Enhanced Interleukin-10 Production in Response to Mycobacterium avium Products in Mononuclear Cells from Patients with Human Immunodeficiency Virus Infection

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Patients with advanced human immunodeficiency virus (HIV) infection are susceptible to infections with Mycobacterium avium complex (MAC). Interleukin (IL)-10 may impair immunity to MAC; therefore, the effect of different MAC preparations on IL-10 production was examined in mononuclear cell cultures from HIV-infected patients. IL-10 levels in cultures for 26 patients were higher than those in 20 control cultures. The highest IL-10 levels were found in cultures from patients with the most advanced HIV disease. Monocytes were the major IL-10 producers, while little IL-10 could be attributed to Th2 lymphocytes. Cultures for patients produced reduced levels of tumor necrosis factor-α and normal levels of IL-12; the production of these cytokines increased after neutralization of IL-10. Circulating IL-10 was higher in HIV-infected patients than in controls, with the highest levels in the AIDS group. Elevated monocyte/macrophage-derived IL-10 production may contribute to the high susceptibility to MAC infection seen in patients with advanced HIV disease.

Cytokine dysregulation is central to the immunopathogenesis of human immunodeficiency virus (HIV) infection, both in relation to regulation of HIV replication and in the development of immunodeficiency [1, 2]. Interleukin (IL)-10 may play an important role in this cytokine dysregulation by its ability to down-regulate cytokine synthesis in monocytes, macrophages, and TH1 lymphocytes [3, 4]. However, present data conflict regarding the level of IL-10 production in peripheral blood mononuclear cells (PBMC) from patients with HIV infection, and the contribution of IL-10 to HIV immunopathogenesis is still unclear [5–10]. A possible overproduction of IL-10 in HIV patients with down-regulation of TH1 responses and inhibition of various macrophage functions might contribute to the increased susceptibility to infections with various intracellular microbes. Mycobacterium avium complex (MAC) is one of the most important intracellular pathogens in patients with advanced HIV infection, and it is associated with high morbidity and mortality [11].

To elucidate the possible role of IL-10 in HIV infection, IL-10 levels in serum and IL-10 production in PBMC from patients with different clinical stages of HIV infection and from controls were quantified in response to 2 different MAC preparations and compared with IL-10 production after stimulation with a nonspecific mitogen. Since IL-10 is produced by a number of cell types, including monocytes, macrophages, and various lymphocyte populations [3, 10, 12, 13], we also assessed the cellular sources of IL-10 in PBMC culture after stimulation with MAC products. Finally, the PBMC response to modulation with IL-10 in terms of production of IL-12 p40 and tumor necrosis factor (TNF)-α was assessed in cultures of samples from patients and controls after stimulation with different MAC preparations.

Materials and Methods

Patients. Blood samples were obtained from 26 HIV-infected patients followed at our department. Patients were classified clinically according to the revised criteria from Centers for Disease Control and Prevention [14], as shown in table 1. At the time of blood sampling, 4 patients had ongoing opportunistic infections: Candida esophagitis (n = 1), MAC (n = 1), and cytomegalovirus (n = 1). One patient probably had visceral leishmaniasis (diagnosed 6 months later). In addition, 1 patient developed manifestations of cryptococcal meningitis 3 months before blood sampling. Fourteen patients were treated with zidovudine at the time of blood sampling; none of the patients received protease inhibitor treatment. Controls were 20 healthy volunteer blood donors, who were HIV seronegative.

Mycobacterial preparations. A mycobacterial isolate was obtained from a cultured blood sample from an AIDS patient, who was attending our department. It was identified as M. avium by
DNA-ribosomal RNA hybridization with a M. avium–specific probe (AccuProbe; Gen-Probe, San Diego) and was cultured in Middlebrook 7H9 broth enriched with ADC (albumin, dextrose, catalase; Difco, Detroit) for 21 days. Preparation of mycobacterial sonicate was accomplished as described by Closs et al. [15]. The protein concentration of the sonicate was 0.12 mg/mL as determined by Lowry’s method [16]. The sonicate designated MAC-SON, was aliquoted and stored at −80°C.

Purified protein derivative from M. avium (MAC-PPD) was a gift from Reidar Bjørlo (National Veterinary Institute, Oslo).

Cell culture. PBMC were obtained from heparinized blood by use of isopaque-ficoll (Lymphoprep; Nycomed Pharma, Oslo) gradient centrifugation within 45 min after blood sampling, as previously described [17]. PBMC were resuspended in RPMI 1640 with 2 mM L-glutamine and 25 mM HEPES buffer (RPMI; Gibco BRL, Paisley, UK) supplemented with gentamicin (40 mg/L) and 10% heat-inactivated pooled human AB serum (culture medium) at a concentration of 2 × 10^6 cells/mL. The fraction of CD14 monocytes in the isolated PBMC was determined by immunomagnetic quantification [18].

PBMC were stimulated with phytohemagglutinin (PHA; Murex Diagnostics, Dartford, UK; final concentration, 1:100), MAC-SON (final concentration, 1:100), or MAC-PPD (final concentration, 1:100), or they were left unstimulated. Cells were cultured (0.2 × 10^6 cells/well) in flat-bottomed 96-well microtiter plates (Costar, Cambridge, MA). In additional wells, in which cells were stimulated with MAC-SON or MAC-PPD, cultures were simultaneously treated with human recombinant IL-10 (specific activity 2.01 × 10^7 U/mg, 100 U/mL) or neutralizing monoclonal anti-human IL-10 (clone 12G8, 5 mg/mL; both reagents provided by S. Narula, Schering-Plough Research Institute, Kenilworth, NJ). Patients’ cells were always cultured in the same microtiter plates as control blood donor cells.

In all preparations of culture medium, the endotoxin level was <10 pg/mL as determined by a quantitative chromogenic limulus amebocyte lysate test (BioWhittaker, Walkersville, MD).

Cultures were harvested at 24 h because the IL-10 level in the stimulated culture supernatants reached a plateau after this point. Plates were centrifuged at 500 g for 10 min, and the supernatants were collected, aliquoted, and stored at −80°C until cytokine analyses.

Separation of T lymphocyte subsets and monocytes. For studies of cytokine production in CD4 and CD8 lymphocytes and monocytes, PBMC were divided into 2 aliquots. One aliquot was depleted for CD14 monocytes, and then CD4 and CD8 lymphocytes were positively selected with antibody-coated immunomagnetic beads (Dynal, Oslo) and anti-mouse Fab antiserum (Detecta-bead CD4/CD8; Dynal) as previously described [19]. The purity of the positively selected CD4 and CD8 lymphocyte subpopulations was 90%–95% as determined by flow cytometry.

The other aliquot (3 × 10^6 PBMC/mL in culture medium), was seeded in 24-well plates (Costar; 1 mL/well), incubated for 1 h at 37°C, washed three times in PBS, and incubated for 10 min on ice. Adherent monocytes were scraped off the surface with a rubber policeman, washed, counted, and resuspended in culture medium. CD14 expression was >80% as determined by flow cytometry.
Cells (10⁶ cells/mL; 0.1 mL/well) were seeded in duplicates as follows: purified CD4 lymphocytes, 90% CD4 lymphocytes plus 10% monocytes, purified CD8 lymphocytes, 90% CD8 lymphocytes plus 10% monocytes, or only monocytes. Addition of monocytes to the lymphocyte cultures was done to accomplish presentation of antigens in the MAC-SON preparation. Cells were stimulated with PHA or MAC-SON and cultured for 24 h, and supernatants were then harvested and stored as described above.

Quantification of IL-10–producing cells by intracellular cytokine staining. PBMC (2 × 10⁶ cells/mL) were cultured in 24-well plates (Costar; 1 mL/well) for 24 h with PHA or MAC-SON in the presence of 2 mM monensin (Sigma, St. Louis) in order to block intracellular transport processes causing cytokine accumulation in the Golgi complex [20]. The plates were incubated for 10 min on ice, and the adherent cells were scraped off the surface with a rubber policeman. Cells were washed and resuspended in PBS with 5% mouse serum (Sigma), 5% human immunoglobulin (Octagam; Octapharma, Vienna), 2% bovine serum albumin (Calbiochem, La Jolla, CA), and 0.1% sodium azide. The cells were then stained (30 min at 4°C) for membrane antigens by fluorescein isothiocyanate (FITC)–conjugated antibodies from Becton Dickinson (San Jose, CA): CD4, clone SK 3; CD8, clone SK 1; CD14, clone Leu-M3. The cells were washed in staining buffer (PBS with 1% fetal bovine serum; Gibco BRL), fixed in 4% (wt/vol) paraformaldehyde (Sigma) in PBS (pH 7.4) for 20 min at 4°C, washed in staining buffer, and stained with phycoerythrin–conjugated anti–IL-10 (PharMingen, San Diego; 0.25 μg/sample) in PBS with 1% heat-inactivated fetal bovine serum (Gibco BRL) and 0.1% (wt/vol) saponin (Sigma; a permeabilization buffer) for 30 min at 4°C.

Last, cells were washed once in permeabilization buffer, resuspended in staining buffer, and analyzed using FACScan with CellQuest software (both from Becton Dickinson). Samples included staining with FITC-conjugated, isotype-matched control antibodies and phycoerythrin-conjugated anti–IL-10 (0.25 μg/sample) that had been preincubated with an excess of recombinant IL-10 (1 μg/sample; Schering-Plough Research Institute). List mode files were collected for 25,000 cells from each sample. Calibration and adjustment of compensation were done with FacsComp software and Calibrite fluorescence beads (both from Becton Dickinson).

Quantification of IL-10, TNF-α, and IL-12 p40. Serum was collected as described elsewhere [21], and circulating IL-10 was quantified by a high-sensitivity ELISA (R&D Systems, Minneapolis). Cytokines in supernatants were quantitated in 96-well microplates (Nunc Maxisorp, Roskilde, Denmark). For the IL-10 ELISA (reagents provided by S. Narula, Schering-Plough Research Institute), wells were coated with monoclonal rat anti-human IL-10 (clone JES3-9D7, 1 μg/mL). The reference standard was recombinant IL-10 (10–625 pg/mL). Subsequent steps included polyclonal rabbit anti-human IL-10 (1:2500), alkaline phosphatase–conjugated donkey anti-rabbit IgG (Jackson ImmunoResearch, West Grove, PA; 1:5000), and p-nitrophenyl disodium phosphate (Sigma; 1 mg/mL in diethanolamine buffer, pH 9.8) for color development.

For the TNF-α ELISA, wells were coated with monoclonal mouse anti-human TNF-α (PharMingen; clone MAb1; 2 μg/mL.

![Figure 2](image-url)
in 0.1 M NaHCO₃, pH 8.2). The reference standard was recombinant TNF-α (PharMingen; 62–4000 pg/mL). Subsequent steps included biotinylated monoclonal mouse anti-human TNF-α (PharMingen; clone Mab11; 4 μg/mL), alkaline phosphatase–conjugated streptavidin (PharMingen; 1:4000), and p-nitrophenyl disodium phosphate as substrate.

IL-12 p40 was quantified by ELISA (R&D Systems). The limit of detection was 0.7 pg/mL for the high-sensitivity IL-10 assay, 5 pg/mL for the IL-10 assay, 15 pg/mL for the IL-12 p40 assay, and 100 pg/mL for the TNF-α assays. Intra- or interassay coefficients of variation were <10% for all assays.

Quantification of lymphocyte subsets in peripheral blood. The numbers of CD4 and CD8 lymphocytes in peripheral blood were determined by immunomagnetic quantification [22].

Statistical methods. Wilcoxon rank sum test was used to compare data for 2 groups of subjects. When >2 groups were compared, the Kruskal-Wallis test was used. If a significant difference was found, Fisher’s least significant difference was computed on the ranks to determine differences between each pair of groups. Responses within the same individuals were compared by the Wilcoxon signed rank test for paired data. Coefficients of correlation (R) were calculated by use of Spearman’s rank test. Data are given as medians and 25th–75th percentiles if not otherwise stated. P values are two sided; P < .05 was considered significant.

Results

IL-10 in Serum

Both asymptomatic HIV-infected patients and AIDS patients had significantly higher levels of IL-10 than did controls (figure 1). Of the 3 AIDS patients with the highest IL-10 levels, 1 probably had visceral leishmaniasis at the time of blood sampling (diagnosed 6 months later; IL-10: 20.3 pg/mL), 1 had an ongoing MAC infection (IL-10: 17.1 pg/mL), and 1 was diagnosed with cryptococcus meningitis 3 months before blood sampling and was receiving maintenance treatment (IL-10: 6.8 pg/mL).

IL-10 Production in PBMC

MAC-PPD and MAC-SON induced significantly higher levels of IL-10 in cell cultures for HIV patients than in cultures for controls: MAC-PPD induced 99 pg/mL (0–218) for HIV patients versus 0 pg/mL (0–0) for controls (P < .001); MAC-SON induced 214 pg/mL (102–386) for HIV patients versus 56 pg/mL (0–118) for controls (P < .001) (figure 2). MAC-PPD–stimulated IL-10 production was significantly higher in symptomatic than in asymptomatic patients (figure 2). A similar trend was seen for MAC-SON (figure 2). No significant correlation was found between IL-10 levels after stimulation with MAC products and CD4 lymphocyte counts in blood (data not shown). In the cultures, similar monocyte proportions were found in cultures from patients (15% [10%–18%]) and controls (12% [8%–19%]).

In contrast, PBMC from HIV-infected patients produced significantly less IL-10 in response to PHA than did cells from blood donor controls (median level, ~48% of controls), as shown in figure 2. Furthermore, there was a significant correlation between PHA-induced IL-10 production and the CD4 lymph-
phocyte count in peripheral blood ($R = .48; P = .01$). Similar levels of stimulated and unstimulated IL-10 production were found in cultures from patients with and without opportunistic infections (data not shown).

**Cellular Sources of IL-10**

**IL-10 production by T lymphocyte subsets and monocytes.** MAC-SON stimulation of purified and separately cultured monocytes and lymphocyte subpopulations from patients and controls revealed that IL-10 was produced only by monocytes and not by CD4 or CD8 lymphocytes (figure 3). In contrast, both CD4 lymphocytes (in the presence of 10% monocytes) and monocytes contributed to the IL-10 production in response to PHA, while no IL-10 production was detected in the CD8 lymphocytes (in the presence of 10% monocytes; figure 3) or in purified CD4 or CD8 lymphocyte cultures (data not shown). Similar patterns were found in cells from both patients and controls, although the monocyte IL-10 production in the patient cultures tended to be higher than that in the controls after MAC-SON stimulation and lower after PHA stimulation.

**Quantification of IL-10 producing cells by intracellular cytokine staining.** To further study the cellular sources of IL-10 in response to various stimuli, we examined the intracellular IL-10 production in PBMC by flow cytometry. In MAC-SON-stimulated cultures, monocytes were the major IL-10-producing cells, and the percentage of monocytes producing IL-10 tended to be higher in patient cultures than in control cultures, as indicated in figure 4. In PHA-stimulated cells, substantial proportions of both monocytes and CD4 and CD8 lymphocytes from both patients and controls produced IL-10 (figure 4).

**Effect of IL-10 Modulation on TNF-α and IL-12 p40 Production in PBMC Cultures after Stimulation with MAC Preparations**

**TNF-α.** MAC-PPD stimulation led to a significantly lower level of TNF-α in cultures for the 26 HIV patients (1162 pg/mL; 360–2947) than in cultures for 19 of the controls (3040 pg/mL; 1180–4152) ($P < .02$). A similar trend was found for MAC-SON (1860 pg/mL [1330–2818] for patients vs. 2430 pg/mL [1210–4661] for controls; $P = .11$). When cultures were stimulated with MAC products in the presence of recombinant IL-10, a significant reduction in TNF-α production in patient (MAC-SON stimulation only) and control cultures was found (figure 5A). Conversely, in cultures for HIV patients stimulated by MAC products and treated with neutralizing anti–IL-10, TNF-α production was significantly increased up to levels found in cultures for the controls (figure 5A). Also, TNF-α production in control cultures was increased after neutralization with anti–IL-10, as shown in figure 5A.

**IL-12 p40.** After stimulation with MAC products, no significant differences in IL-12 p40 levels were found between cultures for HIV patients and controls: MAC-PPD stimulated 124 pg/mL (0–270) in 23 of the patient cultures versus 79 pg/mL (0–259) in 20 control cultures; MAC-SON stimulated 228 pg/mL (42–361) in 23 patient cultures versus 165 pg/mL (102–373) in 20 control cultures. When cultures were stimulated with MAC products in the presence of recombinant IL-10, IL-12 p40 production was significantly reduced to undetectable levels in cultures for both patients and controls (figure 5B). Addition of neutralizing anti–IL-10 had the opposite effect, leading to significantly higher levels of IL-12 p40 cultures for both patients and controls (figure 5B).

**Discussion**

In the present study, we focused on the role of IL-10 in HIV infection. Patients with HIV had significantly higher levels of circulating IL-10 than healthy HIV-seronegative blood donors, with the highest levels occurring in the AIDS group. This is in accordance with data from Ameglio et al. [23], whereas Chehimi et al. [24] did not find any differences between IL-10 levels in HIV patients and controls. Of interest, the 3 patients in our study with the highest serum IL-10 levels (~2- to 6-fold higher than the median level) had ongoing or recent infections with intracellular pathogens. This raises the possibility that increased serum IL-10 levels in HIV infection to some extent may be due to opportunistic infections rather than to HIV-related immunodeficiency per se. If so, differences in patient populations with regard to opportunistic infections may partly explain the conflicting serum IL-10 data found in various studies.

Furthermore, our data show that PBMC from patients with HIV infection, compared with controls, respond to MAC preparations with markedly enhanced IL-10 production. The highest IL-10 levels were found in patients with symptomatic disease, but IL-10 production was similar in patients with or without recent or ongoing opportunistic infections.

As shown by two different methods, monocytes are the predominant source of IL-10 after stimulation with MAC-SON, both in cultures of samples from HIV patients and in control cultures. Several mechanisms may be operative in the induction of monocyte IL-10 production by MAC preparations. First, monocytes and macrophages express several receptors that recognize products from various microorganisms, including mycobacteria [25–27]. Second, lymphocyte activation induced by MAC preparations may affect the level of monocyte IL-10 production via the secretion of various other cytokines [28–31]. Third, IL-10 may be induced in vivo by other monocyte-derived factors that are elevated in HIV infection, such as TNF-α and prostaglandin E2 [32, 33]. Others have shown that in vivo and in vitro, HIV-infected monocytes are important contributors to IL-10 production, spontaneously and after stimulation with HIV or gp120 [10, 13, 24, 34–36]. Also, products from Mycobacterium tuberculosis [37] and Mycobacterium leprae [38] induce IL-10
Figure 4. Quantification of IL-10 producing cells by flow cytometry. Mononuclear cells from blood donor control (A) or HIV-infected patient (B) were cultured for 24 h with phytohemagglutinin (PHA) or sonicate from _M. avium_ complex (MAC-SON) in presence of monensin in order to block intracellular transport processes causing cytokine accumulation in Golgi complex. Cells were stained with monoclonal anti–IL-10–phycoerythrin (PE) conjugate and antibody–fluorescein isothiocyanate conjugates to CD4, CD8, or CD14. Bold lines indicate specific IL-10 staining; thin lines indicate staining with monoclonal anti–IL-10 conjugate after preincubation with excess of recombinant IL-10 (background). Nos. indicate % of cells that stained positively for intracellular IL-10. One representative experiment of 4 is shown.
Figure 5. Effect of modulation with recombinant IL-10 (100 U/mL) or neutralizing anti–IL-10 (5 μg/mL) on production of tumor necrosis factor (TNF-α) (A) and IL-12 p40 (B) in cultures of mononuclear cells from blood donor controls and HIV-infected patients in response to MAC-PPD (purified protein derivative from M. avium complex) or MAC-SON (sonicate from MAC). Cells were cultured for 24 h and tumor necrosis factor-α and IL-12 p40 were quantified by ELISA. Data are given as medians and 25th–75th percentiles. Open bars = no IL-10 modulation; hatched bars = presence of IL-10; cross-hatched bars = presence of anti–IL-10. Cytokine production without vs. with IL-10 or anti–IL-10 modulation were compared by Wilcoxon signed rank test for paired data. P < * .05, and † .01 vs. cytokine levels in cultures without IL-10 or anti–IL-10 modulation.

production in PBMC cultures, with monocytes as the predominant source of IL-10.

In contrast to the cultures stimulated by mycobacterial preparations, PHA-stimulated PBMC cultures from HIV-infected patients, compared with controls, produced lower IL-10 levels, with the lowest levels being in patients with the most advanced disease. Several cell types produced IL-10 in response to PHA, particularly CD4 lymphocytes and monocytes. Thus, the reduced number of CD4 lymphocytes in the patient PBMC cultures might be of importance for their lower IL-10 production. There are discrepant reports concerning IL-10 production in PBMC cultures after PHA stimulation during HIV infection [6, 7, 9, 10, 24, 39], and conflicting results may partly be due to different proportions of CD4 lymphocytes in PBMC cultures in different studies.

Several reports have suggested that a shift in cytokine pattern from Th1 (dominated by interferon-γ and IL-2) to Th2 (dominated by IL-4 and IL-10) contributes to disease progression in patients with HIV [6, 7, 40]; however, other data have not supported this hypothesis [5, 10, 41]. Our findings are clearly relevant to this discussion. We report here that the cellular sources of IL-10 production depend on the nature of the stimulus since IL-10 is only partially (mitogen stimulation) or only to a small extent (stimulation by MAC preparations) derived from CD4 lymphocytes. Production of IL-10 in mixed cell populations, such as PBMC, should be interpreted carefully and does not necessarily depend on Th2 lymphocytes, as has also been suggested by others [5]. We suggest that increased IL-10 release from monocytes and macrophages is of paramount importance for the elevated IL-10 production in HIV infection.

IL-10 normally has a down-regulating effect on production of several other cytokines, such as TNF-α and IL-12 [3, 42, 43]. This led us to examine the interplay between IL-10 and
these two cytokines after stimulation by MAC preparations. Reduced levels of TNF-α were found in cultures for HIV-infected patients compared with cultures for healthy controls, while neutralization of IL-10 led to a normalization of TNF-α production. Addition of IL-10 to the cultures caused a substantial further reduction in TNF-α production. Thus, the elevated IL-10 production might contribute to the down-regulation of TNF-α production in the patient cultures.

Similar levels of IL-12 p40 were found in patient and control cultures after stimulation with MAC preparations. Others have found reduced IL-12 production in cell cultures from HIV-infected patients [44]. Modulation of IL-12 p40 production by addition of IL-10 or neutralizing anti–IL-10 led to a pattern similar to that of TNF-α.

Elevated IL-10 production in HIV patients may well contribute to the reduced immunity to intracellular microorganisms seen in HIV infection, due to the immunosuppressive effects of IL-10. MAC represents one of the most important intracellular pathogens in AIDS patients [11]. The antimycobacterial defenses of macrophages depend on a critical balance between protective and MAC-enhancing cytokines [45–47]. In humans, IL-10 would be expected to reduce the mycobactericidal effect of macrophages by inhibition of various other cytokines, such as TNF-α [3, 42]. However, a recent in vitro study did not find any effect of IL-10 on MAC growth in a human monocyte infection model [48].

In conclusion, our findings clearly demonstrate abnormal IL-10 levels in HIV-infected patients, with markedly higher serum levels and enhanced IL-10 release from PBMC stimulated with MAC preparations and with the most pronounced abnormalities seen in patients with advanced disease. The IL-10 production induced by MAC products in PBMC culture was almost exclusively derived from monocytes. Thus, we suggest that monocytes and macrophages might be major contributors to the increased IL-10 production in patients with HIV infection. This may be an important factor in the development of immunodeficiency in HIV infection. Furthermore, the cytokine imbalance with elevated IL-10 and reduced TNF-α may contribute to the high susceptibility to MAC infection seen in patients with advanced HIV disease.

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