NKG2D-Dependent IL-17 Production by Human T Cells in Response to an Intracellular Pathogen

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We studied the factors that control IL-17 production in human *Mycobacterium tuberculosis* infection. CD4+ cells from healthy tuberculin reactors produced IL-17 in response to autologous *M. tuberculosis*-stimulated monocytes, and most IL-17+ cells were Ag experienced, CD4+CD62L+IL-17+CD11b+ cells, and IL-17 production by CD4+ cells was inhibited by CD4+CD62L+IL-17+CD11b+ cells (10), so that the major cellular subpopulations that produce IL-17 are CD4+ cells. This article must therefore be hereby marked (AI054629, AI073612, and AI063514), the James Byers Cain Research Endowment, was supported by grants from the National Institutes of Health (11). Previously, we found that NK cells express NKG2D and that this was inhibited by anti-NKG2D and that this was described primarily in patients with rheumatoid arthritis and Crohn’s disease (14, 15). NKG2D expression on CD4+ cells has been described in patients with rheumatoid arthritis and Crohn’s disease (14, 15), and CD4+NKG2D+ T cells mediate inflammatory responses (14). Interactions between CD40 on APCs and CD40L on T cells can enhance production of inflammatory cytokines (16), and could modulate IL-17 production. In this study, we evaluated the roles of NKG2D and CD40 in regulating IL-17 production in human *M. tuberculosis* infection. We found that NKG2D expressed on CD4+ cells contributes to IL-23-dependent IL-17 production by CD4+ cells in human *M. tuberculosis* infection.

**Materials and Methods**

**Patient population**

After obtaining informed consent, blood was obtained from 20 healthy tuberculin reactors (persons with a tuberculin skin test diameter of at least 10 mm) and 13 tuberculin-negative donors, and bronchoalveolar lavage fluid was obtained from 5 healthy tuberculin-negative donors. All studies were approved by the Institutional Review Board of the University of Texas Health Center at Tyler.

**Abs and other reagents**

For flow cytometry, we used FITC anti-CD4, FITC anti-CD14, PE anti-CD62L (all from eBiosciences), and allophycocyanin anti-NKG2D (Biolegend). For neutralization, we used mAbs to IL-1 and IL-6 (both from eBiosciences); CD40, IL-23, and TGF-β (all from R&D Systems); NKG2D (Amgen); and isotype control Abs (eBiosciences). γ-Irradiated *M. tuberculosis* H37Rv was obtained from J. Belisle, Colorado State University (Fort Collins, CO).

**Isolation of monocytes, CD4+ cells, and CD8+ cells**

PBMC were isolated by centrifugation over Ficoll-Paque (GE Health). Monocytes and CD4+ and CD8+ cells were isolated by magnetic beads conjugated to anti-CD14, anti-CD4, or anti-CD8 (all from Miltenyi Biotec), respectively. Cell purity was 95%, as measured by flow cytometry.
Culture of alveolar macrophages

Bronchoalveolar lavage was performed by standard methods, and alveolar macrophages were isolated and infected with *M. tuberculosis* H37Ra, as previously described (17). Cells (1.2 × 10⁶) were adhered to each well of 12-well flat-bottom plates for 2 h. Nonadherent cells were removed by washing each well three times with 4 ml of RPMI 1640, and adherent cells were cultured in RPMI 1640 complete medium.

Culture of CD4⁺ cells or CD8⁺ cells and monocytes

Freshly isolated CD4⁺ cells or CD8⁺ cells were cultured in 12-well plates at 2 × 10⁶ cells/well in RPMI 1640 containing penicillin (Life Technologies) and 10% heat-inactivated human serum, with or without 2 × 10⁶ autologous monocytes/well. CD4⁺ cells and monocytes were cultured in the presence or absence of γ-irradiated *M. tuberculosis* H37Rv (10 μg/ml) at 37°C in a humidified 5% CO₂ atmosphere. After 3 days, cell-free culture supernatants were collected, aliquoted, and stored at −70°C until cytokine concentrations were measured. For some experiments, 10 μg/ml neutralizing Abs to IL-1, IL-6, IL-23, or TGF-β were added.

Frequency of CD4⁺CD62L⁺ and CD4⁺CD62L⁻ cells producing IL-17

CD4⁺ cells and autologous monocytes were cultured in the presence or absence of γ-irradiated *M. tuberculosis* H37Rv, and after 48 h, CD4⁺CD62L⁺ and CD4⁺CD62L⁻ cells were isolated, using positive selection with CD62L microbeads. Aliquots of CD4⁺CD62L⁺ and CD4⁺CD62L⁻ cells were plated on ELISPOT plates, and the number of cells that produce IL-17 was detected (eBiosciences). In some experiments, monocytes and either freshly isolated CD4⁺CD62L⁺ or CD4⁺CD62L⁻ cells were cultured in the presence or absence of γ-irradiated *M. tuberculosis* H37Rv. After 48 h, cells were placed on ELISPOT plates, and the number of cells that produce IL-17 was detected.

Detection of intracellular IL-17

To measure intracellular IL-17 in cocultures of CD4⁺ cells and γ-irradiated *M. tuberculosis* H37Rv-stimulated monocytes, we used the intracellular staining kit from eBiosciences. For triple staining, allophycocyanin anti-NKG2D and FITC anti-CD4 were first added. After washing with PBS and 2% FCS, cells were fixed in Cytofix/Cytoperm and washed twice in 1× permeabilization/wash solution. PE anti-IL-17 was then added in staining buffer and 1× permeabilization/wash solution. After incubation at 4°C for 40 min, cells were washed in PBS and 2% FCS, and analyzed by flow cytometry.

Measurement of IL-23, IL-17, and IFN-γ concentrations

Supernatants were stored at −70°C until cytokine concentrations were measured. IL-23, IL-17, and IFN-γ levels were measured by ELISA (eBiosciences).

Statistical analysis

Results are shown as the mean ± SE. For data that were normally distributed, comparisons between groups were performed by Student’s paired or unpaired *t* test, as appropriate. For data that were not normally distributed, the nonparametric Mann-Whitney *U* test was performed.

Results

**IL-17 production by M. tuberculosis-stimulated CD4⁺ and CD8⁺ cells**

IL-17 is generally considered to be produced by CD4⁺ cells, and one recent study showed that IL-17 is produced by CD4⁺ cells, but not CD8⁺ or γδ T cells that are stimulated with *Mycobacterium bovis* bacillus Calmette-Guerin (10). However, CD8⁺ cells also produce IL-17 in response to *Klebsiella pneumoniae* (18). To determine the cellular source of IL-17 in response to *M. tuberculosis*, we isolated CD4⁺ and CD8⁺ cells from PBMC of three healthy tuberculin reactors, and cultured them in the presence or absence of irradiated *M. tuberculosis* for 24–96 h. *M. tuberculosis* induced IL-17 production by CD4⁺ cells at all time points, with maximum concentrations 72 h after stimulation (Fig. 1A). In contrast, *M. tuberculosis* did not elicit IL-17 production by CD8⁺ cells.

To determine whether IL-17 production depends on prior Ag exposure, we isolated CD4⁺ cells from PBMC of 15 tuberculin reactors and 13 tuberculin-negative persons, and cultured them in...
indicating that Ag-experienced, CD4+CD62L− cells from healthy purified protein derivative (PPD)1 donors were cultured with γ-irradiated M. tuberculosis-stimulated autologous monocytes, in the absence or the presence of 10 μg/ml neutralizing Abs to NKG2D or isotype control Abs, and supernatants were collected after 72 h. IL-17 concentrations were measured by ELISA. The horizontal line shows the median, the boxes show the 25th and 75th percentile values, and the whiskers show the 5th and 95th percentile values. B, NKG2D-dependent IL-17 production by CD4+CD62L− cells. Freshly isolated CD4+ cells from healthy tuberculin reactors were cultured with γ-irradiated M. tuberculosis-stimulated autologous monocytes, in the absence or presence of 10 μg/ml neutralizing anti-NKG2D or isotype control Abs. After 48 h, CD4+CD62L− and CD4+CD62L− cells were isolated and incubated overnight in triplicate wells on an ELISPOT plate to detect IL-17-producing cells. Mean values ± SE are shown. C, NKG2D is not expressed by IL-17-producing cells. Freshly isolated CD4+ cells from six healthy PPD+ donors were cultured in the absence or the presence of γ-irradiated M. tuberculosis and autologous monocytes. After 48 h, surface staining was performed with anti-NKG2D and intracellular staining was performed with anti-IL-17 (two left panels). Top right panel. Shows staining with anti-IL-17 and anti-CD56 to detect contaminating NK cells. Bottom right panel. Shows staining with an isotype control Ab for anti-IL-17. A representative figure is shown of five independent experiments. D, Effects of anti-ULBP1 on IL-17 production by CD4+ cells. Freshly isolated CD4+ cells from healthy PPD+ donors were cultured with γ-irradiated M. tuberculosis-stimulated autologous monocytes, in the absence or the presence of 10 μg/ml neutralizing Abs to ULBP1 or isotype control Abs, and supernatants were collected after 72 h. IL-17 concentrations were measured by ELISA.

**CD4+CD62L− cells are the major source of IL-17**

The above findings suggest that CD4+ cells are the major source of M. tuberculosis-induced IL-17. To identify the CD4+ cell subpopulation that produces IL-17, we cultured CD4+ cells from PBMC of 10 healthy tuberculin reactors with autologous monocytes, in the presence or absence of M. tuberculosis. After 48 h, CD62L+ and CD62L− cells were isolated by magnetic selection, and ELISPOT was performed to identify IL-17-producing cells. M. tuberculosis increased the frequency of IL-17+ cells from 7.2 ± 2.5 per 106 to 114 ± 23 per 106 CD4+CD62L− cells, compared with 8.9 ± 2.3 per 106 CD4+CD62L+ cells (p < 0.001; Fig. 1C), indicating that Ag-experienced, CD4+CD62L− cells are the major source for IL-17.

Because some CD62L+ cells can lose CD62L expression during culture, we also determined the capacity of freshly isolated CD4+CD62L− and CD4+CD62L+ cells to produce IL-17 upon culturing with M. tuberculosis. Monocytes from six healthy tuberculin reactors were cultured with freshly isolated CD4+CD62L+ or CD4+CD62L− cells, in the presence or absence of γ-irradiated M. tuberculosis H37Rv. After 48 h, cells were placed on ELISPOT plates, and the number of cells that produce IL-17 was detected. M. tuberculosis increased the frequency of IL-17+ cells from 0 to 86 ± 28 per 105 CD4+CD62L− cells, compared with 38 ± 12 per 105 CD4+CD62L+ cells (p = 0.05).

NKG2D, but not CD40, contributes to M. tuberculosis-induced IL-17 production

Costimulatory molecules play an important role in eliciting cytokine production by T cells. Therefore, we studied the role of the costimulatory molecules, NKG2D and CD40, in IL-17 production. We cultured CD4+ cells and autologous monocytes from PBMC of healthy tuberculin reactors with M. tuberculosis, in the presence of anti-NKG2D or anti-CD40 or isotype control Ab (10 μg/ml). In eight healthy tuberculin reactors, anti-NKG2D reduced IL-17 levels by ~60% (1016 ± 284 vs 423 ± 186 pg/ml, p < 0.001, Mann-Whitney U test; Fig. 2A), compared with isotype control Ab (Fig. 2A). In seven healthy tuberculin reactors, anti-CD40 Ab had no effect on IL-17 production (398 ± 60 vs 403 ± 58 pg/ml).

IL-17 production by CD4+CD62L− cells is NKG2D dependent

We next asked whether NKG2D regulates IL-17 production by Ag-experienced CD4+CD62L− cells. We cultured CD4+ cells and autologous monocytes from PBMC of healthy tuberculin reactors with M. tuberculosis, in the presence of anti-NKG2D or
isotopic control Ab (10 μg/ml). In eight healthy tuberculin reactors, anti-NKG2D reduced the frequency of CD4+CD62– IL-17+ cells from 128 ± 26 per 10^6 cells to 32 ± 8 per 10^6 cells (p = 0.006; Fig. 2B), whereas in four healthy tuberculin reactors, isotype control Ab had no effect (81 ± 20 per 10^6 cells vs 59 ± 18 per 10^6 cells, p = 0.19).

CD4+NKG2D+ cells are not the source of IL-17

The above findings suggest that NKG2D expressed on CD4+ cells is essential for optimal IL-17 production. To determine whether CD4+NKG2D+ cells are the source of IL-17, we cultured CD4+ cells from PBMC of six healthy tuberculin reactors with autologous monocytes, in the presence or absence of M. tuberculosis. After 48 h, cells were stained for CD4, NKG2D, and IL-17. M. tuberculosis increased the number of CD4+NKG2D+ cells from 1.7 ± 0.3% to 3.7 ± 0.7% (p = 0.02). Similarly, M. tuberculosis increased the number of CD4+IL-17+ cells from 0.5 ± 0.3% to 1.2 ± 0.3% (p < 0.001). As shown in the left panels of Fig. 2C, IL-17-producing CD4+ cells are not NKG2D+ cells. Expression of NKG2D was not due to contaminating NK cells in the CD4+ cell preparations, which only contained 0.6% CD56+ cells (Fig. 2C, top right panel).

Effect of anti-ULBP1 on IL-17 production

Previously, we found that among the different NKG2D ligands, only ULBP1 expression was up-regulated on M. tuberculosis-infected monocytes (12). To determine whether NKG2D and ULBP1 interaction is responsible for IL-17 production by CD4+ cells, we cultured CD4+ cells and autologous monocytes from PBMC of healthy tuberculin reactors with M. tuberculosis, in the presence of anti-ULBP1 or isotype control Ab (10 μg/ml). In six healthy tuberculin reactors, anti-ULBP1 significantly inhibited IL-17 production by CD4+ cells from 589 ± 157 to 145 ± 33 pg/ml (p = 0.03; Fig. 2D), and isotype control Ab had no effect (Fig. 2D).

Anti-IL-23 inhibits M. tuberculosis-induced IL-17 production

Previous studies have demonstrated that the initial development of Th17 cells from naive T cells requires TGF-β and IL-6, produced by APCs, and that subsequent expansion of Th17 cells depends on IL-23 (19, 20). To determine whether specific monokines contribute to M. tuberculosis-induced IL-17 production, we cultured M. tuberculosis-activated monocytes from healthy tuberculin reactors with autologous CD4+ cells, in the presence or absence of neutralizing Abs to IL-23, TGF-β, IL-1, and IL-6. In 10 healthy tuberculin reactors, anti-IL-23 significantly abrogated IL-17 production by CD4 cells (1041 ± 149 to 280 ± 89 pg/ml, p < 0.001; Fig. 3A), whereas Abs to TGF-β, IL-1, IL-6, and isotype control IgG2 had no effect (Fig. 3A).

Production of IL-23 by M. tuberculosis-stimulated monocytes and alveolar macrophages

Our results above suggest that IL-23 contributes to M. tuberculosis-induced IL-17 production. Because APCs are believed to be the major cellular source of IL-23 (21, 22), we isolated CD14+ monocytes from PBMC of 14 healthy tuberculin reactors and cultured them in the presence or absence of M. tuberculosis for 48 h. IL-23 concentrations increased 3-fold upon M. tuberculosis stimulation (23.8 ± 6.5 vs 62.7 ± 12 pg/ml, p = 0.01; Fig. 3B).

Because M. tuberculosis first encounters the immune system in the lung, we next evaluated the capacity of alveolar macrophages
from five healthy donors to secrete IL-23 in response to *M. tuberculosis*. Infection with *M. tuberculosis* H37Ra significantly up-regulated IL-23 production (6.4 ± 3.7 vs 32.7 ± 7.9 pg/ml, *p* = 0.01; Fig. 3B).

**CD4+ cells enhance production of IL-23 in a NKG2D-dependent manner**

We hypothesized that NKG2D, expressed by activated CD4+ cells, increased production of IL-23 by *M. tuberculosis*-stimulated monocytes, which in turn increased IL-17 production by CD4+ T cells. To test this hypothesis, we cultured *M. tuberculosis*-stimulated monocytes with autologous CD4+ cells from healthy tuberculin reactors. CD4+ cells enhanced IL-23 production by *M. tuberculosis*-stimulated monocytes, and this effect was markedly reduced by anti-NKG2D (451 ± 69 pg/ml vs 204 ± 70 pg/ml, *p* = 0.03; Fig. 4). Isotype control Abs did not affect IL-23 production (Fig. 4).

**Discussion**

Recent studies in animal models have demonstrated that IL-17 is essential for development of a protective response to vaccination against *M. tuberculosis* (6), but limited information is available on the mechanisms that control IL-17 production in the human response to *M. tuberculosis* infection. In the current study, we found that Ag-experienced CD4+ cells are the major source for IL-17, and that IL-23, but not TGF-β or IL-6, contributes to IL-17 production. Furthermore, addition of CD4+ cells to *M. tuberculosis*-stimulated monocytes increased IL-23 production, and this was mediated through NKG2D. Anti-NKG2D and anti-ULBP1 inhibited IL-17 production, suggesting that NKG2D on CD4+ cells interacts with ULBP1 on monocytes to induce IL-23-dependent IL-17 production in human *M. tuberculosis* infection.

IL-17 is an inflammatory T cell cytokine that causes autoimmune disease (4), but can also contribute to resistance against intracellular pathogens, including viruses, fungi, parasites, and mycobacteria (6, 8, 9, 23–25). IL-17 is not essential for resistance to *M. tuberculosis* (26), but is critical for vaccine-induced protection against challenge with *M. tuberculosis* (6). γδ T cells are the major source for IL-17 in murine infection with several intracellular pathogens, including *M. tuberculosis* (9, 27, 28). However, we found that Ag-experienced CD4+CD62L- cells are the major source for IL-17 in human *M. tuberculosis* infection, confirming recent findings that IL-17 is produced by CD4+, but not CD8+ or γδ T cells, from persons with latent tuberculosis infection (10).

Differentiation of naïve T cells to Th17 cells requires TGF-β and IL-6, but expansion of Th17 cells depends on IL-23. In animal models, IL-23 is essential for IL-17 production during infection by Helicobacter pylori, Pneumocystis carinii, and *K. pneumoniae* (18, 30, 31). However, for mycobacterial infection, findings of others (34, 35), our experimental system evaluated Ag-experienced CD4+ cells, and it is possible that IL-1, IL-6, and TGF-β are important for differentiation of Th17 cells from naïve cells.

Our most surprising finding was that NKG2D enhanced production of IL-23 by APCs, and contributed to IL-17 production by CD4+ cells in response to *M. tuberculosis*. NKG2D expression on CD4+ cells has been described primarily in patients with rheumatoid arthritis and Crohn’s disease (14, 15). CMV can also expand NKG2D+CD4+ cells from CMV-seropositive individuals (36). In these settings, TNF-α and IL-15 induced expression of NKG2D (14, 15), which appeared to act as an alternative costimulatory molecule, because NKG2D+ cells were CD28+ (15, 36).

We found that NKG2D expression on CD4+ cells was enhanced by exposure to monocytes and *M. tuberculosis*, and that production of both IL-23 and IL-17 was inhibited by neutralization of either NKG2D or its ligand on APCs, ULBP1. We have previously shown that, of the multiple ligands for NKG2D, only ULBP1 is up-regulated on the surface of mononuclear phagocytes during *M. tuberculosis* infection (12). During the early stages of mycobacterial infection, mononuclear phagocytes produce soluble mediators, including IL-15 (37), TNF-α (38), and IL-23 (the current study).

We speculate that IL-15 and TNF-α enhance expression of NKG2D on CD4+ cells, which interacts with ULBP1 to increase IL-23 production (Fig. 4), and thus IL-17 production by CD4+CD62L- cells. IL-23 production was significantly increased by the addition of only 4 T cells per 10 monocytes (Fig. 4), a ratio that may mimic the limited number of activated T cells that are present in the lung during the early stages of infection.

In summary, we found that NKG2D expressed on CD4+ cells interacts with ULBP1 on *M. tuberculosis*-infected mononuclear phagocytes to enhance IL-23 secretion by mononuclear phagocytes, which in turn favors IL-17 production by CD4+CD62L- cells. This suggests a novel role for NKG2D in protection against intracellular bacteria such as *M. tuberculosis*.

**Disclosures**

The authors have no financial conflict of interest.

**References**


