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Increased Numbers of Interleukin-12-Producing Cells in Human Tuberculosis

MARTIN E. MUNK,[1]* PETRA MAYER,[1] PETER ANDING,[1] KNUT FELDMANN,[2] AND STEFAN H. E. KAUFMANN[1]

Department of Immunology, University of Ulm, Ulm,[1] and Zentralkrankenhaus der Landesversicherungsanstalt, Gauting,[2] Germany

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Numbers of interleukin-12 (IL-12)-producing cells were quantitated in the peripheral blood of healthy donors and tuberculosis patients by the ELISPOT assay. We observed that (i) stimulation with mycobacteria increases numbers of IL-12 producers from healthy donors and (ii) tuberculosis patients have larger numbers of IL-12 producers than healthy donors. Our data emphasize the importance of IL-12 in immunity to tuberculosis.

The intracellular pathogen *Mycobacterium tuberculosis* is the causative agent of tuberculosis, which is estimated to cause 3 million deaths worldwide annually (2). Resistance to tuberculosis crucially depends on specific T cells which activate intracellular killing of the infectious agent by macrophages (5). CD4 T cells comprise two functionally distinct helper subsets according to their cytokine profiles, namely, Th1 and Th2 cells (3, 4, 7). Predominance of Th1 or Th2 cell responses has an important influence on the outcome of infection with intracellular pathogens (9). The development of the Th cell type is influenced by several cytokines, such as interleukin-4 (IL-4), gamma interferon (IFN-γ), and IL-12, which are produced at the onset of infection (3). IL-12, previously known as natural killer cell stimulatory factor or cytotoxic lymphocyte maturation factor, is an early proinflammatory cytokine which is required for initiation and maintenance of cell-mediated immunity against intracellular microorganisms (10). IL-12 has been identified in tuberculous pleuritis and is produced by alveolar macrophages from tuberculosis patients (12, 13). We quantitated numbers of IL-12-producing cells in the peripheral blood of healthy donors and tuberculosis patients by the ELISPOT assay, which we assume to approximate best the in vivo potential of IL-12 producers. Our data emphasize an important role of IL-12 in immunity to tuberculosis.

Peripheral blood mononuclear cells were drawn on heparin after informed consent from *Mycobacterium bovis* BCG-vaccinated (8 of 10) healthy adult volunteers (Blood Donor Center, Ulm, Germany) and 12 patients with diagnosed pulmonary tuberculosis (mean age ± standard deviation, 40.7 ± 14.2) seen at the Zentralkrankenhaus Gauting. All tuberculosis patients were seronegative for human immunodeficiency virus types 1 and 2 and underwent antituberculosis chemotherapy for less than 30 days. Peripheral blood mononuclear cells were enriched by density gradient centrifugation and washed in RPMI 1640 supplemented with 10% human serum (A^+, Rh^+), HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid), and penicillin-streptomicin (all from Biochrom, Berlin, Germany). Afterwards, cells (5×10^6/ml) were incubated in 250-ml plastic culture flasks (Nunc GmbH, Wiesbaden, Germany) at 37°C for 1 h, and adherent cells (about 90% pure) were recovered by gentle scraping (8).

* Corresponding author. Mailing address: Department of Immunology, University of Ulm, Albert Einstein Allee 11, 89070 Ulm, Germany. Phone: 731-502-3373. Fax: 731-502-3367. Electronic mail address: Martin.Munk@Medizin.Uni-Ulm.De.

Numbers of IL-12 spot-forming cells (SFC) were determined by a modification of the ELISPOT assay described earlier (9). Briefly, nitrocellulose-bottom 96-well plates (Millipore Co., Bedford, Mass.) were coated with anti-human IL-12 (p70) monoclonal antibodies (MAb) (MAb 11.79.15, 1 μg/ml; kindly provided by G. Trinchieri, Wistar Institute, Philadelphia, Pa.) overnight. After being washed with phosphate-buffered saline (PBS) and incubated with blocking buffer (PBS containing 1% bovine serum albumin; Serva Feinbiochemica GmbH, Heidelberg, Germany), freshly isolated adherent cells from healthy donors (1.6×10^5 per well [Fig. 1] and 1×10^5 per well [Fig. 2]) or tuberculosis patients (1×10^5 per well [Fig. 2]) were added in the absence or in the presence of live *M. bovis* BCG (2.5×10^7/ml) or dead *M. tuberculosis* H37Ra (10 μg/ml; Difco, Detroit, Mich.) organisms, IFN-γ (500 U/ml, specific activity = 10^7 U/mg of protein; kindly provided by G. Adolf, Ernst Boehringer-Institut für Arzneimittelforschung, Vienna, Austria), or lipopolysaccharide (LPS) (10 μg/ml; Sigma, St. Louis, Mo.). After 18 h of incubation at 37°C, the wells were washed with PBS containing 0.5% Tween 20 (Fluka, Buchs, Switzerland), and biotin-conjugated anti-human IL-12 (p40) MAb (MAb 8.6.2.1, 0.25 μg/ml; Wistar Institute) was added to each well. After 2 h, the wells were washed and incubated with streptavidin-alkaline phosphatase (1:20,000; Dianova, Hamburg, Germany) for 1 h. Unbound conjugate MAb were washed off, and 5-bromo-4-chloro-3-indolyl phosphate (165 μg/ml)–nitroblue tetrazolium chloride (330 μg/ml) substrate solution (Fluka) was added. Color development was terminated with distilled water, and numbers of IL-12 SFC on dried nitrocellulose wells were determined under a dissection microscope (Carl Zeiss, Aalen-Oberkochen, Germany). The mean from four wells per stimulation is expressed as the number of positive IL-12 SFC per 1.6×10^5 cells or the number of positive IL-12 SFC per 1×10^5 cells.

First, we analyzed numbers of IL-12 SFC among freshly isolated adherent cells from healthy donors. Figure 1 shows that stimulation with dead *M. tuberculosis* organisms or LPS significantly increased numbers of IL-12 SFC from 6 of 10 and 8 of 10 donors, respectively ($P < 0.05$). Live *M. bovis* BCG induced an increase of IL-12 SFC in 5 of 10 donors ($P > 0.05$). Although tuberculosis patients possess mycobacterium-specific T cells as shown in proliferation assays with killed *M. tuberculosis* organisms (mean cpm ± standard deviation, 22,048 ± 22,791) or purified protein derivative (20,593 ± 15,534), the T-cell mitogen phytohemagglutinin and anti-IFN-γ and/or anti-tumor necrosis factor alpha MAb did not influence num-

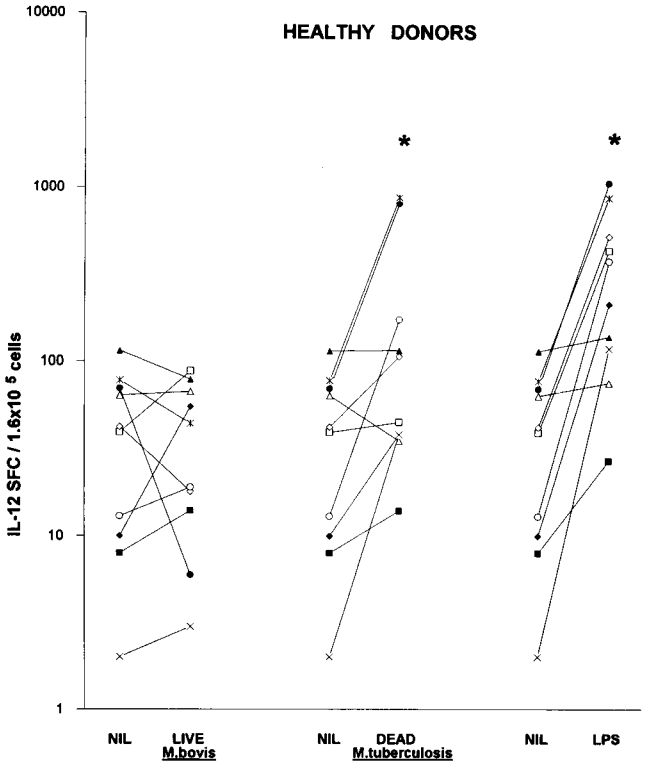

FIG. 1. Numbers of IL-12 SFC among adherent cells from 10 healthy donors. Freshly isolated and selected adherent cells were left unstimulated (NIL) or were pulsed with live *M. bovis* BCG (2.5×10^7/ml) or dead *M. tuberculosis* (10 μg/ml) organisms or with LPS (10 μg/ml) for 18 h. Afterwards, numbers of IL-12 SFC were determined by the ELISPOT assay. Results (mean from four wells per stimulation) are expressed as numbers of IL-12 SFC per 1.6×10^5 cells. *, $P < 0.05$ versus unstimulated cells (tested by the Wilcoxon signed rank test before and after stimulation).

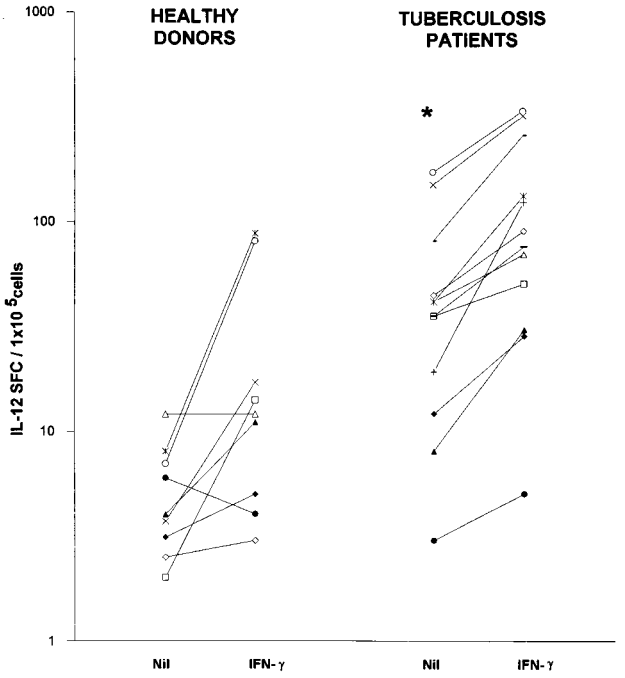

FIG. 2. Numbers of IL-12 SFC among adherent cells from 9 healthy donors and 12 tuberculosis patients. Freshly isolated and selected adherent cells were left unstimulated (Nil) or were stimulated with IFN-γ (500 U/ml) for 18 h. Afterwards, numbers of IL-12 SFC were determined by the ELISPOT assay. Results (mean from four wells per stimulation) are expressed as numbers of IL-12 SFC per 10^5 cells. *, $P < 0.05$ versus unstimulated cells from healthy donors (tested by the Mann-Whitney U test).

bers of IL-12 producers (data not shown). Freshly isolated adherent cells from tuberculosis patients showed an eightfold-larger number of IL-12 SFC (median [range] IL-12 SFC, 38 [3 to 170]) compared with that for healthy donors (5 [2 to 12]) ($P < 0.05$) (Fig. 2). Interestingly, unstimulated adherent cells from tuberculous pleuritis exudate also encompassed large numbers of IL-12 SFC, similar to patient peripheral blood cell levels (data not shown).

IFN-γ induces IL-12 production, and IFN-γ concentration is elevated in tuberculous pleuritis (6). Therefore, we compared the IL-12 SFC response patterns of cells from healthy donors with those from tuberculosis patients after stimulation with IFN-γ. Figure 2 shows that IFN-γ stimulation induced a two- to threefold increase of IL-12 SFC among cells from tuberculosis patients (mean [range], 82 [5 to 324]) and healthy donors (17 [6 to 46]) ($P < 0.05$). These data suggest that IFN-γ induces similar levels of IL-12 SFC in tuberculosis patients and healthy donors.

Our investigation reveals that (i) stimulation with mycobacteria increases numbers of IL-12 SFC from healthy individuals in vitro, (ii) tuberculosis patients have larger numbers of circulating and pleural IL-12 SFC than healthy donors, and (iii) IL-12 SFC from tuberculosis patients and those from healthy donors are stimulated equally by IFN-γ. Mycobacteria induce increased numbers of IL-12 SFC and IL-12 production and thus promote development of Th1-type responses (6). Consequently, IL-12 facilitates activation of infected phagocytes and killing of mycobacterial organisms (5).

Although we used dead *M. tuberculosis* and live *M. bovis* BCG organisms for our in vitro analyses, we assume that viability rather than antigenic diversity accounted for differential IL-12 production. Lymphocytes from tuberculous pleuritis stimulated with *M. tuberculosis* produce more IFN-γ than peripheral blood lymphocytes (1). Furthermore, IL-12 levels are increased in tuberculosis patients (12, 13). Thus, elevated levels of systemic IFN-γ and IL-12 SFC found in tuberculosis patients are important regulatory cytokines related to the promotion of Th1 cell responses. However, the Th1 response is reduced in the peripheral blood of tuberculosis patients, and reversion can be achieved by successful antituberculosis therapy (14). Consistent with our data, depression of the Th1 response argues against a failure of the IL-12 induction arm and suggests impaired T-cell reactivity against tuberculosis. In agreement with published data (11), we have found elevated percentages of circulating monocytes expressing abnormal levels of HLA-DR molecules (data not shown). However, increased numbers of IL-12 SFC found in tuberculosis patients seem to be inversely related to the activation of monocytes in terms of class II major histocompatibility complex determinants. We assume that IL-12-producing cells act as immunomodulators of peripheral and specific T cells. We conclude that peripheral blood and pleural IL-12-producing cells participate in the development of protective Th1 responses in tuberculosis patients. Yet, we cannot formally exclude the possibility that the larger amounts of IL-12 observed in tuberculosis patients indicate susceptibility rather than resistance to tuberculosis.

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REFERENCES

1. **Barnes, P. F., J. S. Abrams, S. Lu, P. A. Sieling, T. H. Rea, and R. L. Modlin.** 1993. Patterns of cytokine production by mycobacterium-reactive human T-cell clones. Infect. Immun. **61:**197–203.
2. **Bloom, B. R., and C. J. L. Murray.** 1992. Tuberculosis: commentary on reemergent killer. Science **257:**1055–1064.
3. **Coffman, R. L., and T. R. Mosmann.** 1991. CD4+ T-cell subsets: regulation of differentiation and function. Res. Immunol. **142:**7–79.
4. **Del Prete, G. F., M. De Carli, C. Mastromauro, R. Biagiotti, D. Macchia, P. Falagiani, M. Ricci, and S. Romagnani.** 1991. Purified protein derivative of *Mycobacterium tuberculosis* and excretory-secretory antigen(s) of *Toxocara canis* expand in vitro human T cells with stable and opposite (type 1 T helper or type 2 T helper) profile of cytokine production. J. Clin. Invest. **88:**346–350.
5. **Kaufmann, S. H. E.** 1993. Immunity to intracellular bacteria. Annu. Rev. Immunol. **11:**129–163.
6. **Kubin, M., J. M. Chow, and G. Trinchieri.** 1993. Differential regulation of interleukin-12 (IL-12), tumor necrosis factor-α, and IL-1γ production in human myeloid leukemia cell lines and peripheral blood mononuclear cells. Blood **83:**1847–1855.
7. **Mosmann, T. R., and R. L. Coffman.** 1989. TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties. Annu. Rev. Immunol. **7:**145.
8. **Munk, M. E., J. De Bruyn, H. Gras, and S. H. E. Kaufmann.** 1994. The *Mycobacterium bovis* 32-kilodalton protein antigen induces human cytotoxic T-cell responses. Infect. Immun. **62:**726–728.
9. **Teixeira, H. C., M. E. Munk, and S. H. E. Kaufmann.** 1995. Frequencies of IFN-γ and IL-4-producing cells during *Mycobacterium bovis* BCG infection in two genetically susceptible mouse strains: role of α/β T cells and NK1.1 cells. Immunol. Lett. **46:**15–19.
10. **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.
11. **Tweardy, D. J., B. Z. Schacter, and J. J. Ellner.** 1984. Association of altered dynamics of monocyte surface expression of human leukocyte antigen DR with immunosuppression in tuberculosis. J. Infect. Dis. **149:**31–37.
12. **Zhang, M., M. K. Gately, E. Wang, J. Gong, S. F. Wolf, S. Lu, R. L. Modlin, and P. F. Barnes.** 1994. Interleukin 12 at the site of disease in tuberculosis. J. Clin. Invest. **93:**1733–1739.
13. **Zhang, M., J. Gong, D. V. Iyer, B. E. Jones, R. L. Modlin, and P. F. Barnes.** 1994. T cell cytokine responses in persons with tuberculosis and human immunodeficiency virus infection. J. Clin. Invest. **94:**2435–2442.
14. **Zhang, M., Y. Lin, D. V. Iyer, J. Gong, J. S. Abrams, and P. F. Barnes.** 1995. T-cell cytokine responses in human infection with *Mycobacterium tuberculosis*. Infect. Immun. **63:**3231–3234.

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