Cytokines Involved in Interferon- γ Production by Human Macrophages

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Abstract

Interferon (IFN)- γ is important to the immune defense against intracellular pathogens and specifically the ability of macrophages to control *Mycobacterium tuberculosis* (MTB). Increasing evidence has accumulated to support the idea that macrophages produce IFN- γ . We describe here the cytokine interactions that determine IFN- γ expression and secretion during MTB infection of human macrophages. Detection of biologically important IFN- γ levels in culture supernatants of MTB-infected human macrophages requires the addition of interleukin (IL)-12. IL-18 augmented IFN- γ production from human macrophages in response to the combination of MTB and supplemental IL-12. Although IL-18 gene expression was generally unchanged, IL-18 protein secretion was enhanced by the combination of MTB and IL-12, and functioned primarily to stimulate IFN- γ release. Importantly, IL-27 induced by MTB infection opposed IFN- γ production by antagonizing IL-18 activity in human macrophages. Neutralization of IL-27 increased the expression of the IL-18 receptor β -chain. Additionally, IL-27 blocked NF- κ B activation in response to IL-18. These results define the signals required for IFN- γ production by human macrophages

and highlight the interactions between cytokines produced during MTB infection. Together, they identify a novel role for IL-27 in regulating macrophage function by disrupting IL-18 activity.

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Introduction

Mycobacterium tuberculosis (MTB) is an intracellular pathogen and the causative agent of tuberculosis. This pathogen has infected one third of the world's population and accounts for an estimated 1.8 million worldwide deaths annually [1]. Interferon (IFN)- γ is pivotal to effective host immune responses that control MTB infection. Mice [2, 3] and humans [4–6] with genetic deficiencies that compromise IFN- γ production or signaling exhibit increased susceptibility to MTB.

IFN- γ signaling in macrophages leads to activation [7] and promotion of host defense mechanisms that control bacterial growth. IFN- γ enhances antigen presentation in macrophages through induction of MHC class II [8], upregulates CD40 expression [9] and directs host events such as fusion of phagosomes with lysosomes. Macrophages have been shown to overcome mycobacterial resistance to phagolysosomal fusion when stimulated with IFN- γ [10]. Additionally, a number of IFN- γ -induced

gene products such as nitric oxide synthase [11] and indoleamine 2,3-dioxygenase [12] limit growth of mycobacterial species.

It has long been known that NK and NKT cells as well as CD4⁺ and CD8⁺ lymphocytes are the primary producers of IFN- γ . Although still contentious, there is an expanding body of evidence that both murine [13–21] and human macrophages [22–25] are capable of producing and secreting IFN- γ both in vitro and in vivo. A primary source of skepticism has been contamination by T or NK cells [26, 27]. To this end, the source of macrophages is an important consideration; protocols that derive macrophages from cells of the bone marrow leave little room for development of alternative cell types [28]. The concern of contaminating cell types has also been satisfied by additional measures such as in situ hybridization [14, 16] or intracellular staining of IFN- γ within cells labeled with markers of macrophage phenotype [13, 15, 24]. These studies are further supported by the observation that recombina-activating gene 2 (RAG-2)^{-/-} mice produce IFN- γ , as do monocytes and dendritic cells obtained from these mice [20]. Nevertheless, data have been published indicating that very small populations of NK or CD8⁺ cells can account for the phenotypes that have been described [29]. However, evidence that macrophage-derived IFN- γ plays a role in defense against MTB [24] as well as mechanistic data describing the regulation of IFN- γ by macrophages and dendritic cells discussed below, provides additional support for this controversial topic in innate immune responses.

Detailed studies have investigated the signals that are required for IFN- γ expression and secretion from murine macrophages. Most of the focus has come to rest upon IL-12 and IL-18 that synergize to yield maximal levels of IFN- γ production in macrophages derived from several sources. Levels of secreted protein in vitro have exceeded 10 ng/ml [16, 18]. In murine macrophages, expression of the IL-12 receptor β 1 increases with exposure to IL-12; this is further enhanced by the addition of IL-18 rendering cells more responsive to IL-12 [30]. Following binding of the receptor, IL-12 induces signaling through STAT4 that is critical to IFN- γ expression in myeloid cells [18, 31]. STAT4^{-/-} mice are defective for IFN- γ production and unable to restrict growth of some intracellular pathogens [31, 32].

IL-18 was originally identified as IFN- γ -inducing factor and characterized for its ability to induce high levels of IFN- γ from Th1 cells [33]. IL-18 is primarily secreted by activated macrophages and Kupffer cells [34]. IL-18 is constitutively expressed in human monocytes and mac-

rophages as a proform [35]. Posttranslational cleavage of proIL-18 by caspase-1 at an aspartic acid residue is required for secretion of the mature and active protein [35]. Cells that are responsive to IL-18 express a heterodimer of the IL-18 receptor (IL-18R) α - and β -chains. The IL-18R α membrane protein is responsible for ligand binding [36]. The β -chain is subsequently recruited to the complex and is required for signaling that leads to activation of NF- κ B and c-Jun N-terminal kinase [37].

We have previously shown that human macrophages secrete IFN- γ in response to IL-12 and MTB [24]. This production is further augmented by neutralization of IL-27 and is critical to restriction of mycobacterial growth [24]. In this work, we have addressed the signals that are required to elicit IFN- γ expression and secretion from primary human macrophages. Although the signals that control IFN- γ production from murine macrophages have been described, an understanding of what is critical for IFN- γ production from human macrophages has not been defined. IL-18 modestly enhances IFN- γ gene expression but has a more profound impact on IFN- γ secretion by human macrophages. Additionally, we have identified the mechanism by which neutralization of IL-27 enhances IFN- γ production in response to IL-12 and MTB.

Materials and Methods

MTB Culture Conditions

MTB strain Erdman, provided by Dr. JoAnne Flynn (University of Pittsburgh School of Medicine), was maintained in Middlebrook broth containing albumin, dextrose, catalase at 37°C with 5% CO₂. γ -Irradiated MTB strain H37Rv was acquired from the Colorado State University TB Vaccine Testing and Research Materials Contract. All operations involving live MTB were performed under standard biosafety level 3 laboratory practices.

Cell Culture

Human peripheral blood mononuclear cells (PBMCs) were obtained from buffy coats by Ficoll (Amersham Biosciences) density gradient centrifugation. Monocytes were subsequently isolated from human PBMCs by OptiPrep (Axis-Shield) density gradient centrifugation as described previously [38] followed by panning in plastic dishes. Monocyte-derived macrophages were removed from the culture dish with PBS (pH 7.4) that contained 5 mM 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 mM glutamine, 25 mM HEPES, 1% human serum and polymyxin B (10 μ g/ml). These isolation and culture conditions routinely result in cells that are $\geq$ 99% positive for CD14, a marker of monocyte/macrophage phenotype [24]. Polymyxin 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.

RT-PCR

Human macrophages (1×10^5 /well) cultivated in 24-well dishes were treated with IL-12 (5 ng/ml) $\pm$ sIL-27R (10 μ g/ml, 10 neutralization dose₅₀; R&D Systems) or medium alone in triplicate. Following 4 h of incubation, macrophages were infected with MTB (approx. MOI 1) or left uninfected. At indicated time points, medium was 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-18 transcripts in macrophages, cDNA was subjected to 40 cycles of amplification in a cycler in a reaction that included 0.4 μ M primer pairs, 1.5 mM MgSO₄, AccuPrime™ Supermix II and AccuPrime™ High Fidelity DNA Polymerase (1 U; Invitrogen). 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 cDNA diluted 25-fold from above, 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.

ELISA Analysis

Human macrophages (5×10^4 /well) were cultivated in 96-well dishes for collection of supernatants to be analyzed for cytokine concentrations. IFN- γ and IL-18 assays (R&D Systems) were performed according to supplied protocols. Standard curves were performed in parallel for determination of cytokine concentrations. The lower limits of detection for the IFN- γ and IL-18 assays were 7.8 and 15.6 pg/ml, respectively. Data are presented as means $\pm$ standard error for combined experiments. The number of experiments included in the data set is specified in the figure legend.

Whole-Cell Extracts

Human macrophages (5×10^5 /well) cultivated in 12-well dishes were treated with IL-27 or medium alone. Following 4 h of incubation, macrophages were stimulated with IL-18 (20 ng/ml) and incubated for an additional 1 h. Macrophages were scraped in cold hypotonic buffer (10 mM HEPES, pH 7.9, 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM DTT) and sonicated on ice to disrupt both cell and nuclear membranes. Cell lysates were clarified by centrifugation at 10,000 g for 10 min at 4°C. Protein concentrations were determined using the DC Protein Assay® (Bio-Rad).

Immunoblot Analysis

Equal amounts of protein were separated on 8% acrylamide SDS-PAGE and transferred to nitrocellulose according to standard techniques. Transcriptionally active NF- κ B was detected with rabbit anti-phospho p65 (Cell Signaling Technologies) and total NF- κ B was detected with mouse anti-p65 (Cell Signaling Technologies). ECL substrate (Amersham Biosciences) was applied to visualize proteins.

Microarray Analysis

RNA (1 μ g) was reverse transcribed to synthesize cDNA according to the manufacturer's instructions (Agilent Technolo-

gies). cDNA was then used to generate Cy-3-labeled cRNA that was recovered using RNeasy columns (Qiagen) and fragmented for hybridization according to the manufacturer's instructions (Agilent Technologies). Human whole genome 4x44K microarrays (Agilent Technologies) were scanned with an Agilent G2565 microarray scanner and individual hybridization intensities acquired using Feature Extraction software version 9.1. Gene expression values within each array (3 for each condition) were evaluated for differential expression between treatment conditions using the J5 statistical test in the Gene Expression Data Analyzer application (GEDA; <http://bioinformatics.upmc.edu/GE2/GEDA.html>). The J5 metric was designed for data sets composed of limited replicates, thereby reducing the chance of generating false positives [39].

Statistical Analysis

An analysis of variance with a Bonferroni multiple comparison test was performed to establish significance between sample groups in the 95% confidence interval.

Results

IL-18 Augments IFN- γ Production by Human Macrophages

We previously reported that human macrophages produce IFN- γ in the presence of IL-12 and MTB [24]. In the murine system, IL-18 induces high levels of IFN- γ secretion in the presence of IL-12. To determine if IL-18 influences the production of IFN- γ from human macrophages, primary monocyte-derived macrophages were treated with increasing concentrations of IL-12 in the presence or absence of IL-18 and infected with MTB for 48 h. As shown in figure 1, IFN- γ production was increased dose-dependently by the addition of IL-12. IFN- γ was not detected in the absence of exogenously added IL-12. At all concentrations of IL-12 tested, IL-18 synergistically augmented production of IFN- γ . The maximal effect was observed in conjunction with 10 ng/ml of IL-12 (fig. 1). In contrast to murine bone marrow-derived or peritoneal exudate macrophages [16, 18], IFN- γ was not detected by human macrophages incubated with IL-12 and IL-18 only (data not shown).

IL-18 Is Secreted by Human Macrophages in Response to MTB

Since exogenously supplied IL-18 augmented IFN- γ production, we evaluated whether or not IL-18 is produced and secreted by human macrophages in response to IL-12 and/or MTB. Macrophages were treated as before with or without IL-12 and infected with MTB; IL-18 expression was evaluated by RT-PCR and ELISA for secreted protein. There was a constitutive presence of IL-18

Fig. 1. IL-18 augments IL-12-induced IFN- γ production. Human monocyte-derived macrophages were treated with IL-12 at the indicated concentrations in the presence or absence of IL-18 (10 ng/ml) and infected with MTB (MOI 1). Supernatants were collected at 48 h and IFN- γ concentrations were determined by ELISA. Data are presented as the means of triplicate samples from an individual experiment representative of typical results $\pm$ standard error.

transcripts detected from macrophages at resting state by qualitative RT-PCR (fig. 2a) and by quantitative real-time PCR (fig. 2b). This was not unexpected; IL-18 is synthesized in an immature form and processed after translation to be secreted [40]. Furthermore, the IL-18 gene was not a member of an activation program of genes induced in human macrophages by a variety of bacteria [41]. Basal levels of IL-18 were present in the supernatants of unstimulated macrophages (fig. 2c). The amount of IL-18 in supernatants increased by infection with MTB, but not further in infected cells by addition of IL-12 at either time point (fig. 2c). These data demonstrate that the IL-18 transcript is expressed by human macrophages and the protein is secreted. Thus, IL-18 could contribute to IFN- γ production after infection with MTB.

IL-18 Is Necessary for Optimal IFN- γ Gene Expression and Secretion by Human Macrophages

In the murine system, IL-12 is sufficient for IFN- γ transcription, but IL-18 is required for high-level secretion by macrophages [18]. Since IL-18 is produced by human macrophages in response to MTB, we evaluated the importance of IL-18 in IL-12- and MTB-induced IFN- γ gene expression and secretion. This was done utilizing a pair of monoclonal antibodies that neutralize IL-18 (anti-

Fig. 2. IL-18 is produced by human macrophages. Human monocyte-derived macrophages were cultured as indicated in the presence of IL-12 (5 ng/ml), soluble receptor to neutralize IL-27 (sIL-27R, 10 μ g/ml) and/or infected with MTB (MOI 1). **a** Macrophages were harvested for RNA isolation at 24 h. RT-PCR was performed as described in Materials and Methods and amplification products were separated on 2% agarose stained with ethidium bromide. Amplification products were not detected in the negative control without reverse transcriptase. **b** Quantitative PCR was performed as described in Materials and Methods. Data are presented as the mean [(C_t IL-18) - (pC_t GAPDH)] $\pm$ standard error and are representative of an experiment performed twice. **c** Supernatants were collected from cultures at 24 or 48 h and analyzed for IL-18 concentration by ELISA. Data are presented as the means of triplicate samples from 5 individual experiments represented by separate donors $\pm$ standard error.

Fig. 3. The role of IL-18 in IFN- γ gene expression by human macrophages. Human monocyte-derived macrophages were treated with IL-12 (5 ng/ml) and/or infected with MTB (MOI 1) as indicated. Isotype control, anti-IL-18R α or anti-human IL-18 were included at 1 μ g/ml where indicated. **a** Macrophages were harvested for RNA isolation at 24 h. Quantitative PCR was performed as described in Materials and Methods. Data are presented as mean log₂ change in gene expression for three combined experiments represented by separate donors $\pm$ standard errors. Values were normalized to the mean expression of GAPDH within a sample group and expressed relative to that of medium alone. **b** Supernatants were collected at 48 h and IFN- γ concentrations determined by ELISA. Data are presented as the means of triplicate samples from at least three combined experiments represented by separate donors $\pm$ standard error. ND = Not detected. Asterisks indicate results that are statistically significant from IL-12 + TB ($p \leq 0.05$).

IL-18) or block access of the cytokine to the receptor subunit responsible for ligand binding (anti-IL-18R α). The efficacy of these antibodies was demonstrated by their ability to block IL-18 augmentation of IFN- γ production induced by IL-12 in monocyte-depleted PBMCs (data not shown).

In MTB-infected macrophage cultures stimulated with IL-12, neutralization of IL-18 modestly reduced IFN- γ gene expression, although this did not reach statistical significance (fig. 3a). Blockade of IL-18R α had a similar effect (fig. 3a). Since IL-18 is reported to have a more significant contribution to IFN- γ protein secretion than transcription in the murine system [18], we evaluated this possibility in human macrophages. Neutralization of IL-18 in MTB-infected macrophage cultures stimulated with IL-12 resulted in a significant decrease in secreted IFN- γ (fig. 3b). This reduction was greater than that observed on transcription, however, IFN- γ secretion was not completely abrogated. A similar result that did not achieve statistical significance was observed by blocking IL-18R α (fig. 3b). Collectively, these results demonstrate that, although not an absolute requirement for IFN- γ production from human macrophages, IL-18 significantly enhances IFN- γ secretion by human macrophages. Furthermore, these data suggest that an MTB-induced pathway independent of IL-18 and IL-12 contributes to IFN- γ production from human macrophages; IFN- γ production is greater than that of IL-12 alone when IL-18 is neutralized (fig. 3b).

Neutralization of IL-27 Upregulates Expression of IL-18R β and Genes Involved in Inflammasome Signaling

We have shown that neutralization of IL-27 with a soluble receptor (sIL-27R) enhances IFN- γ production from a homogenous culture of MTB-infected human macrophages at 24 h after infection [24]. The effect of sIL-27R is dose-dependent and reliant on the presence of IL-12 (fig. 4). Therefore, we explored the mechanism for how neutralization of IL-27 enhances IFN- γ production from human macrophages. Although IL-18 gene expression is increased marginally by sIL-27R in IL-12- and MTB-stimulated cells at 24 h, the difference is not significant (fig. 2). With this in mind, we studied global changes in transcription by microarray analysis to pursue a mechanistic explanation for how neutralization of IL-27 influences IFN- γ expression. RNA from MTB-infected macrophages was compared with those that were also treated with IL-12 and sIL-27R. Microarray data from 3 independent experiments were compared using GEDA software [39].

Table 1. Select genes induced by IL-12 and sIL-27R in MTB-infected macrophages

Gene symbol	Gene name	Fold induction ¹	Description	Ref.
IFNG	interferon- γ	1.41	activator of human macrophages	7
CXCL10	chemokine ligand 10	1.52	interferon-induced chemokine	42
IRAK3	IL-1 receptor-associated kinase 3	1.31	involved in inflammatory-mediated signaling	43
CARD15	NOD2, nucleotide-binding oligomerization domain containing 2	1.28	inflammasome activator in response to muramyl dipeptide	44
PYDC1	POP1, PYD-only protein 1	1.23	regulates inflammatory signaling	44
IL18RAP	IL18RB, IL-18 receptor β	1.25	required for IL-18 responsiveness	37
P2RX4	purigenic receptor P2X4	1.26	extracellular ATP induces IL-18 secretion through purigenic receptors	45

¹ Fold induction of IL-12- and sIL-27R-treated infected macrophages relative to infected macrophages alone.

Fig. 4. IFN- γ production is increased by neutralization of IL-27. Human monocyte-derived macrophages were treated with IL-12 (5 ng/ml) $\pm$ sIL-27R at increasing concentrations and infected with MTB (MOI 1) as indicated. Supernatants were collected at 24 h and IFN- γ concentrations determined by ELISA. Data are presented as the means of triplicate samples from 3 individual experiments represented by separate blood donors $\pm$ standard error. Asterisks indicate comparisons that are statistically significant from the control ($p \leq 0.05$).

Using this approach, 414 genes were identified as differentially expressed between the 2 conditions (online suppl. table 1, www.karger.com/doi/10.1159/000247156). On this gene list, several genes were of particular interest for this study. As anticipated, the combination of supplemental IL-12 and neutralization of IL-27 increased expression of IFN- γ in macrophages infected with MTB

(table 1). Consistent with this observation, a chemokine induced by IFN- γ , CXCL10, was also represented in the list of genes stimulated by IL-12 and sIL-27R. Genes related to inflammasome activation, signaling or responsiveness to IL-18 were also induced (table 1). Of particular interest was the increased expression of the IL-18R β gene in MTB-infected cells that were treated with IL-12 and sIL-27R (table 1). Interestingly, the overall fold change was modest despite meeting criteria for statistical significance. Enhanced IFN- γ production (fig. 1) and another IFN-induced chemokine (CXCL11) has already been demonstrated [24], confirming the microarray results. Therefore, we pursued the microarray result for IL-18R β as an independent validation. IL-18R β gene expression was confirmed independently by quantitative PCR (fig. 5a). While IL-12 and MTB were sufficient to increase IL-18R β expression above that observed at resting state, the combination of IL-12 and sIL-27R caused the greatest increase ($p < 0.001$; fig. 5a).

Given the above results with IL-18R β gene expression, we hypothesized that neutralization of IL-27 may allow human macrophages to be more sensitive to changes in IL-18 concentration. Alternatively, the presence of IL-27 may inhibit signaling induced by IL-18. Since activation of NF- κ B is the dominant consequence of signaling through IL-18R [37], we evaluated the level of NF- κ B activation in response to IL-18 in the presence or absence of IL-27. Following this 5-hour treatment, cellular extracts were prepared and the levels of phosphorylated NF- κ B p65 were evaluated by immunoblot analysis as a functional measure of IL-18 responsiveness. Supplemental IL-18 was sufficient to substantially increase the amount of active, phosphorylated p65 in human macrophages

Fig. 5. Neutralization of IL-27 enhances activation of NF- κ B in response to IL-18. **a** Human monocyte-derived macrophages were treated as indicated with IL-12 (5 ng/ml), sIL-27R (10 μ g/ml), their combination and/or infected with MTB (MOI 1) for 10 h. Quantitative PCR was performed as described in Materials and Methods. Data are presented as mean log₂ change in gene expression $\pm$ standard errors for four independent experiments. Values were normalized to the mean expression of GAPDH with-

in a sample group and expressed relative to that of medium alone. **b, c** Human monocyte-derived macrophages were treated with IL-27 (10 ng/ml) or medium alone for 4h and then stimulated with IL-18 (20 ng/ml) in the presence (**c**) or absence of UV-inactivated MTB (**b**) for an additional 1 h. Cell extracts were prepared as described in Materials and Methods. Equal amounts of protein were loaded in duplicate, separated by SDS-PAGE and immunoblotted for phosphorylated or total NF- κ B p65.

(fig. 5b). Treatment with IL-27, however, markedly decreased NF- κ B activation induced by IL-18 (fig. 5b). In addition, IL-27 was capable of blocking IL-18-mediated p65 phosphorylation even when MTB was present (fig. 5c). In other studies that paralleled the microarray experimental design, neutralization of IL-27 also enhanced the amount of phosphorylated NF- κ B in MTB-infected macrophages, though this effect required the presence of IL-12 (data not shown). These results are consistent with the prediction from gene expression profiles in the microarray analysis that IL-27 antagonizes IL-18.

Discussion

IFN- γ is critical to immune responses that are protective against tuberculosis. Mice with IFN- γ gene disruptions form aberrant ineffective granulomas and cannot contain MTB infection [2, 3]. Similarly, mice depleted of IL-18 produce less IFN- γ and are compromised in their ability to control MTB [46] as well as infections by other intracellular pathogens such as *Salmonella typhimurium* and *Leishmania major* [47, 48]. People with compromised IFN- γ production or responses are more susceptible to MTB [4–6]. Likewise, IL-18, for its significant role in in-

ducing IFN- γ , has been correlated with protective immunity in tuberculosis patients [49]. IL-18 has also been detected in tuberculoid patients that restrict *Mycobacterium leprae* growth, but not in lepromatous patients that exhibit disseminated infection [50]. Collectively, this evidence suggests that IL-18 plays a very significant role in IFN- γ production and immune responses to intracellular pathogens. In this paper we have defined the signals that are required for IFN- γ expression and secretion from human monocyte-derived macrophages as well as how IL-27 antagonizes the contribution of IL-18 in this process.

IL-12 is sufficient for induction of IFN- γ transcription (fig. 3a) and is required for consistent detection of significant levels of IFN- γ in macrophage culture supernatants (fig. 1). This underscores the importance of failed IL-12 production by MTB-infected macrophages [41] and the potential for supplemental IL-12 as an adjunct therapy [24, 41]. Moreover, figure 3 demonstrates that MTB does not induce sufficient levels of biologically active IL-12 p70 since the addition of exogenous IL-12 is necessary to detect significant levels of IFN- γ .

In MTB-infected murine macrophages, it has been reported that IL-18 is necessary for secretion of IFN- γ . Human macrophages increase secretion of IL-18 in response to infection by MTB (fig. 2). They also respond to IL-18

in autocrine/paracrine fashion to augment production of IFN- γ induced by IL-12. This was apparent when IL-18 was neutralized (fig. 3). Transcriptional activation of the IFN- γ gene did not change significantly in the presence of antibodies to block IL-18 (fig. 3a), but IFN- γ found in culture supernatants was reduced 2-fold (fig. 3b). Although IL-18 contributes to optimal IFN- γ secretion, antibodies that blocked IL-18 could not suppress IFN- γ production to the level of IL-12 alone (fig. 3b). Therefore, MTB must also induce important signals for IFN- γ production that are independent of IL-18.

We have previously demonstrated that neutralization of IL-27 augments IFN- γ production from MTB-infected macrophages in response to IL-12 (fig. 4) [24]. Since IL-18 is not produced at a level that is significantly higher when IL-27 is neutralized during infection (fig. 2), we set out to investigate the mechanism for IL-27 influence on IFN- γ production. To answer this question, we evaluated gene expression changes in MTB-infected human macrophages following treatment with IL-12 and sIL-27R by microarray. This analysis identified 414 loci that were differentially expressed (online suppl. table 1); of these, there was a combination of transcripts that were induced as well as those that were repressed. IFN- γ was a gene significantly induced by IL-12 and sIL-27R, validating the microarray analysis. In addition, several of the differentially expressed genes that achieved statistical significance are linked with inflammasome activation (required for IL-18 processing) or responsiveness to IL-18 (table 1). Of particular importance was the increased expression of IL-18R β that mediates ligand-induced signaling and is required for IL-18 responsiveness.

There are many examples of cytokines that influence expression of receptors and signaling components for other cytokines. In one such example, IFN- α and IL-12 induce gene expression for both components of the IL-18 receptor in human NK and T cells [51]. This increase is observed at the level of transcription and confers enhanced binding of NF- κ B to its cognate DNA sequence. In our data with human macrophages, IL-12 induced expression of the gene encoding the IL-18 receptor β chain, but this was further enhanced by neutralization of IL-27 (fig. 5a). This result suggests that IL-27 opposes IL-12 activity in human macrophages and further highlights how IL-27 interacts differently with macrophages and lymphocytes. In contrast, IL-27 augments IFN- γ production induced by IL-12 and IL-18 in human monocyte-depleted PBMCs (data not shown). This is consistent with the ability of IL-27 to synergize with IL-12 in driving IFN- γ production from CD4 $^{+}$ T cells and NK cells [52]. The differ-

ent effects of IL-27 on different cell populations underscore the complexity of IFN- γ regulation and the importance of which cell types are present in a given location when IL-27 is released.

The regulation of the IL-18 receptor by IL-27 that we have defined is functionally important. NF- κ B was activated in response to IL-18 as demonstrated by the phosphorylation of the p65 subunit (fig. 5b). However, IL-27 profoundly diminished the levels of phosphorylated p65 found in IL-18-treated cells, even in the context of macrophage infection by MTB (fig. 5b and c). In addition to influencing IL-18R β expression, it is also possible that IL-27 interferes with IL-18 signaling by influencing expression or activity of receptor-associated signaling machinery at time points other than those investigated here. In this way, IL-27 would also directly oppose the activity of IL-18.

The concept of IFN- γ production by macrophages has been controversial in the literature. Recently, IFN- γ secretion has been ascribed to contaminating NK or CD8 cell populations in the murine system, either in peritoneal exudate cells or in bone marrow derived macrophage cultures [29]. Schleicher et al. [29] point out, however, that alternative sources of macrophages or different stimuli may elicit IFN- γ production by macrophages. This appears to be the case with human monocyte-derived macrophages [25] and with murine macrophages stimulated with *Trypanosoma cruzi* [21]. Bogdan and Schleicher [53], in a later review citing evidence of cell-associated IFN- γ protein, also acknowledge the possibility that macrophages may respond to intracellular IFN- γ through a mechanism by which IFN- γ -loaded endosomes are delivered to phagocytic vacuoles or autophagosomes carrying IFN- γ receptors [53]. Several details support the idea that the IFN- γ observed in our human cell cultures is also derived from macrophages. We employ a density gradient centrifugation step to separate monocytes from lymphoid cells as well as an additional wash step to remove nonadherent cells from our macrophages after differentiation from monocytes. IL-12 is not sufficient to induce consistent IFN- γ production (fig. 3) [24], as would be expected from circulating NK cells. We have also observed IFN- γ accumulation in CD14 $^{+}$ macrophages at the single cell level [24]. Together with recent immunohistochemistry and ELISPOT data [25], we conclude that human macrophages have the potential to produce and respond to IFN- γ in an autocrine/paracrine manner.

The levels of IFN- γ produced by human macrophages are meaningful to macrophage restriction of MTB growth in vitro. This was previously demonstrated [24]. When

IFN- γ was neutralized in a pure culture of MTB-infected macrophages stimulated with IL-12 and sIL-27R, antimycobacterial activity was reversed [24]. This result has been corroborated by use of small interfering RNA that completely abrogates IFN- γ production by human macrophages; the antimycobacterial activity was completely reversed in these macrophages compared with controls (data not shown). IFN- γ production from antigen-presenting cells may be important to an optimal protective response despite a robust Th1 response in the lungs during tuberculosis. This model for enhancing immunity has been termed the 'jump start model' [27] and might be valuable to immune responses for several reasons. Autocrine responses to IFN- γ may be more efficient in activating macrophages. Small amounts of IFN- γ secreted by macrophages and later internalized in an autocrine or paracrine manner may also be useful to generate cytosolic vacuoles with IFN- γ receptors for response to intra-

cellular cytokine, as discussed above. IFN- γ produced by macrophages may also allow for a quicker response to MTB while T cell specific immunity develops. Although NK cells are considered to be a valuable source of IFN- γ early during infection, in the microcosm of infection, the IFN- γ produced by macrophages may contribute greatly to effective macrophage responses that limit MTB growth. In this model, the IL-18 induced by MTB may also contribute to productive macrophage responses.

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