Early Interleukin 12 Production by Macrophages in Response to Mycobacterial Infection Depends on Interferon γ and Tumor Necrosis Factor α

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Summary

Interleukin 12 (IL-12) produced by macrophages immediately after infection is considered essential for activation of a protective immune response against intracellular pathogens. In the murine Mycobacterium bovis Bacillus Calmette-Guérin (BCG) model we assessed whether early IL-12 production by macrophages depends on other cytokines. In vitro, murine bone marrow-derived macrophages produced IL-12 after infection with viable M. bovis BCG or stimulation with LPS, however, priming with recombinant interferon γ (rIFN-γ) was necessary. In addition, IL-12 production by these macrophages was blocked by specific anti-tumor necrosis factor α (TNF-α) antiserum. Macrophages from gene deletion mutant mice lacking either the IFN-γ receptor or the TNF receptor 1 (p55) failed to produce IL-12 in vitro after stimulation with rIFN-γ and mycobacterial infection. In vivo, IL-12 production was induced in spleens of immunocompetent mice early during M. bovis BCG infection but not in those of mutant mice lacking the receptors for IFN-γ or TNF. Our results show that IL-12 production by macrophages in response to mycobacterial infection depends on IFN-γ and TNF. Hence, IL-12 is not the first cytokine produced in mycobacterial infections.

Due to its NK cell and T cell stimulating properties, IL-12 was originally termed NK cell stimulatory factor or cytotoxic lymphocyte maturation factor (1, 2). It is a heterodimer composed of two covalently linked chains, p35 and p40. The light chain (p35) is homologous to IL-6 and G-CSF and is constitutively expressed in several cell types including macrophages. The p40 subunit is homologous to the extracellular part of the IL-6 and G-CSF receptor (3–5). The bioactive p70 heterodimer is produced by monocytes/macrophages and B cells and modulates various functions of mature T and NK cells including cytotoxicity and cytokine production (3, 6–10). It has been shown recently that IL-12 plays a decisive role in host-defense against intracellular pathogens. It is produced by infected monocytes/macrophages as one of the first host responses to infection and, together with TNF, induces IFN-γ production by NK cells (11–13). This early IFN-γ activates macrophages and initiates differentiation of Th1 cells (11, 14–16). Development of a Th1 response and IFN-γ production are central to eradication of various pathogens including Leishmania major (15), Toxoplasma gondii (17), Listeria monocytogenes (11), Mycobacterium tuberculosis (18), Mycobacterium leprae (19), and Schistosoma mansoni (20). On the other hand, in infections characterized by protective Th2 cytokine responses IL-12 downregulates Th2 cell expansion thus exacerbating the disease (21). We here show that the production of IL-12 by Mycobacterium bovis Bacillus Calmette-Guérin (BCG)-infected macrophages in vitro and in vivo depends on prior stimulation with rIFN-γ and is mediated by endogenous TNF-α. The strict dependence of IL-12 secretion on IFN-γ and TNF-α suggests that production of the latter cytokines must precede IL-12 secretion. Thus, macrophage-derived IL-12 cannot be the first cytokine of the sequence leading to protective antimycobacterial immunity mediated by Th1 cells.

Materials and Methods

Mice. C57BL/6 female mice were raised in our own breeding colonies under specific pathogen-free conditions. The mice lacking the IFN-γ receptor (IFN-γR-/-) and those lacking the TNF receptor 1 (Tnfr1-/-) were generated as described in (22–24). Mutant mice were kept under specific pathogen-free conditions.

Microorganisms. M. bovis BCG was grown in Dubos broth (Difco, Detroit, MI) supplemented with BSA and Tween 80 with

1 Abbreviations used in this paper: BCG, Bacillus Calmette-Guérin; BMM, bone marrow-derived macrophages; IFN-γR-/-, mice lacking the IFN-γ receptor; NRS, normal rabbit serum; RT, reverse transcriptase; Tnfr1-/-, mice lacking the TNF receptor 1.
p-nitrophenyl phosphate (Sigma, Munich, Germany) was added. Aliquots were frozen and stored at -70°C. Numbers of viable organisms were determined by plating 1:10 dilutions on Middlebrook Dubos agar plates (Difco). Plates were incubated at 37°C and the numbers of colony-forming units were determined.

Reagents. Murine rIFN-γ was kindly provided by Dr. G. Adolf (Ernst Boehringer-Institut für Arzneimittelforschung, Vienna, Austria). The specific activity was 10^6 U/mg protein. Murine rIL-12 was a gift from Dr. Stan Wolf (Genetics Institute, Cambridge, MA). The specific activity was 5.6 x 10^6 U/mg protein. Aliquots of recombinant cytokines were stored in Click's/RPMI containing 10% FCS. Two rat anti-IL-12 (p40) mAb, C15.6.7 IgG1 and C17.8 IgG2a, were a generous gift of Dr. G. Trinchieri (The Wistar Institute, Philadelphia, PA). LPS from Escherichia coli was obtained from Difco. Murine TNF-α and polyclonal rabbit anti-mouse TNF-α antiserum were purchased from Genzyme (Boston, MA). As a control, normal rabbit serum (NRS) was used. Oligonucleotides for IL-12 (p40) were synthesized on a DNA synthesizer (381A; Applied Biosystems, Foster City, CA). Sense: 449-CGTGCT-CATGGCTGGTGCAAAG; antisense: 761-CTTCATCTGCAA-GTTCTGGC.G.

Macrophage Cultures. Bone marrow-derived macrophages (BMM) were obtained in a serum-free culture medium as described previously (25). BMM were harvested after 9 d and stimulated in IMDM without additives and antibiotics as indicated in Results.

Dot Blot Assay for IL12. Aliquots of culture supernatants were placed into the wells of Millititer filtration plates with 0.45-μm pore size (Millipore, Eschborn, Germany) and incubated at room temperature for 1 h. Subsequently, supernatants were sucked into the membrane filters by using a vacuum filtration holder (Millipore). Wells were blocked with 3% skim milk in 50 mM Tris-HCl buffer, pH 7.5, overnight. After three washes with PBS, aliquots of 200 μl/well of biotinylated anti-IL-12 (p40) mAb C15.6.7 (1 μg/ml) were added. After incubation at room temperature for 2 h, plates were washed three times with PBS and the substrate p-nitrophenyl phosphate (Dianova, Hamburg, Germany) (1:5,000 in 0.1% BSA in PBS) was added. After 30 min of incubation at room temperature, plates were washed three times with PBS and the substrate p-nitrophenyl phosphate (Sigma, Munich, Germany) was added. After 10 min of incubation at room temperature, the reaction was terminated with 0.5 M EDTA, pH 8.0. Aliquots were transferred into flat-bottom microdilution plates and absorbance was measured by a specific ELISA with a detection limit of ~50 pg/ml. Supernatants in which IL-12 was not detectable by ELISA were analyzed by the more sensitive dot-blot assay with a detection limit of 0.5 pg/ml.

ELISA for IL12. IL-12 was measured in a two-site ELISA. The mAb C17.8 IgG2a was used for coating and biotinylated mAb C15.6 IgG1 was employed for detection.

Semiquantitative Reverse Transcriptase (RT)-PCR Analysis and Southern Hybridization of RT-PCR Amplified Products. Semiquantitative RT-PCR analysis of IL-12 and β-actin mRNA was performed as described previously in detail (26). RT-PCR products were fractionated by electrophoresis on 1% agarose gel (0.5 x Tris-borate-EDTA). DNA was partially depurinated by 5-μl vol of 0.25 M HCl for 10-15 min at room temperature and denatured by placing the gel in 5-μl vol of 0.4 M NaOH, 0.6 M NaCl for 15 min. The DNA was blotted on nylon membranes (United States Biochem. Corp., Cleveland, OH) by applying a Vacuum Blotter from Appliedgene (Heidelberg, Germany) using 0.4 M HCl and 0.6 M NaCl denaturing buffer for 1 h. Subsequently the DNA on the nylon membrane was fixed by UV cross-linking (125 mJ/cm^2) with Fluox-Link apparatus (Renner, Darmstadt, Germany) for 3 min. The hybridization probe, 1 μg IL-12 (p40) cDNA, was labeled with biotin according to standard protocols (Gene Images kit, United States Biochem. Corp.) and used for hybridization of target RT-PCR DNA products. The hybridization was performed at 42°C overnight in a hybridization oven (Biomator, Göttingen, Germany). The washing procedure and the chemiluminescent immunodetection protocol were applied according to the manufacturer's descriptions (Gene Images kit). The signal development on x-ray film (XOMAT-AR, Kodak) was performed for 1 h. As molecular weight markers, biotinylated DNA fragments (50-1,000 bp) from Research Genetics (Huntsville, AL) were used.

In Vivo Induction of IL12. To induce IL-12 synthesis in vivo, mice were injected with 5 x 10^6 iv. viable M. bovis BCG. At different time points, spleen cells were prepared and seeded into round-bottom microdilution plates (Nunc, Roskilde, Denmark) at 10^5 cells/well in Clicks/RPMI containing 10% FCS and 5 x 10^-4 M 2-ME. Cells were stimulated with ConA (5 μg/ml), rIFN-γ (500 U/ml), or M. bovis BCG (5 x 10^9/ml). Supernatants were collected after 24 h for determination of IL-12.

Results

To analyze the stimuli that are required for IL-12 production, murine BMM obtained by cultivation in a serum-free medium were used that represent a quiescent macrophage population devoid of contaminating cells like granulocytes or lymphocytes (25). Accumulation of IL-12 in culture supernatants was analyzed by a specific ELISA with a detection limit of 200 pg/ml of IL-12 (p40). Supernatants in which IL-12 was not detectable by ELISA were analyzed by the more sensitive dot-blot assay with a detection limit of ~50 pg/ml. It has been shown previously that the presence of the IL-12 p70 chain correlates with increased levels of the bioactive p70 heterodimer (12).

IL-12 Produced by Macrophages In Vitro After M. bovis BCG Infection or LPS Stimulation Depends on Priming with rIFN-γ. BMM from C57BL/6 mice were primed with rIFN-γ and/or infected with M. bovis BCG or stimulated with LPS. As shown in Table 1, only BMM primed with rIFN-γ for 24 h and subsequently infected with M. bovis BCG or stimulated with LPS for another 24 h produced detectable levels of IL-12. Stimulation with rIFN-γ alone or treatment with M. bovis BCG or LPS alone failed to induce IL-12 synthesis. Incubation of macrophages with LPS before stimulation with rIFN-γ or concomitant treatment of cells with rIFN-γ and LPS for 24 h failed to induce IL-12 production. A kinetics of rIFN-γ priming revealed that rIFN-γ had to be present for at least 8 h before addition of M. bovis BCG or LPS to induce significant IL-12 synthesis (data not shown). It has been shown that LPS-binding protein is required for macrophage stimulation with LPS (27, 28). Because the rIFN-γ used for macrophage priming contained minute concentrations (0.001%) of FCS, we cannot exclude formally contamination of our rIFN-γ preparation with LPS-binding protein. However, we consider the minute FCS concentration insufficient. It appears more likely that BMM stimulation with rIFN-γ induced LPS-binding protein synthesis that then rendered LPS bioactive. Other cytokines tested, including rIL-4 and rIL-6, failed to prime macrophages for IL-12 synthesis (data not shown). In addition, BMM were analyzed for IL-12 mRNA expression by PCR. As shown in Fig. 1 A, IL-12 mRNA was only found in macrophages costimulated with rIFN-γ and M. bovis BCG.
Table 1. Production of IL-12 (p40) by BMM from C57BL/6, IFN-γR−/−, and Tnfr1−/− Mice*

| Stimulus | Production of IL-12 (ng/ml) by mouse strain† |
|----------|---------------------------------------------|
|          | C57BL/6 | IFN-γR−/− | Tnfr1−/− |
| U/ml     | U/ml    | M. bovis BCG | LPS |
| −        | −       | −           | −    |
| −        | −       | +           | −    |
| 10       | −       | −           | −    |
| 100      | −       | −           | −    |
| 10       | −       | +           | −    |
| 100      | −       | −           | +    |
| −        | 10      | −           | −    |
| −        | 100     | −           | −    |
| −        | 10      | +           | −    |
| −        | 100     | +           | −    |

* BMM (10⁵/well) were cultured with or without rIFN-γ. After 24 h, cells were infected with M. bovis BCG organisms (10⁷/well) or treated with LPS (50 ng/ml). Supernatants were harvested after an additional 24 h for detection of IL-12. IL-12 was measured by ELISA (detection limit 200 pg/ml). The amount of IL-12 per ml correlates to 5 × 10⁵ BMM. Data shown are from one of three independent experiments.
† IL-12 was measured by ELISA (detection limit 200 pg/ml). The amount of IL-12 per ml correlates to 5 × 10⁵ BMM. Data shown are from one of three independent experiments.
§ ND: not detectable by the dot-blot assay (detection limit 50 pg/ml).

BMM treated with either rIFN-γ or M. bovis BCG alone did not express IL-12 mRNA. Thus, induction of IL-12 mRNA and protein depended on two signals with IFN-γ as first and mycobacterial infection or LPS as second signal.

Endogenous TNF-α Regulates IL-12 Production by BMM. We have shown previously that TNF-α mediates mycobacterial growth inhibition by nitric oxide (26). To investigate the role of endogenously produced TNF-α in the induction of IL-12 synthesis, a specific polyclonal anti-TNF-α antiserum was used. BMM were primed with rIFN-γ for 24 h and subsequently infected with M. bovis BCG or stimulated with LPS for another 24 h in the presence of anti-TNF-α antiserum or NRS. As shown in Fig. 2, addition of anti-TNF-α antiserum during infection of rIFN-γ–primed BMM with M. bovis BCG or during stimulation with LPS significantly reduced IL-12 production. NRS used as control had no significant effect on IL-12 synthesis by BMM. In parallel, IL-12 mRNA expression was analyzed in BMM stimulated with rIFN-γ and LPS in the presence of anti-TNF-α antiserum or NRS (Fig. 3). The IL-12 mRNA expression was inhibited by incubation of macrophages with anti-TNF-α antiserum although TNF-α itself failed to induce IL-12 synthesis by BMM (Table 1). Hence, induction of IL-12 synthesis by macrophages depended on signaling through both IFN-γ receptor and TNF receptor 1.

BMM from IFN-γR−/− or Tnfr1−/− Mice Fail to Produce IL-12. To further analyze the contribution of IFN-γ and TNF-α to IL-12 production, IFN-γR−/− mice and Tnfr1−/− mice were employed. BMM prepared from these mutant mice were primed for 24 h with increasing concentrations of rIFN-γ and subsequently infected with M. bovis BCG or stimulated with LPS for another 24 h. Neither BMM from IFN-γR−/− nor BMM from Tnfr1−/− mice were able to produce IL-12 at the mRNA or protein level after stimulation with rIFN-γ plus M. bovis BCG or LPS (Table 1 and Fig. 1, B and C). These results verify that IL-12 production by macrophages in vitro exclusively depends on both, IFN-γ and TNF-α and that IFN-γ and TNF-α stimulation cannot be compensated by other cytokines in these mutant mice. Furthermore our data reveal that TNF receptor 1, and not TNF receptor 2, is responsible for the TNF-α effect.

IL-12 Induction In Vivo. C57BL/6 mice were infected with M. bovis BCG. 3 h and 4 d after infection, spleen cells were prepared and analyzed for mRNA encoding the p40 subunit of IL-12 by RT-PCR and Southern hybridization. As shown in Fig. 4, spleen cells from noninfected C57BL/6 mice did not express any IL-12 mRNA, however, IL-12 mRNA was detectable at 3 h and still 4 d after mycobacterial infection. Spleen cells were cultured in vitro with ConA, rIFN-γ, or M. bovis BCG for 24 h and supernatants were collected.
for IL-12 detection. As shown in Table 2, splenocytes from naive mice produced only marginal amounts of IL-12 after in vitro stimulation with \textit{M. bovis} BCG. Infection of mice with \textit{M. bovis} BCG for 3 h significantly increased the capacity of spleen cells to generate IL-12 after in vitro culture with \textit{M. bovis} BCG. At later time points of infection the capacity of spleen cells to synthesize IL-12 was decreased (data not shown). These data demonstrate that mycobacterial infection induces IL-12 expression in vivo. To investigate the influence of IFN-\(\gamma\) and TNF-\(\alpha\) on IL-12 induction in vivo, IFN-\(\gamma\)R\(^{\alpha/}\) and Tnfr1\(^{\alpha/}\) mice were infected with \textit{M. bovis} BCG. Since these mutant mice are highly susceptible to infection with intracellular bacteria (22, 23) spleen cells were analyzed for IL-12 mRNA and protein expression 3 h after infection. Splenocytes from both mutant strains failed to express IL-12 mRNA after infection with \textit{M. bovis} BCG as analyzed by RT-PCR and Southern hybridization (Fig. 4). In vitro, stimulation of spleen cells from mutant mice with ConA, rIFN-\(\gamma\), or \textit{M. bovis} BCG did not induce IL-12 production (Table 2). We conclude that IFN-\(\gamma\) and TNF secretion must precede early IL-12 production by macrophages during \textit{M. bovis} BCG infection.

**Discussion**

According to current view, IL-12 is the first cytokine produced by macrophages infected with intracellular pathogens (11, 15, 17, 29, 30) and it is a requisite cytokine for induction of the Th1 developmental pathway (15, 31). Together with TNF-\(\alpha\), IL-12 stimulates NK cells to generate IFN-\(\gamma\). This early NK cell-derived IFN-\(\gamma\) induces activation of macrophages and differentiation of Th1 cells. In contrast, our data

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**Figure 1.** IL-12 (p40) mRNA expression in BMM from C57BL/6 mice (A), IFN-\(\gamma\)R\(^{\alpha/}\) mice (B), and Tnfr1\(^{\alpha/}\) mice (C). Total cellular RNAs from unstimulated BMM (lane 1), BMM primed with rIFN-\(\gamma\) (500 U/ml) and infected with \textit{M. bovis} BCG (lane 2), BMM primed with rIFN-\(\gamma\) (lane 3), and BMM infected with \textit{M. bovis} BCG (lane 4) were extracted, reverse transcribed, and amplified by PCR with specific primers for IL-12 or \(\beta\)-actin. The amplified products were probed for IL-12 (312 bp) (right) and \(\beta\)-actin (324 bp) (left).

**Figure 2.** Effect of anti-TNF-\(\alpha\) antiserum on IL-12 (p40) production by BMM from C57BL/6 mice. BMM (10^6/well) were primed with rIFN-\(\gamma\) (500 U/ml) for 24 h and subsequently infected with \textit{M. bovis} BCG (10^6/well) or treated with LPS (50 ng/ml) for an additional 24 h in the presence of anti-TNF-\(\alpha\) antiserum (final dilution 1:100). NRS (final dilution 1:100) was used as control. Similar results were obtained in three independent experiments.
suggest that IL-12 is not the first cytokine produced in response to mycobacterial infection. Rather, we conclude from our experiments that rIFN-γ in combination with M. bovis BCG or LPS stimulated TNF-α synthesis in BMM and that both cytokines were then required for IL-12 induction. Consistent with this assumption TNF-α is produced in vitro by macrophages upon stimulation with rIFN-γ and mycobacterial infection and both cytokines are mandatory for activation of antmycobacterial macrophage functions (26). Furthermore, TNF-α production in vivo in response to M. bovis BCG infection is markedly impaired in IFN-γR−/− mutant mice (32). Our results are in contrast to those by Reiner et al. (33) who described IL-12 mRNA expression in BMM treated with LPS alone. However, in this study, macrophages were cultivated in serum-containing medium and hence may have already been primed whereas our experiments were performed under serum-free conditions that yield resting macrophages.

Formal proof for strict dependence on IFN-γ and TNF-α of IL-12 induction in mycobacterial infection in vivo was obtained in experiments using IFN-γR−/− and Tnfr1−/− mutant mice. In contrast to C57BL/6 mice, spleen cells from M. bovis BCG-infected mutant mice lacking either the IFN-γ receptor or the TNF receptor 1 failed to express IL-12 mRNA and to produce IL-12 protein in vitro. We are therefore confident that IL-12 induction in vivo exclusively depends on priming with IFN-γ and TNF-α and that this dependency cannot be compensated by other cytokines. Consistent with our results, depletion of NK cells or IFN-γ by specific mAb reduces IL-12 mRNA expression in schistosome-infected mice (20) and peritoneal macrophages fail to produce IL-12 after infection with L. major in vitro, although inoculation of this pathogen into the peritoneal cavity induces IL-12 production (34). We assume that L. major infection caused IFN-γ and TNF-α production in vivo that primed macrophages for IL-12 synthesis.

Schijns et al. (35) and Swihart et al. (36) have shown that infection of IFN-γR−/− mice with pseudorabies virus or L. major, respectively, leads to Th1 cytokine profiles. We also found IFN-γ production by splenocytes of M. bovis BCG-infected IFN-γR−/− mice (data not shown). In contrast to pseudorabies virus infection, control of M. bovis BCG is IFN-γ dependent and lack of IFN-γ action is obviously not compensated by other cytokines. In the M. bovis BCG system IFN-γ...
Table 2. Production of IL-12 (p40) by Spleen Cells from C57BL/6, IFN-γR<sup>−/−</sup>, and Tnfr1<sup>−/−</sup> mice<sup>*</sup>

| Stimulus        | C57BL/6 0 h | C57BL/6 3 h | IFN-γR<sup>−/−</sup> 0 h | IFN-γR<sup>−/−</sup> 3 h | Tnfr1<sup>−/−</sup> 0 h | Tnfr1<sup>−/−</sup> 3 h |
|-----------------|------------|------------|------------------------|------------------------|------------------------|------------------------|
| Nil             | ND<sup>†</sup> | 1.2        | ND                     | ND                     | ND                     | ND                     |
| ConA            | ND         | 1.6        | ND                     | ND                     | ND                     | ND                     |
| rIFN-γ          | ND         | 2.5        | ND                     | ND                     | ND                     | ND                     |
| M. bovis BCG    | 0.6        | 5.8        | ND                     | ND                     | ND                     | ND                     |

<sup>*</sup> Mice were infected i.v. with 5 x 10<sup>6</sup> viable <i>M. bovis</i> BCG. Spleen cells were prepared at the time points indicated and restimulated in vitro (10<sup>6</sup> cells/well) with ConA (5 μg/ml), rIFN-γ (500 U/ml), or <i>M. bovis</i> BCG (1 x 10<sup>6</sup>/well). After 24 h, supernatants were harvested and analyzed for IL-12 by ELISA.

<sup>†</sup> ND: Not detectable by the DOT-BLOT assay (detection limit 50 pg/ml). Similar results were obtained in three independent experiments.

production may only partially depend on IL-12 and other cytokines may compensate. Another possibility is that IFN-γ production occurs independently from IL-12.

Taken together, our data reveal that IL-12 is not the first cytokine generated after infection with <i>M. bovis</i> BCG. Rather, we assume that IFN-γ primes macrophages for TNF-α production and that both cytokines then induce IL-12 synthesis in response to mycobacterial infection. The cellular source of the IFN-γ produced immediately after mycobacterial infection independent from IL-12 remains to be defined. Whether this immediate early IFN-γ production is stimulated directly by mycobacteria or involves other cytokines such as IL-13 (33, 37) is currently under investigation.

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