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T Cells Enhance Production of IL-18 by Monocytes in Response to an Intracellular Pathogen

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We studied the effect of T cells on IL-18 production by human monocytes in response to Mycobacterium tuberculosis. Addition of activated T cells markedly enhanced IL-18 production by monocytes exposed to M. tuberculosis. This effect was mediated by a soluble factor and did not require cell-to-cell contact. The effect of activated T cells was mimicked by recombinant IFN-γ and was abrogated by neutralizing Abs to IFN-γ. IFN-γ also enhanced the capacity of alveolar macrophages to produce IL-18 in response to M. tuberculosis, suggesting that this mechanism also operates in the lung during mycobacterial infection. IFN-γ increased IL-18 production by increasing cleavage of pro-IL-18 to mature IL-18, as it enhanced caspase-1 activity but did not increase IL-18 mRNA expression. These findings suggest that activated T cells can contribute to the initial immune response by augmenting IL-18 production by monocytes in response to an intracellular pathogen. The Journal of Immunology, 2001, 166: 6749–6753.

Materials and Methods

Patient population

Blood was obtained from nine healthy tuberculin reactors and three healthy tuberculin-negative persons. Bronchoalveolar lavage fluid was obtained from four tuberculin-negative patients who were undergoing bronchoscopy, which was performed to evaluate the possibility of cancer. No patient had clinical or laboratory evidence of tuberculosis or HIV infection. In all cases, the pathologic process was unilateral, and bronchoalveolar lavage was performed in the lung where there was no clinical, bronchoscopic, or radiographic evidence of cancer. Informed consent was obtained from all patients. All studies were approved by the Institutional Review Board of the University of Texas Health Center at Tyler.

Isolation of monocytes and M. tuberculosis-activated T cells

PBMC were isolated from blood by differential centrifugation over Ficoll-Paque (Pharmacia, Piscataway, NJ). PBMC were centrifuged on a Percoll gradient (Amersham Pharmacia Biotech, Uppsala, Sweden), and purified CD14+ cells were isolated from the monocyte fraction by positive selection with magnetic beads conjugated to anti-CD14 (Miltenyi Biotech, Auburn, CA). The positive cells were 94% CD14+, as measured by flow cytometry.

PBMC (2 × 10⁶/ml) were cultured in the presence of heat-killed M. tuberculosis Erdman (10 μg/ml) in a 12-well plate for 4 days. Purified CD3+ cells were isolated by positive selection with magnetic beads conjugated to anti-CD3 (Miltenyi Biotech) and were 96% CD3+ as measured by flow cytometry.

Culture of monocytes and T cells

CD14+ monocytes (5 × 10⁴) were plated in flat-bottom 96-well plates (Becton Dickinson Labware, Franklin Lakes, NJ) in 200 μl of RPMI 1640 (Life Technologies, Grand Island, NY) containing penicillin/streptomycin (Life Technologies) and 10% heat-inactivated human serum, in the presence or absence of 10 μg/ml of heat-killed M. tuberculosis Erdman, provided by Patrick Brennan, Colorado State University (Fort Collins, CO). In some experiments, recombinant IL-12 (a gift from Maurice Gately, Hoffman-LaRoche, Nutley, NJ), IL-2, IL-10, TNF-α, and IFN-γ (all obtained from PharMingen, San Diego, CA), neutralizing Abs to IFN-γ (Genzyme, Cambridge, MA), IL-12, IL-10, TNF-α, or isotype control mouse IgG1 (all obtained from PharMingen) were also added to the cells. In other experiments, monocytes were incubated with 1 μM of the caspase-1 inhibitor Ac-YVAD-cho (Bachem, Torrance, CA), before addition of M. tuberculosis and IFN-γ.

In some experiments, graded numbers of autologous M. tuberculosis-activated T cells were added to 5 × 10⁴ monocytes per well in flat-bottom 96-well plates. In other experiments, 10⁴ monocytes per well were plated in 1 ml of RPMI 1640 and 10% heat-inactivated human serum, with or without 10 μg/ml of heat-killed M. tuberculosis, in 12-well plates with Transwell inserts (Costar, Cambridge, MA). The inserts contained no cells or 2 × 10⁴ M. tuberculosis-activated autologous T cells in RPMI 1640 with 10% heat-inactivated human serum. The insert contained 0.4-μm-diameter pores that allowed diffusion but not cell-to-cell contact.

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Culture of alveolar macrophages
Bronchoalveolar lavage fluid was passed through sterile gauze and centrifuged at 834 × g for 5 min. The cell pellet was resuspended in RPMI 1640, and 1.2 × 10^5 cells were allowed to adhere to each well of flat-bottom 96-well plates. Approximately 90% of the bronchoalveolar lavage cells were macrophages, as judged by Giemsa staining. Nonadherent cells were removed, and adherent cells were >98% alveolar macrophages. These adherent cells were cultured with or without 10 μg/ml of *M. tuberculosis* Erdman.

Measurement of cytokine concentrations
For measurement of IL-18, supernatants from cultured cells were collected after 24 h and stored at −70°C until IL-18 concentrations were measured by ELISA (MBL International, Nagoya, Japan). Concentrations of IL-18 were minimal when cells were cultured in medium alone, and results are expressed as the cytokine concentration in supernatants from *M. tuberculosis*-stimulated cells, minus that in supernatants from unstimulated cells. In some experiments, TNF-α concentrations were measured by ELISA (PharMingen).

Real time PCR for quantification of IL-18 mRNA
Total RNA was extracted from 1.5 × 10^6 monocytes 20 h after culture with heat-killed *M. tuberculosis* using Trizol reagent (Life Technologies). RNA was treated with DNase and Rnase RNase inhibitor (Promega, Madison, WI), extracted with phenol/chloroform, and reverse transcribed using the OligoT15 primer (Promega) and Omniscript reverse transcriptase (Qigen, Valencia, CA).

Primers and probes for the IL-18 gene were designed using Primer Express software (PE Biosystems, Foster City, CA) and synthesized by Operon Technologies (Alameda, CA). The forward and reverse primers were 5′-ATCGCGCTCTATTTAGATAGACT-3′ and 5′-CTCTGGTCGCTATCTTTATACACT-3′, respectively. The probe sequence was 5′-ACTGTAAGATATAATACACCGGACATTTATTT-3′, 5′-fluorescein phosphoramidite-labeled and 3′-TAMRA-labeled. The IL-18 primers and probe spanned an intron, so that they detected IL-18 mRNA but not genomic DNA. The Taqman β-actin control reagents (PE Biosystems) were used as the internal standard. Real time PCR assays were performed in a sealed 96-well microtiter plate (PE Biosystems) on a spectrophotofluorometric thermal cycler (Applied Biosystems 7700 Prism; PE Biosystems). Each cDNA sample was aliquoted into wells containing 1× Taqman Universal PCR master mix (PE Biosystems), 5 pmol of either the IL-18 or β-actin primers, and 1 pmol of the corresponding probe in a total volume of 25 μl. Standard curves for quantification of β-actin cDNA were generated by the appropriate primers and probe to amplify and detect serial 10-fold dilutions of 10 ng/ml of human male DNA (PE Biosystems). Standard curves for quantification of IL-18 cDNA were generated by using IL-18 primers and probe to amplify serial 10-fold dilution of IL-18 cDNA prepared from LPS-stimulated monocytes. Amplification of each sample and each standard curve dilution were performed in triplicate, as follows: 50°C for 2 min, 95°C for 10 min, and 40 cycles of 95°C for 15 s and 60°C for 1 min. The fluorescent signal was measured and plotted during each 60°C annealing and extension step for all samples. Using the cycle threshold (the number of PCR cycles required for the fluorescent dye to be detectable), and the constructed standard curve for each cDNA, the relative amounts of IL-18 and β-actin in each sample were determined.

Measurement of caspase-1 activity
Caspase-1 activity in monocytes was measured by a colorimetric assay (R&D Systems, Minneapolis, MN). In brief, the culture medium was removed, and the cells were washed with PBS, collected with a plastic scraper, and pelleted by centrifugation at 400 × g for 10 min at 4°C. Lysis buffer was added, and the cell lysate was incubated on ice for 10 min, then centrifuged at 10,000 × g for 1 min. Cell lysate (50 μl) was mixed with 50 μl of 2× reaction buffer containing DTT in a 96-well flat-bottom plate. To each reaction well, 5 μl of caspase-1 colorimetric substrate was added and incubated at 37°C for 2 h. Cytosolic caspase-1 activity was assayed by measuring absorbance at 405 nm.

Statistical analysis
Results are shown as the mean ± SE. For data that were normally distributed, comparisons between groups were performed by a paired or unpaired t test, as appropriate. For data that were not normally distributed, the Wilcoxon rank-sum test was used. Values of p < 0.05 were considered statistically significant.

Results
Effect of *T* cells on IL-18 production by *M. tuberculosis*-stimulated monocytes
We hypothesized that T cells activated by *M. tuberculosis* enhanced the capacity of monocytes to produce IL-18. To test this hypothesis, we first measured IL-18 production by monocytes cultured with heat-killed *M. tuberculosis*. Graded numbers of autologous T cells activated by *M. tuberculosis* were added to these monocytes, and IL-18 concentrations were measured. In six healthy tuberculin reactors, monocytes cultured with heat-killed *M. tuberculosis* produced 74 ± 11 pg/ml of IL-18. Addition of activated T cells increased IL-18 production in a dose-dependent manner, with a maximum increase of 7-fold, to 514 ± 118 pg/ml (p = 0.001, Fig. 1). When activated T cells were cultured alone in the absence of monocytes, IL-18 was not detectable.

Effect of soluble factors produced by *T* cells on IL-18 production by monocytes
Next we asked whether soluble factors produced by *M. tuberculosis*-activated T cells contribute to increased IL-18 production. Activated autologous T cells from five healthy tuberculin reactors were cocultured in transwells in 12-well plates containing monocytes exposed to heat-killed *M. tuberculosis*. Activated T cells increased IL-18 production 3- to 8-fold (1012 ± 138 vs 230 ± 31 pg/ml, p = 0.005, Fig. 2). *M. tuberculosis*-activated T cells from healthy tuberculin-negative donors did not significantly enhance IL-18 production by monocytes (data not shown).

Effect of IFN-γ on IL-18 production by monocytes and alveolar macrophages
The above findings suggest that IL-18 production by *M. tuberculosis* activates monocytes to produce increased IL-18. Monocytes were isolated from six healthy tuberculin reactors and were cultured with heat-killed *M. tuberculosis* Erdman. Graded numbers of autologous T cells were added to these monocytes, and IL-18 concentrations were measured. In six healthy tuberculin reactors, monocytes cultured with heat-killed *M. tuberculosis* produced 74 ± 11 pg/ml of IL-18. Addition of activated T cells increased IL-18 production in a dose-dependent manner, with a maximum increase of 7-fold, to 514 ± 118 pg/ml (p = 0.001, Fig. 1). When activated T cells were cultured alone in the absence of monocytes, IL-18 was not detectable.

**FIGURE 1.** Effect of activated T cells on production of IL-18 by *M. tuberculosis*-stimulated monocytes. Monocytes were isolated from six healthy tuberculin reactors and were cultured with 10 μg/ml of heat-killed *M. tuberculosis* Erdman. Graded numbers of *M. tuberculosis*-activated T cells were added to the monocytes. After 24 h, IL-18 concentrations in monocyte supernatants were measured by ELISA. Mean values and SE values are shown.
Because tuberculosis is a disease that primarily affects the lungs, we next wished to evaluate the effects of IFN-γ on IL-18 production by alveolar macrophages, obtained from four patients by bronchoalveolar lavage. Alveolar macrophages produced higher IL-18 concentrations in response to M. tuberculosis than did monocytes, confirming previously published results (12). Alveolar macrophages have undergone differentiation in vivo and may be primed to generate higher concentrations of IL-18 in response to microbial stimuli than monocytes. IFN-γ significantly increased IL-18 production by alveolar macrophages cultured with heat-killed M. tuberculosis (1101 ± 275 vs 431 ± 166 pg/ml, p = 0.01, Fig. 4). Other cytokines had no effect on IL-18 production.

The above results suggested that IFN-γ secreted by M. tuberculosis-activated T cells augmented IL-18 production by monocytes exposed to M. tuberculosis. To test this hypothesis, monocytes from six healthy tuberculin reactors were stimulated with M. tuberculosis in the presence of activated T cells, with or without anti-IFN-γ Abs. Anti-IFN-γ abrogated the capacity of activated T cells to enhance IL-18 production by monocytes (Fig. 5), whereas Abs to TNF-α, IL-12, IL-10, and anti-isotype control IgG had no effect (data not shown).

**Effect of recombinant IFN-γ on IL-18 mRNA expression**

To study the mechanisms by which IFN-γ enhanced M. tuberculosis-induced IL-18 release, we cultured monocytes from four healthy tuberculin reactors with heat-killed M. tuberculosis, with or without IFN-γ, and quantified IL-18 cDNA using real-time PCR. To normalize for the efficiency of RNA extraction and reverse transcription in different samples, data were expressed as the ratio of IL-18 cDNA to β-actin cDNA, expressed in arbitrary units. Addition of IFN-γ did not increase the IL-18/β-actin cDNA ratio (10.5 ± 2.7 vs 9.4 ± 3.7, p = 0.60; Fig. 6), indicating that IFN-γ did not enhance IL-18 mRNA expression.

**Effect of IFN-γ on posttranscriptional control of IL-18 production**

Because IFN-γ increased IL-18 production by monocytes without altering IL-18 mRNA expression, we next determined whether IFN-γ acted through posttranscriptional mechanisms. We evaluated caspase-1 activity because this enzyme controls conversion of pro-IL-18 to mature, bioactive IL-18. Monocytes from five healthy tuberculin reactors were stimulated with M. tuberculosis, with or without IFN-γ. Caspase-1 activity was then measured in monocyte lysates. IFN-γ increased caspase-1 activity from 0.08 ± 0.02 OD units to 0.28 ± 0.06 OD units (p = 0.02, Fig. 7). No caspase-1 activity was detected in lysates of IFN-γ-treated monocytes that were unstimulated with M. tuberculosis.

To confirm that caspase-1 was necessary for IFN-γ-induced IL-18 production, we added graded concentrations of the caspase-1 inhibitor, peptide-Ac-YVAD-cho, to monocytes from three healthy tuberculin reactors stimulated with M. tuberculosis and IFN-γ. The caspase-1 inhibitor reduced IL-18 concentrations from 710 ± 53 to 96 ± 55 pg/ml (p < 0.001). To confirm that this effect was specific for caspase-1 inhibition, we also measured production of TNF-α by M. tuberculosis-stimulated monocytes, release of which is not dependent on caspase-1. Addition of Ac-YVAD did not affect TNF-α concentrations (1253 ± 210 vs 1341 ± 127 pg/ml, p > 0.05).

**Discussion**

This study demonstrates that activated T cells contribute to IL-18 production by monocytes in response to an intracellular pathogen. M. tuberculosis induced minimal IL-18 production by monocytes, but addition of activated T cells markedly enhanced IL-18 production. This effect was observed despite separation of T cells and monocytes by a semipermeable membrane, indicating that it was due to a soluble factor. Addition of recombinant IFN-γ but not other cytokines mimicked the effect of activated T cells, and anti-IFN-γ abrogated the capacity of activated T cells to increase IL-18 production by monocytes. Addition of IFN-γ to alveolar macrophages also up-regulated IL-18 production in response to M. tuberculosis, suggesting that this mechanism operates in the lung during mycobacterial infection. IFN-γ increased IL-18 production by increasing cleavage of pro-IL-18 to mature IL-18, as it enhanced caspase-1 activity but did not increase IL-18 mRNA expression. Our findings suggest that IFN-γ produced by activated T cells optimizes IL-18 production by monocytes in response to infection by intracellular pathogens.

IFN-γ is essential for resistance to many intracellular pathogens, including viruses, fungi, parasites, and mycobacteria (1–6). One of the principal stimuli for IFN-γ production is IL-12 (14, 15), and IL-18 acts in concert with IL-12 to elicit IFN-γ production by T and NK cells. IL-18 also stimulates proliferation and cytolytic activity of NK cells (10, 16–18) and may contribute to generation of
Several lines of evidence suggest that IL-18 is a component of the early immune response to microbial infection. First, IL-18 is produced by dendritic cells and by epidermal keratinocytes, which direct T cell differentiation in response to infection (20, 21). Second, stimulation of monocytes with LPS induces mRNA expression of IL-18 more rapidly than that of IL-12 (22). Third, *Chlamydia* elicits IL-18 production by cell lines derived from mucosal epithelium, where initial contact with microbes occurs (23). The essential contribution of IL-18 to host defenses against intracellular pathogens is underscored by the reduced IFN-γ production and increased severity of disease due to *L. major*, *M. tuberculosis*, and *Cryptococcus neoformans* in mice lacking IL-18 because of a targeted gene deletion (8, 11, 24). In humans, IL-18 production by peripheral blood monocytes is reduced in patients with severe manifestations of infection due to *M. tuberculosis* and *M. leprae*, suggesting that IL-18 contributes to protective immunity against mycobacteria (12, 13).

Production of IL-18 is controlled at the transcriptional and post-translational levels. IL-18 gene expression is enhanced by binding of IFN consensus sequence binding protein and PU.1 to separate promoters (25, 26). IL-18 is produced as a precursor and is converted to the biologically active mature form by caspase-1, an intracellular cysteine protease (27). Microbial infections can up-regulate IL-18 production through different mechanisms. Stimulation of monocytes with *Staphylococcus aureus* or with Sendai virus enhances IL-18 mRNA expression (22, 28), whereas infection of epithelial cells with *Chlamydia trachomatis* increases IL-18 secretion through activation of caspase-1 (23). We found that IFN-γ augmented IL-18 production by up-regulating caspase-1 activity, but not IL-18 mRNA expression. These findings are consistent with those of Puren et al., who reported that IFN-γ did not enhance LPS-induced IL-18 mRNA expression (29). However, they contrast with those of other investigators, who found that IFN-γ increased IL-18 mRNA expression in the murine monocyte RAW264 cell line and in LPS-stimulated monocytes (22, 30). These discrepancies may be explained by three factors. First, regulation of IL-18 production may differ in human monocytes and in a murine monocytic cell line. Second, we used *M. tuberculosis* as a primary stimulus, whereas others used LPS. Third, we used real

![Figure 4](http://www.jimmunol.org/)

**FIGURE 4.** Effect of cytokines on IL-18 production by *M. tuberculosis*-stimulated alveolar macrophages. Alveolar macrophages from four persons were cultured with 10 μg/ml of heat-killed *M. tuberculosis*, with or without various recombinant cytokines. After 24 h, IL-18 concentrations were measured in macrophage supernatants by ELISA. Mean values and SE values are shown.

![Figure 5](http://www.jimmunol.org/)

**FIGURE 5.** Effect of anti-IFN-γ on the capacity of activated T cells to enhance IL-18 production by *M. tuberculosis*-stimulated monocytes. Monocytes were isolated from six healthy tuberculin reactors and cultured with 10 μg/ml of heat-killed *M. tuberculosis*, with or without recombinant cytokines. After 24 h, IL-18 concentrations were measured in cell culture supernatants by ELISA. Mean values and SE values are shown.

![Figure 6](http://www.jimmunol.org/)

**FIGURE 6.** Effect of IFN-γ on *M. tuberculosis*-induced IL-18 mRNA expression. Monocytes were isolated from four healthy tuberculin reactors and cultured with 10 μg/ml of heat-killed *M. tuberculosis* (*M. tb*), with or without recombinant IFN-γ. RNA was isolated and reverse transcribed to cDNA, which was quantified by real time PCR, using primers specific for IL-18 and β-actin. Values are expressed as the ratio of IL-18 cDNA to β-actin cDNA, expressed in arbitrary units. Each spot shows the mean of triplicate determinations.
time PCR to quantify IL-18 mRNA expression, which is likely to be more accurate than the noncompetitive PCR used in previous studies. Although our results clearly show that IFN-γ can elicit IL-18 release in the absence of direct contact between monocytes and T cells, it is possible that such cell-to-cell contact may also contribute to IL-18 production.

During the initial stages of infection with intracellular pathogens, cells of the innate immune system, such as monocytes and macrophages, produce soluble mediators that shape the nature of the subsequent adaptive immune response by T cells. T cells in turn produce cytokines that activate macrophages and stimulate them to produce specific patterns of monokines. Because IFN-γ is central to protection against many organisms, several positive feedback loops have been described that favor production of this cytokine. For example, IFN-γ augments IL-12 production by monocytes in response to M. leprae (31), probably by a priming effect on the IL-12 p40 promoter (32). Our findings suggest the presence of a second positive feedback loop involving IFN-γ and IL-18. Activated T cells markedly enhanced IL-18 production by monocytes in response to M. tuberculosis, and this effect was abrogated by neutralization of IFN-γ. Activated T cells had similar effects on alveolar macrophages, suggesting that this interaction occurs at mucosal surfaces where pathogens are initially encountered. IL-18 production was significantly increased by the addition of only 1–2 T cells per 10 monocytes, suggesting that this interaction occurs early in the immune response, when small numbers of activated T cells are present. Another possibility is that IL-18 may contribute to the adaptive immune response. For example, when healthy tuberculin reactors are exposed to an exogenous M. tuberculosis strain, previously sensitized T cells may quickly induce IL-18 production, leading in turn to more IFN-γ production and therefore rapid elimination of mycobacteria. This speculation is consistent with the relative resistance of healthy tuberculin reactors to reinfection with M. tuberculosis (33).

In summary, our data document another potential link between the innate and adaptive immune responses to an intracellular pathogen. Further studies to delineate these interactions will enhance our understanding of the basis of susceptibility to disease from intracellular organisms, and facilitate development of new strategies for their treatment and prevention.

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References