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Differential Live *Mycobacterium tuberculosis*-, *M. bovis* BCG-, Recombinant ESAT6-, and Culture Filtrate Protein 10-Induced Immunity in Tuberculosis▽

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The high prevalence of *Mycobacterium tuberculosis* makes it imperative that immune responses to evaluate could be predictive of infection. We investigated live *Mycobacterium*- and recombinant antigen-induced cytokine and chemokine responses in patients with active tuberculosis (TB) compared with those of healthy controls from an area where TB is endemic (ECs). *M. tuberculosis*-, *M. bovis* BCG-, ESAT6-, and culture filtrate protein 10 (CFP10)-induced responses were determined in peripheral blood mononuclear cells from patients with pulmonary TB ($n = 38$) and ECs ($n = 39$). The levels of the cytokines gamma interferon (IFN-γ) and interleukin-10 (IL-10) and the chemokines CCL2, CCL3, and CXCL9 were measured. The levels of *M. tuberculosis*- and BCG-induced IFN-γ secretion were significantly reduced ($P = 0.002$ and $P < 0.01$, respectively), while the amount of IL-10 induced by both virulent ($P < 0.01$) and avirulent ($P = 0.002$) mycobacteria was increased in patients with TB. The ESAT6-induced IFN-γ responses were increased in the patients with TB ($P = 0.013$) compared with those in the EC group. When tuberculin skin test (TST)-negative (TST^-; induration, <10 mm) and TST-positive (TST^+) donors were studied separately, both TST^- and TST^+ individuals showed increased IFN-γ responses to *M. tuberculosis* compared with the responses of the patients with TB ($P = 0.037$ and $P = 0.006$, respectively). However, only TST^+ ECs showed reduced IFN-γ responses to ESAT6 ($P = 0.008$) compared with the responses of the patients with TB. The levels of *M. tuberculosis*-induced CCL2 ($P = 0.006$) and CXCL9 ($P = 0.017$) were greater in the patients with TB. The levels of CCL3 secretion in response to *Mycobacterium* and antigen stimulation were comparable between the two groups. While the levels of ESAT6-induced chemokines did not differ between the patients with TB and the ECs, the levels of CFP10-induced CCL2 ($P = 0.01$) and CXCL9 ($P = 0.001$) were increased in the patients. These data indicate differential host IFN-γ, CXCL9, and CCL2 responses to live mycobacteria and mycobacterial antigens and have implications for the identification of potential biomarkers of infection which could be used for the diagnosis of TB.

Tuberculosis (TB) remains an important cause of mortality and morbidity worldwide, causing 2 million deaths annually. Vaccination with *Mycobacterium bovis* BCG provides protection mostly during childhood and primarily against the extrapulmonary forms of the disease (28, 45, 49). It is important to identify immune markers that may be reflective of natural infection in the host and that may therefore be useful in predicting the outcome of the disease.

Host immune responses determine the outcome of *M. tuberculosis* infection. The balance between the key T-cell cytokines gamma interferon (IFN-γ) and interleukin-10 (IL-10), which are produced by macrophages and subsets of T cells, is key to the maintenance of effector responses against the mycobacterium (13, 34). This is further reflected by the relationship between IFN-γ and IL-10, which differs according to the severity of the disease, especially in patients with extrapulmonary *M. tuberculosis* infections (24).

The formation and maintenance of granulomas are essential for the control of mycobacterial infections. Tumor necrosis factor alpha (TNF-α) is critical to an effective response against *M. tuberculosis* and for efficient granuloma formation (38). $TNF^{-/-}$ mice show increased pathology and a more rapid progression of disease during infection with *M. tuberculosis* (6).

IFN-γ- and macrophage-induced TNF-α activates and regulates the C-C chemokines CCL2, CCL3, CCL4, and CCL5 and the C-X-C chemokines CXCL8, CXCL9, CXCL10, and CXCL11 (2) (42). These chemokines have been shown to play an important role in the recruitment and activation of leukocytes at the site of granuloma formation.

CCL2 is the most potent activator of monocytes and is essential for recruitment and migration to the lung. Studies with $CCL2^{-/-}$ mice have investigated the pathology of *M. tuberculosis* infection (26) and have shown that $CCCR2^{-/-}$ mice exhibit faulty granuloma formation and increased susceptibility to disease caused by *M. tuberculosis* (44). IFN-γ-activated chemokines, such as CXCL9 and CXCL10, are induced by *M. tuberculosis* antigens (1, 40).

The circulating levels of cytokines such as IFN-γ, IL-10, transforming growth factor-β, and chemokine CXCL9 are found to be raised in patients with TB (17, 33). Patients with pulmonary TB also display increases in the level of monocyte-activating chemokine CCL2 (20). An increase in the CCL2

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level is associated with monocyte migration to the lung and the reduced progression of disease (26, 43). Recent studies have identified molecular mechanisms which are deficient in patients with pulmonary TB. These include reduced IFN-γ responses at the disease site (21) and a reduced monocyte maturation ability concomitant with a reduction in the levels of markers of antigen presentation, such as major histocompatibility complex class II expression (36). In addition, it has been shown that granulomas differ in cavitary and noncavitary TB (48). This further emphasizes the need to understand the pattern of recognition of mycobacterial antigens in different forms of TB and in transitions from latent to active infection.

Six-kilodalton early secreted antigenic target 6 (ESAT6) and culture filtrate protein 10 (CFP10) are both encoded by region of difference 1 (RD1), which is present in *M. tuberculosis* and *M. bovis* but which is absent from *M. bovis* BCG and most environmental mycobacteria (8, 16). ESAT6 is an immunodominant T-cell-stimulatory antigen and is recognized by specific IFN-γ-secreting T cells that are present in greater numbers in patients with active disease than in those who are uninfected (37, 47). The importance of these recombinant antigens is in their immune recognition by T cells specific to infected individuals. They are a primary component of new diagnostic assays and are meant to detect latent infections in individuals because of their lack of cross-reactivity with *M. bovis* BCG, which is used for vaccination (27). Commercial tests that use IFN-γ-release assays are increasingly available as diagnostic and predictive tests for newer-generation vaccines. ESAT6- and CFP10-induced IFN-γ responses have been shown to be useful in discriminating infected individuals from healthy controls (1, 9, 46). Demissie et al. have shown that immune responses to ESAT6 are more likely to be associated with active infection and that a dormancy-related antigen (HspX or α-crystallin) might be a better marker of latency in patients with TB, while cross-reactive antigens, such as the antigen 85A complex, do not distinguish between active and latent TB (11).

Antigen recognition and binding to cell surface receptors result in cytokine activation and intracellular signaling cascades within the host cell (14, 39). Virulent *M. tuberculosis* organisms are efficient at evading macrophage host defenses, such as phagolysosome fusion; downregulation of the activation of proinflammatory cytokines, such as TNF-α; inhibition of antigen presentation; and also host apoptotic responses (19, 25). In contrast, avirulent mycobacteria, such as the *M. bovis* BCG strain, are processed along the endosomal pathway, activating stronger responses of proinflammatory cytokines, such as TNF-α, IL-2, and IL-6, than the responses activated by virulent mycobacteria (7). In addition, the uptake and processing of live and dead mycobacteria differ in host cells (18). Therefore, although both mycobacteria and mycobacterial antigens can stimulate host immunity, it is likely that their immunogenicities may differ due to processing and presentation of antigens.

There is as yet a limited understanding of how host cell responses differ when the host is challenged with live mycobacteria as opposed to mycobacterial antigens. We investigated the differential cytokine responses to live mycobacteria (virulent *M. tuberculosis* and avirulent *M. bovis* BCG strains) and compared them with those to the *M. tuberculosis* antigens ESAT6 and CFP10. BCG vaccination is widespread in Pakistan and since 1981 has routinely been administered at birth through the Expanded Program for Immunization (51). Therefore, the levels of cytokine and chemokine secretion by peripheral blood mononuclear cells (PBMCs) in response to different stimuli were analyzed in patients with pulmonary TB and were compared with those in healthy vaccinated controls from areas where TB is endemic (ECs). Our results highlight the variability of host responses to mycobacteria and mycobacterial antigens.

MATERIALS AND METHODS

Subject selection. This was a cross-sectional study comparing the PBMC responses of patients with TB with those of BCG-vaccinated healthy donors. Thirty-eight patients with pulmonary TB were recruited from the outpatient clinics of Aga Khan University Hospital and Medical College (AKUH) and Masoomeen Hospital, Karachi, Pakistan. All the subjects were unrelated and were from different parts of urban Karachi. All study subjects were examined, evaluated, and recruited by infectious diseases consultants. The patients were either newly diagnosed with the disease or had taken less than 7 days of antituberculous therapy. All samples were taken after the participants has provided written informed consent. The study received the approval of the Ethical Review Committee of AKUH. The patients had no significant comorbid conditions, including diabetes mellitus, chronic renal failure, or chronic liver disease, and also were not receiving any corticosteroid therapy. Although Pakistan is a setting with a low prevalence of human immunodeficiency virus (HIV) infection, all patients were screened for HIV and tested negative.

The diagnosis of TB was based on clinical examination, chest X ray, Zeihl-Neelsen staining of sputum for acid-fast bacilli (AFB), culture for AFB, and/or the clinical response to treatment (as assessed by the resolution of fever and cough and weight gain). All patients with pulmonary TB had a positive chest X ray, with the findings being consistent with active TB, as evaluated by one of the consulting physicians. All patients showed a favorable clinical response to antituberculous therapy. The patients were diagnosed as having minimal, moderate, or advanced pulmonary TB by using modified classifications of the National Tuberculosis Association of the United States on the basis of the extent of lung tissue involvement (10, 22). Among the patients with pulmonary TB, 10 had minimal disease, while 28 had moderate disease. The patient group comprised 24 females and 14 males, and the mean age was 28.2 ± 12.3 years.

BCG-vaccinated asymptomatic healthy staff at AKUH (mean age, 28.1 ± 6.6 years) with no known exposure to TB were used as ECs. The healthy control group comprised 22 females and 17 males. No member of the control group had a household member with TB, nor did they have any relationship to any of the patients recruited in the study. All volunteers had a normal chest X ray. The tuberculin skin test (TST) was performed by the intradermal administration of 5 tuberculin units on the volar surface of the right arm, and the result was read by a single reader 48 h later. An induration of ≥10 mm was used as a cutoff for a positive response. Both TST-negative (TST^-; $n = 21$) and TST-positive (TST^+; $n = 18$) ECs were included in the study.

***Mycobacterium* culture.** *M. tuberculosis* H37Rv was acquired from ATCC. *M. bovis* BCG (Montreal vaccine strain) was used as described previously (19). All strains were grown to logarithmic phase in 7H9 Middlebrook medium supplemented with 0.02% glycerol, 10% albumin-dextrose-catalase Middlebrook enrichment, and 0.5% Tween 80 (all from Difco Laboratories, Detroit, MI). Aliquots of mycobacteria at 2×10^6 CFU/ml were frozen in growth medium containing 15% glycerol and were stored at −70°C. For the infection assay, aliquots of mycobacteria were freshly thawed, washed three times in phosphate-buffered saline, and diluted as required for the infection. To avoid clumping of the mycobacteria, the cell suspension was briefly sonicated and was then allowed to stand for 5 min to allow the large clumps to settle, leaving behind a suspension of single cells (19). The mycobacterial inoculum was also plated out for each assay to determine bacterial viability, which was greater than 80% in each case.

Reagents. Recombinant antigens ESAT6 and CFP10 were obtained through the TB Vaccine Testing and Research Materials Contract (contract NO1-A1-40091), NIH, NIAID, awarded to Colorado State University.

Isolation and stimulation of PBMCs. PBMCs were obtained by gradient separation of whole blood with Histopaque (Gibco-BRL). The cells were counted with a hemacytometer and plated at 2×10^5 per well in a 24-well tissue culture plate. Bacterial inocula were made in a volume of 250 μl RPMI medium, which was added to each well containing 2×10^5 PBMCs. Subsequently, the cell culture

volume of each well was made up to 1 ml containing 10% autologous serum in RPMI medium.

The time course and the dose-response of the *M. tuberculosis* and BCG infection of PBMCs was performed with PBMCs from healthy donors ($n = 10$). Two concentrations of the mycobacteria, 2×10^5 CFU/ml (infection ratio, 1) and 10^6 CFU/ml (infection ratio, 5), were employed. The levels of IFN-γ secretion in response to *M. tuberculosis* and *M. bovis* BCG infection of PBMCs from healthy control donors were determined at 18 and 48 h postinfection.

The BCG-induced IFN-γ responses did not show any significant difference between infection with 2×10^5 CFU/ml (1,627 ± 2,169 pg/ml) and 10^6 CFU/ml (1,831 ± 2,088 pg/ml) at 18 h. At 48 h, the trend was for the level of IFN-γ secretion to be greater with infection with 2×10^5 CFU/ml (3,794 ± 2,621 pg/ml) than with infection with 10^6 CFU/ml (2,703 ± 2,581 pg/ml), but there was no significant difference between the doses. Similar trends were observed with the dose-response curve for *M. tuberculosis*-stimulated PBMCs, except that the trend in the level of IFN-γ secretion was lower in response to the virulent bacilli at 2×10^5 CFU/ml (1,347 ± 3,107 pg/ml) than in response to the virulent bacilli at 10^6 CFU/ml (1,604 ± 1,796 pg/ml), confirming previous results (19).

For the remaining experiments, PBMCs were infected with *M. tuberculosis* or *M. bovis* BCG at 2×10^5 CFU/ml to obtain an infection ratio of 1 per cell. Although an increase in the level of *Mycobacterium*-induced cytokine secretion was observed at 48 h poststimulation compared with that observed at 18 h poststimulation, we found that the levels at the earlier time point were more suitable for use for the study of cytokine secretion, as there was a greater change in the levels during the earlier period.

A dose-response curve for the ESAT6 and CFP10 antigen stimulation experiments was also determined with concentrations of 2.5, 5, and 10 μg/ml. On the basis of the trends in IFN-γ secretion observed in response to antigen stimulation of PBMCs, we determined that a concentration of 5 μg/ml measured at 18 h poststimulation was optimal for these experiments (data not shown).

PBMCs were also stimulated with ESAT6 (5 μg/ml) and CFP10 (5 μg/ml). All supernatants were collected at 18 to 20 h poststimulation for the measurement of cytokine and chemokine levels. Samples were centrifuged to collect any cellular debris, aliquoted, and stored at −70°C until they were tested.

ELISAs for IFN-γ, IL-10, CCL2, CCL3, and CXCL9. IFN-γ and IL-10 secretion was detected in cellular supernatants by using standards and enzyme-linked immunosorbent assay (ELISA) reagents obtained from Endogen (Rockford, IL). Cytokine levels were measured by a sandwich ELISA technique, according to the manufacturer's instructions and as reported previously (20). Recombinant human cytokine was used to obtain a dose-response curve with a range of detection of from 3.9 to 1,000 pg/ml. All experimental samples were tested in duplicate.

CCL2, CCL3, and CXCL9 standards and monoclonal antibody pairs for capture and detection were obtained from R&D Systems (Abingdon, United Kingdom). All measurements were carried out according to the manufacturer's recommendations and as described previously (20). Recombinant human cytokine was used to obtain a dose-response curve with ranges of detection of from 6.25 to 500 pg/ml for CXCL9 and 6.25 to 1,000 pg/ml for CCL2.

Statistical analysis. Data are represented by box plots that illustrate the 25th and 75th quartiles and the medians, shown as horizontal bars. Nonparametric statistical analysis was performed by using the Kruskal-Wallis and Mann-Whitney U tests, as required. Spearman's rank correlation was also performed. All analyses were performed with Statistical Package for Social Sciences software (SPSS, Inc.).

RESULTS

Increased *M. tuberculosis*- and BCG-induced IFN-γ and IL-10 levels in patients with TB. The *M. tuberculosis*- and *M. bovis* BCG-induced IFN-γ responses in the patients with TB were compared with those in healthy ECs. The levels of IFN-γ and IL-10 secretion in the absence of a stimulant were negligible in both the test and the control groups. The *M. tuberculosis*-induced IFN-γ responses were significantly reduced ($P = 0.002$) in the patients with TB than in the controls (Fig. 1A). Similarly, BCG induced IFN-γ responses were also reduced in the patients with TB ($P < 0.01$) compared with the responses in the controls (Fig. 1B).

We next compared the levels of IL-10 secretion in patients and controls. In contrast to the findings for IFN-γ, *M. tuberculosis*-induced IL-10 levels were significantly raised in patients compared with the levels in the controls ($P < 0.01$) (Fig. 1C). Similarly, BCG-induced IL-10 levels were also greater in the patients with TB than in the controls ($P = 0.002$) (Fig. 1D). The magnitude of IL-10 induced by *M. tuberculosis* in the patient group was severalfold greater than that induced by BCG stimulation of PBMCs ($P = 0.047$).

Mycobacterial antigens ESAT6 and CFP10 induced IFN-γ and IL-10 secretion in patients with TB. To investigate whether the *M. tuberculosis*-induced chemokine responses were reflective of those induced by recombinant antigens from the bacilli, we stimulated PBMCs with both ESAT6 and CFP10. The ESAT6-induced IFN-γ responses were significantly increased ($P = 0.013$) in the patients with TB (median, 41 pg/ml) compared with those in the controls (median, 2 pg/ml) (data not shown), corresponding with previous findings (9). The magnitude of IFN-γ secretion in response to ESAT6 in the healthy control group was also severalfold lower than that in response to *M. tuberculosis* ($P = 0.043$).

The level of ESAT6-induced IL-10 secretion was greater in the patients with TB than in the ECs (medians, 3 and 0 pg/ml, respectively; $P = 0.033$). Although the IFN-γ responses induced by CFP10 stimulation of PBMCs were stronger than those elicited by ESAT6 stimulation of PBMCs, the responses were comparable between the patients with TB (median, 225 pg/ml) and ECs (median, 135 pg/ml). Similarly, the level of IL-10 induced by CFP10 was also higher than that induced by ESAT6, but the levels were not significantly different between the patients with TB and the ECs (medians, 468 and 301 pg/ml, respectively).

***M. tuberculosis*- and ESAT6-induced IFN-γ in TST^- and TST^+ healthy controls.** Previous studies have described the utility of ESAT6-stimulated IFN-γ responses for the detection of latent TB (4) (12). Our healthy EC group comprised subjects with both negative and positive TST reactions to purified protein derivative, as determined by a positive induration greater than or equal to 10 mm. We determined both the *M. tuberculosis*- and the ESAT6-induced IFN-γ responses in both TST^- and TST^+ individuals. As shown in Fig. 2A, there was no difference in the *M. tuberculosis*-induced IFN-γ response between TST^- and TST^+ healthy controls. Compared with the patients with TB, both TST^- and TST^+ healthy controls secreted significantly greater levels of IFN-γ upon stimulation with *M. tuberculosis* ($P = 0.037$ and $P = 0.006$, respectively). In the same groups, the ESAT6-induced IFN-γ responses of the TST^+ controls were significantly reduced ($P = 0.008$) compared with those of the patients with TB (Fig. 2B). The ESAT6-induced IFN-γ levels in the TST^- controls and the patients with TB were comparable.

***Mycobacterium*-induced chemokine levels are raised in patients with TB.** We subsequently investigated the *Mycobacterium*-induced chemokine levels in the patients with TB and the controls. The levels of secretion of CCL2, CCL3, and CXCL9 in the absence of stimulants were comparable between the patient and the control groups (data not shown). Both *M. tuberculosis* and BCG stimulated CCL2 secretion in all patients with TB, while responses to either mycobacterium were present in only a limited number (five or six) of ECs, and the responses occurred in both TST^- and TST^+ ECs. The level of *M. tuberculosis*-induced CCL2 was significantly greater in the

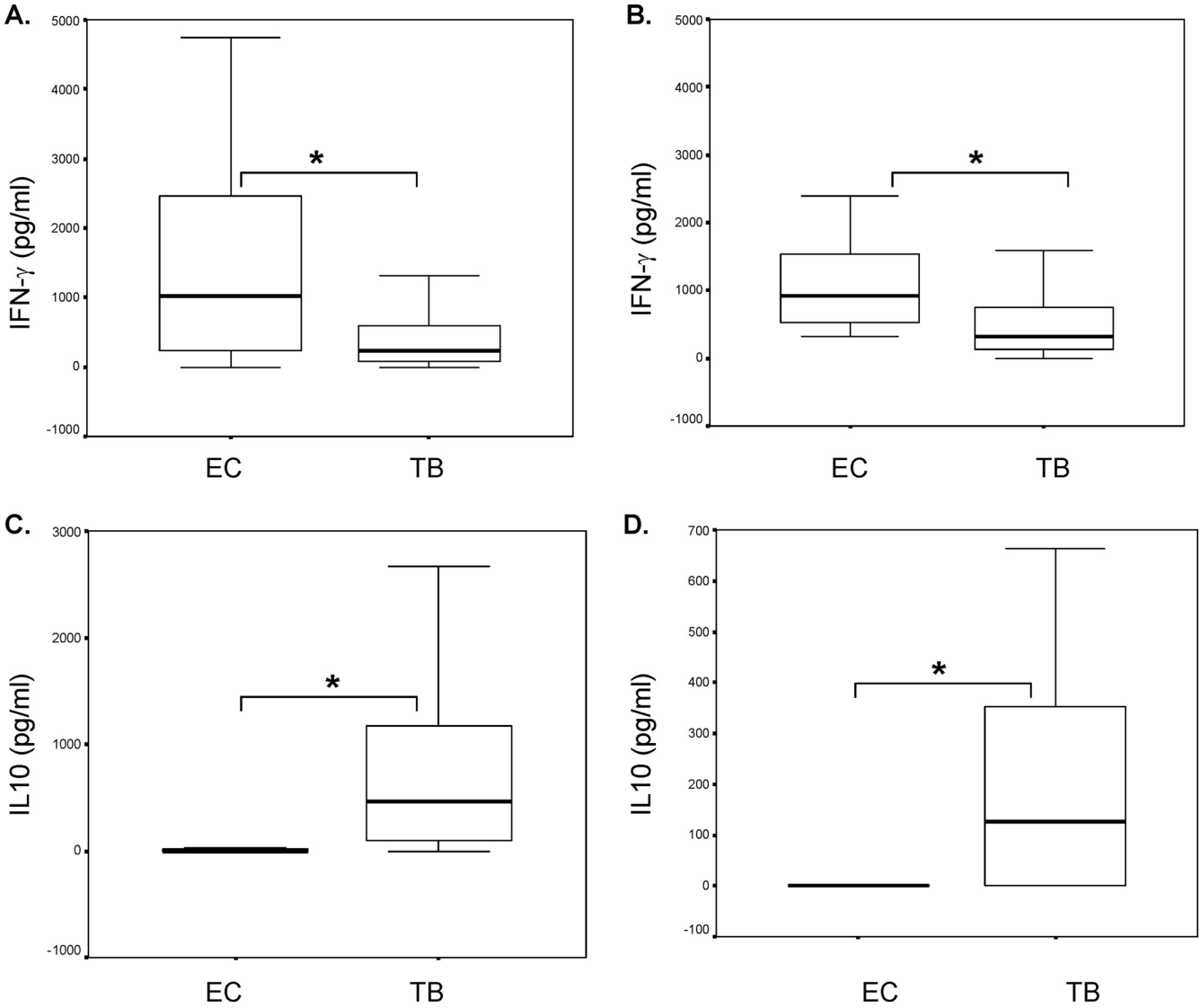

FIG. 1. Differential *Mycobacterium*- and mycobacterial antigen-induced IFN-γ responses in patients with TB. PBMCs (2×10^5) from the patients with TB and the ECs were infected with *M. tuberculosis* or BCG at 2×10^5 CFU/well. Supernatants were harvested at 18 h poststimulation for the measurement of IFN-γ and IL-10 levels. The data are shown as the means of individual experiments from the patients with TB ($n = 38$) and ECs ($n = 39$). The box plots represent the data for each group after the level of cytokine secretion from unstimulated cells was subtracted. The whiskers indicate the 25th and 75th quartiles, while a line indicating the median separates the two. *, significant differences between groups ($P < 0.05$). (A) IFN-γ induction by *M. tuberculosis*; (B) IFN-γ induction by BCG; (C) IL-10 induction by *M. tuberculosis*; (D) IL-10 induction by BCG.

patients with TB than in the controls ($P = 0.006$), Fig. 3A. The level of BCG-induced CCL2 secretion also showed a trend to be greater in the patients with TB, but the levels were not significantly different between the patients and the controls ($P = 0.074$). The level of *M. tuberculosis*-induced CCL2 secretion in the patients with TB was significantly greater than that in response to BCG infection ($P < 0.01$) (data not shown).

The levels of *M. tuberculosis*-induced CCL3 were comparable between the patients and the controls (medians, 1,306 and 1,800 pg/ml, respectively), and the levels of BCG-induced CCL3 were also comparable between the two groups (medians, 1,592 and 1,919 pg/ml, respectively).

The level of *M. tuberculosis*-induced CXCL9 was significantly greater in the patients with TB ($P = 0.017$) than in the controls (Fig. 3B). The level of BCG-induced CXCL9 secretion was negligible in both the ECs and the patients with TB (medians 0 and 0, pg/ml, respectively) (data not shown).

ESAT6- and CFP10-induced chemokines in patients with TB. The levels of ESAT6-induced CCL2 did not differ between the patients with TB and the healthy controls (medians, 1,046 and 443 pg/ml, respectively). However, the level of CFP10-induced CCL2 was significantly greater in the patients with TB than in the healthy controls ($P = 0.01$) (Fig. 4A).

The levels of ESAT6-induced CCL3 were comparable between the patients with TB and the ECs (medians, 1,567 and 1,590 pg/ml, respectively). CFP10 induced higher levels of

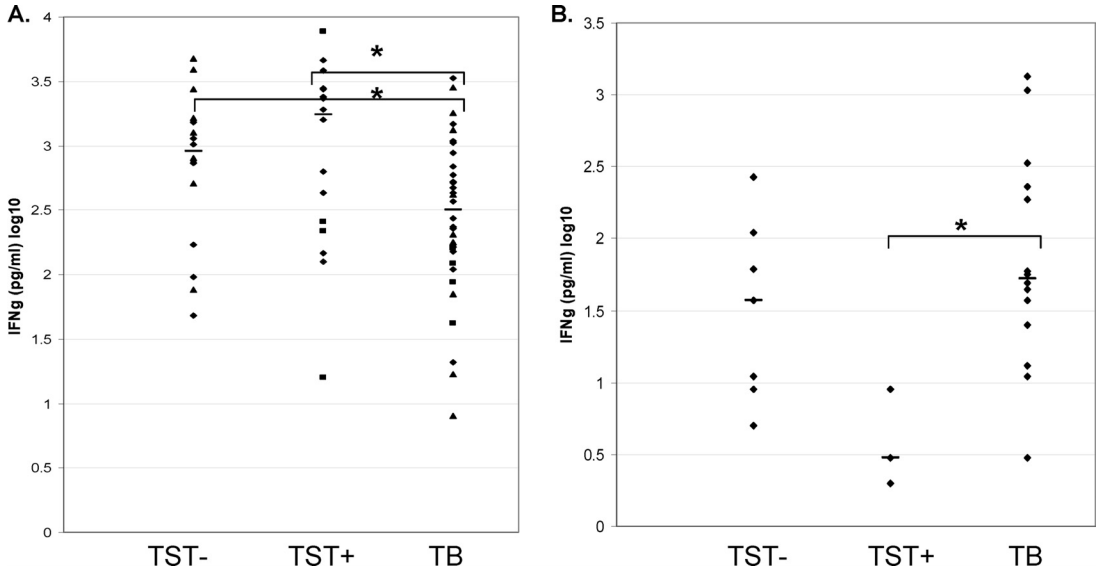

FIG. 2. Differential IFN-γ responses in TST^+ and TST^- controls. Data for PBMCs from the TST^+ and TST^- controls and the patients with TB after stimulation with *M. tuberculosis* (A) or mycobacterial antigen ESAT6 (B) at 5 μg/ml and measurement of the levels of IFN-γ (IFNg) secretion at 18 h postinfection are shown. Each data point is represented on the scatter graph, and median values are indicated by a horizontal line.

CCL3 in the patients with TB, but the level was not significantly greater than that in the ECs (medians, 2,076 and 2,200 pg/ml, respectively).

The levels of ESAT6-induced CXCL9 were similar between the patients and the controls (medians, 0 and 0 pg/ml, respectively). However, the level of CFP10-induced CXCL9 was significantly greater ($P = 0.001$) in the patients than in the controls (Fig. 4B).

DISCUSSION

Our results indicate that the IFN-γ responses to a live mycobacterial infection caused by either virulent *M. tuberculosis* or the avirulent BCG vaccine strain differ from those to mycobacterial antigens ESAT6 and CFP10.

The *Mycobacterium*-induced IFN-γ suppression observed in the patients with pulmonary TB corresponds to the findings of

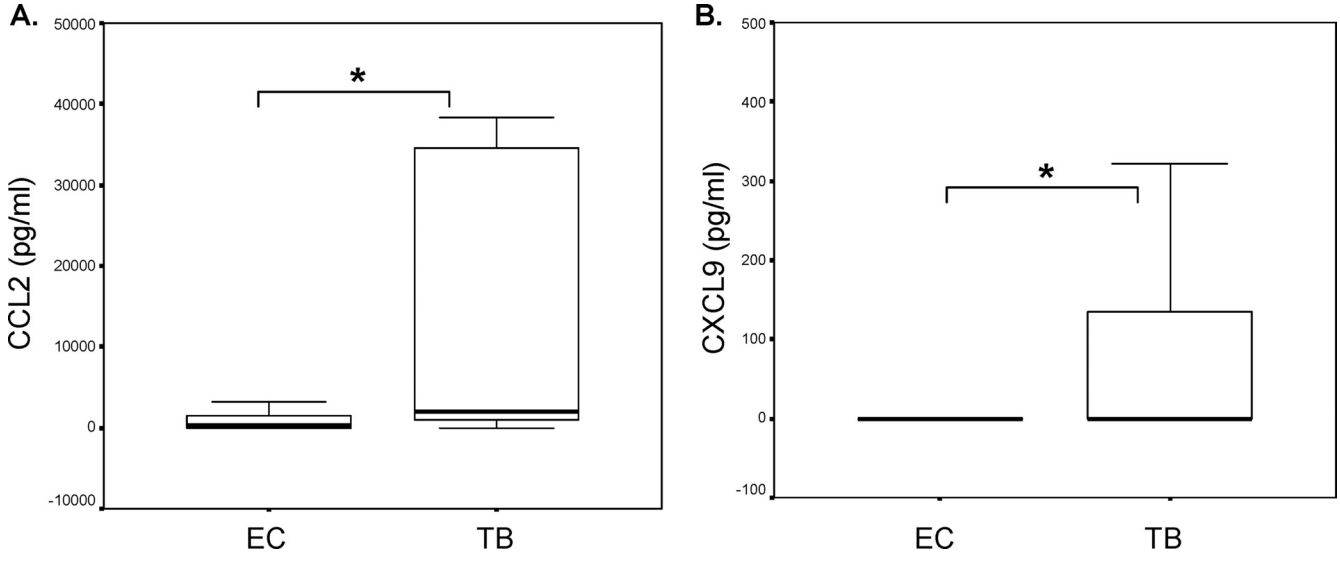

FIG. 3. Increased levels of CCL2 (A) and CXCL9 (B) secretion in response to *M. tuberculosis* in patients with active TB. PBMCs from the patients with TB and the ECs were infected with *M. tuberculosis*, as described in the legend to Fig. 1. The supernatants were harvested at 18 h poststimulation for measurement of cytokine levels. The means of individual experiments with PBMCs from the patients with TB ($n = 38$) and ECs ($n = 39$) are shown. The box plots represent data from each group after the levels of CCL2 and CXCL9 secretion from unstimulated cells were subtracted. The whiskers indicate the 25th and 75th quartiles, while a line indicating the median separates the two. *, significant differences between groups ($P < 0.05$).

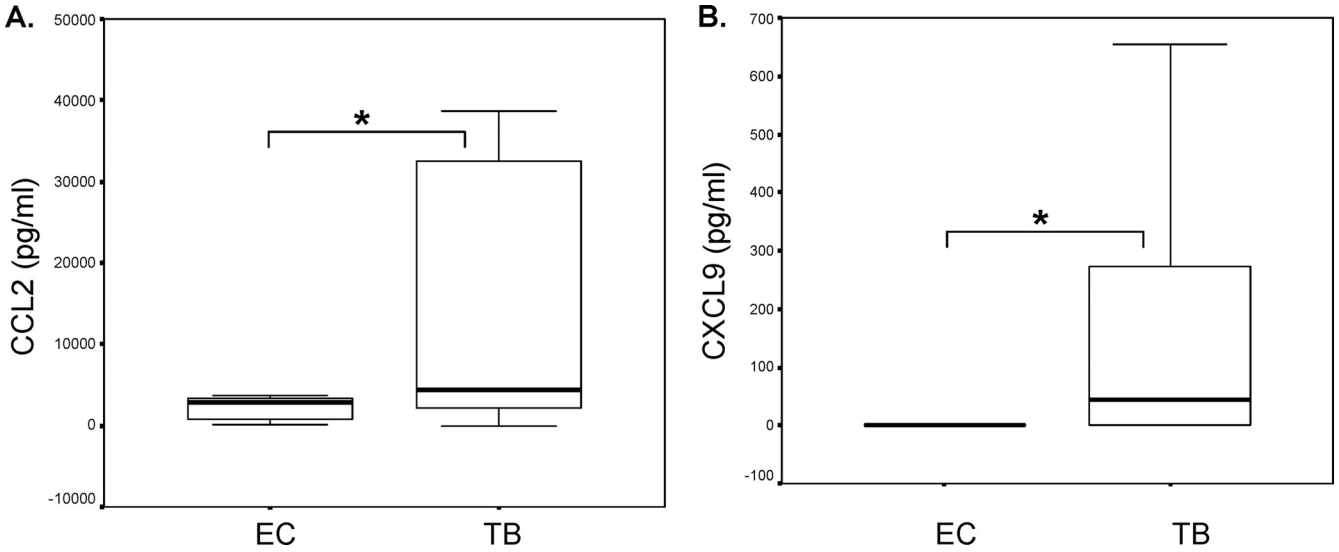

FIG. 4. CFP10 is a more potent stimulator of CCL2 (A) and CXCL9 (B) in patients with TB. PBMCs from the patients with TB and the ECs were stimulated with CFP10 at 5 μg/ml for 18 h, and the chemokine levels in the cell supernatants were measured. The means of individual experiments with PBMCs from the patients with TB ($n = 38$) and the ECs ($n = 39$) are shown. The box plots represent data from each group after the level of CXCL9 secretion from unstimulated cells was subtracted. The whiskers indicate the 25th and 75th quartiles, while a line indicating the median separates the two. *, significant differences between groups ($P < 0.05$).

previous studies, which indicate the lowering of T-cell-mediated immunity in patients with TB (41). The increased levels of *M. tuberculosis*- and BCG-induced IL-10 in the patients with TB also corroborate previous data (41).

We found the ESAT6-induced IFN-γ responses to be increased in the individuals with active TB. This also corresponds to the findings of studies that have indicated that ESAT6-induced IFN-γ may be an indicator of antigen-specific responses in infected individuals (35, 37). The levels of ESAT6-induced IL-10 were not different between the controls and the patients. Also, the levels of CFP10, which has previously been shown to also elicit *M. tuberculosis*-specific IFN-γ responses in infected individuals, were not found to be different between the patients with TB and the healthy ECs.

Previous studies have correlated TST positivity with mycobacterial antigen-induced IFN-γ responses (4, 31) but have shown that this correlation is decreased in BCG-vaccinated donors (9). When we compared the IFN-γ responses of TST^+ and TST^- individuals, they were not significantly different, as has previously been shown in areas with high rates of TB transmission (3). As all subjects were BCG vaccinated according to Pakistan immunization guidelines, TST positivity cannot be attributed to BCG vaccination. As Pakistan has a very high incidence of TB at 181/100,000 population (50), the possibility of exposure to *M. tuberculosis* cannot be ruled out, nor can exposure to environmental mycobacteria. Recent studies of household contacts of patients with TB show that the time postexposure at which the IFN-γ response to *M. tuberculosis* is measured determines the cytokine profile obtained (23).

However, when the responses of both TST^+ and TST^- healthy controls were separately compared with those of the patients with active TB, it was found that the TST^+ individuals made significantly larger amounts of IFN-γ in response to *M. tuberculosis* than the patients with TB. These data indicate that *M. tuberculosis*-specific immunity was primed in TST^+ controls, probably due to latent infection. When the responses to ESAT6 were evaluated, it was found that the IFN-γ responses of the TST^+ controls were suppressed compared with those of patients with active TB. These data indicate that protective immunity to *M. tuberculosis* infection may be engendered by antigens/epitopes other than the RD1 antigen ESAT6. Reports by Demissie et al. have indicated that while ESAT6 may elicit responses indicative of active TB infection, the responses to other antigens, such as α-crystallin, may be more useful for the detection of latent infection (11). This has implications for vaccine development, as ESAT6 is one of several candidate antigens being employed in trials in which its protective efficacy against *M. tuberculosis* is being tested (5, 32).

Recent literature has indicated an important role for CCL2 as an adjunct biomarker to be used with IFN-γ for assessment of the response to *M. tuberculosis* infection (40). CCL2 levels are increased in patients with pulmonary TB (20), and here we have shown that the CCL2 level was increased in response to both *M. tuberculosis* infection and BCG infection. In addition, both ESAT6- and CFP10-induced CCL2 levels were increased in the patients with TB. The magnitude of CCL2 elicited in response to *M. tuberculosis* was significantly greater than that elicited in response to BCG, indicating activation by specific antigenic components. Of the recombinant antigens, the magnitude of CCL2 secretion in response to CFP10 was found to be greater than that in response to ESAT6. This further suggests a role of CCL2 as a biomarker for the identification of *M. tuberculosis* infection.

We found the level of *M. tuberculosis*-induced CXCL9 secretion to be increased in the patients with TB, but BCG did not induce CXCL9. Previous studies have shown increased levels of CXCL9 in the PBMCs of patients with TB stimulated with a recombinant fusion protein, ESAT6-CFP10 (1). Our data indicate that the levels of ESAT6-induced CXCL9 were not different between the patients and the controls, but the

levels of CFP10-induced CXCL9 were increased in the patients with TB. Therefore, it may be the CFP10 component of the ESAT6-CFP10 recombinant protein that elicits CXCL9 activation.

These data are important, as an increasing amount of information in the literature states the need to study biomarkers, in addition to IFN-γ, for the testing of individuals for infection with *M. tuberculosis*. However, ESAT6 and CFP10 remain the antigens of choice. Recent studies compared the IFN-γ response to H37Rv-encoded antigens, in addition to those of RD1, including the antigen 85A complex, antigen 85A complex peptides, and heparin-binding hemagglutinin adhesin (29, 30). However, reports from regions where TB is endemic indicate that the use of these antigens for the diagnosis of active TB remains limited (3, 15). Therefore, it is important to use multiple antigens in screening assays in order to elicit a larger number of antigenic responses. This may be particularly important in regions of endemicity with a larger number of latently infected individuals. Such exposed individuals would respond differently from uninfected community controls, regardless of their TST positivity. Thus, it would not be possible to evaluate them by using the standard criteria expected of the IFN-γ assays used in regions where TB is not endemic.

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