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)[1]-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-$\gamma R^{o/o}$) and those lacking the TNF receptor 1 (Tnfr1$^{o/o}$) 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-$\gamma R^{o/o}$, mice lacking the IFN-γ receptor; NRS, normal rabbit serum; RT, reverse transcriptase; Tnfr1$^{o/o}$, mice lacking the TNF receptor 1.

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shaking. 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 10^7 U/mg protein. Murine rIL-12 was a gift from Dr. Stan Wolf (Genetics Institute, Cambridge, MA). The specific activity was 5.6×10^6 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 rTNF-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, Inc., Foster City, CA). Sense: 449-CGTGCTCATGGCTGGTGCAAAG; antisense: 761-CTTCATCTGCAAGTTCTTGGGC.

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 IL-12 (p40). 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 streptavidin alkaline phosphatase (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, München, 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 $Å_{405}$ was measured in an Immunoreader NJ 2000 (Intermed). The IL-12 content was calculated by using rIL-12 as a standard with medium alone as a blank.

ELISA for IL-12. 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 × Tris-borate-EDTA). DNA was partially depurinated by 5-gel vol of 0.25 M HCl for 10–15 min at room temperature and denatured by placing the gel in 5-gel 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 Appligene (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/cm²) with Fluo-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 (Biometra, 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 IL-12. To induce IL-12 synthesis in vivo, mice were injected with 5×10^6 i.v. 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 5×10^{-5} M 2-ME. Cells were stimulated with ConA (5 μg/ml), rIFN-γ (500 U/ml), or *M. bovis* BCG (5×10^6/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 p40 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$^{o/o}$, and Tnfr1$^{o/o}$ Mice**

Stimulus				Production of IL-12 (ng/ml) by mouse strain‡		
rIFN-γ	rTNF-α	*M. bovis* BCG	LPS	C57BL/6	IFN-γR$^{o/o}$	Tnfr1$^{o/o}$
U/ml	*U/ml*					
–	–	–	–	ND§	ND	ND
–	–	+	–	ND	ND	ND
–	–	–	+	ND	ND	ND
10	–	–	–	ND	ND	ND
100	–	–	–	ND	ND	ND
10	–	+	–	10.0	ND	ND
100	–	+	–	16.8	ND	ND
10	–	–	+	7.8	ND	ND
100	–	–	+	14.9	ND	ND
–	10	–	–	ND	ND	ND
–	100	–	–	ND	ND	ND
–	10	+	–	ND	ND	ND
–	100	+	–	ND	ND	ND

* BMM (10^5/well) were cultured with or without rIFN-γ. After 24 h, cells were infected with *M. bovis* BCG organisms (10^6/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 5×10^5 BMM. Data shown are from one of three independent experiments.
§ ND: not detectable by the dot-blot assay (detection limit 50 pg/ml).
Similar results were obtained in three independent experiments.

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$^{o/o}$ or Tnfr1$^{o/o}$ Mice Fail to Produce IL-12. To further analyze the contribution of IFN-γ and TNF-α to IL-12 production, IFN-γR$^{o/o}$ mice and Tnfr1$^{o/o}$ 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$^{o/o}$ mice nor BMM from Tnfr1$^{o/o}$ 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

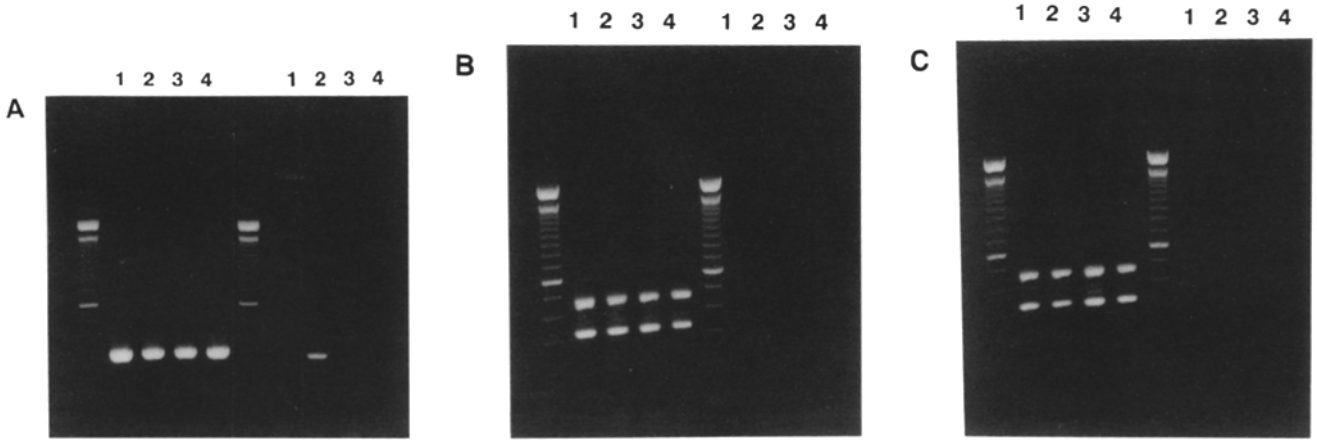

Figure 1. IL-12 (p40) mRNA expression in BMM from C57BL/6 mice (*A*), IFN-γR$^{o/o}$ mice (*B*), and Tnfr1$^{o/o}$ mice (*C*). Total cellular RNAs from unstimulated BMM (lane *1*), BMM primed with rIFN-γ (500 U/ml) and infected with *M. bovis* BCG (lane *2*), BMM primed with rIFN-γ (lane *3*), and BMM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

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 *M. bovis* BCG. Infection of mice with *M. bovis* BCG for 3 h significantly increased the capacity of spleen cells to generate IL-12 after in vitro culture with *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-γ and TNF-α on IL-12 induction in vivo, IFN-γR$^{o/o}$ and Tnfr1$^{o/o}$ mice were infected with *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 *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-γ, or *M. bovis* BCG did not induce IL-12 production (Table 2). We conclude that IFN-γ and TNF secretion must precede early IL-12 production by macrophages during *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-α, IL-12 stimulates NK cells to generate IFN-γ. This early NK cell–derived IFN-γ induces activation of macrophages and differentiation of Th1 cells. In contrast, our data

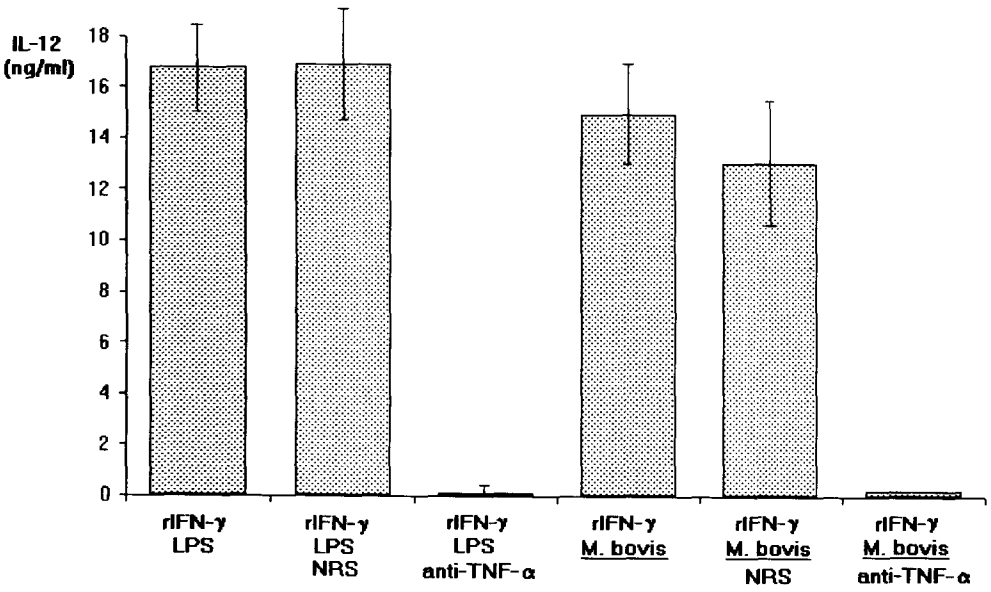

Figure 2. Effect of anti-TNF-α antiserum on IL-12 (p40) production by BMM from C57BL/6 mice. BMM (10^5/well) were primed with rIFN-γ (500 U/ml) for 24 h and subsequently infected with *M. bovis* BCG (10^6/well) or treated with LPS (50 ng/ml) for an additional 24 h in the presence of anti-TNF-α antiserum (final dilution 1:100). NRS (final dilution 1:100) was used as control. Similar results were obtained in three independent experiments.

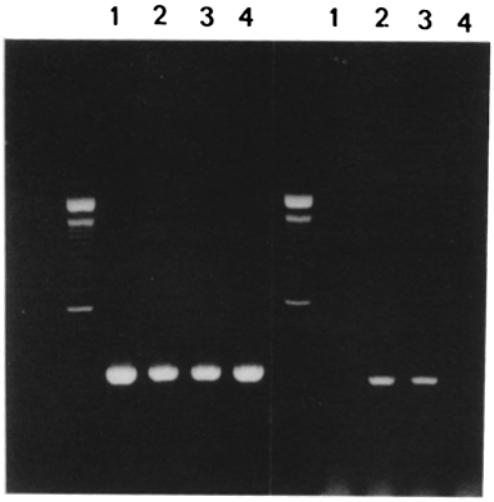

Figure 3. Effect of anti-TNF-α antiserum on IL-12 (p40) mRNA expression by BMM from C57BL/6 mice. Total cellular RNAs from unstimulated BMM (lane *1*), BMM primed with rIFN-γ (500 U/ml) and treated with LPS (50 ng/ml) (lane *2*), BMM primed with rIFN-γ and treated with LPS in the presence of NRS (final dilution 1:100) (lane *3*), and BMM stimulated with rIFN-γ and treated with LPS in the presence of anti-TNF-α antiserum (final dilution 1:100) (lane *4*) were extracted, reverse transcribed, and amplified by PCR with specific primers for IL-12 or β-actin. The amplified products were probed for IL-12 (312 bp) (*right*, lanes *1*–*4*) and β-actin (324 bp) (*left*, lanes *1*–*4*).

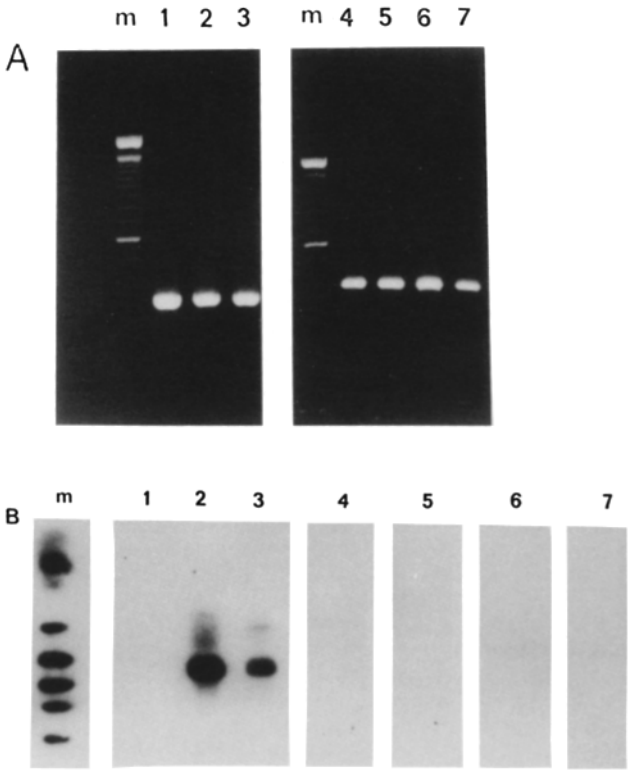

Figure 4. Analysis of IL-12 (p40) mRNA expression in spleen cells of *M. bovis* BCG-infected C57BL/6 mice by RT-PCR analysis and Southern hybridization with IL-12 (p40) cDNA. (*A*) β-actin RT-PCR of spleen cells from *M. bovis* BCG-infected C57BL/6 mice. (Lane *m*) DNA molecular weight marker (100–2,000). (Lane *1*) β-actin RT-PCR from spleen cells of an uninfected C57BL/6 mouse; (lane *2*) β-actin RT-PCR from spleen cells of a *M. bovis* BCG-infected C57BL/6 mouse 3 h after infection; (lane *3*) β-actin RT-PCT from spleen cells of a *M. bovis* BCG-infected C57BL/6 mouse 4 d after infection; (lane *4*) β-actin RT-PCR from spleen cells of an uninfected IFN-γR$^{o/o}$ mouse; (lane *5*) β-actin RT-PCR from spleen cells of a *M. bovis* BCG-infected IFN-γR$^{o/o}$ mouse 3 h after infection; (lane *6*) β-actin RT-PCR from spleen cells of an uninfected Tnfr$^{o/o}$ mouse; (lane *7*) β-actin RT-PCR from spleen cells of a *M. bovis* BCG-infected Tnfr1$^{o/o}$ mouse 3 h after infection. (*B*) Reprobing RT-PCR amplified IL-12 from spleen cells by Southern hybridization. (Lane *m*) DNA molecular weight marker (50–1,000). (Lanes *1*–*7*) Reprobed IL-12 RT-PCR amplified products (312 bp) from cDNA preparations corresponding to cDNA samples of lanes *1*–*7* in *A*, respectively.

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 antimycobacterial macrophage functions (26). Furthermore, TNF-α production in vivo in response to *M. bovis* BCG infection is markedly impaired in IFN-γR$^{o/o}$ 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$^{o/o}$ and Tnfr1$^{o/o}$ 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$^{o/o}$ 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$^{o/o}$ 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$^{o/o}$, and Tnfr1$^{o/o}$ mice**

	Production of IL-12 (ng/ml) by mouse strain					
	C57BL/6		IFN-γR$^{o/o}$		Tnfr1$^{o/o}$	
Stimulus	0 h	3 h	0 h	3 h	0 h	3 h
Nil	ND‡	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

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