Elevated Levels of Interferon-inducible Protein-10 (IP)-10/CXCL10, but not of Interferon-γ, in Patients with Pulmonary Tuberculosis

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Mycobacterial strains are potent inducers of cytokines/chemokines by mononuclear phagocytes, which constitute an important cellular component of the first line of defense in the innate immune system. Interferon (IFN)-γ-inducible protein (IP-10 or CXCL10) is a potent chemoattractant; however, little is known about the IP-10 profiles attributable to the Th1 regulation associated with active tuberculosis (TB). In this study, we investigated the production of IP-10, interleukin (IL)-12 p40, and IFN-γ by the peripheral blood mononuclear cells (PBMCs) of patients with active pulmonary TB in response to in vitro stimulation with Triton X-100 soluble proteins (TSPs) or the 30-kDa antigen. The TSP antigens used in the present study were isolated and purified from Mycobacterium tuberculosis H37Rv (virulent strain), M. tuberculosis H37Ra (avirulent strain), and Mycobacterium bovis BCG. The results were compared with those obtained for healthy tuberculin reactors (HTRs). Concordant with earlier studies, IFN-γ production was significantly depressed in the PBMCs from TB patients compared with those in the HTR group. However, the IP-10 levels in the PBMCs from TB patients were significantly elevated 18 h after stimulation compared to those in the PBMCs from HTRs. IP-10 release was correlated in a significant manner with the release of IFN-γ in the HTRs, but this was not the case for the TB patients. Collectively, these data suggest that TB patients show altered regulation of Th1-driving cytokine and chemokine production in response to a variety of mycobacterial antigens.

Key Words: Tuberculosis, Mycobacterium tuberculosis, Interferon-γ-inducible protein, Interferon-γ, 30-kDa antigen, TSP antigen

INTRODUCTION

Tuberculosis (TB) remains the leading cause of morbidity and mortality due to an infectious agent worldwide, although the understanding of its immunopathogenesis remains incomplete (35). The immune response mounted to TB infection is generally successful in containing, but not eliminating, the pathogen. Acute active TB can result in a small percentage of infections, probably due to the lack of initiation of appropriate immune responses. During TB infection, Mycobacterium tuberculosis persists in macrophages within granulomas in the organs of infected hosts (9). With respect to host immune responses, macrophage-mycobacterium interactions play crucial roles in bacterial growth inhibition/killing, the recruitment of accessory...
immune cells for a local inflammatory response, and the presentation of antigens to T cells for the development of acquired immunity. In addition, interactions between macrophages and other effector cells occur in a milieu of both cytokines and chemokines (29).

Chemokines serve to attract and activate other inflammatory effector cells, including lymphocytes (29). A variety of experiments have shown that excessive or insufficient production of cytokines and chemokines may significantly contribute to the pathophysiology of TB. During TB, the mononuclear phagocyte migratory activity is a highly regulated process that requires a defined repertoire of chemokines/receptors, and dysregulated expression of these proteins may alter inflammatory effector cells’ recruitment and activation (4). A more complete understanding of the roles that cytokines and chemokines play in the protection against or exacerbation of TB would enhance the development of preventive and therapeutic strategies against *M. tuberculosis*.

The active TB is characterized by a profound and prolonged suppression of *M. tuberculosis*-specific T-cell responses, as shown by the decreased production of the cytokines interferon (IFN)-γ and interleukin (IL)-2 (12,31,32). IFN-γ rapidly induces the expression of genes in macrophages and other cell types (25), and one of these genes encodes the 10-kDa secreted protein CXCL10, which shares significant homology with a family of chemotactic and mitogenic proteins (24). CXCL10, which is also called IFN-γ-inducible protein 10 (IP-10), is a member of the CXC-chemokine family. It is expressed in lymphocytes and monocytes (15), and is involved in trafficking monocytes and activated Th1 cells to inflamed foci through interaction with the CXCR3 chemokine receptor. High levels of IP-10 have been found in the delayed-type hypersensitivity reaction to tuberculin purified protein derivative (PPD) (15), in lymph nodes and lung tuberculous granulomas (8), in the pleural effusions of TB patients (27), in the plasma of TB patients (2,14), and in patients co-infected with TB and HIV who are experiencing immune reconstitution syndrome (5). However, little is known about the mycobacterial antigen-induced IP-10 responses in the context of IFN-γ production during TB.

In the present study, we analyzed the IP-10 secretion profiles of peripheral blood mononuclear cells (PBMCs) from active pulmonary TB patients after *in vitro* stimulation with various mycobacterial antigens (Ags), including the 30-kDa Ag, and Triton X-100 soluble proteins (TSP) from *M. tuberculosis* H37Rv, *M. tuberculosis* H37Ra, and *M. bovis* BCG strains. These profiles were compared with those of healthy tuberculin reactors (HTR). The results show that the expression of IP-10 is prominently up-regulated in TB patients compared to HTRs. We also demonstrate that IP-10 release is significantly correlated with the release of IFN-γ in HTRs but not in TB patients.

**MATERIALS AND METHODS**

1. **Subjects**

Whole blood was obtained by venipuncture from a total of ten patients and ten HTRs. All subjects gave their written informed consent before being enrolled in the study. The HTR subjects were recruited from medical college students at Chungnam National University, Korea, and they had no previous history of clinical TB. Skin reactions in healthy volunteers of more than 10 mm after an intradermal test with 2 TU of PPD-RT23 (Statens Serum Institut, Copenhagen, Denmark) were considered positive. Each of these HTRs had received *Mycobacterium bovis* bacillus Calmette-Guérin (BCG) vaccination when a child.

Active pulmonary TB patients participated in this study within 1 month of beginning first-line anti-tuberculosis drug medication at Konyang University Hospital (Daejeon, Korea). All of the patients in the present study had parenchymal TB, and none had miliary or pleural TB. All patients had a positive sputum culture for *M. tuberculosis*. They had no previous history of diabetes mellitus or steroid therapy, and all were HIV-negative. Extensive clinical histories were obtained from the subjects and included data on age, sex, medical history, chest radiographic findings, sputum staining and culture results, drug sensitivities, treatment, and outcome. This study was reviewed and approved by the Institutional Research Board of Konyang University Hospital.
2. Isolation of the Triton X-100 soluble proteins (TSP)-Rv, TSP-Ra, and TSP-BCG, and purification of the 30-kDa Ag

*M. tuberculosis* H37Rv (ATCC 27294), *M. tuberculosis* H37Ra, and *M. bovis* BCG Pasteur-1173P2 were grown in Sauton's synthetic medium at 37°C for 6 weeks. To obtain a whole cell paste used for TSP Ag isolation, cells were harvested by centrifugation at 10,000 rpm for 30 min, washed twice with Tris-buffered saline (TBS). After centrifugation, 462 grams, 356 grams, and 325 grams (wet weight) of *M. tuberculosis* H37Rv, *M. tuberculosis* H37Ra, and *M. bovis* BCG bacilli were then obtained, respectively. The *M. tuberculosis* H37Rv culture supernatant was filter-sterilized using a 0.22-µm membrane filter, and then used for purification of the 30-kDa Ag.

The TSP-Rv, TSP-Ra, and TSP-BCG Ags were isolated as previously described by Kim *et al.* (16). Briefly, the cells were suspended in 100 ml TBS that contained 1% Triton X-100, 10 mM phenylmethylsulfonyl fluoride (PMSF), 0.05% NaN₃, and 1 mM EDTA and incubated with shaking for 18 h at 37°C. The Triton X-100 extracts were centrifuged at 15,000 × g for 1 h, and the supernatants were precipitated with ammonium sulfate (20% to 80% saturation). Precipitation was performed by repeated dilution and phase separation. The resultant aqueous phase was cleansed by the repeated addition of 11.4% Triton X-114 to a final concentration of 2%. After three rounds of 0% to 80% ammonium sulfate precipitation, each isolated TSP Ag was stored in sterile aliquots at -20°C.

The 30-kDa Ag was purified from the culture filtrate proteins (CFP) of *M. tuberculosi*s H37Rv as described by Lee *et al.* (19). In brief, the native 30-kDa Ag was purified from the CFP by anion-exchange chromatography (1st) (Macro-Prep High Q supported column; Bio-Rad, Hercules, CA, USA), ammonium sulfate precipitation, hydrophobic interaction chromatography (HIC), and anion-exchange chromatography (2nd) (UNO-Q6 column; Bio-Rad). The final purified Ag was dialyzed against phosphate-buffered saline (PBS; pH 7.2), applied to a column with immobilized polymyxin B (Detoxi-Gel Endotoxin Removing Gel; Pierce, Rockford, IL, USA) to reduce the level of endotoxin, filter sterilized, and then frozen at -20°C.

The endotoxin content of each TSP Ag and 30-kDa Ag preparation was < 0.02 ng/mg protein, as determined by the *Limulus* amebocyte lysate assay (QCL1000; BioWhittaker, Walkersville, MD, USA). The protein concentrations were estimated using the bicinchoninic acid protein assay kit (Pierce), with bovine serum albumin (BSA) as the standard.

3. Isolation of human PBMCs and Ag stimulation

PBMCs were isolated by density sedimentation over Histopaque-1077 (Sigma Chemical Co., St. Louis, MO, USA). The PBMCs were suspended at a density of 1×10⁶ viable cells/ml in complete medium [RPMI 1640 (Gibco-BRL, Gaithersburg, MD, USA) supplemented with 10% fetal bovine serum (Gibco-BRL), Gaithersburg, MD, USA] supplemented with 10% fetal bovine serum (Gibco-BRL), sodium pyruvate, non-
essential amino acids, 100 IU/ml penicillin G, and 100 µg/ml streptomycin]. PBMCs were then stimulated with 1.0 µg/ml 30-kDa Ag and incubated at 37 °C in a humidified, 5% CO2 atmosphere until used for supernatant collection. Lipopolysaccharide (LPS) (1 µg/ml; Sigma Chemical Co.) and phytohemagglutinin (PHA) (1 µg/ml; Sigma Chemical Co.) were used as positive controls for the Ag stimulation in this study.

4. Enzyme-linked immunosorbent assay

A sandwich enzyme-linked immunosorbent assay (ELISA) was used to detect human IFN-γ, IL-12 p40, and IP-10 (BD PharMingen, San Diego, CA, USA) in culture supernatants. Assays were performed as recommended by the manufacturers. The cytokine concentrations in the samples were calculated using standard curves generated for recombinant cytokines, and the results are expressed in pg/ml. The difference between duplicate wells was consistently less than 10% of the mean.

5. Statistical analysis

For statistical analysis, the data obtained from independent experiments were presented as the mean ± SD and analyzed using the Student's t-test or linear regression analysis. Differences were considered to be significant at p<0.05.

**Figure 2.** IFN-γ (A) and IL-12p40 (B) production by PBMCs from TB patients in response to TSP-Rv, TSP-Ra, TSP-BCG, and 30-kDa Ag. IFN-γ and IL-12p40 production by PBMCs from HTRs and TB patients were determined after in vitro stimulation with TSP-Rv, TSP-Ra, TSP-BCG, and 30-kDa Ag (1 µg/ml for each Ag). The supernatants were harvested at 18 h (for IL-12p40) or 96 h (for IFN-γ) for the assessment of cytokines by ELISA. The values are shown as the mean ± SD of the supernatant samples. *p<0.05 and **p<0.01, as compared with the HTRs (Student's t-test). Us, unstimulated.
RESULTS

1. Purification of TSP-Rv, TSP-Ra, TSP-BCG, and 30-kDa Ag

The TSP-Rv, TSP-Ra, and TSP-BCG proteins were isolated by extraction with 1% Triton X-100, 20% to 80% ammonium sulfate precipitation, three rounds of phase separation with Triton X-114, and three rounds of 0% to 80% ammonium sulfate precipitation from *M. tuberculosis* H37Rv, *M. tuberculosis* H37Ra, and *M. bovis* BCG, respectively. The 30-kDa Ag was purified from CFP of *M. tuberculosis* H37Rv by: 1) anion-exchange chromatography, 0 to 40% ammonium sulfate precipitation, and HIC; and 2) anion-exchange chromatography. The SDS-PAGE analysis of the isolated TSP-Rv, TSP-Ra, and TSP-BCG Ags and purified 30-kDa Ag are shown in Fig. 1. Most of the TSP Ags showed a diverse banding pattern in the 10- to 100-kDa molecular weight (MW) range. We recovered approximately 10.1 mg of TSP-Rv, 13.9 mg of TSP-Ra, and 4.92 mg of TSP-BCG from 462 g, 356 g, and 325 g of *M. tuberculosis* H37Rv, *M. tuberculosis* H37Ra, and *M. bovis* BCG bacilli, respectively. As judged by SDS-PAGE, the purified 30-kDa Ag was at least 90% pure. The 40.5 mg of 30-kDa Ag was

Figure 3. IP-10 production by PBMCs from TB patients in response to TSP-Rv, TSP-Ra, TSP-BCG, and 30-kDa Ag. The IP-10 production levels by PBMCs from HTRs and TB patients were determined after *in vitro* stimulation with TSP-Rv, TSP-Ra, TSP-BCG, and 30-kDa Ag (1 µg/ml for each Ag). The supernatants were harvested at 18 h (A) and 96 h (B), and the IP-10 protein levels were measured by ELISA. The values are shown as the mean ± SD of supernatant samples. *p<0.05 and **p<0.01, as compared with the HTRs (Student's *t*-test). Us, unstimulated.
recovered from 985 mg of CFP of *M. tuberculosis* H37Rv.

2. IFN-γ and IL-12 p40 production by PBMCs from TB patients and HTRs after *in vitro* stimulation with TSP-Rv, TSP-Ra, TSP-BCG, and 30-kDa Ag

The induction of a potent Th1-type immune response is important for protective immunity against TB (9). Thus, we investigated the Th1 immune response to mycobacterial antigens by measuring IFN-γ and IL-12 p40 production in response to the TSP-Rv, TSP-Ra, TSP-BCG, and 30-kDa Ag in the PBMCs from TB patients and HTRs. The kinetics of IFN-γ and IL-12 p40 production in response to these Ags were monitored, so as to determine the time required to achieve maximal IFN-γ and IL-12 p40 levels (data not shown). Although the IFN-γ levels in response to the TSP-Ra in TB patients were heterogeneous among individuals (range, 8.7–1266.0 pg/ml), the mean IFN-γ concentrations in the PBMCs of the TB patients were significantly depressed compared with the corresponding values in the PBMCs of the HTRs at 96 h after *in vitro* stimulation with TSP-Rv, TSP-Ra, TSP-BCG, and 30-kDa Ag (Fig. 2A). Meanwhile, the production levels of IL-12 p40 following stimulation with TSP-Rv, TSP-Ra, TSP-BCG, and 30-kDa Ag did not show significant differences between the HTRs and TB patients (Fig. 2B). These data clearly show that the production of IFN-γ, but not of IL-12 p40, by the PBMCs of TB patients is down-regulated by a variety of mycobacterial Ags.

3. IP-10 production by PBMCs from TB patients and HTRs after *in vitro* stimulation with TSP-Rv, TSP-Ra, TSP-BCG, and 30-kDa Ag

To investigate IP-10 production by PBMCs in response to diverse Ags, we first determined the kinetics of IP-10 production induced by the 30-kDa Ag in PBMCs from HTRs. We found that IP-10 release in response to the 30-kDa Ag was increased in a time-dependent manner (data not shown). The production of IP-10 by each TSP Ag and 30-kDa Ag was then compared between HTRs and TB patients at two time-points, 18 h and 96 h. At 18 h after *in vitro* stimulation with TSP-Rv, TSP-Ra, TSP-BCG, and 30-kDa Ag, the production of IP-10 was significantly increased in the TB patients compared to the HTRs. However, there were no significant differences in IP-10 production between the TB patients and HTRs after 96 h stimulation (Fig. 3A and B). We found no differences in the patterns of IP-10 production for the different TSP Ags or 30-kDa Ag used to stimulate the PBMCs. Interestingly, the production of IP-10 by each individual correlated significantly with the production of IFN-γ in response to the TSP-Rv and 30-kDa Ag in the HTRs (n=10, r=0.84, and p<0.05 for the 30-kDa Ag; n=9, r=0.74, and p<0.05 for TSP-Rv), but not found in TB patients (n=10, r=0.19, and NS for the 30-kDa Ag; n=10, r=0.000, and NS for TSP-Rv). NS, not significant.

Figure 4. Linear regression analysis of IFN-γ and IP-10 production in TB patients and HTRs. The levels of IP-10 and IFN-γ release were assayed by ELISA after stimulation with the TSP-Rv and 30-kDa Ags for 96 h. A significant correlation was found between IP-10 and IFN-γ production after *in vitro* stimulation with 30-kDa Ag or TSP-Rv Ag in HTRs (n=10, r=0.84, and p<0.05 for the 30-kDa Ag; n=9, r=0.74, and p<0.05 for TSP-Rv), but not found in TB patients (n=10, r=0.19, and NS for the 30-kDa Ag; n=10, r=0.000, and NS for TSP-Rv). NS, not significant.
kine and chemokine production in response to a variety of mycobacterial Ags.

**DISCUSSION**

In the present study, we demonstrate that IFN-γ production is significantly depressed, whereas the IP-10 levels are significantly elevated in the PBMCs from TB patients compared with those from HTRs. Notably, IP-10 release was correlated in a significant manner with the release of IFN-γ in HTRs but not in TB patients. IFN-γ, which is produced by both CD4 and CD8 T cells, as well as by NK cells in TB patients, is regarded as a key cytokine in the control of *M. tuberculosis* infection. Although IFN-γ production alone is not sufficient to control *M. tuberculosis* infection, studies with IFN-γ knockout mice showing high susceptibility to virulent *M. tuberculosis* have stressed its roles in the protective response to this pathogen (6,10). Our previous study and several other studies have shown that IFN-γ production by PBMCs is reduced in TB patients (7,13,31,33). Consistent with these earlier findings, in the present study, we observed depressed levels of IFN-γ in response to a variety of mycobacterial Ags, such as TSP-Rv, TSP-Ra, TSP-BCG, and 30-kDa Ag, in the TB patients.

In contrast, the production of IL-12 p40 by PBMCs from TB patients was not down-regulated by the mycobacterial Ags. Based on the results of our previous study, which found that active TB patients showed significantly decreased IL-12 production after stimulation with the 30-kDa Ag or 32-kDa Ag (31), the present study seems to have yielded a controversial result. There may be ethnic or other environmental differences leading to variable IL-12 p40 responses to individual *M. tuberculosis* antigens. A recent study from Indonesia (30) demonstrated that depressed IFN-γ production was not due to decreased IL-12/IL-23 production. Considering the fact that IL-12 is a heterodimeric cytokine (p70), consisting of p40 and p35 subunits (26), and that p40 can pair with either p35 or p19 to form IL-12 (p40/p35) and IL-23 (P40/P19), respectively, the p40 levels against mycobacterial Ags may not accurately represent the IL-12 status. As a case in point, Liu et al. (22) have shown that IFN regulatory factor (IRF)-1 plays a role in the transcriptional activation of the IL-12 p35 gene, but not of the p40 gene, by physically interacting with an inverted IRF element within the IL-12 p35 promoter upon IFN-γ activation. In addition, Goodridge et al. (11) have demonstrated distinct signaling mechanisms that regulate the LPS-mediated induction of IL-12 p40 and IL-12 p35 in macrophages. As the IL-12 profile and its underlying mechanism have not been investigated as much as for IFN-γ, further studies with a larger sample size are needed to elucidate the role of IL-12 in the contribution of initial anergy during TB.

IP-10 was originally identified as an IFN-γ-inducible gene in monocytes, fibroblasts, and endothelial cells. Previous studies have demonstrated that active TB is associated with increased IP-10 levels in the plasma, and therefore the measurement of IP-10 levels has been suggested as a diagnostic marker of TB (2,27). Besides of IFN-γ, other cytokines such as IFN-α and -β or TNF-α have been implicated to induce the production of IP-10 as well (3,28). In addition, IP-10 and other cytokines such as MCP-1 and IL-6 act together to lead ultimate immune responses (17,23). Our findings support earlier observations that IP-10 production is up-regulated in TB patients (2,27), although our data are unique in that they show the IP-10 profiles in cultured PBMCs after stimulation with various mycobacterial antigens. In contrast to the increased PBMC IP-10 levels at 18 h after stimulation with Ags, the IP-10 levels at 96 h did not show significant differences between TB patients and HTRs. Considering that IP-10 is induced by IFN-γ (24), increased IFN-γ secretion by PBMCs from HTRs may affect the up-regulation of IP-10 production at 96 h.

The present data also demonstrate the lack of correlation between IFN-γ and IP-10 production in TB patients, although the significant correlation in HTRs implies that Th1-type cytokine and chemokine production against a variety of mycobacterial Ags is not optimally regulated in TB patients. Previous studies have shown altered regulation of cytokine production in pulmonary TB, which suggests that dysregulated cytokine production contributes to the immunopathogenesis of human TB (19,20,21). In addition, we have previously demonstrated that the chemokine CXCL8/IL-8
levels are depressed significantly in multidrug-resistant TB patients compared with early TB patients, which indicates the differential regulation of chemokine production among the different clinical stages of human TB (21). However, the correlation between IFN-γ and IP-10 production in PBMCs from TB patients and HTRs has not been reported. Further studies will clarify the underlying molecular mechanisms for the altered regulation of IFN-γ and IP-10, which may affect the host defense during TB infection.

The present study reinforces the notion that various mycobacterial Ags act as stimulants and give similar patterns of responses in each group of samples. Research has focused on the roles of various secreted protein Ags of *M. tuberculosis* in the immune response to infection, since these proteins are of great importance for the development of a vaccine against TB (9). The PPD Ag or CFPs of *M. tuberculosis* have been widely used for the study of TB immunity since these protein preparations contained most of the proteins released into the host cytoplasm when mycobacterium infected the host cells. Previous studies have shown that various *M. tuberculosis* Ags induce the secretion of a large number of cytokines, including IL-1, IL-2, IL-10, IL-12, and TNF-α, by monocytes and macrophages (13,19). The 30-kDa Ag is a strong inducer of IFN-γ, IL-12, IL-10, and TNF-α in human monocytes and PBMCs (1,20,21,33).

*M. tuberculosis* TSP is a mixture of the whole native antigen fraction from *M. tuberculosis* without massive degradation of proteins by Triton X-100, and is fractionated by Triton X-114 phase partitioning (16). Although TSP preparations may not contain much of CFP of mycobacterium, we have previously demonstrated that the TSP from *M. tuberculosis* induces comparable levels of IL-12 and TNF-α, and a similar dependency on the PI 3-K and ERK1/2 pathways as those induced by *M. tuberculosis* (34). The present data show that TSP antigens from avirulent strains *M. tuberculosis* H37Ra and *M. bovis* BCG elicit comparable production of cytokines, including IP-10, IL-12 p40, and IFN-γ, as compared to those induced by the TSP antigen from the virulent *M. tuberculosis* H37Rv strain. This suggests that the major protein fractions of *M. tuberculosis* complex strains are similar in terms of inducing activities for crucial cytokines/chemokines for protective immune responses against mycobacteria.

In conclusion, our results demonstrate that IP-10 levels are elevated significantly in the PBMCs from TB patients compared with those from HTRs. IP-10 release is correlated in a significant manner with the release of IFN-γ in HTRs but in TB patients. Collectively, these data indicate that TB patients show altered regulation of Th1-driving cytokine and chemokine production levels in response to a variety of mycobacterial antigens.

**REFERENCES**


21) Lee JS, Song CH, Lim JH, Kim HJ, Park JK, Paik TH, Kim CH, Kong SJ, Shon MH, Jung SS, Jo EK: The production of tumour necrosis factor-alpha is decreased in peripheral blood mononuclear cells from multidrug-resistant tuberculosis patients following stimulation with the 30-kDa antigen of Mycobacterium


