Identification of Cytokines in Whole Blood for Differential Diagnosis of Tuberculosis versus Pneumonia

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Differentiating tuberculosis (TB) from pneumonia remains a challenge. We evaluated the cytokine profiles of whole blood cells from patients with TB (n = 38) or pneumonia (n = 30) and from healthy individuals (n = 30) before and after stimulating cells with ESAT-6 or lipopolysaccharide (LPS). When the percent change in the levels of gamma interferon (IFN-γ) after stimulation with ESAT-6 was used in receiver operating characteristics (ROC) analysis (a graphic method to determine the diagnostic accuracy of a test) to identify a patient with TB, the area under the curve (AUC) was 90.4%, and a cutoff point of a 3.59% change produced a corresponding sensitivity, specificity, and accuracy of over 80%. When the change in IFN-γ after stimulation of blood cells with LPS was used to identify a patient with pneumonia, the AUC reached 89.1%, and a cutoff point of 3.59% produced a sensitivity, specificity, and accuracy of approximately 80% each. When the change in interleukin-12 (IL-12) after stimulation of blood cells with LPS was used to identify a patient with pneumonia, the AUC was 85.2%, and a cutoff point of 2.08% gave a sensitivity, specificity, and accuracy of 80.0%, 78.9%, and 79.4%, respectively. We conclude that the percent change in IFN-γ after stimulation of whole blood cells with ESAT-6 may differentiate patients with TB from patients with pneumonia. The percent change in IFN-γ and IL-12 after LPS stimulation of whole blood cells could differentiate patients with pneumonia from patients with TB.

Differentiating pulmonary tuberculosis (TB) from pneumonia caused by other infectious organisms remains a serious challenge. Examination of sputum smears for acid-fast bacilli (AFB) is relatively insensitive, and culture for Mycobacterium tuberculosis takes days to weeks to produce a result (10). The use of the tuberculin skin test (TST) to diagnose TB is limited in regions where a large fraction of the population has been vaccinated with Mycobacterium bovis bacillus Calmette-Guerin (BCG) (11). Therefore, recent research has focused on identifying M. tuberculosis-specific antigens. One of these, early secretory antigen target-6 (ESAT-6), is encoded by a genetic segment that is absent from BCG (25), and two commercial tests have been developed on the basis of the enhanced ability of ESAT-6 (and other antigens) to simulate production of gamma interferon (IFN-γ) in blood samples from patients with TB (18). Although useful for the diagnosis of TB, a study examining the utility of such tests for differentiating TB from other pulmonary diseases has demonstrated rather low specificity and sensitivity (76% and 79%, respectively), presumably because of the background of underlying respiratory disease in the patients (17).

The objective of this study was to determine whether other cytokine markers could be identified that would be more useful for the differential diagnosis of TB and pneumonia from blood samples. We used microarrays coated with antibodies against tumor necrosis factor alpha (TNF-α), interleukin-6 (IL-6), IL-8, IL-10, monocyte chemoattractant protein 1 (MCP-1), IFN-γ, IL-1β, IFN-induced protein 10 (IP-10), and IL-12 to evaluate the cytokine profiles of whole blood cells collected from patients with TB or pneumonia and from healthy individuals. The cytokine profiles were determined before and after stimulation with ESAT-6 (to magnify the cytokine response in patients with TB) and lipopolysaccharide (LPS) to amplify the response in patients with pneumonia in terms of both the absolute levels and the percent differences before and after stimulation.

MATERIALS AND METHODS

Patients. This study was approved by the local Institutional Review Board, and informed consent was obtained from all participants. From January 2007 to December 2007, 38 patients with pulmonary TB, 30 patients with pneumonia, and 30 healthy participants were recruited from Tri-Service General Hospital in Taiwan. Patients with pulmonary TB were enrolled according to the following inclusion criteria: primary TB defined by the presence of middle or lower lung infiltration with or without ipsilateral hilar lymphadenopathy, secondary TB defined by the presence of unilateral or bilateral upper lung cavitiation or infl-
rection of the right upper lobe apical and posterior segments or the left lobe apical-posterior segment, or a definitive diagnosis of TB established by isolation of tubercle bacilli in culture or identification of specific nucleic acid sequences (TB-PCR) from sputum or bronchoalveolar lavage fluid (BALF) (6). Patients with pneumonia were enrolled according to the following inclusion criteria: the presence of community-acquired pneumonia diagnosed by the American Thoracic Society (ATS) guideline (19), the presence of pulmonary infiltrate in chest films that mimicked pulmonary TB but negative results of sputum acid-fast smear and TB culture, and resolution of the pulmonary infiltrate within 1 week after antibiotic therapy. Patients with pneumonia were excluded if their chest films showed a delay of resolution lasting more than 1 month or if they had a history of pulmonary TB. The healthy control participants were enrolled from patients undergoing physical examinations. Any healthy participants who had a history of pulmonary infection or TB, type 2 diabetes, diseases causing immunosuppression (e.g., collagen vascular disease), or malignant tumors were excluded from the study.

Preparation and stimulation of samples. Three-milliliter blood samples were collected from all participants (before intensive anti-TB therapy in the patients with TB) in the presence of dry lithium heparin and were aliquoted into three samples, added into a 48-well cell culture plate, and stimulated by adding 1 μl of 1 μg/μl LPS or 1 μg/μl ESAT-6 to the wells containing 999 μl of blood from the first two samples, respectively. An equal volume (1 μl) of normal saline was added to the third unstimulated sample. After stimulation, the plates were placed in a carbon dioxide incubator for 24 h and then centrifuged at 3,000 rpm for 10 min. The supernatants were collected, and a 10-μl plasma sample was used for analysis.

Identification of cytokine markers. Initial cytokine expression was evaluated with a commercially available protein array membrane (RayBio Human Cytokine Antibody Array C, Series 1000; RayBiotech Inc.) for which detection was enhanced with a chemiluminescence kit (Amersham Pharmacia Biotech). This assay consisted of antibodies against 174 different cytokines and chemokines spotted in duplicate onto three membranes. Detection was performed according to the manufacturer’s instructions. Samples from three patients with TB and three healthy controls were stimulated and prepared as described above. From a preliminary protein array membrane assay, we identified TNF-α, IL-8, IL-10, MCP-1, IFN-γ, IL-1β, IP-10, and IL-12 as the candidate marker cytokines for differential diagnosis.

Cytokine assay. Protein array chips were subsequently prepared for the cytokine assay. Briefly, monoclonal antibodies against human cytokines TNF-α, IL-6, IL-8, IL-10, MCP-1, IFN-γ, IL-1β, IP-10, and IL-12 (R&D Systems, Inc.) were dissolved in sterile phosphate-buffered saline (PBS) at a concentration of 500 μg/ml and printed by the Microsys 5100 Microarray system (Cartesian Technology) onto silylated glass slides with reactive acetaldehyde groups (CSS-100 silylated glass slides; CEL Associates, Houston, TX) in four by seven microarrays. PBS plus 1% bovine serum albumin (BSA) and 0.05% Tween-20 (PBSB) and biotinylated antibody against human cytokines were also printed onto the slides to serve as negative and positive controls, respectively. The anticytokine arrays were printed at 4°C for 24 h and then placed in PBS containing 2% bovine serum albumin (2% PBSB) at room temperature for 30 min. Afterwards, the anticytokine arrays were washed with PBS containing 0.025% Tween-20 (0.025% PBSB) for 1 min and with PBS for 2 min before being dried by centrifugation. Blood samples were collected from all of the participants, stimulated, and prepared as described above. Ten-microliter plasma samples were added onto the corresponding blocks of cytokine arrays at room temperature and removed after 30 min. The cytokine arrays were washed in 0.025% PBSB for 3 min and dried by centrifugation. A mixture of nine biotinylated antibodies against the selected nine test human cytokines (R&D Systems, Inc.) was diluted with PBSB to give a final concentration of 40 μg/ml (1:200 dilution). This diluted mixture was added onto the cytokine arrays at room temperature for 30 min and then removed. The arrays were washed with 0.025% PBSB for 3 min and with PBS wash buffer for 3 min before being dried by centrifugation. Then, Cy5-conjugated streptavidin (Jackson ImmunoResearch laboratories, Inc.) diluted at 1:1,000 with PBSB was added onto the corresponding blocks of cytokine array at room temperature for 20 min and then removed. The arrays were washed as described above and dried by centrifugation. The cytokine arrays were examined with a microarray scanner (Genepix 4000B), and the intensity of fluorescence at an absorbance wavelength of 635 nm was measured and analyzed by GenePix Pro, version 6.0, software. The fluorescence values of unstimulated specimens (background values) were subtracted from the values for stimulated specimens in all cases.

Statistical analysis. Because of the skewed distribution of residuals, continuous variables were expressed as median and interquartile range (IQR). In addition, numbers and percentages were computed and are shown for the categorical variables. A Kruskal-Wallis test was used to examine the differences in age and laboratory data among the three groups of participants; once a significant result was reported, a Mann-Whitney U test was then performed for posthoc tests. For analysis of the nine cytokines and the corresponding changes after stimulation with either ESAT-6 or LPS, the age-adjusted differences among various groups were tested using Friedman’s test. Friedman’s test was also used to analyze the differences in expression levels of cytokines among the various stimulations for a given patient group. In order to distinguish patients with TB from those with pneumonia, a receiver operating characteristic (ROC) curve was used to select an appropriate cutoff point giving similar sensitivity and specificity values. The chosen cutoff point will be applied to distinguish patients with TB and pneumonia in the future. An α value of 0.05 indicated significance; when posthoc tests were necessary, the significance level was changed to 0.017 (α/3 = 0.005/3, or 0.017) to compensate for the increased probability of making a type I error when multiple comparisons are made. All statistical analysis was performed using SAS, version 9.1.3, software (SAS Inc., Cary, NY).

RESULTS

The demographic characteristics of the 98 participants are summarized in Table 1. The three groups were similar with respect to distribution by sex and smoking habit, but the participants in the healthy control group were significantly younger than those in the pneumonia or TB groups. The group with pneumonia had higher white blood cell counts, more neutrophils, and fewer lymphocytes while the hemoglobin and monocyte counts in the patients with pneumonia and TB were significantly lower than those in the healthy participants.

Comparison of cytokine levels between the control and TB groups. The differences in the levels of nine cytokines in whole blood among the three groups under different conditions of stimulation are shown in Table 2. The unstimulated samples of whole blood from the patients with TB displayed higher levels of IL-8, MCP-1, IP-10, and IFN-γ than the control group after controlling for the difference in ages. When the whole-blood samples were stimulated with ESAT-6, the samples from the patients with TB displayed higher levels of IL-6, IL-8, MCP-1, and IFN-γ than those from the control group. When the whole-blood samples were stimulated with LPS, the samples from the patients with TB displayed higher levels of IL-8 and MCP-1.

Comparison of cytokines between the control and pneumonia groups. After adjustment for the difference in ages, the unstimulated whole-blood samples from the patients with pneumonia had significantly higher levels of IL-8, IL-1β, and MCP-1 than those from the healthy participants. When samples were stimulated with ESAT-6, those from the patients with pneumonia had higher levels of MCP-1 than those from the healthy participants. When the samples were stimulated with LPS, the patients with pneumonia had higher levels of IL-8 than the healthy participants.

Comparison of cytokines between the TB and pneumonia groups. No differences in the levels of the nine cytokines were observed between the TB and pneumonia groups when the samples were either unstimulated or stimulated with ESAT-6. After stimulation with LPS, however, the samples from the patients with pneumonia had higher levels of IL-10 and IFN-γ than those from the patients with TB.

Percent changes in levels of cytokines after stimulation: patients with TB versus healthy participants. In addition to the higher absolute levels of these cytokines after stimulation with ESAT-6, the samples from the patients with TB also showed significantly greater changes in the levels before and
after stimulation for cytokines IL-6, IL-8, IL-10, MCP-1, and IFN-γ than the samples from the control group (Table 3). In contrast, there were no significant differences between the patients with TB and the healthy participants with respect to the changes in the levels of cytokines after stimulation with LPS except for IL-12, which showed a significantly greater change in the healthy participants than in the patients with TB group.

**Percent changes in levels of cytokines after stimulation: patients with pneumonia versus healthy participants.** Stimulation of the samples from the pneumonia group with ESAT-6 produced a change in the level of MCP-1 that was significantly greater than that in the control group. After stimulation with LPS, the changes in the levels of IL-8 and IL-10 were significantly greater than those from the patients with pneumonia. After stimulation with LPS, the samples from the patients with pneumonia showed significantly greater changes in secretion of IL-12 and IFN-γ (Table 3).

**ROC analysis to distinguish the patients with pneumonia and TB.** An ROC curve is a way to present the characteristics of a diagnostic test such that the y axis represents the sensitivity and the x axis represents the specificity obtained when the cutoff value that separates healthy individuals from ill ones varies. An example of the ROC analysis for the percent change in IFN-γ is included as Table S1 in the supplemental material. As the cutoff value varies, the corresponding sensitivity and specificity change. The cutoff at which the values for the sensitivity and specificity are closest to each other (in this example, 3.59%) was selected as the optimal cutoff point.

**DISCUSSION**

The production of cytokines has been an area of active research in the search for biomarkers for diagnosis and monitoring of therapy for TB. Sputum levels of cytokines, such as IFN-γ, TNF-α, IL-8, and IL-6, have been reported to correlate with disease activity during active pulmonary TB (22). Several investigations of the value of cytokines measured in pleural fluids have been reported (1, 13, 20). However, sputum and pleural fluids are not as easily obtained as blood samples. In this study, we report the identification of several cytokine markers that can be measured in whole blood and used to differentiate TB from pneumonia.
We performed a preliminary protein array membrane assay comparing stimulated samples from patients with TB and healthy individuals to identify candidate marker cytokines for differential diagnosis of TB and pneumonia. The markers we identified included TNF-α, IL-6, IL-8, MCP-1, IFN-γ, IL-1β, IP-10, and IL-12. TNF-α and interleukins 6, 8, and 1β are proinflammatory cytokines associated with a variety of infections. TNF-α has been shown to play a role along with IFN-γ in the formulation and maintenance of granuloma (15, 16). IL-6 is secreted by both macrophages and T cells and recruits and activates monocytes (7). MCP-1 targets monocytes and T cells (5, 21) and has been reported to be elevated in the plasma of patients with TB (14). IFN-γ is a well-established marker for TB (18). IL-10 is a downregulatory cytokine that inhibits overproduction of the proinflammatory cytokines (8). Interferon-induced protein 10 (IP-10) is a CXC chemokine produced primarily by monocytes and T cells. IL-12 is a Th1 cytokine that has been hypothesized to be decreased in patients with the early stage of active TB and increased after therapy (9, 24). Interleukins 6, 8, 10, 12, and IFN-γ in plasma or bronchoalveolar lavage fluid (BALF) have all been evaluated recently as possible markers for the prognosis of patients with pneumonia (4, 26). Thus, all of these markers have the potential to provide useful information about the infection status of patients with TB.

Our results showed significant differences in the expression

| Cytokine and condition | Healthy individuals (n = 30) | Patients with pneumonia (n = 30) | Patients with tuberculosis (n = 38) | P valueb | Posthoc testc |
|------------------------|----------------------------|---------------------------------|---------------------------------|----------|--------------|
| IL-6                   | Unstimulated               | 10.7 (10.7, 10.8) A             | 10.7 (10.7, 11.3) A             | 0.131    | NA           |
|                        | ESAT-6                    | 11.6 (10.8, 13.7) B             | 14.1 (11.1, 49.3) B             | 0.014*   | H < T        |
|                        | LPS                       | 175.9 (60.4, 245.7) C           | 42.8 (24.1, 172.1) C            | 0.161    | NA           |
| IL-8                   | Unstimulated               | 748.0 (735.0, 760.2) A          | 778.4 (748.6, 1100.6) A         | 0.003*   | H < P = T    |
|                        | ESAT-6                    | 797.6 (763.1, 997.0) B          | 1461.0 (1244.6, 2341.5) B       | <0.001*  | H < T        |
|                        | LPS                       | 1064.6 (899.1, 1920.3) C        | 3249.2 (1738.0, 4743.1) C       | <0.001*  | H < P = T    |
| IL-10                  | Unstimulated               | 30.2 (28.8, 32.9) A             | 31.3 (30.4, 37.8) A             | 0.869    | NA           |
|                        | ESAT-6                    | 31.7 (30.0, 34.1) A             | 35.8 (31.7, 49.4) B             | 0.236    | NA           |
|                        | LPS                       | 34.5 (30.8, 39.5) B             | 48.1 (36.6, 60.5) C             | 0.015*   | T < P        |
| IL-12                  | Unstimulated               | 183.5 (177.9, 196.3) A          | 184.9 (180.5, 212.0) A          | 0.254    | NA           |
|                        | ESAT-6                    | 184.0 (179.8, 201.1) B          | 188.7 (183.2, 212.7) B          | 0.384    | NA           |
|                        | LPS                       | 199.3 (190.7, 240.7) C          | 191.7 (194.3, 237.3) C          | 0.097    | NA           |
| IL-1β                  | Unstimulated               | 26.6 (26.5, 27.1) A             | 27.0 (26.8, 30.2) A             | 0.004*   | H < P        |
|                        | ESAT-6                    | 27.8 (26.9, 34.4) B             | 30.5 (27.4, 41.1) B             | 0.470    | NA           |
|                        | LPS                       | 171.5 (100.8, 325.9) C          | 82.9 (46.8, 225.7) C            | 0.735    | NA           |
| MCP-1                  | Unstimulated               | 2792.5 (2781.8, 2863.3) A       | 3154.6 (2924.7, 3851.7) A       | 0.001*   | H < P = T    |
|                        | ESAT-6                    | 2804.9 (2796.4, 2985.2) B       | 3961.7 (3500.9, 4559.9) B       | <0.001*  | H < P = T    |
|                        | LPS                       | 2831.1 (2805.8, 3575.6) C       | 4028.7 (3464.8, 5207.9) B       | <0.001*  | H < T        |
| IP-10                  | Unstimulated               | 171.5 (167.6, 183.1) A          | 186.4 (178.0, 255.0) A          | 0.016*   | H < T        |
|                        | ESAT-6                    | 193.0 (175.2, 236.3) B          | 215.6 (185.5, 278.2) B          | 0.125    | NA           |
|                        | LPS                       | 975.0 (402.5, 2716.9) C         | 279.6 (207.2, 390.1) C          | 0.145    | NA           |
| TNF-α                  | Unstimulated               | 23.6 (22.9, 27.2) A             | 23.9 (23.1, 25.5) A             | 0.712    | NA           |
|                        | ESAT-6                    | 25.7 (23.4, 30.8) B             | 26.5 (23.7, 31.6) B             | 0.074    | NA           |
|                        | LPS                       | 76.0 (44.5, 158.5) C            | 45.3 (28.3, 414.8) C            | 0.220    | NA           |
| IFN-γ                  | Unstimulated               | 25.8 (24.9, 27.4) A             | 29.0 (27.7, 32.5) A             | 0.005*   | H < T        |
|                        | ESAT-6                    | 26.0 (25.1, 27.3) AB            | 29.3 (27.8, 34.6) A             | 0.002*   | H < T        |
|                        | LPS                       | 27.1 (25.3, 28.6) B             | 36.4 (29.7, 43.2) B             | 0.007*   | T < P        |

*a Different letters denote significant difference between two stimulations (P < 0.017).

b Friedman’s test, adjusted for age, was used. *, significantly different among the three groups (P < 0.05).

c Friedman’s test, adjusted for age, was used. An adjusted α (α’ = 0.05/3 = 0.017) was applied. P, pneumonia patients; T, tuberculosis patients; H, healthy individuals; NA, not applicable.
of various cytokines by unstimulated whole blood cells from patients with TB, pneumonia, and healthy individuals. The patients with TB displayed higher absolute levels of IL-8, MCP-1, IP-10, and IFN-γ than the healthy participants after controlling for the difference in ages. The patients with pneumonia showed significantly higher levels of IL-8, IL-1β, and MCP-1 than the healthy participants. However, no significant differences in the absolute levels of any measured cytokines were found between the TB and pneumonia groups. These data suggest that the unstimulated cytokine profile does not provide information that can be used to distinguish patients with TB from those with pneumonia.

### TABLE 3. Percent change of cytokine levels after ESAT-6 and LPS stimulation

| Cytokine and condition | Healthy individuals (n = 30) | Patients with pneumonia (n = 30) | Patients with tuberculosis (n = 38) | P value<sup>a</sup> | Posthoc test<sup>c</sup> |
|------------------------|----------------------------|--------------------------------|-----------------------------------|-----------------|-----------------|
| IL-6                   |                            |                                |                                   |                 |                 |
| ESAT-6                 | 7.41 (0.14, 27.64)         | 22.80 (2.10, 220.86)           | 278.58 (91.39, 1063.65)           | 0.005*          | H = P < T       |
| LPS                    | 1546.64 (458.75, 2180.30)  | 275.55 (123.39, 1186.82)       | 282.82 (87.05, 1870.94)           | 0.161           | NA              |
| IL-8                   |                            |                                |                                   |                 |                 |
| ESAT-6                 | 6.93 (1.36, 30.83)         | 72.40 (26.01, 122.55)          | 147.61 (68.36, 281.16)            | 0.001*          | H = P < T       |
| LPS                    | 42.90 (20.38, 147.42)      | 188.23 (111.04, 335.74)        | 127.17 (44.84, 189.81)            | 0.011*          | H < P           |
| IL-10                  |                            |                                |                                   |                 |                 |
| ESAT-6                 | 4.01 (−1.25, 6.89)         | 8.06 (1.49, 21.61)             | 9.98 (4.92, 27.62)                | 0.033*          | H < T           |
| LPS                    | 11.29 (2.43, 17.03)        | 36.07 (15.89, 75.31)           | 18.33 (5.38, 84.51)               | 0.002*          | H < P           |
| IL-12                  |                            |                                |                                   |                 |                 |
| ESAT-6                 | 1.92 (0.53, 4.21)          | 1.06 (0.40, 1.87)              | 1.06 (0.42, 2.68)                 | 0.476           | NA              |
| LPS                    | 11.08 (2.92, 26.97)        | 5.20 (2.32, 6.80)              | 0.11 (−1.62, 1.41)                | <0.001*         | T < P = H       |
| IL-1β                  |                            |                                |                                   |                 |                 |
| ESAT-6                 | 2.50 (0.48, 29.41)         | 4.45 (0.66, 21.53)             | 45.23 (8.98, 111.57)              | 0.107           | NA              |
| LPS                    | 5.23 (2.74, 11.21)         | 205.15 (36.26, 712.37)         | 252.76 (76.70, 778.94)            | 0.888           | NA              |
| MCP-1                  |                            |                                |                                   |                 |                 |
| ESAT-6                 | 0.76 (0.06, 2.30)          | 19.67 (4.69, 26.19)            | 23.63 (8.36, 37.14)               | 0.005*          | H < P = T       |
| LPS                    | 1.41 (0.61, 23.35)         | 23.36 (4.18, 40.56)            | 5.60 (−0.77, 26.03)               | 0.103           | NA              |
| IP-10                  |                            |                                |                                   |                 |                 |
| ESAT-6                 | 9.49 (2.68, 38.43)         | 4.82 (0.63, 18.40)             | 4.19 (1.13, 24.74)                | 0.888           | NA              |
| LPS                    | 429.15 (121.25, 1496.97)   | 37.76 (13.07, 83.70)           | 32.37 (1.60, 95.00)               | 0.065           | NA              |
| TNF-α                  |                            |                                |                                   |                 |                 |
| ESAT-6                 | 3.97 (0.72, 13.77)         | 5.47 (2.07, 11.34)             | 10.75 (6.97, 114.28)              | 0.072           | NA              |
| LPS                    | 231.95 (78.85, 563.07)     | 57.04 (11.52, 1475.99)         | 25.02 (1.32, 418.69)              | 0.097           | NA              |
| IFN-γ                  |                            |                                |                                   |                 |                 |
| ESAT-6                 | 2.03 (−0.30, 2.71)         | 0.73 (−0.03, 1.84)             | 7.77 (4.38, 11.71)                | <0.001*         | H = P < T       |
| LPS                    | 3.59 (1.10, 9.89)          | 15.39 (4.03, 36.07)            | 0.00 (−4.52, 2.21)                | 0.018*          | T < P           |

<sup>a</sup> Percent change was calculated by the following formula: \(\frac{\text{value detected after stimulation} - \text{value detected in saline}}{\text{value detected in saline}} \times 100\).  
<sup>b</sup> Friedman's test, adjusted for age, was used. * significantly different among the three groups (\(P < 0.05\)).  
<sup>c</sup> Friedman's test, adjusted for age, was used. An adjusted \(\alpha (\alpha = 0.05/3 = 0.017)\) was applied. P, pneumonia patients; T, tuberculosis patients; H, healthy individuals; NA, not applicable.

### TABLE 4. Accuracies and cutoff points of ROC curves for percent changes in cytokine levels

| Parameter | Value for the cytokine by major outcome of ROC analysis (% [95% CI])<sup>a</sup> | AUC | Cutoff point | Sensitivity | Specificity | PPV | NPV | Accuracy |
|-----------|-------------------------------------------------------------------|-----|-------------|-------------|-------------|-----|-----|----------|
| IFN-γ     | Tuberculosis: 73.2 (60.6, 85.9) Pneumonia: 68.5 (55.8, 81.2)      | 90.4 (81.7, 99.2) | 3.59        | 84.2        | 83.3        | 86.5 | 80.6 | 85.8     |
| IL-6      | Tuberculosis: 73.2 (60.6, 85.9) Pneumonia: 68.5 (55.8, 81.2)      | 89.1 (81.7, 96.6) | 3.59        | 84.2        | 83.3        | 86.5 | 80.6 | 85.8     |
| IL-12     | Tuberculosis: 73.2 (60.6, 85.9) Pneumonia: 68.5 (55.8, 81.2)      | 85.2 (75.9, 95.2) | 3.59        | 84.2        | 83.3        | 86.5 | 80.6 | 85.8     |

<sup>a</sup> The major outcome refers to either TB or pneumonia as the real diagnostic disease.
Several recent assays have been introduced that effectively diagnose TB on the basis of the secretion of cytokines (e.g., IFN-γ) after whole-blood samples are stimulated with *M. tuberculosis*-specific antigens, such as ESAT-6. When ESAT-6 was added to the whole-blood samples in our study, those from the patients with TB secreted higher levels of IL-6, IL-8, MCP-1, and (as expected) IFN-γ than the stimulated samples from healthy individuals.

The samples from the patients with pneumonia secreted higher levels of MCP-1 than those from the healthy individuals after stimulation with ESAT-6. However, no differences were observed between the patients with TB and those with pneumonia under this condition, suggesting that the cytokine profile after stimulation with ESAT-6 cannot differentiate TB from pneumonia.

We performed a similar analysis of the cytokine profiles after samples were stimulated with LPS, an antigen associated frequently with pulmonary infections caused by nonmycobacteria. When LPS was added to the whole-blood samples, those from the patients with TB showed higher levels of IL-8 and MCP-1 than those from the healthy individuals, and those from the patients with pneumonia showed higher levels of IL-8 than those from the healthy control group. Furthermore, the samples from the patients with pneumonia showed higher levels of IL-10 and IFN-γ than those from the TB group after the LPS stimulation. However, ROC analysis of the results for IL-10 and IFN-γ suggested that the secreted level of neither cytokine was adequate to distinguish patients with pneumonia from those with TB.

Because the absolute levels of cytokines produced by the blood samples before and after stimulation could be influenced by variations in the number of immune cells in the samples, we analyzed the percent change in the levels of cytokines between the unstimulated and stimulated blood samples. We observed that, after stimulation with ESAT-6, the percent changes in the levels of IL-6, IL-8, IL-10, MCP-1, and IFN-γ in the samples from the patients with TB were significantly greater than those in the samples from the healthy participants, and a higher percent change in the level of MCP-1 was found in the blood from the patients with pneumonia than in the samples from healthy individuals. Importantly, the samples from the patients with TB showed higher percent changes in IL-6, IL-8, and IFN-γ than those from the pneumonia group, suggesting that IL-6, IL-8, and IFN-γ were potential markers to differentiate between TB and pneumonia. The ROC curves generated with the percent change of IFN-γ suggested that IFN-γ was a good biomarker to discriminate TB from pneumonia when whole blood cells were stimulated by ESAT-6. In contrast, the ROC analysis revealed that the percent change in levels of IL-6 and IL-8 were not adequate discriminatory tools for TB.

After stimulation with LPS, there were no significant differences between the patients with TB and the healthy participants according to the percent changes in levels of any of the cytokines except IL-12. The percent change in the levels of IL-12 in patients with TB was lower than that in either of the other groups. Under the same conditions, the percent changes in levels of IL-8 and IL-10 in the patients with pneumonia were significantly greater than those in the healthy participants. Moreover, the percent changes in levels of IL-12 and IFN-γ in the samples from the patients with pneumonia were significantly higher than those in the samples from patients with TB, suggesting that IL-12 and IFN-γ were potential markers to differentiate between TB and pneumonia under this condition.

The ROC analysis of percent change in levels of IFN-γ and IL-12 suggested that both cytokines were good biomarkers to discriminate pneumonia from TB when whole blood cells were stimulated by LPS.

To our knowledge, this is the first study to evaluate the value of cytokines produced in response to ESAT-6 and LPS to differentiate TB from pneumonia. However, several other studies have analyzed the expression of cytokines in patients with TB, and our results for IL-10 and IL-8 are not in complete agreement with some of these reports. Al-Attiyah and Mustafa investigated the secretion of cytokines by peripheral blood mononuclear cells from patients with TB (2). These authors reported that the secretion of IL-6, but not IL-8, was enhanced by stimulation with the antigens tested. We observed a similar result for IL-6 after stimulation with ESAT-6. In contrast, we did observe a significant difference in the secretion of IL-8 by whole blood cells from patients with TB compared to healthy individuals in the presence and absence of stimulation. Other investigators have reported that the levels of IP-10 and IFN-γ in blood samples from patients with TB are significantly higher than those in samples from healthy individuals both before and after stimulation with ESAT-6 and that MCP-1 expression is significantly higher in patients with TB after stimulation (3, 23). Our results for the levels of MCP-1 and IFN-γ are in agreement with these reports. However, we did not observe a significant difference between the patients with TB and healthy individuals in the levels of IP-10 either before or after stimulation. Hughes et al. used intracellular cytokine cytometry to evaluate the response of whole-blood samples to ESAT-6 and also found variability in the secretion of IL-10 (12). Not all patients responded by secreting IL-10, and 2 of the 16 patients with TB who secreted IL-10 did not secrete IFN-γ. This variability may depend on whether the immune response mounted by the patients was primarily of the Th2 or Th1 type.

Another interesting observation from our study was the elevated level of IFN-γ produced by specimens from patients with pneumonia before and after stimulation with ESAT-6 relative to the specimens from the healthy individuals. We have not found reports in the literature that describe stimulation of cytokines by ESAT-6 in patients with pneumonia, and we do not have an explanation for this finding.

There were several limitations to this preliminary study. First, specimens from patients with pneumonia were not included in the original screen to identify biomarkers. Thus, potential cytokine markers that could be used to identify patients with pneumonia may have been missed, and this list of markers cannot be considered all-inclusive. We chose ESAT-6 and LPS as stimuli because they are antigens produced by *M. tuberculosis* and Gram-negative bacteria, respectively, and we hypothesized that they had the greatest likelihood of generating a specific response. However, they would be expected to bind to and stimulate different types of blood cells, and the current study did not determine whether the differences in response to stimulation we observed were the result of differences in the production of cytokines or differences in the size of the populations of responding cells. Finally, the number of patients in the three groups was relatively small. Therefore, the
performance of the markers we have identified must be confirmed in larger trials.

In summary, we found that when whole blood cells were stimulated with ESAT-6, the percent change in IFN-γ was a suitable biomarker to differentiate patients with TB from those with pneumonia. Similarly, the percent changes of IFN-γ and IL-12 appeared to be potential biomarkers for differentiating patients with pneumonia from those with TB when whole blood cells were stimulated with LPS. Our study demonstrates that the use of ex vivo whole-blood cytokine profiling after stimulation with ESAT-6 provides an alternative method to detect TB in BCG-vaccinated populations. Therefore, the technology used in this study may have the potential to be developed as a platform for detecting latent TB infection as well as for community or outbreak screening for TB in the future.

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