

## Cytokine Profiles in Immunocompetent Persons Infected with *Mycobacterium avium* Complex

Ramakrishna Vankayalapati,<sup>1</sup> Benjamin Wizel,<sup>1,2</sup> Buka Samten,<sup>1</sup> David E. Griffith,<sup>1,3</sup> Homayoun Shams,<sup>1</sup> Margaret R. Galland,<sup>1</sup> C. Fordham von Reyn,<sup>6</sup> William M. Girard,<sup>3</sup> Richard J. Wallace, Jr.,<sup>3,5</sup> and Peter F. Barnes<sup>1,3,4</sup>

<sup>1</sup>Center for Pulmonary and Infectious Disease Control and Departments of <sup>2</sup>Immunology, <sup>3</sup>Medicine, <sup>4</sup>Cell Biology, and <sup>5</sup>Microbiology, University of Texas Health Center, Tyler; <sup>6</sup>Infectious Disease Section, Department of Medicine, Dartmouth-Hitchcock Medical Center, Lebanon, New Hampshire

To evaluate the immunologic factors that contribute to protection against *Mycobacterium avium* complex (MAC), cytokine production by peripheral blood mononuclear cells (PBMC) from human immunodeficiency virus–negative persons with pulmonary MAC (MAC patients) and healthy control subjects with a delayed hypersensitivity skin test response to *M. avium* sensitin (MAS-positive control subjects) was measured. In MAC patients, mycobacterium-stimulated PBMC produced higher concentrations of interleukin (IL)–10 but lower concentrations of interferon (IFN)– $\gamma$ , IL-12, and tumor necrosis factor (TNF)– $\alpha$ , compared with PBMC from MAS-positive control subjects. Immunolabeling for intracellular IL-10 revealed that this cytokine was produced by both monocytes and T cells. Alveolar macrophages produced TNF– $\alpha$  and IL-10 in response to MAC, which suggests that these cytokines are produced in the lungs of patients with pulmonary disease caused by this pathogen. Our findings suggest that IFN– $\gamma$ , TNF– $\alpha$ , and IL-12 contribute to protection against MAC, whereas IL-10 is immunosuppressive.

*Mycobacterium avium* complex (MAC) includes *M. avium* and *M. intracellulare*, which are widely distributed in soil and water throughout the world. Because MAC is ubiquitous in the environment, exposure to these organisms is inevitable. Despite widespread exposure, disease is unusual in immunocompetent hosts. However, during the past 2 decades, MAC has been recognized as an increasingly common cause of progressive pulmonary disease in the United States. This disease has a bimodal clinical presentation. One form resembles pulmonary tuberculosis radiographically and primarily affects male smokers with preexisting chronic obstructive pulmonary disease, whereas the other form is associated with mid-lung interstitial infiltrate and bronchiectasis in older non-smoking women without underlying pulmonary abnormalities [1, 2].

*M. avium* is a major cause of morbidity and mortality in patients with advanced human immunodeficiency virus (HIV)

infection, in whom it causes disseminated disease involving multiple organs [3, 4]. Because patients with advanced HIV infection have multiple abnormalities in numbers and functions of T cells, the specific immunologic defects that predispose to disease from *M. avium* remain undefined. The nature of immune dysfunction that predisposes to MAC lung disease in HIV-negative persons is also unknown. Understanding these defects is important to the development of immunotherapeutic modalities to treat and prevent disease due to MAC in persons with or without HIV infection.

To gain insight into the immunologic factors that contribute to protection against MAC, we studied healthy persons who were infected with MAC and HIV-negative persons with pulmonary disease due to these organisms. We reasoned that healthy infected persons mount an effective immune response to MAC, whereas persons with pulmonary disease do not. We evaluated the immune response in these 2 groups by comparing the capacity of peripheral blood mononuclear cells (PBMC) to proliferate and produce cytokines in response to MAC.

### Materials and Methods

**Study population.** Blood was obtained from 26 patients with a diagnosis of pulmonary disease due to MAC (MAC patients). A diagnosis was made on the basis of guidelines recommended by the American Thoracic Society [5]. In all cases, sputum culture results were positive, chest abnormalities were present on radiographs, and disease was confined to the lungs. Of the 26 patients, 9 had cavitary disease and 17 had nodular bronchiectasis. None of the patients had risk factors for HIV infection. Twenty-one pa-

Received 22 August 2000; revised 16 October 2000; electronically published 20 December 2000.

Informed consent was obtained from all patients. All studies were approved by the institutional review board of the University of Texas Health Center at Tyler.

Financial support: Center for Pulmonary and Infectious Disease Control, the Cain Foundation for Infectious Disease Research, and the National Institutes of Health (heat-killed *Mycobacterium intracellulare* strain 1067 was provided through grant AI-05074). P.F.B. holds the Margaret E. Byers Cain Chair for Tuberculosis Research.

Reprints or correspondence: Dr. Peter F. Barnes, CPIDC, University of Texas Health Center, 11937 US Hwy. 271, Tyler, TX 75708-3154 (peter.barnes@uthct.edu).

The Journal of Infectious Diseases 2001;183:478–84

© 2001 by the Infectious Diseases Society of America. All rights reserved.  
0022-1899/2001/18303-0015\$02.00

tients were women and 5 were men, and they had a median age of 69 years (range, 39–85 years). At the time that blood samples were obtained, 10 patients had received no treatment and 16 had been treated for 1–21 months with antimycobacterial agents.

Skin testing was done on healthy control subjects to evaluate the delayed-type hypersensitivity response to *M. avium* sensitin and tuberculin, as described elsewhere [6, 7]. The *M. avium* sensitin 10/2 (filling lot 68; provided by Kaare Haslov, Statens Serum Institute, Copenhagen). We excluded from the study persons in whom the tuberculin skin test reaction was  $\geq 5$  mm and persons in whom the *M. avium* sensitin skin test response was 1–9 mm. The remaining subjects were divided into those with positive ( $\geq 10$  mm in diameter) or negative (0 mm in diameter) *M. avium* skin test results. Blood was obtained from 19 healthy control subjects who had positive *M. avium* skin test responses (median diameter, 21 mm; range, 11–35 mm) and from 3 persons with negative skin test responses. These groups are hereafter referred to as MAS-positive and MAS-negative control subjects, respectively.

Bronchoalveolar lavage (BAL) fluid was obtained from 8 patients who underwent bronchoscopy to evaluate the possibility of cancer. None of these patients had clinical or laboratory evidence of tuberculosis, MAC disease, or HIV infection. In all cases, the pathologic process was unilateral, and BAL was done in lungs with no bronchoscopic or radiographic evidence of cancer.

**Isolation of PBMC and CD14<sup>+</sup> monocytes.** PBMC were isolated from blood by differential centrifugation over Ficoll-Paque (Pharmacia). PBMC were centrifuged on a Percoll gradient (Amersham Pharmacia Biotech), and purified CD14<sup>+</sup> cells were isolated from the monocyte fraction by positive selection with magnetic beads conjugated to anti-CD14 (Miltenyi Biotech). The positive cells were 94% CD14<sup>+</sup>, as measured by flow cytometry.

**Cell culture conditions.** PBMC ( $2 \times 10^5$ ) or CD14<sup>+</sup> monocytes ( $5 \times 10^4$ ) were plated in flat-bottomed 96-well plates (Becton Dickinson Labware) in 200  $\mu$ L of RPMI 1640 (Life Technologies) containing penicillin and streptomycin (Life Technologies) and 10% heat-inactivated human serum in the presence or absence of 10  $\mu$ g/mL heat-killed *M. intracellulare* strain 1067, which was previously isolated from a patient with pulmonary disease due to MAC. In some experiments, heat-killed *M. avium* strain 104 (provided by Luiz Bermudez, Kuzell Institute, San Francisco) and *M. tuberculosis* Erdman (provided by Patrick Brennan, Colorado State University, Fort Collins) were used.

BAL fluid was passed through sterile gauze and centrifuged at 834 g for 5 min. The cell pellet was resuspended in RPMI and  $1.2 \times 10^5$  cells were allowed to adhere to flat-bottomed, 96-well plates. About 90% of the BAL cells were alveolar macrophages, as judged by Giemsa staining. Non-adherent cells were removed, and the adherent cells were >98% alveolar macrophages. These adherent cells were cultured in the presence or absence of 10  $\mu$ g/mL MAC.

**Lymphocyte proliferation.** PBMC ( $2 \times 10^5$ /well) were cultured in the presence or absence of heat-killed MAC or *M. tuberculosis* Erdman. Cells were incubated for 5 days at 37°C in an atmosphere containing 5% CO<sub>2</sub>, and 1  $\mu$ Ci [<sup>3</sup>H]thymidine was added for the final 18–20 h of culture, after which, radioactivity incorporation was measured in a beta counter (Wallac). Results are expressed as the mean counts per minute for triplicate wells.

**Measurement of cytokine concentrations.** Preliminary experi-

ments showed that MAC-stimulated cells yielded maximal concentrations of tumor necrosis factor (TNF)- $\alpha$ , interleukin (IL)-10, and IL-18 after a 1-day culture. Maximal concentrations of IL-4, IL-12, and interferon (IFN)- $\gamma$  were obtained after culture for 2, 2, and 4 days, respectively. Supernatants of cultured PBMC, CD14<sup>+</sup> monocytes, and alveolar macrophages were collected at optimal time points for measurement of each cytokine and were stored at -70°C until cytokine concentrations were measured by ELISA. Paired antibodies were used to detect TNF- $\alpha$ , IL-10, IFN- $\gamma$ , and IL-4 (PharMingen). ELISA kits were used to measure levels of IL-18 (MBL International) and IL-12 p70 (R&D Systems). The lower limits of detection for the ELISAs were 5 pg/mL for IL-12 and IFN- $\gamma$ , 8 pg/mL for IL-4, IL-10, and TNF- $\alpha$ , and 13 pg/mL for IL-18. Cytokine concentrations were minimal when cells were cultured in medium alone, and results are expressed as the cytokine concentration in supernatants from MAC-stimulated cells minus that in supernatants from unstimulated cells.

**Immunolabeling of intracellular cytokines.** Immunolabeling to detect intracellular TNF- $\alpha$ , IL-10, and IFN- $\gamma$  was done by using the Cytofix/Cytoperm Plus kit (PharMingen). Controls for each experiment included cells that were unstained, cells to which fluorescein isothiocyanate (FITC)-conjugated or phycoerythrin (PE)-conjugated mouse IgG had been added, and cells that had been subjected to single staining either with the antibody to the surface marker or to the cytokine being evaluated.

PBMC or macrophages were cultured with heat-killed MAC for 1 day to detect intracellular TNF- $\alpha$  and IL-10 and for 4 days to detect intracellular IFN- $\gamma$ . To induce intracellular accumulation of newly synthesized proteins, 0.7  $\mu$ L/mL Golgi Stop (PharMingen) was added for 6 h to the cells in culture. Cells were then harvested, and immunolabeling was performed. For double staining, anti-CD3 (FITC; Dako) or PE-cyanine 5-conjugated (PC5; Immuntotech), anti-CD4 (PE; Dako), anti-CD8 (PE; Dako), or anti-CD14 (PC5 or PE; Becton Dickinson) were first added. After being washed with PBS and 5% fetal calf serum (FCS), cells were fixed in Cytofix/Cytoperm and were washed twice in 1 $\times$  permeabilization/wash solution. FITC anti-TNF- $\alpha$ , PE anti-IL-10, or PE anti-IFN- $\gamma$  (all from PharMingen) were then added in staining buffer and 1 $\times$  permeabilization/wash solution. After incubation at 40°C for 40 min, cells were washed in PBS and 5% FCS and were analyzed by flow cytometry, using a fluorescence-activated cell sorter (EPICS C; Coulter).

**Statistical analysis.** Data are shown as the mean  $\pm$  SE. Differences between groups were compared by use of the unpaired Student's *t* test.

## Results

**Proliferative responses to MAC.** In preliminary experiments, we determined that *M. avium* strain 104 and *M. intracellulare* strain 1067 elicited similar proliferative responses by PBMC from MAS-positive control subjects and from MAC patients. We selected the *M. intracellulare* strain for use in subsequent experiments. To determine the capacity of T cells to recognize mycobacterial antigens, we measured proliferative responses of PBMC to killed MAC and to *M. tuberculosis*. The proliferative response of PBMC from 22 MAC patients was

about half that of PBMC from 19 MAS-positive control subjects ( $P = .07$ ; figure 1). The proliferative response of PBMC from MAC patients to *M. tuberculosis* was also about half that of PBMC from MAS-positive control subjects ( $P = .01$ ; figure 1). Because MAS-positive control subjects had negative tuberculin skin test results and were unlikely to be infected with *M. tuberculosis*, proliferation to *M. tuberculosis* probably resulted from the recognition of antigens common to both mycobacteria. The negative tuberculin test results suggest that a proliferative response by peripheral blood lymphocytes is not sufficient for delayed-type hypersensitivity, which may require other factors that permit lymphocytes to home to tissue sites where tuberculin is present. MAS-negative control subjects showed minimal proliferation to MAC (mean counts per minute,  $2389 \pm 706$ ), which suggests that proliferative responses reflected the response of T cells sensitized by prior exposure to MAC.

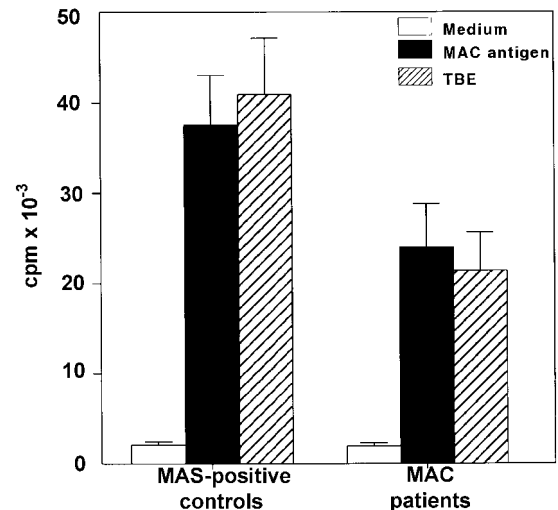
**Cytokine production by PBMC from patients and control subjects.** Because IFN- $\gamma$  contributes to host defenses against *M. tuberculosis* and *M. avium* in animals [8–10], we evaluated the capacity of PBMC to produce this cytokine after stimulation with MAC. PBMC from 19 MAC patients produced  $541 \pm 155$  pg/mL of IFN- $\gamma$ , whereas PBMC from 15 MAS-positive control subjects produced  $1644 \pm 305$  pg/mL ( $P = .003$ ; figure 2A). PBMC from MAS-negative control subjects produced only  $115 \pm 23$  pg/mL of IFN- $\gamma$ , which suggests that IFN- $\gamma$  secretion depended on T cells sensitized by prior infection with MAC. IL-4 was not detected in supernatants of PBMC from MAC patients or from MAS-positive control subjects.

Because studies in animals have shown that TNF- $\alpha$  is essential for host defense against *M. tuberculosis* and *M. avium* [11, 12], whereas IL-10 is immunosuppressive [13–15], we evaluated the capacity of PBMC to produce these cytokines in response to MAC. PBMC from MAC patients produced lower TNF- $\alpha$  concentrations than did those from MAS-positive control subjects ( $269 \pm 20$  vs.  $794 \pm 15$  pg/mL;  $P = .006$ ). In contrast, PBMC from MAC patients produced higher concentrations of IL-10 than did those from MAS-positive control subjects ( $1559 \pm 485$  vs.  $432 \pm 73$  pg/mL;  $P = .03$ ).

Production of IFN- $\gamma$ , TNF- $\alpha$ , and IL-10 by PBMC from MAC patients with cavitary disease and those with nodular bronchiectasis were not significantly different (data not shown).

IL-12 and IL-18 are produced by monocytes and contribute to production of IFN- $\gamma$  by T cells and NK cells [16]. To determine whether the reduced IFN- $\gamma$  production by MAC patients was due to reduced production of IL-12 and IL-18, we measured the capacity of PBMC to produce these cytokines (figure 2B). PBMC from 10 MAC patients produced lower concentrations of IL-12 than did PBMC from MAS-positive control subjects ( $8 \pm 5.4$  vs.  $55 \pm 18$  pg/mL;  $P = .03$ ). Concentrations of IL-18 were similar in patients and control subjects ( $29 \pm 4$  vs.  $49 \pm 14$  pg/mL;  $P = .20$ ).

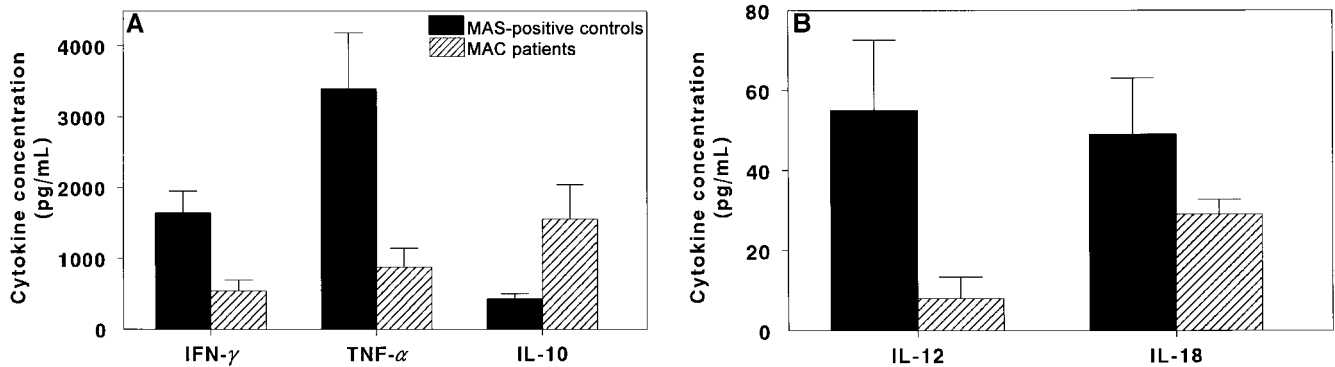
**T cell subpopulations producing IFN- $\gamma$ .** To identify the T cell



**Figure 1.** Proliferative responses of mycobacterium-stimulated peripheral blood mononuclear cells (PBMC). PBMC were isolated from 19 *Mycobacterium avium* sensitin-positive (MAS+) control subjects and from 22 *M. avium* complex (MAC) patients and were cultured in the presence of medium alone, 10  $\mu$ g/mL heat-killed MAC (MAC antigen), or 10  $\mu$ g/mL heat-killed *M. tuberculosis* Erdman (TBE). Proliferation was measured by radiolabeled thymidine incorporation. Mean values ( $\pm$ SE) are shown. cpm, counts per minute.

subpopulations that produce IFN- $\gamma$  in response to MAC, we cultured PBMC from 3 MAC patients and 4 MAS-positive control subjects with MAC for 4 days. Double immunolabeling was performed with anti-IFN- $\gamma$  in combination with anti-CD4 or anti-CD8. The percentages of CD4<sup>+</sup> and CD8<sup>+</sup> cells were similar in patients and control subjects (CD4<sup>+</sup> cells: 61%  $\pm$  3% in patients vs. 57%  $\pm$  5% in control subjects; CD8<sup>+</sup> cells: 28%  $\pm$  7% in patients vs. 34%  $\pm$  11% in control subjects). Both CD4<sup>+</sup> and CD8<sup>+</sup> cells expressed intracellular IFN- $\gamma$ . In 4 MAS-positive control subjects, 47%  $\pm$  16% of CD4<sup>+</sup> cells were positive for IFN- $\gamma$ , compared with 18%  $\pm$  3% in 3 MAC patients. Similarly, 39%  $\pm$  17% of CD8<sup>+</sup> cells were positive for IFN- $\gamma$  in the MAS-positive control subjects, compared with 17%  $\pm$  3% in the MAC patients. Although the percentages of CD4<sup>+</sup> and CD8<sup>+</sup> cells that were IFN- $\gamma$  positive were higher in MAS-positive control subjects, these differences were not statistically significant, probably because of the small number of subjects tested.

**Cellular sources of TNF- $\alpha$  and IL-10.** When PBMC are stimulated with *M. tuberculosis*, monocytes are the major source of TNF- $\alpha$  and IL-10 [17, 18]. However, some mycobacterial antigens can elicit production of these cytokines from T cells [19, 20]. To identify the source of these cytokines in PBMC stimulated with MAC, we combined surface immunolabeling with staining to detect intracellular cytokines in cells from 5 MAS-positive control subjects and 3 MAC patients. For each experiment, PBMC were divided into 2 aliquots. One aliquot was cultured with MAC for 24 h, and single and double immunolabeling were performed by using anti-CD3, anti-TNF-



**Figure 2.** Production of interferon (IFN)- $\gamma$ , tumor necrosis factor (TNF)- $\alpha$ , and interleukin (IL)-10 (A) and of IL-12 and IL-18 (B) by *Mycobacterium avium* complex (MAC)-stimulated peripheral blood mononuclear cells (PBMC). PBMC were isolated from *M. avium* sensitin-positive (MAS+) control subjects and from MAC patients and were cultured in the presence of 10  $\mu$ g/mL heat-killed MAC. Data are shown for results from 15 MAS-positive control subjects and 22 MAC patients (A) and for 8 MAS-positive control subjects and 10 MAC patients (B). Cytokine concentrations were measured by ELISA. Mean values ( $\pm$ SE) are shown.

$\alpha$ , and anti-IL-10. A second aliquot of PBMC was centrifuged over Percoll, and CD14<sup>+</sup> monocytes were isolated by positive selection with magnetic beads conjugated to anti-CD14. These monocytes, which were >90% CD14<sup>+</sup> by flow cytometry, were cultured with MAC for 24 h, and immunolabeling was performed as for PBMC, except that anti-CD14 was substituted for anti-CD3.

Single staining with anti-TNF- $\alpha$  revealed that the percentage of cells expressing intracellular cytokine was 10-fold higher in monocytes than in PBMC (44%  $\pm$  10% vs. 4.4%  $\pm$  1.3%;  $P = .006$ ). The percentage of cells expressing intracellular IL-10 was also higher in monocytes (23%  $\pm$  8% vs. 3.4%  $\pm$  1.4%;  $P = .04$ ).

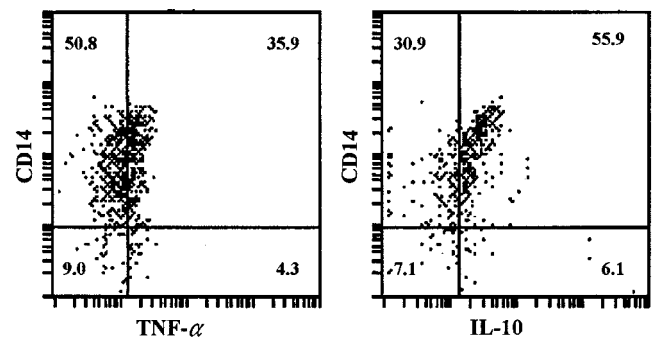
Double immunolabeling of PBMC revealed that 79%  $\pm$  6% of the cells were CD3<sup>+</sup> cells, 4.4%  $\pm$  1.5% were TNF- $\alpha$ -positive CD3<sup>+</sup> cells, and 2.8%  $\pm$  0.6% were IL-10-positive CD3<sup>+</sup> cells. In the monocyte preparations, 37%  $\pm$  12% of the cells were TNF- $\alpha$ -positive CD14<sup>+</sup> cells, and 5.3%  $\pm$  2.3% were TNF- $\alpha$ -positive CD14<sup>-</sup> cells. In addition, 27%  $\pm$  9% of the cells were IL-10-positive CD14<sup>+</sup> cells, and 7.0%  $\pm$  3.3% were IL-10-positive CD14<sup>-</sup> cells. Figure 3 shows a representative result. These findings confirm the results of single immunolabeling, demonstrating that higher percentages of monocytes than of T cells expressed intracellular TNF- $\alpha$  and IL-10. However, because the percentage of T cells in PBMC is higher than that of monocytes, both cell populations contribute significantly to production of these cytokines.

**Cytokine production by alveolar macrophages.** Because MAC causes lung disease in immunocompetent persons, we wished to determine whether the cytokines produced by blood monocytes were produced by alveolar macrophages. We obtained alveolar macrophages from 8 persons without mycobacterial disease and cultured them in the presence or absence of heat-killed MAC. Minimal amounts of TNF- $\alpha$  and IL-10 were produced in the absence of MAC. However, TNF- $\alpha$  concentrations were mark-

edly elevated (mean levels, 2698  $\pm$  364 pg/mL) in supernatants of MAC-stimulated macrophages from all patients (figure 4). IL-10 concentrations were also high (mean level, 2550  $\pm$  490 pg/mL) in supernatants of macrophages from 6 patients. These findings demonstrate that TNF- $\alpha$  and IL-10 are likely to be produced in the lungs of MAC patients.

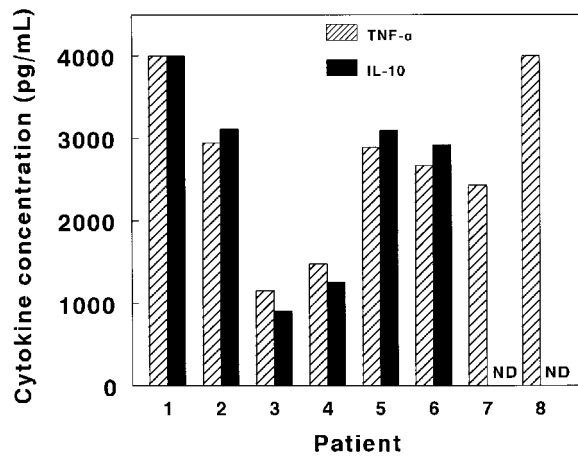
**Discussion**

MAC causes disseminated disease in patients with HIV infection and pulmonary disease in persons without obvious immunodeficiency. Although many studies have evaluated the immune response to MAC infection in animals, limited data are available for humans. *M. avium* sensitin is a valuable tool to evaluate prior infection with MAC in healthy individuals. Persons with skin test responses to this sensitin are likely, for sev-



**Figure 3.** Intracellular staining for tumor necrosis factor (TNF)- $\alpha$  and interleukin (IL)-10 in monocytes stimulated with *Mycobacterium avium* complex (MAC). Purified monocytes were isolated from an *M. avium* sensitin-positive control subject and were cultured with 10  $\mu$ g/mL heat-killed MAC for 24 h. Double immunolabeling was done with anti-CD14 in combination with anti-TNF- $\alpha$  and anti-IL-10. The percentages of positive cells in each quadrant are shown.





**Figure 4.** Production of tumor necrosis factor (TNF)- $\alpha$  and interleukin (IL)-10 by alveolar macrophages in response to *Mycobacterium avium* complex (MAC). Macrophages from bronchoalveolar lavage fluid of 8 patients without MAC disease were cultured in the presence of 10  $\mu$ g/mL heat-killed MAC. Cytokine concentrations were measured by ELISA. ND, not done.

eral reasons, to have been previously infected with MAC. First, patients with culture-proven MAC pulmonary disease show selective skin test reactivity to *M. avium* sensitin [7]. Second, PBMC from MAS-positive control subjects proliferated and produced IFN- $\gamma$  in response to MAC, whereas PBMC from MAS-negative control subjects did not, which suggests that T cells sensitized to MAC antigens were only present in MAS-positive control subjects. Third, skin test reactions to mycobacterial preparations, such as tuberculin and lepromin, are considered to be evidence of infection with *M. tuberculosis* and *M. leprae*, respectively.

The current study represents the first evaluation of cytokine production in immunocompetent persons with pulmonary MAC disease and in healthy control subjects with MAC infection. In MAC patients, mycobacterium-stimulated PBMC (compared with PBMC from MAS-positive control subjects) produced higher concentrations of IL-10 but lower concentrations of IFN- $\gamma$ , IL-12, and TNF- $\alpha$ . The percentages of CD4<sup>+</sup> and CD8<sup>+</sup> T cells producing IFN- $\gamma$  were reduced in MAC patients, and IL-10 was produced by both monocytes and T cells. Alveolar macrophages produced TNF- $\alpha$  and IL-10 in response to MAC, suggesting that these cytokines are produced in the lungs of patients with pulmonary disease from this pathogen. Our findings suggest that IFN- $\gamma$ , TNF- $\alpha$ , and IL-12 contribute to protection against MAC, whereas IL-10 is immunosuppressive.

In the immune response to mycobacterial infection, macrophages contribute to host defenses and to limiting the inflammatory response through secretion of proinflammatory cytokines, such as TNF- $\alpha$ , and anti-inflammatory cytokines, such as IL-10. TNF- $\alpha$  is essential for the development of protective immunity against tuberculosis and MAC in mice: animals with

disrupted genes for TNF- $\alpha$  or the 55-kDa TNF- $\alpha$  receptor show marked susceptibility to tuberculosis [11] and develop fatal MAC infection [12]. In addition, recombinant TNF- $\alpha$  reduces growth of MAC in mice [21]. Unlike TNF- $\alpha$ , IL-10 inhibits antimycobacterial defenses: mice lacking the IL-10 gene have lower bacterial burdens of *M. bovis* bacille Calmette-Guérin [13], and anti-IL-10 antibodies enhance resistance to MAC [14, 15].

In the immune response to *M. tuberculosis* in humans, TNF- $\alpha$  is a double-edged sword. TNF- $\alpha$  contributes to the capacity of alveolar macrophages to inhibit growth of *M. tuberculosis* [22], perhaps by inducing apoptosis [23]. However, TNF- $\alpha$  production by monocytes is higher in tuberculosis patients than in healthy control subjects [24, 25], and increased TNF- $\alpha$  production is associated with fever and cachexia in tuberculosis patients [24]. IL-10 production by PBMC in tuberculosis patients is variable—some investigators have reported increased production [26] and others have not [18, 27]. Our current findings in MAC infection differ significantly from those in human tuberculosis. We demonstrated predominant production of IL-10 over TNF- $\alpha$  by PBMC from MAC patients, a finding that is consistent with the protective role of TNF- $\alpha$  and the immunosuppressive role of IL-10 in murine models. Reduced TNF- $\alpha$  production in MAC patients, compared with tuberculosis patients, is consistent with the clinical manifestations of these infections, with MAC disease being more slowly progressive and having less prominent fever and weight loss.

IFN- $\gamma$  plays a pivotal role in immune defenses against mycobacteria, and IL-12 is a major stimulus for IFN- $\gamma$  production [28, 29]. Mice lacking IFN- $\gamma$  or IL-12 due to targeted gene deletions have increased susceptibility to *M. tuberculosis* [8, 9, 30] and to MAC [10, 31]. Administration of IL-12 confers protection to animals infected with MAC, and protection is dependent on production of IFN- $\gamma$  [10]. Most studies of the role of IFN- $\gamma$  and IL-12 in immune defenses against nontuberculous mycobacteria in humans involved kindreds with rare genetic susceptibility to severe extrapulmonary and disseminated mycobacterial disease. For example, genetic defects that cause reduced IL-12 production by monocytes or defective expression of receptors for IFN- $\gamma$  or IL-12 predispose to severe manifestations of mycobacterial disease [32–36]. In addition, administration of IFN- $\gamma$  ameliorated disease in small numbers of patients with multidrug-resistant tuberculosis [37] and disseminated and pulmonary MAC disease [38, 39].

Our current results confirm and extend these observations, demonstrating that IFN- $\gamma$  and IL-12 contribute to protection against MAC in persons without evident immunodeficiency. Production of IFN- $\gamma$  and IL-12 by PBMC from MAC patients was reduced by 70% and 85%, respectively, compared with findings in MAS-positive control subjects. The percentages of CD4<sup>+</sup> and CD8<sup>+</sup> cells in MAC-stimulated PBMC were similar in patients and control subjects, suggesting that reduced T cell numbers did not explain decreased IFN- $\gamma$  production. However, it

is possible that reduced numbers of NK cells in MAC patients may contribute to this phenomenon.

To account for reduced production of TNF- $\alpha$ , IL-12, and IFN- $\gamma$  by PBMC from MAC patients, we speculate that the primary abnormality in these individuals is excessive production of IL-10, which can exhibit multiple immunosuppressive effects. First, IL-10 inhibits the capacity of *M. avium*-infected monocytes to produce TNF- $\alpha$ , reducing apoptosis and enhancing mycobacterial survival [40, 41]. Second, IL-10 inhibits IL-12 production by monocytes exposed to mycobacteria [42, 43], and reduced IL-12 production leads to decreased IFN- $\gamma$  secretion. Third, IL-10 directly down-regulates expression of costimulatory molecules on mycobacterium-infected monocytes, inhibiting IFN- $\gamma$  production by T cells [44]. Enhanced IL-10 production in MAC patients parallels findings in patients with lepromatous leprosy and ineffective immunity to *M. leprae* [45, 46]. However, IL-4 production is also increased in lepromatous leprosy, but not in MAC patients. Transforming growth factor- $\beta$  reduces IFN- $\gamma$  production in tuberculosis patients [26], and further studies should evaluate the contribution of this cytokine to the abnormalities observed in MAC patients.

Mycobacterial antigens are potent stimuli for IL-10 production. CD14<sup>+</sup> monocytes are the major source of IL-10 produced by PBMC in response to *M. tuberculosis* and *M. leprae* [18, 46], and IL-10 mRNA is detected predominantly in CD14<sup>+</sup> CD3<sup>-</sup> cells in the pleural fluid of patients with tuberculous pleuritis [47]. However, mycobacterium-reactive T cells from patients with tuberculosis or leprosy, when cultured in vitro, can also produce IL-10 [20, 48, 49]. We found that MAC elicited IL-10 production by both monocytes and T cells. Although the percentage of IL-10-containing cells was 10-fold higher in monocytes than in T cells, the number of T cells in PBMC is 5–10-fold higher than that of monocytes, so that both cell populations are likely to contribute significantly to IL-10 production. Our findings differ from those of Müller et al. [50], who reported that monocytes were the major source of IL-10 when PBMC from healthy control subjects and HIV-infected patients were stimulated with MAC. These subjects were not tested for MAC infection, and only 1 HIV-infected patient had MAC disease. In immunocompetent MAC patients and MAS-positive control subjects, sensitized T cells may contribute significantly to IL-10 production. In contrast, immunocompetent persons who are not infected with MAC and HIV-infected persons with defective T cell function may lack sensitized T cells that produce IL-10.

In summary, we found that, in MAC patients, mycobacterium-stimulated PBMC produced more IL-10 but less IFN- $\gamma$ , IL-12, and TNF- $\alpha$  than did PBMC from MAS-positive control subjects. This pattern of cytokine production differs from those previously described for patients with other mycobacterial infections, such as tuberculosis and leprosy. We speculate that excessive IL-10 production inhibits secretion of TNF- $\alpha$ , IL-12, and IFN- $\gamma$ , increasing susceptibility to development of pulmonary disease from MAC. Understanding the mechanisms

responsible for increased IL-10 production may facilitate the development of immunomodulatory strategies to treat and prevent disease due to MAC.

#### Acknowledgments

We thank Luiz Bermudez (Kuzell Institute, San Francisco) for providing *Mycobacterium avium* strain 104, Kaare Haslov (Statens Serum Institute, Copenhagen) for providing the *M. avium* sensitin, Patrick Brennan for providing *M. tuberculosis* Erdman, and John Belisle (Colorado State University, Fort Collins) for preparing heat-killed *M. intracellulare* strain 1067. We also thank the patients with *M. avium* complex disease who volunteered to participate in the study.

#### References

- Wallace RJ Jr, Zhang Y, Brown BA, et al. Polyclonal *Mycobacterium avium* complex infections in patients with nodular bronchiectasis. *Am J Respir Crit Care Med* **1998**;158:1235–44.
- Prince DS, Peterson DD, Steiner RM, et al. Infection with *Mycobacterium avium* complex in patients without predisposing conditions. *N Engl J Med* **1989**;321:863–8.
- French AL, Benator DA, Gordin FM. Nontuberculous mycobacterial infections. *Med Clin North Am* **1997**;81:361–79.
- Horsburgh CR Jr. *Mycobacterium avium* complex infection in the acquired immunodeficiency syndrome. *N Engl J Med* **1991**;324:1332–7.
- Wallace RJ Jr, Glassroth J, Griffith DE, Olivier KN, Cook JL, Gordin F. Diagnosis and treatment of disease caused by nontuberculous mycobacteria. *Am J Respir Crit Care Med* **1997**;156 (Suppl 2):S1–S25.
- von Reyn CF, Barber TW, Arbeit RD, et al. Evidence of previous infection with *Mycobacterium avium*-*Mycobacterium intracellulare* complex among healthy subjects: an international study of dominant mycobacterial skin test reactions. *J Infect Dis* **1993**;168:1553–8.
- von Reyn CF, Williams DE, Horsburgh CR Jr, et al. Dual skin testing with *Mycobacterium avium* sensitin and purified protein derivative to discriminate pulmonary disease due to *M. avium* complex from pulmonary disease due to *Mycobacterium tuberculosis*. *J Infect Dis* **1998**;177:730–6.
- Flynn JL, Chan J, Triebold KJ, Dalton DK, Stewart TA, Bloom BR. An essential role for interferon-gamma in resistance to *Mycobacterium tuberculosis* infection. *J Exp Med* **1993**;178:2249–54.
- Cooper AM, Dalton DK, Stewart TA, Griffin JP, Russell DG, Orme IM. Disseminated tuberculosis in interferon-gamma gene-disrupted mice. *J Exp Med* **1993**;178:2243–7.
- Silva RA, Pais TF, Appelberg R. Evaluation of IL-12 in immunotherapy and vaccine design in experimental *Mycobacterium avium* infections. *J Immunol* **1998**;161:5578–85.
- Flynn JL, Goldstein MM, Chan J, et al. Tumor necrosis factor- $\alpha$  is required in the protective immune response against *Mycobacterium tuberculosis* in mice. *Immunity* **1995**;2:561–72.
- Ehlers S, Benini J, Kutsch S, Endres R, Rietschel ET, Pfeffer K. Fatal granuloma necrosis without exacerbated mycobacterial growth in tumor necrosis factor receptor p55 gene-deficient mice intravenously infected with *Mycobacterium avium*. *Infect Immun* **1999**;67:3571–9.
- Murray PJ, Young RA. Increased antimycobacterial immunity in interleukin-10-deficient mice. *Infect Immun* **1999**;67:3087–95.
- Bermudez LE, Champi J. Infection with *Mycobacterium avium* induces production of interleukin-10 (IL-10), and administration of anti-IL-10 antibody is associated with enhanced resistance to infection in mice. *Infect Immun* **1993**;61:3093–7.
- Denis M, Ghadirian E. IL-10 neutralization augments mouse resistance to systemic *Mycobacterium avium* infections. *J Immunol* **1993**;151:5425–30.
- Okamura H, Tsutsui H, Kashiwamura S, Yoshimoto T, Nakanishi K. Inter-

- leukin-18: a novel cytokine that augments both innate and acquired immunity. *Adv Immunol* **1998**;70:281–312.
17. Barnes PF, Chatterjee D, Abrams JS, et al. Cytokine production induced by *Mycobacterium tuberculosis* lipoarabinomannan: relationship to chemical structure. *J Immunol* **1992**;149:541–7.
  18. Lin Y, Zhang M, Hofman FM, Gong J, Barnes PF. Absence of a prominent Th2 cytokine response in human tuberculosis. *Infect Immun* **1996**;64:1351–6.
  19. Aung H, Toosi Z, Wisniewski JJ, et al. Induction of monocyte expression of tumor necrosis factor alpha by the 30-kDa antigen of *Mycobacterium tuberculosis* and synergism with fibronectin. *J Clin Invest* **1996**;98:1261–8.
  20. Barnes PF, Abrams JS, Lu S, Sieling PA, Rea TH, Modlin RL. Patterns of cytokine production by mycobacterium-reactive human T cell clones. *Infect Immun* **1993**;61:197–203.
  21. Bermudez LE, Stevens P, Kolonoski P, Wu M, Young LS. Treatment of experimental disseminated *Mycobacterium avium* complex infection in mice with recombinant IL-2 and tumor necrosis factor. *J Immunol* **1989**;143:2996–3000.
  22. Hirsch CS, Ellner JJ, Russell DG, Rich EA. Complement receptor-mediated uptake and tumor necrosis factor-alpha-mediated growth inhibition of *Mycobacterium tuberculosis* by human alveolar macrophages. *J Immunol* **1994**;152:743–53.
  23. Balcewicz-Sablinska MK, Keane J, Kornfeld H, Remold HG. Pathogenic *Mycobacterium tuberculosis* evades apoptosis of host macrophages by release of TNF-R2, resulting in inactivation of TNF-alpha. *J Immunol* **1998**;161:2636–41.
  24. Cadranet J, Philippe C, Perez J, et al. In vitro production of tumour necrosis factor and prostaglandin E<sub>2</sub> by peripheral blood mononuclear cells from tuberculosis patients. *Clin Exp Immunol* **1990**;81:319–24.
  25. Ogawa T, Uchida H, Kusumoto Y, Mori Y, Yamamura Y, Hamada S. Increase in tumor necrosis factor alpha- and interleukin-6-secreting cells in peripheral blood mononuclear cells from subjects infected with *Mycobacterium tuberculosis*. *Infect Immun* **1991**;59:3021–5.
  26. Hirsch CS, Toossi Z, Othieno C, et al. Depressed T cell interferon-gamma responses in pulmonary tuberculosis: analysis of underlying mechanisms and modulation with therapy. *J Infect Dis* **1999**;180:2069–73.
  27. McDyer JF, Hackley MN, Walsh TE, Cook JL, Seder RA. Patients with multidrug-resistant tuberculosis with low CD4<sup>+</sup> T cell counts have impaired Th1 responses. *J Immunol* **1997**;158:492–500.
  28. Scott P. IL-12: initiation cytokine for cell-mediated immunity. *Science* **1993**;260:496–7.
  29. Trinchieri G. Interleukin-12 and its role in the generation of Th1 cells. *Immunol Today* **1993**;14:335–7.
  30. Cooper AM, Magram J, Ferrante J, Orme IM. Interleukin 12 is crucial to the development of protective immunity in mice intravenously infected with *Mycobacterium tuberculosis*. *J Exp Med* **1997**;186:39–45.
  31. Doherty TM, Sher A. IL-12 promotes drug-induced clearance of *Mycobacterium avium* infection in mice. *J Immunol* **1998**;160:5428–35.
  32. Frutch DM, Holland SM. Defective monocyte costimulation for IFN-gamma production in familial disseminated *Mycobacterium avium* complex infection: abnormal IL-12 regulation. *J Immunol* **1996**;157:411–6.
  33. Jouanguy E, Lamhamedi-Cherradi S, Lammas D, et al. A human IFNGR1 small deletion hotspot associated with dominant susceptibility to mycobacterial infection. *Nat Genet* **1999**;21:370–8.
  34. Altare F, Durandy A, Lammas D, et al. Impairment of mycobacterial immunity in human interleukin-12 receptor deficiency. *Science* **1998**;280:1432–5.
  35. De Jong R, Altare F, Haagen IA, et al. Severe mycobacterial and *Salmonella* infections in interleukin-12 receptor-deficient patients. *Science* **1998**;280:1435–8.
  36. Newport MJ, Huxley CM, Huston S, et al. A mutation in the interferon-gamma-receptor gene and susceptibility to mycobacterial infection. *N Engl J Med* **1996**;335:1941–9.
  37. Condos R, Rom WN, Schluger NW. Treatment of multidrug-resistant pulmonary tuberculosis with interferon-gamma via aerosol. *Lancet* **1997**;349:1513–5.
  38. Holland SM, Eisenstein EM, Kuhns DB, et al. Treatment of refractory disseminated nontuberculous mycobacterial infection with interferon gamma. *N Engl J Med* **1994**;330:1348–55.
  39. Chatte G, Panteix G, Perrin-Fayolle M, Pacheco Y. Aerosolized interferon gamma for *Mycobacterium avium* complex lung disease. *Am J Respir Crit Care Med* **1995**;152:1094–6.
  40. Fratazzi C, Arbeit RD, Carini C, Remold HG. Programmed cell death of *Mycobacterium avium* serovar 4-infected human macrophages prevents the mycobacteria from spreading and induces mycobacterial growth inhibition by freshly added, uninfected macrophages. *J Immunol* **1997**;158:4320–7.
  41. Balcewicz-Sablinska MK, Gan H, Remold HG. Interleukin 10 produced by macrophages inoculated with *Mycobacterium avium* attenuates mycobacteria-induced apoptosis by reduction of tumor necrosis factor-alpha activity. *J Infect Dis* **1999**;180:1230–7.
  42. Sieling PA, Wang XH, Gately MK, et al. IL-12 regulates T helper type 1 cytokine responses in human infectious disease. *J Immunol* **1994**;153:3639–47.
  43. Gong J, Zhang M, Modlin RL, et al. Interleukin-10 downregulates *Mycobacterium tuberculosis*-induced Th1 responses and CTLA-4 expression. *Infect Immun* **1996**;64:913–8.
  44. Rojas RE, Balaji KN, Subramanian A, Boom WH. Regulation of human CD4<sup>+</sup>αβ T-cell-receptor-positive (TCR+) and gamma delta TCR+ T-cell responses to *Mycobacterium tuberculosis* by interleukin-10 and transforming growth factor beta. *Infect Immun* **1999**;67:6461–72.
  45. Yamamura M, Uyemura K, Deans RJ, et al. Defining protective responses to pathogens: cytokine profiles in leprosy lesions. *Science* **1991**;254:277–9.
  46. Sieling PA, Abrams JS, Yamamura M, et al. Immunosuppressive roles for interleukin-10 and interleukin-4 in human infection: in vitro modulation of T cell responses in leprosy. *J Immunol* **1993**;150:5501–10.
  47. Barnes PF, Lu S, Abrams JS, Wang E, Yamamura M, Modlin RL. Cytokine production at the site of disease in human tuberculosis. *Infect Immun* **1993**;61:3482–9.
  48. Gerosa F, Nisii C, Righetti S, et al. CD4<sup>+</sup> T cell clones producing both interferon-gamma and interleukin-10 predominate in bronchoalveolar lavages of active pulmonary tuberculosis patients. *Clin Immunol* **1999**;92:224–34.
  49. Verhagen CE, van der Pouw Kraan TCTM, Buffing AAM, et al. Type 1- and type 2-like lesional skin-derived *Mycobacterium leprae*-responsive T cell clones are characterized by coexpression of IFN-gamma/TNF-alpha and IL-4/IL-5/IL-13, respectively. *J Immunol* **1998**;160:2380–7.
  50. Müller F, Aukrust P, Lien E, Haug CJ, Froland SS. Enhanced interleukin-10 production in response to *Mycobacterium avium* products in mononuclear cells from patients with human immunodeficiency virus infection. *J Infect Dis* **1998**;177:586–94.