Interleukin-12 and Interleukin-27 Regulate Macrophage Control of \textit{Mycobacterium tuberculosis}

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\textit{Mycobacterium tuberculosis} is an intracellular pathogen that persists within macrophages and remains a considerable global threat to human health. The purpose of this study was to investigate how interleukin (IL)–12 and IL-27 regulate human macrophage interactions with \textit{M. tuberculosis}. Quantitative measurement of transcripts showed that IL-12 or \textit{M. tuberculosis} induced IL-27 gene expression in human macrophages. Furthermore, IL-27 receptor subunits were shown by reverse transcription–polymerase chain reaction and flow cytometry to be expressed and present at the cell surface. Neutralization of IL-27 in the presence of IL-12 reduced viable \textit{M. tuberculosis} recovered from macrophages. Antimycobacterial activity was accompanied by a heightened inflammatory response that included tumor necrosis factor, IL-6, interferon-\(\gamma\), and a subset of chemokines. These results implicate IL-12 and IL-27 in regulating human macrophages, and IL-27 derived from macrophages during infection impedes control of \textit{M. tuberculosis} growth.

Tuberculosis is the clinical manifestation of interactions between the human host and the intracellular bacterial pathogen \textit{Mycobacterium tuberculosis}. \textit{M. tuberculosis} is responsible for \(\sim\) 8.8 million new infections and claims the lives of nearly 1.6 million people worldwide each year [1]. Following entry into the host lung, \textit{M. tuberculosis} is taken up by macrophages and dendritic cells. \textit{M. tuberculosis} resists phagolysosomal fusion and persists within these phagocytic cells. Subsequent cell-mediated immune responses lead to formation of a granuloma composed of macrophages, dendritic cells, T and B lymphocytes, and fibroblasts [2]. Granulomas contribute to host protection by acting as a barrier to prevent bacterial dissemination, shielding surrounding tissue from inflammation, creating a low-oxygen environment, and providing an ecosystem rich in antimicrobial effectors [3].

Vital to granuloma formation are the signals provided by macrophages and dendritic cells in response to infection. Tumor necrosis factor (TNF) produced by macrophages early following infection is crucial in the formation and maintenance of granulomas in mice and humans [4–7]. This is in part the result of induction of chemokine expression in macrophages by TNF [8]. Chemokines serve as essential signals that recruit macrophages and T cells to sites of infection. Interleukin (IL)–12 production by dendritic cells is critical for a strong T cell response that serves as the predominant source of interferon (IFN)–\(\gamma\) [9].

IL-12 is a heterodimeric cytokine that is composed of p40 and p35 polypeptide chains. IL-12 contributes to cell-mediated immune responses by promoting differentiation of naive T cells and IFN-\(\gamma\) production [9]. Furthermore, IL-12 has long been shown to play an essential role in effective immunity against \textit{M. tuberculosis} [10–12]. IL-27 is also involved in driving commitment of naive T cells to a Th1 phenotype and synergizes with IL-12 to yield high levels of IFN-\(\gamma\) production in T cells [13–15]. IL-27 is a structurally similar [16] heterodimeric cytokine composed of the Epstein-Barr virus–induced gene 3 (EBI3) product and the p28 polypeptide [14].

Despite the defined role of IL-27 in promoting inflammation, Th1 responses, and IFN-\(\gamma\) production, there is an increasing body of evidence that demon-
strates IL-27 inhibits inflammation. Several studies have shown that animals deficient in IL-27 or its receptor chains are able to reduce microbial burdens during some infections, including infections due to *M. tuberculosis*, but suffer adverse outcomes from extreme inflammatory responses [17–19]. These studies and others have demonstrated hyperimmune responses that typically involve T cell proliferation and cytokine production that culminates with tissue damage [17–20]. The role of IL-27 in the induction of T cell immunity, therefore, may be secondary to its role in regulating inflammation. This suggests that short-term blockade of IL-27 signaling may be beneficial during infection and could be considered an attractive therapeutic for treatment of chronic infections.

Although macrophages and dendritic cells are the predominant producers of IL-12 and IL-27, accumulating evidence in the murine system has shown that these cells are also capable of autocrine response to these molecules [21, 22]. IL-12 receptor chains are found in resting macrophages [21]. A primary consequence of IL-12–induced signaling in murine macrophages is production of IFN-γ and cellular activation [22]. There is also evidence that murine macrophages express the receptor for IL-27 [23]. However, the effects that IL-12 and IL-27 may exert directly on human macrophages during the course of infection have not been explored.

In the context of reports that IL-12 can activate mouse macrophages, we investigated the effects of IL-12 and IL-27 on human macrophages during the course of infection by *M. tuberculosis*. We present evidence for autocrine/paracrine regulation of antimycobacterial activity and antagonist effects of IL-12 and IL-27. The results presented here extend our understanding of the effects of IL-12 and IL-27 on human macrophages and offer insight into an immunotherapeutic approach to control *M. tuberculosis* infection.

**MATERIALS AND METHODS**

**Mycobacterium culture conditions.** *M. tuberculosis* strain Erdman, provided by Dr. JoAnne Flynn (University of Pittsburgh School of Medicine), was maintained in Middlebrook broth containing albumin, dextrose, and catalase (ADC) at 37°C in 5% CO2. For macrophage infections, recovered bacteria were plated on Middlebrook 7H10 agar that contained 10% oleic acid-albumin-dextrose-catalase. All operations involving live *M. tuberculosis* were performed under standard biosafety level 3 laboratory practices.

**Cell culture.** Human peripheral blood mononuclear cells were obtained from buffy coats by sequential Ficoll (Amersham Biosciences) and OptiPrep (Axis-Shield) density gradient centrifugation as described previously [24], followed by panning in plastic dishes. Monocyte-derived macrophages were removed from the culture dish with PBS (pH 7.4) that contained 5 mmol/L EDTA and 4 mg/mL lidocaine. The cells were washed with PBS and plated onto Primaria (Becton Dickinson-Falcon) culture dishes in DMEM supplemented with 2 mmol/L glutamine, 25 mmol/L HEPES, 1% human serum, and polymixin B (10 μg/mL). Polymixin B was included in the culture medium to abrogate effects of possible LPS contamination from the recombinant IL-12 (R&D Systems). No other antibiotics were included in macrophage cultures.

**Reverse transcription–polymerase chain reaction (RT-PCR).** Macrophages (5 × 10^5 macrophages/well) cultivated in 96-well dishes were treated with IL-12 (5 ng/mL), sIL-27R (10 μg/mL), or medium alone in triplicate. Following 4 h of incubation, macrophages were infected with *M. tuberculosis* (MOI 1) or left uninfected. At indicated time points, media were removed from cultures, the cells were lysed with TriReagent (Molecular Research Center), and RNA was isolated according to commercial product protocol. First-strand cDNA synthesis was performed using SuperScript III reverse transcriptase (Invitrogen) with 750 ng RNA according to protocol. For detection of IL-27 receptor transcripts in macrophages, cDNA was subjected to 40 cycles of amplification in a cycler in a reaction that included 0.4 μmol/L primers pairs, 1.5 mmol/L MgSO4, AccuPrime Supermix II, and AccuPrime High Fidelity DNA Polymerase (1 U [Invitrogen]). The primers used were WSX-1F, GCCAAAGATGCCTTCCAG; WSX-1R, CACACATCTTGGAGCAAGAG; gp130F, CAAAGCTACTCCCTTAAG; gp130R, CCACTACAGCACTTTTC; β-actinF, GCACCACACTTCTCAATGAG; β-actinR, ATAGCAGAGGATGAGAAC. The contents of PCR reactions were separated on 2% agarose gels and stained with ethidium bromide.

**Quantitative PCR.** Real-time cycling of reactions that included diluted cDNA, gene-specific primer-probe sets (Applied Biosystems), and iQ Supermix (Bio-Rad) was performed in triplicate using an iQ5 cycler (Bio-Rad). Glyceraldehyde phosphate dehydrogenase was used as an internal reference gene.

**Flow cytometry.** Macrophages were mixed for 15 min at room temperature with monoclonal antisera to block Fc receptors. For surface expression of receptors, macrophages were immunolabeled with mouse monoclonal anti-CD14 conjugated with FITC (AbD Serotec), mouse monoclonal anti-gp130 conjugated with phycoerythrin (R&D Systems), goat polyclonal anti-WSX1 (R&D Systems), or an appropriate isotype control. Immunobound anti-WSX1 was visualized with mouse anti-goat IgG conjugated with FITC (Santa Cruz Biotechnology) following fixation for 15 min with PBS that contained 1% paraformaldehyde. For intracellular labeling of IFN-γ, the cells were paraformaldehyde fixed and permeabilized with Hanks’ Balanced Salt Solution that contained 0.2% saponin and 0.05% NaN3. IFN-γ was detected with mouse monoclonal antibody conjugated with carboxyfluorescein (R&D Systems). Cells were collected with a FACScan flow cytometer (Becton Dickinson) and...
the results analyzed with CellQuest software. A minimum of 10,000 gated events were collected for each sample group.

**Macrophage infection and enumeration of M. tuberculosis.** Human macrophages cultivated in 96-well dishes (5 × 10⁴ macrophages/well) were treated with IL-12, sIL-27R, both with or without anti-IFN-γ (R&D systems) or isotype control, or medium alone in triplicate. Following 4 h of incubation, macrophages were infected with *M. tuberculosis* (MOI 1) or left uninfected. Infected cultures were incubated ~72 h at 37°C in 5% CO₂. Culture supernatants were removed and macrophages were permeabilized with 1% saponin to release bacteria. Ten-fold serial dilutions were plated on Middlebrook 7H10 agar and incubated 21 days at 37°C with 5% CO₂.

**ELISA analysis.** TNF-α, IL-6, IL-1β, IFN-γ, I-TAC, and MCP-1 Duo sets were purchased from R&D Systems, and assays were performed according to supplied protocols. Cytokine concentrations for experimental samples analyzed in duplicate were determined from standard curves performed in parallel. Data are presented as mean values (±SEs) for 2 independent experiments.

**Statistical analysis.** A 2-way analysis of variance or Student *t* test was used to establish significance in the 95% confidence interval between individual sample groups.

**RESULTS**

**IL-27 production by human macrophages.** Although the function of IL-12 and IL-27 on T cell–mediated immune responses has been studied, the effect(s) of these molecules on the human cells that produce them has been undefined. Macrophages produce significantly less IL-12 in response to *M. tuberculosis* than other pathogens such as gram-negative bacteria [25, 26]. Therefore, *M. tuberculosis* infection offers a unique system to study macrophage responses to IL-12 supplied exogenously and how IL-27 might alter those responses.

To address these issues, we first evaluated IL-27 expression by human macrophages in response to IL-12 or *M. tuberculosis* infection. In response to IL-12, macrophages increased expression of EBI3 and p28 over time, as revealed by quantitative PCR (figure 1). Transcript levels for EBI3 peaked at 4 h, and the rate of expression was maintained above that of the unstimulated control through 24 h. Similarly, EBI3 transcripts increased rapidly in response to *M. tuberculosis*. Early during infection, the combination of IL-12 and *M. tuberculosis* resulted in a level of EBI3 expression greater than that of either stimulus individually.

Transcript levels for p28 increased in response to IL-12, peaked at 4 h, and by 8 h decreased to a level comparable to that for the unstimulated control (figure 1). Similarly, p28 transcripts increased over time in response to *M. tuberculosis*, peaked at 8 h, but were maintained at a high level through 24 h of infection. Macrophages exposed to IL-12 and *M. tuberculosis* expressed p28 transcript levels that were higher than those from macrophages exposed to either agent alone at all points in the time course.

**IL-27 receptor expression by human macrophages.** Because human macrophages express IL-27 genes, we investigated whether these cells are equipped to respond to this cytokine. The IL-27 receptor is a heterodimer composed of WSX-1, the binding chain, and gp130, the signal transducing chain [13–15, 23]. Transcripts for both chains of the receptor were detected in resting macrophages by RT-PCR (figure 2A). In addition, transcripts encoding the receptor chains continued to be expressed at 24 h for all conditions tested (figure 2A). To confirm expression of the receptor at the cell surface, macrophages were immunolabeled for WSX-1 or gp130 and ana-
analyzed by flow cytometry. As shown in figure 2B, the population of cells is strongly positive for CD14 (>99% of cells), indicating a monocyte/macrophage phenotype. These cells at resting state were positive for WSX-1 and gp130 surface expression above any background staining with an isotype control (figure 2B). These data demonstrate that human macrophages express the receptor for IL-27 and are thus capable of responding to the ligand.

**Macrophage infection and neutralization of IL-27.** Human macrophages produce IL-27 and have receptors for the cytokine, so we examined the effects of IL-27 on macrophages during in-
fection. Macrophages were stimulated with IL-12, soluble receptor for IL-27 (sIL-27R), their combination, or left untreated for 4 h before infection with M. tuberculosis. Following 24 h of infection, combined treatment with IL-12 and sIL-27R was most effective at slowing M. tuberculosis growth relative to other conditions (figure 3A). By 72 h, the number of viable M. tuberculosis was lowest with the combined treatment (figure 3A). Because the largest impact on M. tuberculosis growth was observed at 72 h, this time point was used for subsequent experiments. Treatment with IL-12 and sIL-27R alone resulted in a modest reduction in M. tuberculosis recovered from macrophages following 72 h of infection, compared with the untreated control. However, the combination of IL-12 and sIL-27R consistently resulted in the greatest reduction in colony-forming units (CFU) (figure 3B). These CFU differences are specific for macrophage–M. tuberculosis interactions and are not the result of bacterial replication in the extracellular environment or a loss in macrophage viability (data not shown). These results suggest that IL-27 acts directly on macrophages to block antimycobacterial activity, whereas IL-12 augments this activity.

Transcriptional analysis of the macrophage inflammatory response to M. tuberculosis. Next, we determined whether the pattern of gene expression for the inflammatory response of macrophages was altered after treatment with IL-12 and sIL-27R. Proinflammatory genes were selected on the basis of their established roles in immune responses to M. tuberculosis. Quantitative analysis of transcripts demonstrated that TNF was induced rapidly by IL-12 or M. tuberculosis (figure 4A). Over time, their combination resulted in a level of expression that was greater than that of either stimulus alone (figure 4A). IL-12, sIL-27R, and M. tuberculosis all increased transcript levels for IFN-γ independently (figure 4A). We also examined transcript levels for the chemoattractant I-TAC, because chemokines participate in granuloma formation through the recruitment of monocytes, macrophages, and lymphocytes [3]. IL-12, sIL-27R, and M. tuberculosis rapidly stimulated I-TAC expression independently (figure 4B). These results show that early following infection, IL-12 and sIL-27R induce transcripts for proinflammatory molecules important to immune defense against M. tuberculosis. Transient increases in transcripts for IFN-γ and I-TAC in response to sIL-27R alone may be due to neutralization of baseline levels of IL-27. In the absence of IL-12, this transcription may not be sustained over time.

Cytokine and chemokine production in the presence of IL-12 and sIL-27R. Proinflammatory cytokines can be regulated at the level of both transcription and translation. Therefore, we determined whether protein secretion paralleled the gene expression changes we observed. M. tuberculosis induced TNF and IL-6 accumulation in culture supernatants following infection that was further increased by IL-12 and even more by the addition of sIL-27R at 2 h (TNF), 8 h (TNF), and 24 h (IL-6; figure 5A). Infected cells continued to produce TNF and IL-6 through 48 h of infection (data not shown). At 48 h, the combination of IL-12 and sIL-27R stimulated the most IL-1β production in infected cells (data not shown). Importantly, IFN-γ was
detected in culture supernatants 24 h after infection in IL-12–treated cells (figure 5A). Consistent with findings of the gene expression analysis, this amount of protein was increased nearly 2-fold by neutralization of IL-27 and continued to increase through 48 h.

To further confirm IFN-γ production by macrophages, we measured cytokine accumulation by intracellular staining and flow cytometry. As shown in figure 5B, there was a shift in fluorescence for CD14+ macrophages treated with IL-12, sIL-27R, and heat-killed M. tuberculosis, compared with the unstimulated control. This indicates intracellular accumulation of IFN-γ that is consistent with ELISA results (figure 5A). Because IFN-γ is produced in infected macrophages treated with IL-12 and sIL-27R, we investigated whether this cytokine was central to the antimycobacterial activity demonstrated in figure 3. Addition of neutralizing antibodies to IFN-γ reversed much of the effect on M. tuberculosis growth mediated by macrophages treated with IL-12 and sIL-27R (figure 5C).

Next we examined the presence of I-TAC and MCP-1 protein in culture supernatants of human macrophages. IL-12 induced a dramatic increase in I-TAC secretion from infected macrophages at 24 h (figure 5C). A high level of production of this chemokine was maintained through 48 h of infection. Additionally, IL-12 induced an increase in MCP-1 production in infected cells (data not shown). These results show that treatment of infected macrophages with IL-12 results in elevated production of TNF, IL-6, IFN-γ, and I-TAC that is further augmented at different times by neutralization of IL-27.

**DISCUSSION**

Macrophages are central to the initiation of inflammatory responses to M. tuberculosis and the primary effector cells for clearance of the bacterium. We have shown that human macrophages respond to IL-12 and IL-27 to regulate control of M. tuberculosis. Human macrophages infected with M. tuberculosis produce low levels of IL-12 [25, 26] but respond to supplemental IL-12 with enhanced cytokine synthesis. This demonstrates that IL-12 can activate human macrophages similar to murine macrophages [27]. Another consequence of IL-12 treatment, however, is induction of IL-27 expression that interferes with antimycobacter-
IL-12 and IL-27 Effects on Macrophages

IL-27 has both proinflammatory and anti-inflammatory properties, either of which may predominate in a particular system. We hypothesized that IL-27 would oppose IL-12–mediated activation of human macrophages and clearance of M. tuberculosis. Treatment with IL-12 and a soluble receptor for IL-27 (sIL-27R) during infection reduced the growth of bacteria recovered from macrophages after 3 days (figure 3A and 3B). This was the result of a macrophage-mediated mechanism to slow the growth of M. tuberculosis following 24 h of infection.

Manipulations of IL-12 and sIL-27R also influenced proinflammatory cytokines and chemokines produced by macrophages. IL-12 impressively augmented IFN-γ, IL-6, and I-TAC production from infected cells. I-TAC is produced by macrophages that also produce high amounts of IL-12 in response to gram-negative bacteria [29] and by primary human monocytes in response to IFN-γ [30]. Importantly, neutralization of IL-27 augmented levels of TNF early during infection (figure 5A), IFN-γ at 24 h (figure 5A), and IL-1β later during infection (data not shown). Neutralization of IL-27 increased IL-12–driven TNF protein release but not transcription. TNF, however, is regulated posttranscriptionally [31], suggesting that IL-27 may influence translation or processing of this molecule. In the microenvironment of an infection, small changes in these factors are likely to influence macrophage responses and activation states. TNF and IFN-γ activate macrophages to eliminate M. tuberculosis [32, 33]. The kinetics of this response may be also important to the antimycobacterial mechanism: increased production of TNF and IFN-γ early during infection may allow macrophages to better combat M. tuberculosis at a critical time.

This report adds significantly to the idea that human macrophages can produce IFN-γ. IFN-γ production by human alveolar macrophages from patients with pulmonary sarcoidosis was reported nearly 2 decades ago [34]. A subsequent report of M. tuberculosis–induced IFN-γ production by human alveolar macrophages demonstrated that >99% of their adherent cells were macrophages [35]. We showed here that the combination of M. tuberculosis, sIL-27R, and supplemental IL-12 induced IFN-γ from macrophages detected in culture supernatants by ELISA and in cells by flow cytometry. The latter confirmation emphasized that IFN-γ is produced by CD14+ macrophages and not by a contaminating cell type. I-TAC, an IFN-γ–induced gene, was produced only under conditions in which IFN-γ was present. In addition, an antibody that neutralizes IFN-γ restored M. tuberculosis growth in macrophages. Together, these data underscore the biological activity of IFN-γ derived from macrophages.

IFN-γ is critical to an effective immune response to M. tuberculosis. This is highlighted by studies involving IFN-γ–deficient mice that are markedly more susceptible to infection and fail to develop a normal granulomatous response that contains the bacterial infection [36, 37]. Likewise, people with genetic defects that confer impaired IFN-γ signaling are more susceptible to mycobacterial infections [38–41]. Consistent with these reports, we showed in figure 3C that IFN-γ is necessary for the antimycobacterial activity when IL-27 is neutralized (figure 3).

Therefore, in the absence of lymphocytes, it is reasonable that even measurably small amounts of IFN-γ in the microenvironment of infection could boost macrophage restriction of M. tuberculosis. IFN-γ, however, is probably not sufficient, because comparable levels of IFN-γ were produced later during infection by macrophages treated with IL-12 and M. tuberculosis. Supplementation of IL-12 early after infection reduces pathologic effects in the lungs of mice with reduced cellular infiltration and smaller, fewer granulomas [42]. However, the bacterial burden in the lungs was unaffected. It is possible in that study IL-27 helped to protect mice from pathologic effects in the lungs, consistent with the role of IL-27 in regulating inflammation. However, the presence of IL-27 may have prevented development of the macrophage-mediated inhibition of M. tuberculosis growth reported here.

Although we have shown treatment with IL-12 and neutralization of IL-27 influences macrophage responses to M. tuberculosis, the contributions of these results should not be limited to the involvement of macrophages. The full scope of acquired immunity to M. tuberculosis could be influenced by the heightened inflammatory response shown in this report. For example, TNF is essential for normal granuloma formation [4–7]. The production of chemokines, such as IL-12–induced I-TAC, is important in the recruitment of lymphocytes to the site of infection for granuloma formation. Future studies involving animal models of disease will further address the effects of neutralizing IL-27 on cellular recruitment to the lungs, granuloma formation, and global containment of M. tuberculosis. The heightened inflammatory response in vivo may limit the bacterial burden and contribute to more-effective granuloma formation.

Furthermore, this study suggests that neutralization of IL-27 activity may offer therapeutic value during infection. Elimination of IL-27 signaling leads to immunopathologic conditions in mouse models of infection with several microbial pathogens. However, temporary blockade of IL-27 with a self-limited therapy, such as a soluble receptor, may avoid these negative effects. In the wake of multidrug resistant M. tuberculosis, therapeutic strategies that reduce inhibitory responses and direct the immune response appropriately are desirable. Immunotherapeutic approaches involving IL-12 and neutralization of IL-27 may augment current therapies. The results presented here extend our knowledge of the effects of heterodimeric cytokines on myeloid cells that make this approach more realistic and provide a mechanistic explanation of how this therapy might work.
References


