Characterization of Human Cellular Immune Responses to Novel *Mycobacterium tuberculosis* Antigens Encoded by Genomic Regions Absent in *Mycobacterium bovis* BCG

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Comparative genomics has identified several regions of differences (RDs) between the infectious *Mycobacterium tuberculosis* and the vaccine strains of *Mycobacterium bovis* BCG. We aimed to evaluate the cellular immune responses induced by antigens encoded by genes predicted in 11 RDs. Synthetic peptides covering the sequences of RD1, RD4 to RD7, RD9 to RD13, and RD15 were tested for antigen-induced proliferation and secretion of Th1 cytokine, gamma interferon (IFN-γ), by peripheral blood mononuclear cells (PBMC) obtained from culture-proven pulmonary tuberculosis (TB) patients and *M. bovis* BCG-vaccinated healthy subjects. Among the peptide pools, RD1 induced the best responses in both donor groups and in both assays. In addition, testing of TB patients’ PBMC for secretion of proinflammatory cytokines (tumor necrosis factor alpha [TNF-α], interleukin 6 [IL-6], IL-8, and IL-1β), Th1 cytokines (IFN-γ, IL-2, and TNF-β), and Th2 cytokines (IL-4, IL-5, and IL-10) showed differential effects of RD peptides in the secretion of IFN-γ and IL-10, with high IFN-γ/IL-10 ratios (32 to 5.0) in response to RD1, RD5, RD7, RD9, and RD10 and low IFN-γ/IL-10 ratios (<1.0) in response to RD12, RD13, and RD15. Peptide-mixing experiments with PBMC from healthy subjects showed that secretion of large quantities of IL-10 in response to RD12 and RD13 correlated with inhibition of Th1 responses induced by RD1 peptides. In conclusion, our results suggest that *M. tuberculosis* RDs can be divided into two major groups—one group that activates PBMC to preferentially secrete IFN-γ and another group that activates preferential secretion of IL-10—and that these two groups of RDs may have roles in protection against and pathogenesis of TB, respectively.

*Mycobacterium tuberculosis* is among the most successful human pathogens and is the prime cause of human tuberculosis (TB) in the world. Globally, about 2 billion people are infected with *M. tuberculosis*, 8 to 10 million develop active disease, and 2 million die from TB every year (53). An overwhelming majority of TB patients reside in the developing countries, which suffer from marked poverty, lack of healthy living conditions, and inadequate medical facilities (53).

The global control of TB requires effective vaccines and reagents for specific diagnosis. The only vaccine currently available for humans is *Mycobacterium bovis* bacillus Calmette-Guérin (BCG), a live attenuated strain of *M. bovis*. In spite of being the most used vaccine in the world, with proven efficacy against childhood TB meningitis and miliary TB (41, 49), BCG has failed to reduce the global burden of TB (43). The efficacy of BCG against pulmonary TB in adults has varied between 0 and 80% (16). Furthermore, vaccination with *M. bovis* BCG faces two additional problems. First, it induces a delayed-type hypersensitivity response that cannot be distinguished from infection with *M. tuberculosis*, and therefore, it compromises the use of purified protein derivative (PPD) of *M. tuberculosis* in skin tests for diagnostic or epidemiological purposes (25, 26). Second, *M. bovis* BCG, as a live vaccine, is contraindicated in human immunodeficiency virus-infected patients for fear of causing disease (11). PPD, the commonly used diagnostic reagent, is nonspecific because of the presence of antigens cross-reactive with *M. bovis* BCG and environmental mycobacteria (14, 25). Thus, there is a need to identify *M. tuberculosis*-specific antigens to develop new protective vaccines and specific diagnostic reagents against TB.

Comparative genome analyses of the *M. tuberculosis* genome and *M. bovis* BCG have shown that 16 genomic regions of *M. tuberculosis* are deleted or lacking in some or all strains of *M. bovis* and/or *M. bovis* BCG (9). Among these regions of differences (RDs), 11 regions (RD1, RD4 to RD7, RD9 to RD13, and RD15) covering 89 open reading frames (ORFs) of *M. tuberculosis* H37Rv are absent in all *M. bovis* BCG substrains currently used as vaccines to protect against TB in different parts of the world (9, 29). Immunological evaluation of the proteins encoded by these regions was predicted to identify antigens of *M. tuberculosis* important for developing specific diagnostic reagents and vaccines to control TB (6, 10, 27, 28). In addition, these *M. tuberculosis*-specific proteins may also have roles in mediating the immunological mechanisms of virulence and pathogenesis in TB (10, 25).

Although the host effector/immunological mechanisms that provide protection against TB are not fully understood, resistance to mycobacterial infections is primarily mediated by the interaction of antigen-specific T cells and macrophages (17, 28). This interaction is often dependent on the interplay of cytokines produced by these cells. Although a broad spectrum of cytokines may contribute to protection, the Th1 helper type 1 (Th1) response, dominated by gamma interferon (IFN-γ) secretion, is considered a principal mediator of protective immu-
nity against TB (4, 17, 33, 34). In addition, the secretion of IFN-γ in response to M. tuberculosis-specific antigens, like ESAT6 and CFP10, has been shown to have diagnostic significance (7, 15, 21, 28). On the other hand, the Th2 responses, characterized by the secretion of interleukin 4 (IL-4), IL-5, and IL-10, are associated with a lack of protection (8, 17). In particular, IL-10 is associated with reduced resistance and chronic progressive TB (51). Furthermore, IL-10 deactivates macrophages and downregulates the secretion of Th1 cytokines (51). In addition, innate-immune-response-related proinflammatory cytokines, IL-6, IL-12, and tumor necrosis factor alpha (TNF-α), initiate events that curb mycobacterial growth by recruiting monocytes into the lesions and activating them (15). The proinflammatory cytokine TNF-α, mainly secreted by activated macrophages, contributes to antitubercular action and limits disease pathology (17). Therefore, it was considered useful to study cytokine responses in TB patients to proteins encoded by genes predicted in RDs of M. tuberculosis to understand the possible roles of these antigens in protection against and pathogenesis of TB, as well as to identify new groups of antigens with potential in the diagnosis and development of subunit vaccines.

To immunologically characterize the proteins encoded by genes predicted in RDs of M. tuberculosis that are deleted or absent in all M. bovis BCG strains, we used in this study overlapping synthetic peptides corresponding to proteins predicted to be encoded by genes of RD1, RD4 to RD7, RD9 to RD13, and RD15. The peptides corresponding to each RD were pooled and tested with peripheral blood mononuclear cells (PBMC) isolated from TB patients and M. bovis BCG-vaccinated healthy subjects in antigen-induced proliferation and cytokine assays to detect the secretion of innate-immune-response-related proinflammatory cytokines—IL-6, IL-8, TNF-α, and IL-1β—and specific-immune-response-related Th1 cytokines—IFN-γ, IL-2, and TNF-β—and Th2 cytokines—IL-4, IL-5, and IL-10. To our knowledge, this is the first comprehensive study to evaluate the cellular immune responses of M. tuberculosis RDs absent in all strains of M. bovis BCG.

MATERIALS AND METHODS

Patients. Heparinized venous blood was collected from newly diagnosed and culture-confirmed pulmonary TB patients (n = 48) attending the Chest Diseases Hospital, Kuwait. Buffy coats were obtained from M. bovis BCG-vaccinated and PPD-positive healthy subjects (n = 45) donating blood at the Central Blood Bank, Kuwait. The groups of healthy donors and TB patients were serologically negative for human immunodeficiency virus infection and included Kuwaiti, as well as non-Kuwaiti, citizens. Informed consent was obtained from all the subjects, and the study was approved by the Ethical Committee of the Faculty of Medicine, Kuwait University, Kuwait.

Complex mycobacterial antigens and synthetic peptides of M. tuberculosis. The complex mycobacterial antigens used in this study were whole-cell killed M. tuberculosis H37Rv and M. bovis BCG (1, 39) and M. tuberculosis culture filtrate (MT-CF) and cell walls (MT-CW) provided by P. J. Brennan (Colorado State University, Fort Collins) through the repository of TB research materials at the National Institute of Allergy and Infectious Diseases (NII contract no. AI-25147). A total of 1,648 peptides (25-mers overlapping neighboring peptides by 10 amino acids) spanning the sequence of putative proteins encoded by genes in the RD1, RD4 to RD7, RD9 to RD13, and RD15 genomic regions were designed based on the amino acid sequence deduced from the nucleotide sequences of the respective genes (Table 1). These peptides were commercially synthesized by Thermo Hybaid GmbH (Ulm, Germany) using fluorocarbonyl andistry, as described previously (38, 39). The stock concentrations (5 mg/ml) of the peptides were prepared in normal saline (0.9%) by vigorous pipetting, and the working concentrations were prepared by further dilution in tissue culture medium RPMI 1640, as previously described (38, 39).

Isolation of PBMC and stimulation for antigen-induced proliferation and cytokine secretion. PBMC were isolated from the blood of TB patients and healthy subjects by flotation on Lymphoprep gradients using standard procedures (2, 35). In brief, 2 x 10^7 PBMC suspended in 50 µl were seeded into the wells of 96-well tissue culture plates (Nunc, Denmark). Antigens in 50 µl complete medium were added at optimal concentrations to the wells in triplicate. Whole bacilli were used at 10 µg/ml (wet weight) and MT-CW at 1 µg/ml. All other antigens and peptides were used at an optimal concentration of 5 µg/ml. The cells in the control wells did not receive any mycobacterial antigen/peptide. The final volume of the culture in each well was adjusted to 200 µl. The plates were incubated at 37°C in a humidified atmosphere containing 5% CO2 and 95% air. On day 6, the culture supernatants were harvested. The radioactivity incorporated was obtained as cpm. The average cpm was calculated from triplicate cultures stimulated with each antigen or peptide-stimulated cultures/cpm in cultures lacking antigen or peptide.

Table 1. Synthetic peptides corresponding to ORFs predicted in the RDs present in M. tuberculosis H37Rv but absent in all strains of M. bovis BCG

<table>
<thead>
<tr>
<th>Region deleted</th>
<th>ORF designation</th>
<th>No. of ORFs</th>
<th>No. of synthetic peptides</th>
</tr>
</thead>
<tbody>
<tr>
<td>RD1</td>
<td>ORF2–ORF11, ORF14, ORF15</td>
<td>12</td>
<td>220</td>
</tr>
<tr>
<td>RD4</td>
<td>Rx0221–Rx0223c</td>
<td>3</td>
<td>80</td>
</tr>
<tr>
<td>RD5</td>
<td>Rx3117–Rx3121</td>
<td>5</td>
<td>72</td>
</tr>
<tr>
<td>RD6</td>
<td>Rv1506c–Rv1516c</td>
<td>11</td>
<td>236</td>
</tr>
<tr>
<td>RD7</td>
<td>Rv2346c–Rv2353c</td>
<td>8</td>
<td>167</td>
</tr>
<tr>
<td>RD9</td>
<td>Rv3617–Rv3623</td>
<td>7</td>
<td>108</td>
</tr>
<tr>
<td>RD10</td>
<td>Rv1255c–Rv1257c</td>
<td>3</td>
<td>71</td>
</tr>
<tr>
<td>RD11</td>
<td>Rv3425–Rv3429</td>
<td>5</td>
<td>84</td>
</tr>
<tr>
<td>RD12</td>
<td>Rx2072c–Rx2075c</td>
<td>4</td>
<td>83</td>
</tr>
<tr>
<td>RD13</td>
<td>Rx2645c–Rv2660c</td>
<td>16</td>
<td>225</td>
</tr>
<tr>
<td>RD15</td>
<td>Rv1963c–Rv1977</td>
<td>15</td>
<td>302</td>
</tr>
</tbody>
</table>

*RD designations are according to Behr et al. (9).
*a ORF designations for RD1 are according to Amouyli et al. (5), whereas for other RDs, the ORF designations are according to Behr et al. (9).
Based on the median delta IFN-γ and percentages of responders in IFN-γ assays, the antigens were considered strong (delta IFN-γ > 10, with >70% responders), moderate (delta IFN-γ > 3.0 but <10, with 50 to 70% responders), or weak (delta IFN-γ < 3.0, with <50% responders) stimulators of PBMC.

**Immunossays for the quantitation of cytokines by FlowCytomix kits.** The supernatants, collected from the cultures of PBMC of TB patients (n = 17) before the 1H-thymidine pulse, were assayed to determine the concentrations of secreted cytokines by using FlowCytomix kits (Bender Medsystems Inc., Austria) according to the manufacturer's instructions. These kits allow simultaneous quantification of 10 cytokines, including Th1 cytokines, IFN-γ, IL-2, and TNF-β; Th2 cytokines, IL-10, IL-4, and IL-5; and the proinflammatory cytokines, TNF-α, IL-6, IL-8, and IL-1β. In brief, FlowCytomix technology is based on spectrally discrete microspheres that are used as the solid phase in an immunosassay. The beads are internally dyed with Starfire Red, a far-red (685- to 690-nm)-emitting fluorochrome that is excited by UV, argon, or HeNe lasers. The test samples were analyzed by flow cytometry using Coulter Epics FC500 (Beckman Coulter Inc.). For each analysis, up to 10,000 events were acquired. The mean concentration of each cytokine was expressed as pg/ml. By using the above-mentioned kits, the minimum detectable concentrations of IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, TNF-α, TNF-β, and IFN-γ were 4.5 pg/ml, 8.9 pg/ml, 6.4 pg/ml, 5.3 pg/ml, 4.7 pg/ml, 6.4 pg/ml, 6.9 pg/ml, 7.8 pg/ml, 3.2 pg/ml, and 7.0 pg/ml, respectively. In response to antigenic stimuli, the values of E/C that were ≥2 were considered positive responses (4). E/C is defined as follows: E/C = cytokine concentration in antigen-stimulated cultures/cytokine concentration in cultures lacking antigen.

In the experiments where the concentrations of cytokines in control cultures lacking antigen were not detectable, the E/C values were determined by dividing the concentration of a given cytokine in antigen-stimulated cultures with the minimum detectable concentration of the same cytokine.

**Statistical analysis.** The antigen-induced proliferation and IFN-γ secretion results with antigens and peptide pools showing strong to moderate responses were statistically analyzed for significant differences between TB patients and healthy subjects using a nonparametric Mann-Whitney U test for two independent samples. P values of <0.05 were considered significant.

**RESULTS**

**Antigen-induced proliferation of PBMC in response to complex mycobacterial antigens and RD peptides.** In antigen-induced proliferation assays, PBMC from all TB patients and healthy subjects responded to one or more complex mycobacterial antigens (data not shown). In addition, all complex mycobacterial antigens were strong stimulators of PBMC from TB patients and healthy subjects, as indicated by high median SI values (14.3 to 25.3 and 19 to 22, respectively) and percentages of responders (77 to 90% and 96 to 98%, respectively) (Fig. 1A and B). Furthermore, the proliferation of PBMC from TB patients was strong in response to the RD1 peptide pool (median SI values, 11, and 75% responders); moderate in response to peptide pools of RD4, RD6, RD7, and RD9 (median SI values, 3.0 to 3.7, and 50 to 57% responders); and weak to peptide pools of RD5, RD10, RD11, RD12, RD13, and RD15 (median SI values, <3.0, and <50% responders) (Fig. 1C). In healthy subjects, only peptide pools of RD1 and RD7 induced moderate responses (SI values, 4.4 and 3.1, and responders, 62 and 51%, respectively), whereas all other peptide pools were weak stimulators of PBMC (median SI values, <3.0, and <50% responders) (Fig. 1D). The statistical analysis of antigen-induced proliferation responses showed that none of the complex mycobacterial antigens induced responses that were significantly different in TB patients than in healthy subjects (P > 0.05), whereas among the peptide pools inducing strong to moderate responses, only RD1 and RD4 induced significantly better responses (P < 0.05) in TB patients than in healthy subjects (Fig. 1A, B, C, and D).

**Estimation of antigen-induced IFN-γ secretion by PBMC in response to complex mycobacterial antigens and RD peptides by ELISA.** Antigen-induced IFN-γ was secreted by PBMC from all TB patients and healthy subjects in response to one or more complex mycobacterial antigens (data not shown). Furthermore, all complex mycobacterial antigens were strong stimulators of IFN-γ secretion by PBMC of TB patients (median delta IFN-γ, 12 to 18 U/ml, and responders, 73 to 89%) (Fig. 2A and healthy subjects (median delta IFN-γ, 42 to 50 U/ml, and responders, 98 to 100%) (Fig. 2B). The statistical analysis of data showed that healthy subjects secreted significantly more IFN-γ than TB patients in response to all complex mycobacterial antigens (P < 0.05). In response to peptide pools, only RD1 peptides induced moderate responses in both TB patients (median delta IFN-γ, 4.0 U/ml, and 56% responders) and healthy subjects (median delta IFN-γ, 7.0 U/ml, and 62% responders), with no statistical difference between the two groups (P > 0.05). The peptide pools of all other RDs were weak stimulators of IFN-γ secretion in both TB patients and healthy subjects (median delta IFN-γ, <3.0 U/ml, and <50% responders) (Fig. 2C and D, respectively).

**Estimation of antigen-induced secretion of cytokines by PBMC in response to complex mycobacterial antigens and RD peptides by FlowCytomix assays.** Antigen-induced secretion of the proinflammatory Th1 and Th2 cytokines was studied with PBMC in 17 randomly selected TB patients from the group described above in response to the complex mycobacterial antigens and peptide pools of RDs. The secretion of the proinflammatory cytokine IL-6 by PBMC was observed in response to all of the complex mycobacterial antigens and peptide pools of RDs (data not shown), whereas IL-1β and TNF-α were secreted only in the presence of the complex mycobacterial antigens (data not shown), and IL-8 secretion was not enhanced by stimulation of PBMC with any antigen (data not shown).

Among the Th1 and Th2 cytokines, the secretion of TNF-β was induced by complex mycobacterial antigens and peptide pools of RD1, RD6, and RD13 (data not shown), whereas IL-2, IL-4, and IL-5 secretion by PBMC could not be detected in response to any of the antigens and RD peptides (data not shown). However, strong antigen-induced IFN-γ secretion was observed in response to all complex mycobacterial antigens, with the percentages of responders ranging from 76 to 88%. The peptide pools of RD1, RD4, RD5, RD6, RD7, RD9, and RD10 induced secretion of IFN-γ with 53 to 31% positive responders, whereas RD11, RD12, RD13, and RD15 were weak inducers of IFN-γ secretion, as indicated by 19 to 25% positive responders. Strong antigen-induced secretion of IL-10 was observed with all the complex mycobacterial antigens, with 76 to 100% positive responders. Among the peptide pools, RD6, RD12, RD13, and RD15 showed antigen-induced secretion of IL-10, with 38 to 47% positive responders, whereas RD1, RD7, RD9, RD10, and RD11 induced IL-10 secretion from PBMC of less than 25% of patients.

The determination of relative concentrations of Th1 and Th2 cytokines by calculating IFN-γ/IL-10 ratios indicated distinct Th1 biases in response to MT-CF and peptide pools of RD1, RD5, RD7, RD9, and RD10 (ratio of IFN-γ to IL-10 =
32 to 5.0), whereas in response to other complex mycobacterial antigens and peptide pools of RDs, no Th2 biases were observed (ratio of IFN-γ/H9253 to IL-10/H11005 ratio of 1.1 to 0.4).

**Antigen-induced IL-10 secretion and effects of RD12 and RD13 peptides on antigen-induced proliferation and IFN-γ secretion by PBMC of healthy subjects.** To further evaluate IL-10 secretion in response to complex mycobacterial antigens and peptide pools in healthy subjects, randomly selected culture supernatants from PBMC of 18 M. bovis BCG-vaccinated healthy subjects were tested for IL-10 concentrations using ELISA. The analysis of the results showed that PBMC of healthy subjects secreted IL-10 in response to all complex mycobacterial antigens (median concentrations, 284 to 846 pg/ml), but in response to RD peptides, only RD12 and RD13 showed antigen-induced IL-10 secretion (median concentrations, 215 and 140 pg/ml, respectively) (Fig. 3A and B).

To determine if secretion of IL-10 in response to RD12 and RD13 peptides could have any effect on the response of PBMC to Th1 cell-stimulating antigens, experiments were performed with PBMC from healthy subjects (n = 10) in the presence of four different concentrations (0.2, 1.0, 5.0, and 25 μg/ml) of these RD peptides, either alone or in combination with RD1. The results of antigen-induced proliferation and IFN-γ secretion assays showed that when tested alone, RD12 and RD13 failed to induce proliferation or IFN-γ secretion at all concentrations, whereas RD1 peptides showed optimal responses in both assays at 5 μg/ml (Fig. 4A; data are shown for proliferation only). Further experiments with PBMC from the same donors at the optimal (5 μg/ml) (Fig. 4B) and suboptimal (1 μg/ml) (Fig. 4C) concentrations showed that addition of either RD12 or RD13 peptides to the PBMC cultures, at concentrations equal to those of RD1 peptides, inhibited RD1-induced proliferation and IFN-γ secretion at both concentrations (Fig. 4B and C; data are shown for proliferation only). These inhibitory effects were not due to a toxic effect of RD12 and RD13 on PMBC, because the cells were >98% viable (as determined by the trypan blue exclusion technique) at the end of the culture period in cultures with and without RD12 and RD13.

**FIG. 1.** Antigen-induced proliferation of PBMC of TB patients and healthy subjects in response to complex mycobacterial antigens (A and B, respectively) and RD peptides (C and D, respectively). PBMC obtained from pulmonary TB patients (n = 48) and M. bovis BCG-vaccinated healthy subjects (n = 45) were cultured in the presence of complex mycobacterial antigens and pools of RD peptides. Each point represents the SI by PBMC from an individual donor in response to a given antigen. The median SIs are represented by horizontal bars, and the percentages of positive responders to each antigen are presented. The dashed lines indicate cutoff SI values of ≥3.
DISCUSSION

Cellular immune responses were analyzed with PBMC from pulmonary TB patients and M. bovis BCG-vaccinated healthy subjects using assays that assessed antigen-induced cell proliferation and secretion of a battery of proinflammatory Th1 and Th2 cytokines. The antigens used were complex mycobacterial antigens and peptide pools corresponding to the M. tuberculosis genomic regions RD1, RD4 to RD7, RD9 to RD13, and RD15, which are considered absent in M. bovis BCG (9). The study of cellular immune responses with respect to the target molecules recognized is important for the understanding of protective and pathological immune mechanisms in TB and identification of antigens suitable for the diagnosis and development of new vaccines (17, 26). To our knowledge, this is the first comprehensive study to evaluate the cellular immune responses to 11 RDs of M. tuberculosis that are considered absent in all strains of M. bovis BCG (9).

All of the complex mycobacterial antigenic preparations induced strong antigen-induced proliferation and IFN-γ responses in both donor groups, but compared to M. bovis BCG-vaccinated healthy subjects, TB patients secreted significantly smaller quantities of IFN-γ (P < 0.05) in response to all preparations of complex mycobacterial antigens. Similar results have been reported previously (12, 46, 54). However, among RD peptides, only RD1 induced strong to moderate responses in both donor groups and in both assays. Some of the other RD peptides induced moderate responses in TB patients (RD4, RD6, RD7, and RD9) and healthy subjects (RD7) in antigen-induced proliferation assays, but all of the other RD peptides induced weak responses in antigen-induced proliferation and IFN-γ responses in both donor groups. These results suggest that only RD1 contains immunodominant antigens. It has been shown that the RD1 genomic region of M. tuberculosis has three major T-cell antigens, i.e., PPE68, ESAT6, and CFP10 (18, 28). When tested alone, ESAT6 and CFP10 induce strong antigen-induced proliferation and IFN-γ responses in TB patients but weak responses in M. bovis BCG-vaccinated healthy subjects residing in Kuwait, which could be attributed to previous exposure to M. tuberculosis (2, 3, 4, 26, 32), whereas, PPE68 induces strong responses in both TB patients and M. bovis BCG-vaccinated healthy subjects (32). The testing of M. bovis BCG-vaccinated healthy subjects previously uninfected with M. tuberculosis (as indicated by nonresponsiveness to ESAT6/CFP10; n = 33) showed moderate stimulation of their PBMC with RD1 (median delta IFN-γ, 6.0 U/ml). The major antigen that contributes to RD1 reactivity in non-M. tuberculosis-infected M. bovis BCG-vaccinated healthy subjects is PPE68 (32). This is because the immunodominant epitopes of

![FIG. 2. IFN-γ secretion by PBMC of TB patients and healthy subjects in response to complex mycobacterial antigens (A and B, respectively) and RD peptides (C and D, respectively). PBMC obtained from pulmonary TB patients (n = 48) and M. bovis BCG-vaccinated healthy subjects (n = 45) were cultured in the presence of complex mycobacterial antigens and pools of RD peptides, and the supernatants collected on day 6 were tested for concentrations of secreted IFN-γ (U/ml). Each point represents the concentration of the cytokine secreted by PBMC from an individual donor in response to a given antigen. The median concentrations of IFN-γ are represented by horizontal bars, and the percentages of positive responders in response to each antigen are presented.](http://iai.asm.org/)
PPE68 (amino acids 127 to 136) are completely identical in PPE68 and several PPE family proteins that are present in *M. tuberculosis*, as well as in *M. bovis* BCG (32, 42). We have recently shown that PBMC from PPE68-reactive *M. bovis* BCG-vaccinated healthy subjects respond equally well to the peptide amino acids 127 to 136 (32). As the peptide pool of RD1 used in this study contained the sequence of amino acids 127 to 136 of PPE68, the moderate responses obtained to RD1 in *M. bovis* BCG-vaccinated subjects are not surprising. These findings further suggest that specificity of *M. tuberculosis* regions at the genomic level, as is the case with RD1, may not necessarily translate into specificity at the antigen/epitope level.

Infection with *M. tuberculosis* induces the production of multiple innate immune response-related proinflammatory cytokines with chemotactic activities for monocytes and lymphocytes (44). These chemotactic molecules ensure the recruitment of appropriate cells at the appropriate time to the site of disease activity. Our results showed that PBMC of all patients (n = 17) spontaneously secreted the proinflammatory cytokines IL-6 and IL-8, while the PBMC of 88% patients secreted detectable concentrations of IL-1β and TNF-α (data not shown), indicating the in vivo antigen priming of PBMC. These results confirm the previous findings indicating spontaneous expression of the messages (47) and secretion of the proteins of the proinflammatory cytokines by PBMC of TB patients (20, 40). Furthermore, the stimulation of PBMC with complex mycobacterial antigens induced antigen-specific secretion of IL-6, TNF-α, and IL-1β, but not of IL-8, whereas the peptide pools of all RDs induced antigen-specific secretion of IL-6 only (data not shown). Thus, these results suggest that the peptide pools of various RDs do not have different effects on inducing the secretion of innate-immune-response-related proinflammatory cytokines.

The spontaneous secretion by PBMC of one or more Th1 and Th2 cytokines was observed in the majority (60% and 94%, respectively) of TB patients (data not shown). This observation indicates a mixed state of Th1/Th2 phenotypes of cells with a shift toward Th2 cytokines. These results are compatible with previous findings reporting dominance of Th2 cytokines in TB patients (4, 43). The mixed pattern of Th1/Th2 cytokines may be due either to the activation of Th0 cells or to simultaneous activation of both Th1 and Th2 cells, resulting in a Th0 phenotype of the cytokines released. However, the Th2 dominance may play a role in the pathogenesis of the disease, as suggested previously (22). A reversal from pathological Th2 cytokine dominance toward protective Th1 cytokines has been observed in TB patients after successful chemotherapy and clinical healing (4, 22).

When tested for antigen-induced secretion of Th1 and Th2 cytokines, PBMC of TB patients secreted TNF-β in response to the complex mycobacterial antigens and peptide pools of RD1, RD6, and RD13 (data not shown). However, in spite of strong antigen-induced proliferation responses to complex mycobacterial antigens and the RD1 peptide pool, IL-2 was not detected in any of the antigen-stimulated cultures (data not shown). This could have been due to utilization of IL-2 by T cells proliferating in response to antigenic stimuli (48). In addition, the secretion of the Th2 cytokines IL-4 and IL-5 was not detected in response to the mycobacterial antigens and the peptide pools (data not shown). Like IL-2, IL-4 consumption in ex vivo cell cultures has also been described previously (50). The lack of antigen-specific secretion of IL-5 by PBMC of TB patients supports previous observations (4, 23).

Among the Th1 and Th2 cytokines, IFN-γ and IL-10 are considered the main cytokines responsible for protection against and pathogenesis of TB, respectively (4, 13, 17, 20, 31). Antigen-induced secretions of both of these cytokines by PBMC were observed in response to all complex mycobacterial antigens. However, variations in the concentrations of secreted IFN-γ and IL-10 were seen with the complex mycobacterial antigens used, i.e., MT-CF induced the highest concentration of IFN-γ (1,452 pg/ml) and the lowest concentration of IL-10 (100 pg/ml), whereas other complex mycobacterial antigens, i.e., MT-CW, whole-cell *M. tuberculosis*, and whole-cell *M. bovis* BCG induced lower concentrations of IFN-γ (800 to 1,220 pg/ml) and higher concentrations of IL-10 (1,052 to 1,738 pg/ml). IL-10 has multiple effects that interfere with the func-

**FIG. 3.** IL-10 secretion by PBMC of *M. bovis* BCG-vaccinated healthy subjects in response to complex mycobacterial antigens (A) and RD peptides (B). PBMC obtained from *M. bovis* BCG-vaccinated healthy subjects (n = 18) were cultured in the presence of complex mycobacterial antigens and pools of RD peptides. The culture supernatants were collected on day 6 and tested for concentrations of secreted IL-10 (pg/ml). Each point represents the concentration of IL-10 secreted by PBMC from an individual donor in response to a given antigen. The median concentration of each cytokine is represented by a horizontal bar.
tions of protective cells and cytokines (52), thereby helping mycobacteria to survive intracellularly, despite the abundant production of IFN-γ (24). On the other hand, the absence of IL-10 accelerates mycobacterial clearance (52). Therefore, it is imperative to avoid or minimize stimulation of IL-10 production when identifying antigens of *M. tuberculosis* to design a vaccine against TB. The high IFN-γ and low IL-10 concentrations in response to MT-CF support the suggestion that secreted *M. tuberculosis* antigens could be more suitable for developing new vaccines against TB (25, 27).

In response to various peptide pools, preferential secretion of IFN-γ was observed with RD1, RD5, RD7, RD9, and RD10 (IFN-γ/IL-10 ratio, 32 to 5.0), whereas IL-10 was preferentially secreted in response to RD12, RD13, and RD15 (IFN-γ/IL-10 ratio, 0.6 to 0.4), and both IFN-γ and IL-10 were secreted in response to RD4 and RD6 (IFN-γ/IFN-α ratios, 2.0 and 1.1, respectively). The IFN-γ/IL-10 ratio provides a useful objective marker of disease activity in TB and can be important in disease management (19, 46). In these studies, the authors have shown that in response to mycobacterial antigens, high IFN-γ/IL-10 ratios strongly correlate with protection and TB cure, whereas low ratios correlate with disease severity. In light of these observations, our results suggest that RD4, RD6, RD12, RD13, and RD15 may play roles in the pathogenesis of TB, and RD1, RD5, RD7, RD9, and RD10 in mediating protection, and thus could be useful in searching for new vaccine candidates.

To investigate the effects of peptide pools of RDs, which induced mainly IL-10 secretion, on antigen-induced proliferation and IFN-γ responses, we cultured PBMC of healthy subjects in the presence of peptide pools of RD1 alone or along with peptide pools of RD12 or RD13 at suboptimal (1-μg/ml) and optimal (5-μg/ml) concentrations. The addition of RD12 and RD13 peptides inhibited RD1-induced proliferation and IFN-γ responses at both concentrations. The significant inhibition of responses at suboptimal, as well as optimal, concentrations of peptides rules out the possibility that the effect could have been nonspecific because of increased peptide concentrations.

In conclusion, our study shows that among the 11 RDs tested, RD1 induces the best Th1 responses in both TB patients and BCG-vaccinated healthy subjects, suggesting that antigens present in RD1 could be useful in designing new vaccines against TB. In the past, it has been shown that the two RD1-encoded antigens, ESAT6 and CFP10, are promising vaccine candidates in animal models of TB (28). In addition, ESAT6 and CFP10 have also been demonstrated to be useful in the specific detection of infection with *M. tuberculosis* in IFN-γ assays (15, 21). The preferential secretion of IFN-γ in response to peptide pools of RD5, RD7, RD9, and RD10 suggests that the individual antigens of these RDs may also be identified and evaluated for vaccine and diagnostic potential to improve upon the efficacy of RD1-encoded antigens.

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