Increased Production of Interleukin-10 by Human Blood Monocytes Stimulated with *Mycobacterium avium-intracellulare* complex

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Abstract

Macrophages produce various cytokines in response to mycobacteria, including interleukin 10 (IL-10) and tumor necrosis factor alpha (TNF-α). IL-10 has been shown to down-regulate numerous macrophage functions, including microbicidal activity against intracellular bacteria and parasites. IL-10 also inhibits interferon-gamma (IFN-γ) production and antigen-specific proliferation of Th1 cells mediating immunologic resistance to mycobacterial infection. In contrast, TNF-α activates macrophages and may augment their mycobactericidal activity. In this study, peripheral blood mononuclear cells (PBMC) or blood monocytes obtained from healthy tuberculin reactors were stimulated in vitro with heat-killed *Mycobacterium tuberculosis* or heat-killed *M. avium-intracellulare* complex (MAC) to produce IL-10 and TNF-α. We studied a total of 26 clinical isolates of *M. tuberculosis* and 28 isolates of MAC. MAC-stimulated PBMC and monocytes released significantly larger amounts of IL-10 than those cells stimulated with *M. tuberculosis*. However, there was no difference in induction of TNF-α production between MAC and *M. tuberculosis*. When TNF-α activity was neutralized by the addition of anti-TNF-α mAb in culture, MAC still induced more IL-10 secretion than did *M. tuberculosis*. These findings suggest that increased production of IL-10 by MAC-stimulated monocytes may play a role in the intractable disease caused by these organisms.

Introduction

Tuberculosis (TB) remains an unresolved public health problem in developing countries. According to WHO, TB is responsible for 3 million deaths per year and 8 million new cases develop annually worldwide¹⁹. In Western countries, the decline in number of TB cases halted in the mid-eighties, and in the United States the number of TB cases has increased 18%¹⁹. Several reasons have been suggested for this increase in frequency of TB, including the spread of HIV and the appearance of multi-drug resistant strains of *M. tuberculosis*. Furthermore, recent studies have reported that *Mycobacterium avium-intracellulare* complex (MAC) are common opportunistic pathogens in AIDS patients and immunocompromised hosts. *M. tuberculosis* and MAC are intracellular pathogens that are preferentially taken up by mononuclear phagocytes and multiply in these cells. The pathogenicity...
of these organisms depends on the interplay between cell-mediated, but not humoral, immune response and the virulence of the pathogen. Healthy individuals infected with mycobacteria can develop a cellular immune response to this pathogen and successfully control its growth. Cell-mediated immunity is characterized by activation of CD4+ T cells which secrete cytokines such as interferon-gamma (IFN-γ), activating macrophages to kill bacilli. Previous studies have demonstrated that monocytes/macrophages infected with mycobacteria secrete both pro-inflammatory cytokines, such as interleukin-6 (IL-6), tumor necrosis factor-alpha (TNF-α), and IL-12, and inhibitory cytokines, such as IL-10 and transforming growth factor-beta (TGF-β). These cytokines may regulate the cellular immune response and growth of mycobacteria. IL-10 was first described as a product of "T helper-2 (Th2)" cell which suppresses IFN-γ production by Th1 cells. IL-10 was subsequently shown to exert stimulatory effects on B-Cells, for example, by increasing expression of class II major histocompatibility antigens and by enhancing their viability. Since IL-10 also inhibits many functions of mononuclear phagocytes, including antigen-presenting capacity, production of inflammatory cytokines and intracellular parasite killing, it is thought to be a type of macrophage deactivating factor. A recent study found that administration of anti-IL-10 antibody augmented resistance of mice to Mycobacterium avium infections, suggesting that IL-10 may play a role in the pathogenesis of mycobacterial infections. On the other hand, TNF-α activates macrophages and may augment mycobactericidal activity. In this study, we compared the production of IL-10 and TNF-α by human blood mononuclear cells and blood monocytes stimulated in vitro with clinically isolated strains of M. tuberculosis or MAC.

Materials and Methods

Mycobacteria

M. tuberculosis and MAC (M. avium and M. intracellulare) were isolated from the sputa of patients with pulmonary tuberculosis and atypical mycobacteriosis, respectively. Mycobacteria were cultured in Dubos' medium, killed by autoclaving, then washed and resuspended in phosphate buffered saline (PBS) at 10 mg/ml (wet weight). We obtained one mycobacterial strain from each patient, preparing a total of 26 strains of M. tuberculosis and 28 strains of MAC (14 strains of M. avium and 14 strains of M. intracellulare). The species of each strain was identified by a DNA-DNA hybridization method using a commercial kit (DDH Mycobacteria, Kyokuto, Japan). Endotoxin was not detected in the mycobacterial preparations with the Limulus Amoebocyte Lysate assay (detection limit, 50 pg/ml).

Cells and culture conditions for cytokine production

Venous blood from four healthy tuberculin reactors was drawn into heparinized syringes, diluted 1:1 in RPMI-1640 (Dainippon Pharmaceutical Co, Japan), layered onto Lymphocyte Separation Medium (Organon Teknica, Japan), and centrifuged for 30 min at 300 g. Peripheral blood mononuclear cells (PBMC) were isolated from the interface and washed three times in RPMI 1640. Then PBMC were suspended at 5–8 × 10^6/ml in RPMI-1640 with 10% heat-inactivated fetal bovine serum (FBS; BioWHITTAKER, Walkersville, MD), and 3 ml were incubated in plastic dishes (JIMRO, Japan) for 1 h at 37°C. Non-adherent cells were removed by washing dishes. Adherent cells were incubated with 3 ml PBS containing 5% FBS with 3 mM ethylenediaminetetraacetic acid and 2 mM HEPES for 10 min on ice. The adherent cells from the dish consisted of >90% CD14+ cells or monocytes, as determined by flow cytometry. PBMC (1 × 10^6/ml) or monocytes (0.5 × 10^6/ml) were cultured in RPMI-1640 containing 10% heat-inactivated pooled human serum with antibiotics (100 μg/ml of streptomycin and 100 U/ml of penicillin) and L-glutamine (2 mM) at 37°C humidified in a 5% CO₂ atmosphere. Previous studies reported that IL-10 production by activated monocytes is enhanced...
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by TNF-α1). So cells were cultured in 96-well flat-bottomed culture dishes (FALCON 3072, BECON DICKINSON, NJ: 200 μl/well) with the heat-killed mycobacterial preparations in the presence or absence of 10 μg/ml anti-TNF-α mAb (mouse IgG1, HAYASHIBARA, Japan). Culture supernatants were collected at 8 hours, 1 day, 3 days and 5 days after stimulation and then stored at −20°C until assay.

Enzyme-linked immunosorbent assay (ELISA) for IL-10 and TNF-α

IL-10 concentration in the culture supernatants was measured by ELISA using IL-10 specific monoclonal antibodies. ELISA plates (Nunc-Immuno® Plate) were coated overnight at 4°C with anti-human IL-10 mAb (rat IgG1, clone JES3-9D7, Pharmingen, San Diego, CA) at 2 μg/ml (50 μl/well). After blocking with 5% FBS in PBS, samples and standard dilutions of recombinant IL-10 (Pharmingen) were added at 100 μl/well and then incubated for 1 h at room temperature. Biotinylated rat anti-IL-10 mAb (Pharmingen) was used at 1 μg/ml and incubated for 1 h at room temperature. After washing, the plates were incubated with streptavidin/peroxidase (Sigma) diluted at 1:1000 for 45 min at room temperature and developed with a substrate solution. OD was read at 405 nm using an ELISA reader. The sensitivity of the ELISA for IL-10 was 35 pg/ml. Concentrations of TNF-α were determined using an ELISA kit (R & D Systems, Minneapolis, MN) in the same culture supernatants, with a limit of detection of 4.8 pg/ml TNF-α.

Statistics

All values are presented as mean ± SEM. Statistical significance of differences between groups was determined using Student’s t-test.

Results

Production of IL-10 by PBMC stimulated with mycobacteria

Fig. 1A shows the kinetics of IL-10 production by PBMC from a healthy subject stimulated with M. tuberculosis (26 strains) or MAC (28 strains). IL-10 production by PBMC was first detected at day 1 and was maximal at day 3. To determine the optimal concentration of heat-killed mycobacteria

Fig. 1 IL-10 production by PBMC stimulated with M. tuberculosis or MAC.

(A): PBMC (1 × 10^6/ml) were cultured for 8 hours to 5 days with an optimal concentration of heat-killed mycobacteria (M. tuberculosis 100 μg/ml, MAC 50 μg/ml).

(B): PBMC (1 × 10^6/ml) were cultured with different concentrations of heat-killed mycobacteria for 3 days. IL-10 concentrations in culture supernatants were measured by ELISA. Vertical bars represent the mean ± SEM. Asterisks indicate statistical significance (*p<0.05, **p<0.01).
yielding maximal IL-10 production, PBMC were stimulated with different concentrations of the mycobacterial preparation. The optimal concentrations of PBMC were 100 µg/ml for M. tuberculosis and 50 µg/ml for MAC (Fig. 1B). In subsequent experiments, these concentrations were used for stimulation. Fig. 1 also shows that MAC induced greater levels of IL-10 production than did M. tuberculosis; the differences achieved statistical significance during days 1 to 5 of culture (p<0.05) and at concentrations of 25 to 100 µg/ml (p<0.05 or p<0.01). There was no difference in induction of IL-10 production by M. avium and M. intracellulare (data not shown).

**IL-10 production by monocytes from a healthy subject**

PBMC included various types of cells, such as T cells, B cells, and monocytes. We next isolated monocytes from PBMC using the plastic adherence method, and stimulated monocytes with M. tuberculosis or MAC. In contrast to PBMC, monocytes rapidly secreted IL-10 and reached maximal levels at day 1 (Fig. 2A). Optimal concentrations of mycobacteria for monocytes were the same as for PBMC (Fig. 2b). As shown in Fig. 2, MAC induced higher levels of IL-10 production by monocytes than did M. tuberculosis for every period of culture and every mycobacterial concentration studied (p<0.05 or p<0.01).

**IL-10 and TNF-α production by PBMC or monocytes from different individuals**

As shown in Fig. 3, MAC induced significantly larger amounts of IL-10 production than did M. tuberculosis in all subjects tested (p<0.01). On the other hand, M. tuberculosis- and MAC-induced TNF-α production by PBMC were comparable in each of the subjects (Fig. 4).

**Effect of neutralizing TNF-α antibodies on IL-10 production by PBMC stimulated with mycobacteria**

To determine whether TNF-α affects on mycobacteria-stimulated IL-10 induction, PBMC were cultured with mycobacterial preparations in the presence of anti-TNF-α antibodies, control antibodies (mouse IgG1) or medium alone. Although anti-TNF-α antibodies, but not control antibodies, markedly reduced IL-10 production by PBMC stimulated with mycobacteria, MAC still induced significantly higher levels of IL-10 than did M. tuberculosis (Fig. 5: p<0.05 or p<0.01).

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Fig. 2. IL-10 production by monocytes stimulated with M. tuberculosis or MAC.
(A): Monocytes (0.5 × 10^6/ml) were cultured for 8 hours, 1 day and 3 days with an optimal concentration of heat-killed mycobacteria (M. tuberculosis 100 µg/ml, MAC 50 µg/ml).
(B): Monocytes (0.5 × 10^6/ml) were cultured with different concentrations of heat-killed mycobacteria for 1 day. IL-10 concentrations in culture supernatants were measured by ELISA. Vertical bars represent the mean ± SEM. Asterisks indicate statistical significance (*p<0.05, **p<0.01).
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Fig. 3  IL-10 production by PBMC or monocytes from different individuals. PBMC (1 × 10⁶/ml) and monocytes (0.5 × 10⁶/ml) from each of 4 donors were cultured with an optimal concentration of heat-killed mycobacteria (M. tuberculosis 100 µg/ml, MAC 50 µg/ml) for 3 days and 1 day, respectively. IL-10 concentrations in culture supernatants were measured by ELISA. Vertical bars represent the mean ± SEM. Asterisks indicate statistical significance (*p<0.01).

Fig. 4  TNF-α production by PBMC stimulated with M. tuberculosis or MAC. PBMC (1 × 10⁶/ml) from the same subjects as in Fig. 3 were cultured with heat-killed mycobacteria (M. tuberculosis 100 µg/ml, MAC 50 µg/ml) for 1 day and 3 days. TNF-α concentrations in culture supernatants were measured by ELISA. Vertical bars represent the mean ± SEM.

Discussion

Our findings show that human blood monocytes produce more IL-10 in the presence of heat-killed MAC bacilli than in the presence of M. tuberculosis (Figs. 1, 2, 3). It is known that mononuclear phagocytes and T cells produce IL-10⁴,⁵. In our laboratory, cell separation experiments confirmed that monocytes, but not T lymphocytes, produced IL-10 when stimulated with M. tuberculosis (unpublished observations). Recently, Barnes et al. detected IL-10 mRNA predominantly in CD3⁻ and CD14⁺ cells but not in CD3⁺ pleural fluid cells obtained from patients with tuberculous pleuritis⁶. These observations suggested that mononuclear phagocytes may be the main source of IL-10 in M. tuberculosis infection. Activated macrophages also produce TNF-α, which plays a role in the pathogenesis of mycobacterial diseases. TNF-α inhibits mycobacterial growth⁷ and is required for granuloma
Fig. 5 Effect of anti-TNF-α mAb on IL-10 production by PBMC stimulated with M. tuberculosis or MAC.

PBMC (1 × 10^6/ml) from one subject were cultured with an optimal concentration of heat-killed mycobacteria (M. tuberculosis 100 μg/ml, MAC 50 μg/ml) for 3 days in the presence or absence of anti-TNF-α mAb (10 μg/ml) or control mouse IgG (10 μg/ml). IL-10 concentrations in culture supernatants were measured by ELISA. Vertical bars represent the mean ± SEM. Asterisks indicate statistical significance (*p<0.05, **p<0.01).

formation, which limits the extent of mycobacterial infection15). On the other hand, TNF-α may have immunopathological effects such as fever, weight loss and tissue necrosis. In this study, PBMC produced similar levels of TNF-α in the presence of either M. tuberculosis or MAC (Fig. 4). The mechanism by which MAC produces much more IL-10 than does M. tuberculosis is unclear. Since no difference was found in TNF-α induction between M. tuberculosis and MAC (Fig. 4), the difference in IL-10 production does not appear to be mediated by TNF-α, which upregulates IL-10 expression in PBMC. Moreover, when we neutralized TNF-α activity by the addition of anti-TNF-α mAb to culture, MAC still secreted IL-10 than did M. tuberculosis (Fig. 5), suggesting that TNF-α-independent mechanisms account for the differences in IL-10 secretion. Some structural differences in lipoarabinomannans from mycobacteria are associated with its differential capacity to induce the secretion of TNF-α in macrophages16). Furthermore, there are differences between M. tuberculosis and MAC in terms of the structure of mycolic acid subclass and mycoloyl glycolipid, a characteristic component of the cell wall in acid-fast bacteria17). We speculate that certain heat-stable cell-wall component(s) of MAC may be responsible for augmentation or enhanced production of IL-10 by activated macrophages.

It is well known that immunological resistance to mycobacterial infections is mediated by cooperative interaction between T lymphocytes and macrophages. IL-10 inhibits both IFN-γ production and antigen-specific proliferation of Th1 cells in the presence of macrophages6,8). Moreover, IL-10 has been shown to down-regulate a number of macrophage functions, including cytokine production6) and microbicidal activity against intracellular parasites7). Recently, Bermudez & Champsi8) and Denis & Ghadirian9) examined independently the involvement of IL-10 in murine M. avium infections. They demonstrated that IL-10 production by spleen cells from mice infected with M. avium increased as infection progressed. In addition, they showed that treatment of infected mice with neutralizing anti-IL-10 antibody resulted in diminished bacterial growth in the spleens. Tsuyuguchi et al. found
that PBMC of patients with MAC infection exhibited decreased in vitro lymphocyte proliferation and IL-2 production after stimulation with purified protein derivative (PPD)\textsuperscript{18}. They also showed that heat-killed whole MAC organism or its lipid component impaired the capacity of human PBMC to proliferate in vitro in response to PPD and mitogens\textsuperscript{19}. Another role of IL-10 in immunosuppression during human mycobacterial infections was suggested by Sieling et al\textsuperscript{20}. They demonstrated that PBMC of leprosy patients and healthy donors released IL-10 antibodies enhanced PBMC proliferation in patients infected with \textit{M. leprae}, suggesting that \textit{M. leprae}-triggered release of IL-10 suppresses T cell proliferative responses. We also observed that neutralizing anti-IL-10 antibody significantly enhanced the proliferative response of PBMC to the mycobacterial preparations studied here (data not shown). Taken together, our findings suggest that increased production of IL-10 by MAC-stimulated monocytes play a role in the intractable disease caused by these organisms.

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*Mycobacterium avium-intracellulare* complex (MAC) による
ヒト末梢血単球からのIL-10産生

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要 旨
結核菌を含む抗酸菌感染にともない，局所にはマクロファージを主体とするリノバ球，好中球等の集積からなる肉芽腫が形成され結核菌が貪食される。貪食された菌体は種々の方法により殺菌から逃れようとするが，有効な感染防御免疫の成立には，T細胞とマクロファージの活性化によって産生されるTh1系サイトカインにより，強力な細胞免疫が誘導されることが必要である。抗酸菌感染症，特に*M. avium-intracellulare* complex (MAC) 感染においては，しばしば細菌性免疫の抑制が起こることが報告されているが，その機構は未だ明らかではない。今回我々は健常人からヒト末梢血単核球（PBMC）と単球（monocyte）を分離し，臨床分離株である結核菌株26菌株，MAC菌株28菌株を用いて刺激を加え，その上清中の抑
制性サイトカインであるIL-10の産生を中心に検討を加えた。IL-10産生は，PBMCでは第一日目がピークであり，monocyteでは第三日目であった。いずれにおいてもMAC菌体は，結核菌体に比べて有意にIL-10の産生を促進させた（p<0.01）。MAC菌によってIL-10が多量に誘導されることでは，MAC菌自身が生存することに有利な状況を作り，生体防御の免疫機能を低下させ，AIDS末期に感染を引き起こし，又一般にMAC感染症の難治化を引き起こす要因の1つとなることが考えられる。又，IL-10産生に関しては，TNF-αの関与する機序があると考えられるが，結核菌群とMAC菌群間でTNF-αの産生量に差は認められなかったことから，IL-10産生の調節に関しては他のメカニズムの存在が考えられる。