis virus internal ribosomal entry sequence was cloned between the IL-23 p19 subunit and the IL-12/23 p40 subunit in pAd/CMV/V5 (Invitrogen Life Technologies). This construct was used to create adenovirus secreting active IL-23 as previously described (20).

Flow cytometry

Lung and spleen cells were obtained by crushing through cell strainers as described previously (19). For intracellular staining, lung and spleen cells were stimulated with 500 ng/ml ionomycin and 50 ng/ml PMA, in the presence of 3 μM monensin (Sigma-Aldrich) for 4–6 h. Cells were stained for surface markers with anti-CD4 (clone RM4-5), and anti-(TCR)δ chain (clone GL3) Abs, fixed with 4% paraformaldehyde, permeabilized, and stained with anti-IL-17 (clone TC11-18H10) and anti-IFN-γ (clone XMG1.2; BD Pharmingen) Abs. Cells were analyzed using a FACSCalibur flow cytometer (BD Biosciences) and FlowJo software (Tree Star).

For purification, lung and spleen cells were stained with anti-CD3, anti-CD4, and anti-γδ TCR, and sorted on a FACSAria flow cytometer (BD Biosciences) in the BSL3 facility.

Results

IL-17 production in response to M. tuberculosis*-infected DC*

Infection with a low dose of *M. tuberculosis* via aerosol results in the slow priming of IFN-γ-producing CD4 T cells, with responses detected in the mediastinal lymph node by ~2 wk postinfection (21, 22). These cells, along with *M. tuberculosis*-specific CD8 T cells, traffic to the lungs between 2 and 3 wk postinfection, and control but do not eliminate the infection. We were interested in examining the production of IL-17 by the T cells in response to *M. tuberculosis* infection, based on reports that IL-23 may be an important mediator of control in this infection (15, 23).

Cells obtained from the lungs were stimulated with infected DC in an ELISPOT format for IL-17. In the ELISPOT assay, IL-17 production was easily detected in lung cells stimulated with *M. tuberculosis*-infected (but not uninfected) DC (Fig. 1). IL-17 production could also be detected in the absence of DC when lung cells were stimulated with culture supernatant from infected DC. In contrast to IL-17 production, IFN-γ production was reduced without exogenous infected DC as APC, and was not induced by the DC supernatant alone. IL-17 production from lung cells could also be triggered by DC transfected with adenovirus that expressed IL-23. Addition of neutralizing anti-IL-12/23p40 Ab to the infected DC supernatants inhibited the production of IL-17 (Fig. 1*C*).

These results led us to hypothesize that IL-17 production was predominantly triggered by exposure to cytokines produced by infected DC (likely IL-23) and not by ligation of the TCR. To examine this, naive spleen and lung cells were stimulated with *M. tuberculosis*-infected DC, and found to produce IL-17 by ELISPOT (Fig. 2). This response was mediated by soluble factors independent of contact with DC, because the supernatant from the infected DC (but not uninfected DC) could also induce IL-17 production by naive spleen and lung cells (Fig. 2*A*). Recombinant IL-23 was also capable of inducing IL-17 in lung cells from naive mice (Fig. 2*A*).

To determine whether the IL-17 production was from CD4 T cells, naive spleen and lung cells were separated into two fractions using microbeads: $CD4^+CD8^+$ T cells and $CD4^-CD8^-$ cells. These populations were stimulated with supernatant from *M. tuberculosis*-infected DC in an ELISPOT for IL-17 (Fig. 2*B*). IL-17-producing cells were detected only in the $CD4^-CD8^-$ pool, indicating that the IL-17 production seen in naive cells under these

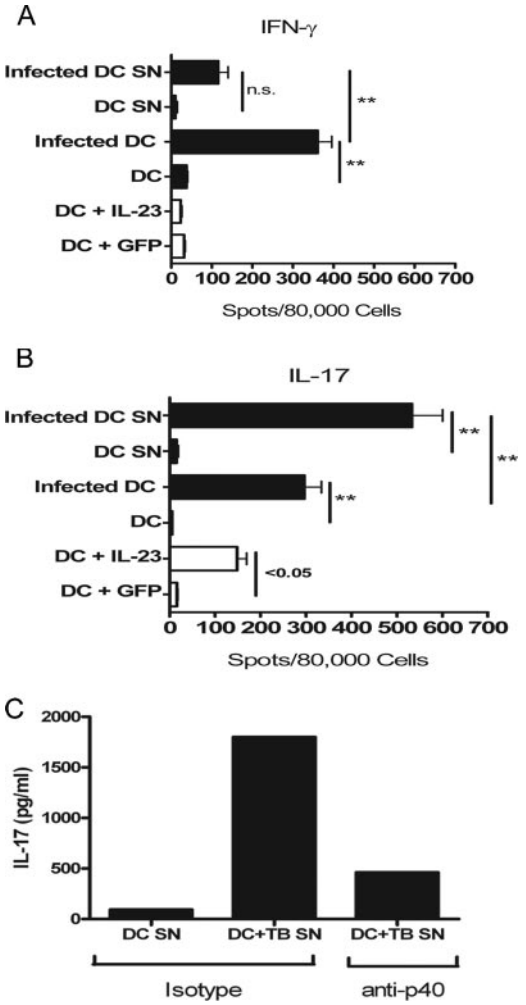

FIGURE 1. Cytokine expression in *M. tuberculosis*-infected lung. At 4 wk of infection, whole lungs ($n = 4$) were pressed through cell strainers, washed with PBS, RBC lysed, and then the cells (lung cells) were resuspended in T cell medium. Lung cells were then incubated with either uninfected DC (DC), *M. tuberculosis*-infected DC, or cultured supernatants (SN) from these DC or recombinant adenovirus expressing IL-23 or GFP. Cells were incubated for 48 h in an IFN-γ (*A*) or IL-17 (*B*) ELISPOT. **, $p < 0.001$. This experiment was repeated three times with similar results. *C*, Lung cells were stimulated with supernatants from infected DC (DC+TB SN) as above with the addition of neutralizing anti-IL-12/23p40 (anti-p40) or isotype Ab. After 48 h of incubation, the concentration of IL-17 was determined in the culture supernatant by Bioplex Suspension Array.

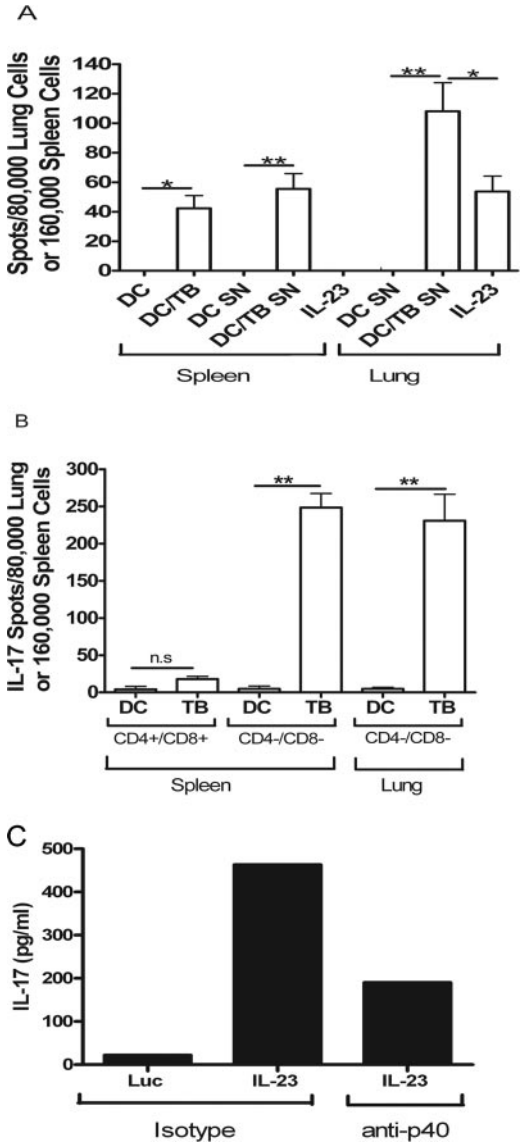

FIGURE 2. IL-17 production by naive cells is induced by IL-23 and supernatant from infected DC. *A*, Naive cells from spleen and lungs of mice ($n = 4$) were incubated with either uninfected DC (DC) or infected DC (DC/TB) or with cultured supernatants (DC SN or DC/TB SN) or 10 ng/ml rIL-23, for 48 h in an IL-17 ELISPOT. *B*, Spleen and lung cells from uninfected mice were purified with MACS beads into two pooled fractions: $CD4^+$ and $CD8^+$ T cells ($CD4^+CD8^+$) and the remaining fraction ($CD4^-CD8^-$). Sufficient $CD4^+CD8^+$ cells could not be recovered from lungs of uninfected mice. These fractions were then stimulated with either uninfected DC supernatant (DC) or infected DC supernatant (TB) in an IL-17 ELISPOT. *, $p < 0.01$; **, $p < 0.001$. This experiment was repeated once with similar results. *C*, Naive spleen cells were depleted of $CD4^+$ and $CD8^+$ T cells by magnetic bead separation. The depleted spleen cells were stimulated with DC transfected with adenovirus expressing either luciferase or IL-23 in the presence of IL-12/23p40 Ab or isotype. The concentration of IL-17 was determined at 48 h by Bioplex Suspension Array.

conditions was not from $CD4^+$ or $CD8^+$ T cells (Fig. 2*B*). Production of IL-17 protein from the $CD4^-CD8^-$ pool could be detected by incubation with adenovirus expressing IL-23, and this production was inhibited by addition of neutralizing anti-IL-12/23p40 Ab (Fig. 2*C*).

Production of IL-23 by M. tuberculosis*-infected DC induces IL-17 from lymphocytes*

Because the supernatant from *M. tuberculosis*-infected DC was so effective at inducing IL-17 production, even from naive cells, the inflammatory cytokines produced in the first 24 h following DC infection were characterized. As expected, *M. tuberculosis* infection induced proinflammatory cytokines and chemokines, including IL-1α, IL-1β, G-CSF, CCL3/MIP-1α, keratinocyte-derived chemokine, CCL5/RANTES, and TNF-α (Table I). There was appreciable production of the shared subunit of IL-12 and IL-23 (p40), but only modest IL-12p70 was induced upon infection. These results were consistent with the secretion of IL-23 during infection with *M. tuberculosis*.

To confirm that IL-23 production from infected DC was necessary for IL-17 induction, naive $CD90^+$ spleen cells were stimulated with supernatants from *M. tuberculosis*-infected wild-type DC, IL-12p35$^{-/-}$ DC, and IL-12/23p40$^{-/-}$ DC. Absence of

Table I. *Cytokine expression by infected DC*[a]

Cytokine	DC SN (pg/ml)	TB/DC SN (pg/ml)
IL-1α	5	331
IL-1β	21	503
IL-2	16	8
IL-3	3	18
IL-4	0.1	0.9
IL-5	4	20
IL-6	45	5,472
IL-10	9	54
IL-12p40	282	1,164
IL-12p70	10	85
IL-17	11	29
G-CSF	50	10,824
KC	5	941
MIP1α	3,000	10,635
RANTES	1,794	4,029
IFN-γ	1	17
TNF-α	25	785

[a] DC were cultured for 6 days. The medium was replaced, and the cells were plated at 1×10^6/ml, and then infected with *M. tuberculosis* at a multiplicity of infection of 3. After 18 h, the cells were pelleted by centrifugation and the concentration (in picograms per milliliter) of cytokines and chemokines in the culture supernatant (SN) was analyzed by Bioplex Suspension Array. This experiment was repeated once with similar results. KC, Keratinocyte-derived chemokine; TB, tuberculosis.

IL-12p70 (IL-12p35$^{-/-}$) had no impact on IL-17 production. In contrast, supernatant from *M. tuberculosis*-infected IL-12/23p40$^{-/-}$ DC (lacking IL-12 and IL-23) did not induce IL-17 production (Fig. 3*A*). To further confirm the role of IL-23 in stimulation of IL-17 from naive spleen cells, an adenovirus producing IL-23 was used to infect spleen cells. In response to this stimulation, CD90$^+$ (Thy 1.2) spleen cells produced IL-17, and there was no production of IL-17 from the CD90$^-$ cells (data not shown). The IL-17 production from CD90$^+$ cells was inhibited by addition of neutralizing anti-IL-12/23 p40 Ab (Fig. 3*B*). None of these conditions induced IFN-γ from the naive cells (data not shown).

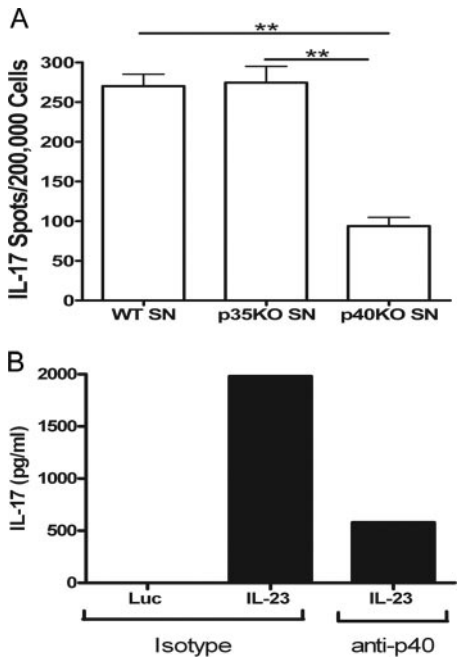

FIGURE 3. Production of IL-17 by lymphocytes is dependent on IL-12/23p40 expression in DC. *A*, Magnetic bead-purified naive spleen CD90$^+$ cells were incubated with cultured supernatant from infected wild-type, IL-12p35$^{-/-}$, or IL-12/23p40$^{-/-}$ DC in an IL-17 ELISPOT. **, $p < 0.001$. *B*, Naive spleen CD90$^+$ cells were stimulated with DC transfected with adenovirus expressing either luciferase (Luc) or IL-23, and either neutralizing anti-IL-12/23p40 Ab (anti-p40) or isotype Ab was also added to the cultured cells. The concentration of IL-17 in the culture supernatant was determined at 48 h by Bioplex Suspension Array.

γδ T cells are a source of IL-17 in naive mice

It has recently been shown that γδ T cells are involved in a homeostatic loop involving IL-23-induced IL-17 (17). Because the source of IL-17 was in the lymphocyte subset, and most of the IL-17 was not produced by CD4$^+$ or CD8$^+$ T cells in naive mice, we hypothesized that γδ T cells were one possible source of IL-17. CD90$^+$ spleen cells from uninfected wild-type and γδ KO mice were compared for IL-17 production by ELISPOT.

The number of IL-17-producing cells was significantly reduced in the γδ$^{-/-}$ mice compared with wild-type CD90$^+$ cells (Fig. 4*A*). This activity was also inhibited by anti-IL-12/23p40 Ab (Fig. 4*A*). Similarly, when the γδ KO CD90$^+$ spleen cells were stimulated with AdIL-23, there was a reduced frequency of IL-17-producing cells compared wild-type CD90$^+$ cells (Fig. 4*B*). These results support that γδ T cells were a substantial source of IL-17 produced in response to IL-23 from *M. tuberculosis*-infected DC.

γδ T cells as well as other non-CD4$^+$ T cells are major producers of IL-17 in response to M. tuberculosis *infection*

Although γδ T cells were a major source of IL-17 in uninfected mice, the contribution of these cells to IL-17 production during *M. tuberculosis* infection was unknown. The data from the γδ KO

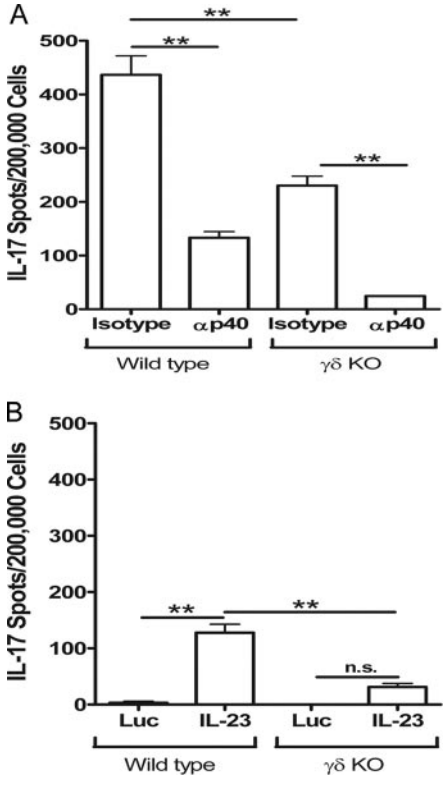

FIGURE 4. IL-17 production is reduced in cells from γδ-deficient mice. CD90$^+$ spleen cells from naive wild-type and γδ$^{-/-}$ (KO) mice ($n = 4$) were stimulated with *M. tuberculosis*-infected DC supernatant in an IL-17 ELISPOT in the presence of either isotype control or neutralizing IL-12/23p40 Ab (*A*), and with either DC plus Ad luciferase (Luc) or DC plus Ad IL-23 (IL-23) (*B*). **, $p < 0.001$.

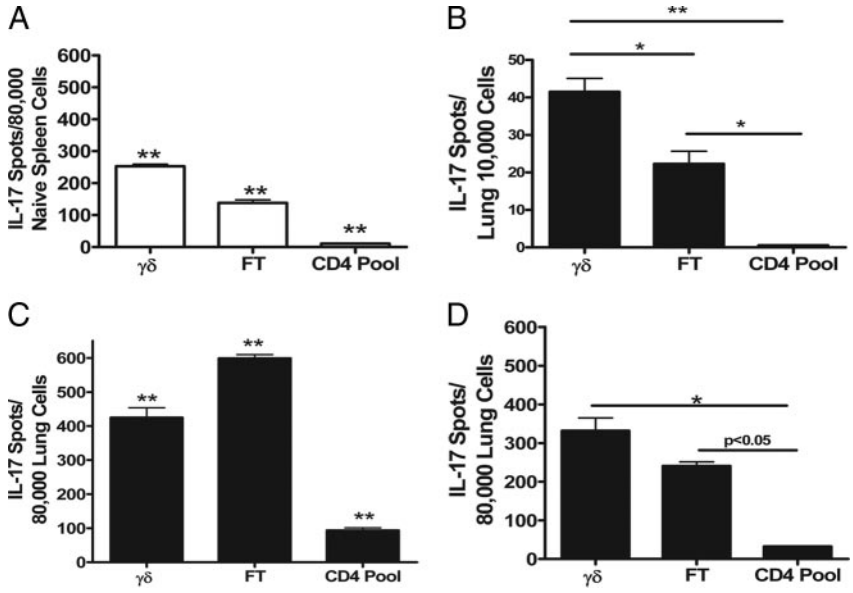

FIGURE 5. Both γδ and other non-CD4 T cells contribute to IL-17 production in naive and *M. tuberculosis*-infected mice. *A–D*, Naive spleen (*A*), lungs at 2 wk (*B*), lungs at 4 wk (*C*), and lungs at 52 wk postinfection (*D*) were purified by magnetic bead separation into three groups: enriched γδ cells, a pool of CD4/CD8/B220/CD11b cells (CD4 Pool), and the remaining cells (flow through (FT)). These cells were stimulated with supernatants from *M. tuberculosis*-infected DC for 48 h in an IL-17 ELISPOT. *, $p < 0.01$; **, $p < 0.001$. The naive and 4 wk postinfection time points were repeated once with similar results.

mice suggested that there were subsets in addition to γδ T cells that produced IL-17. We followed up these studies in *M. tuberculosis*-infected mice, and purified cells from the lungs and spleen of infected mice at various time points (2, 4, and 52 wk postinfection), using microbeads, into fractions containing 1) $CD4^+$, $CD8^+$, B cells and macrophages ("CD4 pool"); 2) γδ T cells; or 3) all other cells ("flow through"). This latter fraction contained NK cells, NK T cells, and other cell types (data not shown). These cells were stimulated with supernatant from uninfected or infected DC, and tested for IL-17 production by ELISPOT (Fig. 5). The γδ T cell fraction and the flow-through fraction both contained cells capable of producing IL-17 in response to DC supernatant, at a higher frequency than $CD4^+$ T cells. Thus, γδ T cells as well as other non-$CD4^+$ or -$CD8^+$ cells can produce IL-17 during *M. tuberculosis* infection.

To confirm the contribution of γδ T cells to IL-17 production during *M. tuberculosis* infection, $CD4^+$ and γδ cells were purified from spleens at 2 and 4 wk postinfection, and from the lungs at 4 wk postinfection by FACSAria (Fig. 6*A*). Both cell types were stimulated with infected or uninfected DC, as well as supernatant from these DC in an ELISPOT format.

Throughout the course of infection in the spleen, the γδ cells were the most frequent producers of IL-17 in response to cultured supernatant (Fig. 6*B*), even at 4 wk postinfection. Although there were IL-17-producing CD4 T cells, the frequency was low (Fig. 6*B*).

At 4 wk postinfection in the lung, the frequency of IL-17-producing cells in the γδ subset was much higher than in the $CD4^+$ T cell subset (Fig. 6*C*). The proportion of γδ cells, expressed as a percentage of $CD3^+$ cells, ranged between 2 and 3% in the lung throughout infection, whereas CD4 cells were between 47 and 54% of $CD3^+$ cells (Fig. 6*D*). When the differing proportion of these cell types was taken into account, γδ cells remained the dominant producers of IL-17 (Fig. 6*E*). The induction of IL-17 by infected DC supernatant was unaffected by the absence of IL-12 p35 but was ablated by the absence of IL-12/IL-23 p40, confirming a requirement for IL-23 (data not shown).

No IFN-γ production could be detected by cells from spleen or lung (in ELISPOT) at 2 wk postinfection, which is consistent with the kinetics of the T cell response to low dose *M. tuberculosis* infection (14). However, in contrast to IL-17 production, IFN-γ production at 4 wk postinfection in the lung and spleen was dominated by $CD4^+$ cells (data not shown). This is consistent with initial IL-17 production before significant T cell priming and IFN-γ production. In contrast to IL-17 production by γδ cells, production of IFN-γ by $CD4^+$ T cells required *M. tuberculosis*-infected DC (data not shown).

FACS analysis confirmed the ELISPOT findings. Lung cells from naive mice and mice 8 wk postinfection were stimulated with ionomycin and PMA, and IL-17 was detected by intracellular cytokine staining. The percentage of IL-17-positive γδ cells was greater than CD4 T cells at both time points (Fig. 7). CD4 T cell staining for IFN-γ is increased in infected mice compared with naive mice (data not shown). In contrast, CD4 T cell staining for IL-17 remained low throughout the course of infection (Fig. 7).

Discussion

IL-17 production by CD4 T cells defines a recently described subset of Th cells, the Th17 cells, which appear to contribute to inflammation and autoimmune responses. These cells have been reported to be present and expanded in the lungs during *M. tuberculosis* infection (16), although the role of these cells in this infection remains unknown. The current study demonstrates that another T cell subset, the γδ T cells, dominate the early production of IL-17 in response to IL-23 in *M. tuberculosis* infection, and these cells continue to produce this cytokine throughout the infection. We also demonstrate that naive γδ T cells are major producers of IL-17 in response to IL-23. This supports an innate response for γδ T cells in stimulating inflammation in response to bacterial infection.

In analyzing the IL-17 expression in the lung during infection, we found anti-CD3/CD28 Ab stimulation to be a poor inducer of IL-17 compared with stimulation with ionomycin/PMA (data not shown). Supernatant from *M. tuberculosis*-infected DC or IL-23 alone could induce IL-17 in cells. This suggested that TCR engagement could be bypassed and appears to be insufficient for IL-17 secretion. In contrast, anti-CD3/CD28 Ab is a strong inducer of IFN-γ from these lung cells, although primarily from CD4 T cells.

Production of IL-17 by γδ T cells in response to IL-23 appears to be an innate response, because this subset from naive mice responded with substantial IL-17 production when stimulated with infected DC, supernatant from infected DC, or rIL-23. The ratio of γδ to CD4 cells in naive mice remained similar throughout the course of infection. At the earliest time points examined, there was

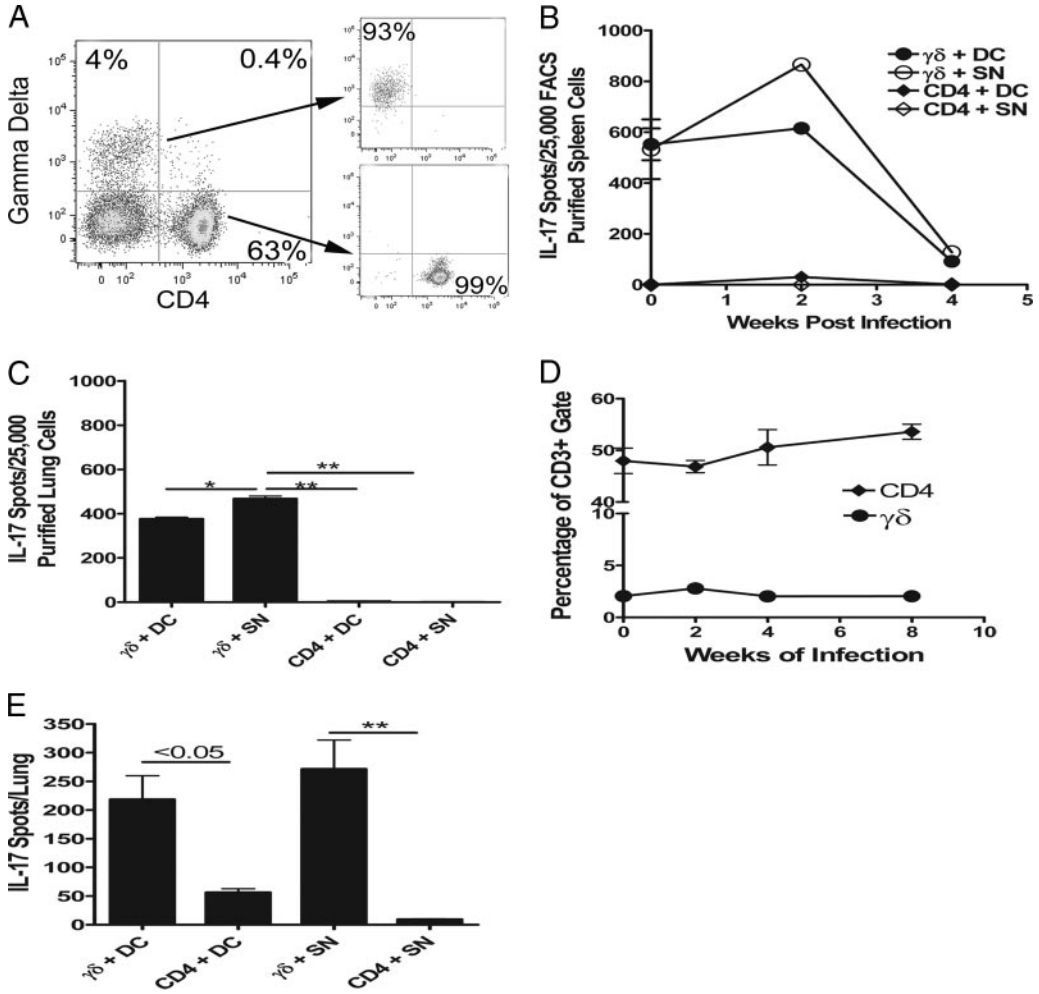

FIGURE 6. IL-17 production is higher in purified γδ cells compared with CD4 T cells. Lungs and spleens from *M. tuberculosis*-infected mice ($n = 4$) were sorted by flow cytometry into purified γδ TCR-positive and $CD4^+$ cells at various time points. *A*, A representative sort is shown for wk 4 infected spleen. *B* and *C*, The cells from spleen at various time points (*B*) and lungs at 4 wk postinfection (*C*) were stimulated with infected DC (DC) or cultured supernatants from infected DC (SN) in an IL-17 ELISPOT. *D*, Percentages of γδ and $CD4^+$ cells in the lung throughout infection. *E*, Comparison of IL-17 production adjusted to the ratio of γδ and CD4 for whole lung at 4 wk postinfection. ∗, $p < 0.01$; ∗∗, $p < 0.001$. The 4 wk time point was repeated once with similar results.

no IFN-γ produced in the lungs, but substantial IL-17, indicating that this is an early event in infection. The IL-17 production may be important for initiating early inflammatory events. At later time points, IL-17 production from CD4 T cells increased, and both subsets contributed to the total amount of IL-17 in the lungs.

The role of IL-17 in *M. tuberculosis* infection, either beneficial or detrimental, is not yet clear. At least in the mouse model, IL-23 is not required for control of this infection, nor is it necessary for IFN-γ production in the presence of IL-12 (16). However, the use of *M. tuberculosis* as a model system allows one to observe the cell subsets that can produce this cytokine, perhaps in part because of the slow induction of an IFN-γ-producing subset of T cells. It has been demonstrated that IFN-γ inhibits development of the Th17 cells (4, 5), and there is little IFN-γ present in the lungs during the initial 2 wk of infection with *M. tuberculosis*. The effects of IFN-γ on IL-17 production by γδ T cells are not known. Therefore, the lung environment early in infection is conducive to IL-17 production. Even after CD4 T cell priming and substantive production of IFN-γ, we found γδ T cell (and other non-$CD4^+$ T cell) production of IL-17, and the contribution from γδ cells was consistently greater than that from the CD4 T cells. After T cell priming, the immune response is characterized by a strong IFN-γ response; this may explain the relatively weak (compared with IFN-γ) IL-17 response from $CD4^+$ T cells. Therefore, during an immune response dominated by IFN-γ (or perhaps even IL-4), γδ cells may assume a more dominant role in producing IL-17.

How might early induction of IL-17 contribute to the immune response against lung pathogens, including *M. tuberculosis*? IL-23 induction of IL-17 from lung γδ cells has recently been implicated in steady-state granulopoiesis, which balances influx and apoptosis of neutrophils in the lung (17). We propose that this feedback mechanism between lung APCs and γδ cells acts as a proinflammatory network during infection. Neutrophils are found in the tuberculous granulomas of mice, humans, and nonhuman primates. The role of neutrophils in granuloma formation and lung inflammation in *M. tuberculosis* is not well understood. There is also an accumulation of γδ cells in the lungs of mice infected with the tuberculosis vaccine strain BCG at day 7, before that of Ag-specific αβ T cells at ~3 wk (9). γδ KO mice infected with *M. tuberculosis* did not demonstrate differences in bacterial load compared with wild-type mice, although granuloma structure was less organized (12, 24). At higher infectious doses, there was extensive influx of neutrophils and foamy macrophages in the γδ KO mice (12, 24). A general early proinflammatory role during infection

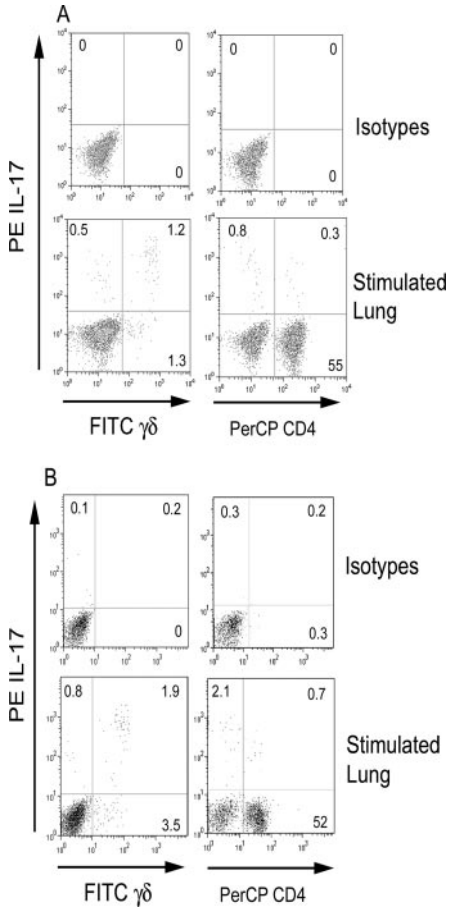

FIGURE 7. Intracellular IL-17 staining of γδ and CD4 T cells. Lung cells were harvested ($n = 4$), pooled, and stimulated with ionomycin and PMA for 4 h. Lung cells from naive wild-type mice (*A*) or mice infected with *M. tuberculosis* for 8 wk (*B*) were stained with Abs recognizing CD3, CD4, γδ TCR, and (intracellular) IL-17. The percentages of positive cells in the CD3 gate are expressed in each quadrant. The cells were first gated by forward and side scatter plot, and then on the CD3-positive gate. Shown are the γδ and $CD4^+$ cells with intracellular IL-17 expression.

and a late anti-inflammatory role for γδ cells during infections has been proposed (25).

Activation of resting Vγ1 γδ T cells is mediated by soluble factors from BCG-infected DC (26). These factors cause γδ T cells to up-regulate CD69 expression, and enhance cytotoxicity and DC IL-12 production by secretion of IFN-γ. The IFN-γ production was most likely IL-12p70 mediated; however, we found only low-level IL-12p70 production from *M. tuberculosis*-infected DC.

Our results are consistent with a role for γδ cells as early responders that promote inflammation by production of IL-17. These cells do so before CD4 T cell priming (and IFN-γ production). It has been suggested that the early expression of cytokines by γδ cells may fit the Th1/Th2 paradigm, implying a role in influencing CD4 T cell development. Early in the course of *Listeria monocytogenes* infection, γδ cells express IFN-γ, whereas early in *Nippostrongylus brasiliensis* infection, γδ cells express IL-4 (27). We show here that γδ cells produce IL-17 early in infection, thereby mirroring the recent incorporation of Th17 cells to the paradigm. In this case, the IL-17 most likely promotes an influx of cells to the lungs, although a direct role for influencing CD4 T cell development may emerge in *M. tuberculosis* infection. It is interesting to note that this IL-17 production coexists with CD4 T cell production of IFN-γ at later stages of infection.

It has also recently been shown that activated γδ T cells can present Ag to CD4 cells in a model of alternative Ag presentation function (28, 29). It is tempting to speculate that, early in infection, γδ cells are activated in the lung by mycobacterial Ag and cytokines such as IL-23. These activated γδ cells then in turn produce IL-17 aiding inflammation and up-regulate surface costimulatory molecules and MHC class II to facilitate Ag presentation to subsequently infiltrating αβ T cells.

The data presented in this study demonstrate that, in addition to the newly recognized Th17 CD4 T cell subset, γδ T cells as well as other non-CD4/CD8 cells can produce IL-17 in response to factors, including IL-23, produced by *M. tuberculosis*-infected DC. These cells, present in naive mice as well as *M. tuberculosis*-infected mice, may play an important role in initiating inflammation and recruiting cells to the site of infection. There may be additional roles for IL-17-secreting cells during infections that are specific to these non-classically restricted T cells.

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Disclosures

The authors have no financial conflict of interest.

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