INFECTION AND IMMUNITY, May 1997, p. 1936–1938 Vol. 65, No. 5
0019-9567/97/$04.00+0

Interleukin-12 Secretion by *Mycobacterium tuberculosis*-Infected Macrophages

CHRISTOPH H. LADEL,[1] GUDRUN SZALAY,[1] DETLEV RIEDEL,[1] AND STEFAN H. E. KAUFMANN[1,2]*

Department of Immunology, University of Ulm, Ulm,[1] and Max Planck Institute for Infection Biology, Berlin,[2] Germany

Received 19 September 1996/Returned for modification 6 December 1996/Accepted 3 February 1997

Infection with *Mycobacterium tuberculosis* or phagocytosis of large latex beads induced interleukin-12 (IL-12) production in macrophages. In contrast, tumor necrosis factor (TNF) was produced only in response to *M. tuberculosis* infection, not after phagocytosis of latex beads. Comparable results were obtained with cells from immunocompetent C57BL/6 and gamma interferon receptor-deficient mutant mice. Thus, phagocytosis by mechanisms not specific for *M. tuberculosis* was a sufficient trigger for IL-12 secretion, emphasizing the central role of this cytokine in the initiation of anti-infective immunity.

The early cytokine response by the innate immune system has a decisive influence on the host response against infectious agents (4, 25). The early cytokines fulfill two major functions. First, they mobilize the innate immune system to achieve rapid control of the infectious agent. Second, they perform an instructive role in the acquired immune response by promoting appropriate expression of T-cell functions (4). Tumor necrosis factor (TNF), which is produced by infected macrophages, contributes to early restriction of microbial growth by the innate immune system (5). Early interleukin-12 (IL-12) favors TH1 cell development, whereas early IL-4 promotes TH2 cell differentiation (11, 25). Because gamma interferon (IFN-γ)-secreting TH1 cells are vital for protection against intracellular pathogens, rapid IL-12 induction is essential for protective immunity against these infections. Although the central role of IL-12 in protection against intracellular pathogens, including *Listeria monocytogenes*, *Leishmania major*, and *Mycobacterium tuberculosis*, has been established (2, 9, 13, 26), the requirements for IL-12 induction remain insufficiently understood, and different pathways have been described (6, 10, 11, 23, 24). To determine factors involved in IL-12 induction in tuberculosis, we performed in vitro studies with viable and heat-killed *M. tuberculosis* organisms as well as with inert particles. To further analyze IFN-γ involvement in IL-12 induction, these experiments used immunocompetent C57BL/6 mice as well as IFN-γ receptor-deficient gene deletion mutant (IFN-γR$^{-/-}$) mice.

C57BL/6 and IFN-γR$^{-/-}$ mice were bred and maintained at the central animal facilities of the University of Ulm under strict specific-pathogen-free conditions. Breeding pairs of the IFN-γR$^{-/-}$ mouse mutants were kindly provided by M. Aguet, Lausanne, Switzerland (14). Bone marrow cells from the femora of 8- to 12-week-old mice were cultured in Dulbecco modified Eagle medium (Gibco BRL, Paisley, Scotland) supplemented with 10% fetal calf serum (FCS; PAA, Linz, Austria), 5% horse serum (Seromed, Berlin, Germany), and 30% conditioned medium from L929 cells at 37°C in CO_2 for 9 days as described previously (5). The resulting bone marrow-derived macrophages (BMM) were used as source of macrophages. Single-cell suspensions were prepared from spleens by teasing through stainless steel meshes as described previously (16). Spleen cells were seeded into 96-well plates at 2×10^5 cells/well and cultured in Iscove modified Dulbecco medium supplemented with 10% FCS, 10 mM glutamine, 0.05 mM mercaptoethanol, and 1 μg of indomethacin per ml. BMM were plated into 96-well plates at 5×10^4 cells/well in Dulbecco modified Eagle medium supplemented with 10% FCS, 10 mM glutamin, and 1 mM sodium pyruvate 24 h before stimulation. Cells were stimulated with live *M. tuberculosis* H37Rv (infection ratio of 1:1), 10^6 heat-killed *M. tuberculosis* (HKM; obtained by heating a culture of *M. tuberculosis* for 2 h at 80°C), or two different-size (1.2 or 3.1 μm in diameter) latex beads (Sigma, Dreieich, Germany) at a concentration of 10^6 per well. BMM and spleen cells were cultured for 4 and 2 days, the respective time points of maximal responses. Afterwards, supernatants were removed and IL-12 (p40 and p70, monoclonal antibody clones C17.8 and C15.6) or TNF concentrations were determined by enzyme-linked immunosorbent assay (ELISA) or bioassay, respectively, as described in detail elsewhere (16, 17). To assess the metabolic activity of cultured cells independent of proliferation, cells were incubated in the presence of MTT (dimethylthiazol-diphenyltetrazolium bromide) (Sigma) after removal of supernatants (on day 2 or 4 for spleen cells or BMM, respectively) for another 4-h period. This measurement provides information about the metabolic cell activity independent of proliferation (12, 18). Quantifications were performed with a SpectraMax apparatus (Molecular Devices, Sunnyvale, Calif.), using the SoftMax ProSoftware (Molecular Devices).

BMM from C57BL/6 and IFN-γR$^{-/-}$ gene disruption mutant mice produced IL-12 after stimulation with live *M. tuberculosis*, HKM, and 3.1-μm-diameter latex beads (Fig. 1A). In both IFN-γR$^{-/-}$ and C57BL/6 mice, highest IL-12 levels were induced by viable *M. tuberculosis* organisms. TNF was detectable only after stimulation with viable *M. tuberculosis* organisms or HKM, whereas latex beads of either size failed to induce TNF secretion (Fig. 1B). Comparable results were obtained after stimulation of spleen cells from C57BL/6 or IFN-γR$^{-/-}$ mice (Fig. 2). Determination of metabolic activities by the MTT assay is considered an in vitro marker of metabolic cell activity independent of proliferation (12). Because under the culture conditions used, BMM showed no or only marginal proliferation (data not shown), metabolic activity of BMM or spleen cells was evaluated by MTT cleavage. A significant metabolic activity was observed after stimulation of BMM (Fig. 3A) as well as of naive spleen cells (Fig. 3B) in response to all stimuli used. Since MTT cleavage is caused by oxidative metabolism of cells as well as bacteria, cells infected with live

* Corresponding author. Mailing address: Department of Immunology, University of Ulm, D-89070 Ulm, Germany. Phone: 0731/502-3361. Fax: 0731/502-3367. E-mail: Stefan.Kaufmann@Medizin.Uni-Ulm.DE.

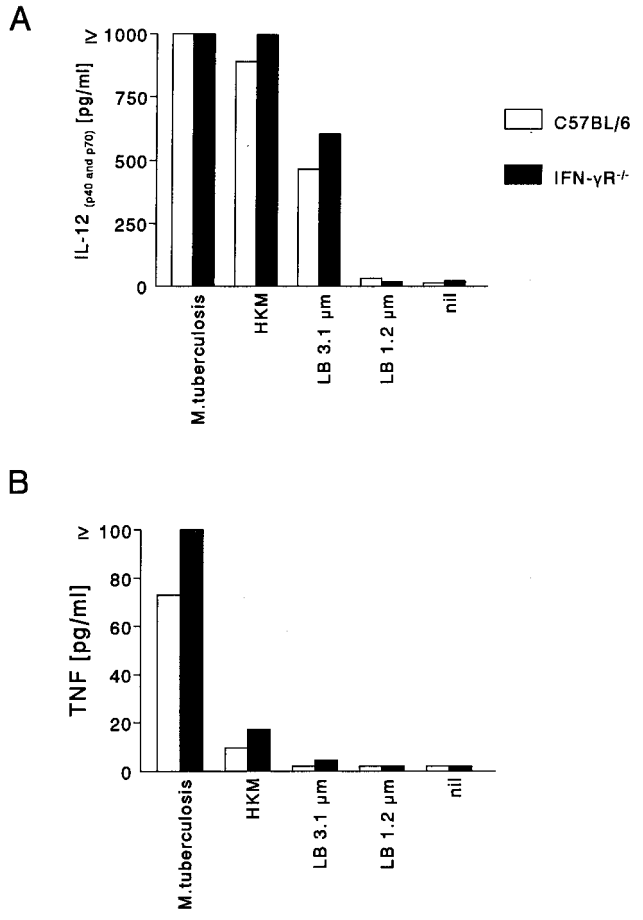

FIG. 1. Cytokine secretion by BMM after in vitro culture with *M. tuberculosis* or latex beads (LB). BMM were cultured for 4 days with the stimuli indicated. Cytokine activities in supernatants were analyzed by ELISA (IL-12; A) or bioassay (TNF; B). Data represent the means of triplicates of a representative experiment repeated twice with comparable results (standard deviation, <10%).

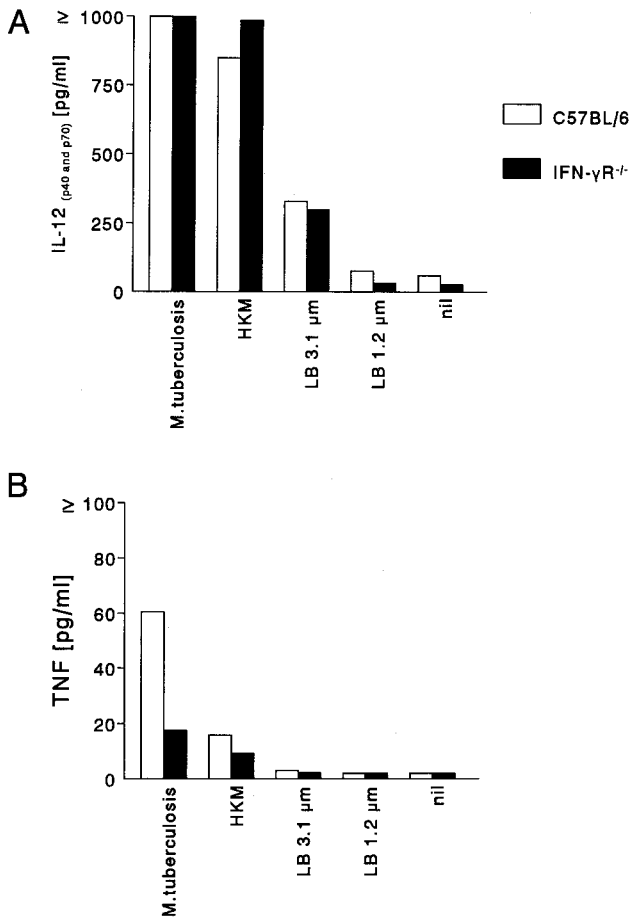

FIG. 2. Cytokine secretion by spleen cells after in vitro culture with *M. tuberculosis* or latex beads (LB). Spleen cells were cultured for 2 days with the stimuli indicated. Supernatants were analyzed by ELISA (A) and bioassay (B). Data represent the means of triplicates of a representative experiment repeated twice with comparable results (standard deviation, <10%).

bacteria could not be analyzed by this method due to intracellular survival of *M. tuberculosis* organisms. Our data show that cells are metabolically active in vitro under conditions which do not induce IL-12 or TNF secretion. We further assume that the failure to digest inert latex beads caused long-lasting activation of BMM and spleen cells independent of their size. In contrast, only the larger (3.1-μm-diameter) latex beads were capable of inducing measurable amounts of IL-12; the smaller (1.2-μm-diameter) ones failed to do so. Induction of IL-12 by inert particles suggests a role of phagocytosis in IL-12 induction. It remains to be established whether phagocytosis of particles involved scavenger receptors or other mechanisms (15). Failure to stimulate IL-12 secretion in murine peritoneal exudate cells by small latex beads has been described elsewhere (23), whereas Fulton et al. found IL-12 induction in human peripheral blood cells by larger latex beads (10). Our data suggest dependency on the size of latex beads of IL-12 induction. Differential uptake of particles of different size has been described previously (20). Taken together, these findings suggest that phagocytosis by mechanisms which are not unique to *M. tuberculosis* organisms promoted IL-12 induction. In addition, receptor-mediated signalling induced by mycobacterium-specific ligands could synergize with this type of phagocytosis in macrophage activation (1, 21, 22, 26, 27). The most likely candidate for receptor-mediated IL-12 induction by *M. tuberculosis* is lipoarabinomannan (10, 22, 27). This glycolipid has been shown to participate in the uptake of *M. tuberculosis* via the mannose receptor (22). This receptor, however, cannot be made responsible for engulfment of latex beads, which may be taken up by scavenger receptors (15). Therefore, *M. tuberculosis* can induce IL-12 through different pathways, with at least two receptors as likely candidates for IL-12 induction (21, 22).

Previous experiments from this laboratory suggested IFN-γ dependency of IL-12 induction by *M. bovis* BCG in BMM from IFN-γR$^{-/-}$ mutants which were grown in serum-free medium (6). We assume that in absence of serum, BMM remained in a highly quiescent stage, causing a need for IFN-γ in IL-12 secretion by these cells. The data described here show that IL-12 can be induced in serum-cultured BMM from IFN-γR$^{-/-}$ mice, suggesting that IFN-γ dependency can be overcome by stimuli present in the serum. In contrast to IL-12, TNF was not induced by latex beads, and HKM stimulated only small amounts of TNF in BMM. In spleen cells, significant TNF secretion was observed only after infection with viable *M. tuberculosis*, and marginal amounts of TNF were observed after HKM stimulation. These findings suggest differential and apparently independent activation pathways for TNF and IL-12. Because the spleen comprises virtually all leukocyte populations, it is possible that discrete populations were responsible for IL-12 and TNF secretion by splenocytes. In particular, we

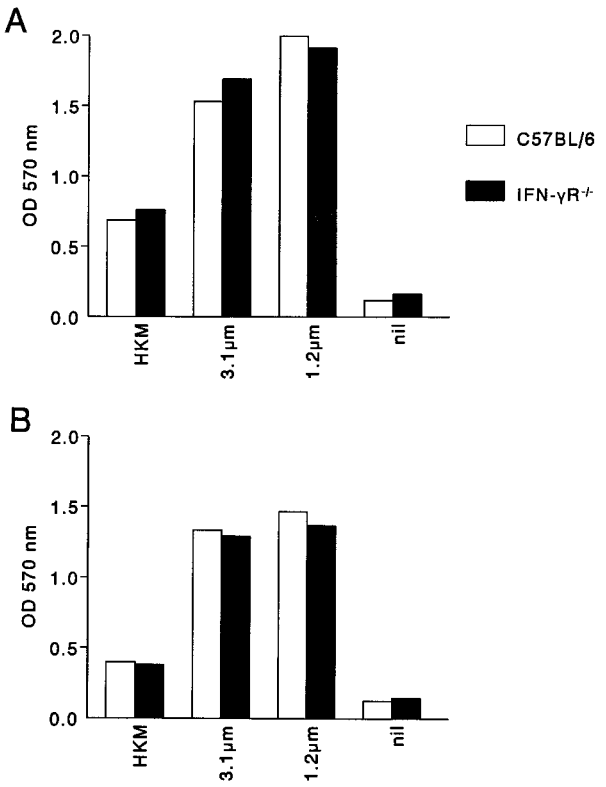

FIG. 3. Metabolic activity of BMM (A) on day 4 and spleen cells (B) on day 2 after stimulation with latex beads and different mycobacterial antigens. Data from a representative experiment repeated twice are shown.

cannot exclude IL-12 secretion by B cells or dendritic cells, which are a well-known source of this cytokine (11, 25).

Secretion of IL-12 and TNF in response to microbial infection represents a major defense mechanism of the innate immune system against intracellular pathogens (2, 9, 11, 13, 26). TNF is primarily involved in further amplification of the innate immune system, encompassing macrophages and natural killer cells, whereas the function of IL-12 is directed mostly toward polarization of the TH0 cells toward the TH1 pole. Both TNF produced by macrophages and IFN-γ from TH1 cells have been shown to be essential for control of experimental tuberculosis of mice (3, 7, 8). Our data showing that IL-12 can be induced by particulate substrates like beads or heat-killed bacteria as well as virulent tubercle bacilli themselves emphasize the central role of this cytokine in the initiation of anti-infective immunity against intracellular bacteria, including *M. tuberculosis*.

This work received financial support from the Sonderforschungsbereich 322 "Lympho-Hämopoese" (project B7) and BMBF Project "Mykobakterielle Infektionen."

We are grateful to K. Tell for monoclonal antibody production, A. Dreher for excellent technical support, and R. Mahmoudi for secretarial help. We thank G. Adolf, M. Aguet, G. Trinchieri, and S. Wolf for helpful reagents.

REFERENCES

1. **Cleveland, M. G., J. D. Gorham, T. L. Murphy, E. Toumanen, and K. M. Murphy.** 1996. Lipoteichoic acid preparations of gram-positive bacteria induce Interleukin-12 through a CD14-dependent pathway. Infect. Immun. **64:**1906–1912.
2. **Cooper, A. M., A. D. Roberts, E. R. Rhoades, J. E. Callahan, D. M. Getzy, and I. M. Orme.** 1995. The role of interleukin-12 in acquired immunity to *Mycobacterium tuberculosis* infection. Immunology **85:**423–432.
3. **Cooper, A. M., D. K. Dalton, T. A. Steward, J. P. Griffin, D. G. Russell, and I. M. Orme.** 1993. Disseminated tuberculosis in interferon γ gene-disrupted mice. J. Exp. Med. **178:**2243–2247.
4. **Fearon, D. T., and R. M. Locksley.** 1996. The instructive role of innate immunity in the acquired immune response. Science **272:**50–54.
5. **Flesch, I. E. A., and S. H. E. Kaufmann.** 1991. Mechanisms involved in mycobacterial growth inhibition by gamma-interferon activated bone marrow macrophages: role of reactive nitrogen intermediates. Infect. Immun. **59:**3213–3218.
6. **Flesch, I. E. A., J. H. Hess, S. Huang, M. Aguet, J. Rothe, H. Bluethmann, and S. H. E. Kaufmann.** 1995. Early interleukin-12 production by macrophages in response to mycobacterial infection depends on interferon-γ and tumor necrosis factor-α. J. Exp. Med. **181:**1615–1622.
7. **Flynn, J. L., J. Chan, K. J. Triebold, D. K. Dalton, T. A. Stewart, and B. R. Bloom.** 1993. An essential role for interferon-γ in resistance to *Mycobacterium tuberculosis* infection. J. Exp. Med. **178:**2249–2254.
8. **Flynn, J. L., M. M. Goldstein, J. Chan, K. J. Triebold, K. Pfeffer, C. J. Lowenstein, R. Schreiber, T. W. Mak, and B. R. Bloom.** 1995. Tumor necrosis factor-α is required in the protective immune response against *Mycobacterium tuberculosis* in mice. Immunity **2:**561–572.
9. **Flynn, J. L., M. M. Goldstein, K. J. Triebold, J. Sypek, S. Wolf, and B. R. Bloom.** 1995. IL-12 increases resistance of BALB/c mice to *Mycobacterium tuberculosis* infection. J. Immunol. **155:**2515–2524.
10. **Fulton, S. A., J. M. Johnsen, S. F. Wolf, D. S. Sieburth, and W. H. Boom.** 1996. Interleukin-12 production by human monocytes infected with *Mycobacterium tuberculosis*: role of phagocytosis. Infect. Immun. **64:**2523–2531.
11. **Gazzinelli, R. T.** 1996. Molecular and cellular basis of interleukin-12 activity in prophylaxis and therapy against infectious diseases. Mol. Med. Today **2:**258–267.
12. **Gerlier, D., and N. Thomasset.** 1986. Use of MTT colorimetric assay to measure cell activation. J. Immunol. Methods **94:**57–63.
13. **Heinzel, F. P., D. S. Schoenhaut, R. M. Rerko, L. E. Rosser, and M. K. Gately.** 1993. Recombinant interleukin 12 cures mice infected with *Leishmania major*. J. Exp. Med. **177:**1505–1509.
14. **Huang, S., W. Hendriks, A. Althage, S. Hemmi, H. Bluethmann, R. Kamijo, J. Vilcek, R. M. Zinkernagel, and M. Aguet.** 1993. Immune response in mice that lack the interferon-γ receptor. Science **259:**1742–1745.
15. **Krieger, M., and J. Herz.** 1994. Structures and functions of multiligand lipoprotein receptors: macrophage scavenger receptors and LDL receptor-related protein (LRP). Annu. Rev. Biochem. **63:**601–637.
16. **Ladel, C. H., S. Daugelat, and S. H. E. Kaufmann.** 1995. Immune response to *Mycobacterium bovis* bacille Calmette Guérin infection in major histocompatibility complex I and II deficient mutant mice: contribution of CD4 and CD8 T cells to acquired resistance. Eur. J. Immunol. **25:**377–384.
17. **Ladel, C. H., C. Blum, and S. H. E. Kaufmann.** 1996. Control of natural killer cell-mediated innate resistance against the intracellular pathogen *Listeria monocytogenes* by γ/δ T lymphocytes. Infect. Immun. **64:**1744–1749.
18. **Mosmann, T.** 1983. Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. J. Immunol. Methods **65:**55–63.
19. **Pearson, A. M.** 1996. Scavenger receptors in innate immunity. Curr. Opin. Immunol. **8:**20–28.
20. **Pratten, M., and J. B. Lloyd.** 1986. Pinocytosis and phagocytosis: the effect of size of a particulate substrate on its mode of capture by rat peritoneal macrophages cultured in vitro. Biochim. Biophys. Acta **881:**307–313.
21. **Savedra, R., R. L. Delude, R. R. Ingalls, M. J. Fenton, and D. T. Golenbock.** 1996. Mycobacterial lipoarabinomannan recognition requires a receptor that shares components of the endotoxin signalling system. J. Immunol. **157:** 2549–2554.
22. **Schlesinger, L. S., T. M. Kaufman, S. Iyer, S. R. Hull, and L. K. Marchinado.** 1996. Differences in mannose receptor-mediated uptake of lipoarabinomannan from virulent and attenuated strains of *Mycobacterium tuberculosis* by human macrophages. J. Immunol. **157:**4568–4575.
23. **Skeen, M. J., M. A. Miller, T. M. Shinnick, and H. K. Ziegler.** 1996. Regulation of murine macrophage IL-12 production. J. Immunol. **156:**1196–1206.
24. **Song, F., G. Matsuzaki, M. Mitsuyama, and K. Nomoto.** 1996. Differential effects of viable and killed bacteria on IL-12 expression of macrophages. J. Immunol. **156:**2979–2984.
25. **Trinchieri, G.** 1995. Interleukin-12: a proinflammatory cytokine with immunoregulatory functions that bridge innate resistance and antigen-specific adaptive immunity. Annu. Rev. Immunol. **13:**251–276.
26. **Tripp, C. S., M. K. Gately, J. Hakimi, P. Ling, and E. R. Unanue.** 1994. Neutralization of IL-12 decreases resistance to *Listeria* in SCID and C.B-17 mice. J. Immunol. **152:**1883–1887.
27. **Venisse, A., J.-J. Fournie, and G. Puzo.** 1995. Mannosylated lipoarabinomannan interacts with phagocytes. Eur. J. Biochem. **231:**440–447.

Editor: P. J. Sansonetti