Peptide-Specific T Cell Response to *Mycobacterium tuberculosis*: Clinical Spectrum, Compartmentalization, and Effect of Chemotherapy

Robert John Wilkinson, Hans Martin Vordermeier,* Katalin Andrea Wilkinson,* Ann SjoÈlund, Carlos Moreno,* Geoffrey Pasvol, and Juraj Ivanyi*

The T cell repertoire of 59 patients with untreated tuberculosis was compared with that of 46 bacille Calmette-Guérin–vaccinated controls by assaying the proliferative responses to six permissively recognized peptides from the 16-, 19-, and 38-kDa molecules of *Mycobacterium tuberculosis*. A trend from higher to lower reactivity following this order: vaccinated controls > lymph node disease > localized extrapulmonary > pulmonary > pleural was seen for 4 of the peptides (P < .03). The decreased response of blood lymphocytes from patients with pleural tuberculosis was partially accounted for by sequestration of peptide-responsive cells within the pleural fluid. Chemotherapy “reversed” the depressed proliferative responses of patients with pulmonary and pleural tuberculosis depending on the peptide origin, being greatest for peptides of 16 kDa, transient for those of 19 kDa, and least for those of 38 kDa. These data demonstrate antigen specificity in the decreased responsiveness of patients with tuberculosis.

Vaccination using bacille Calmette-Guérin (BCG) is only partially effective in preventing tuberculosis [1, 2]. The reasons for this are multiple. One possibility is that the antigenic specificity and phenotype of CD4+ T cell response to this vaccine differs from that induced by infection by *Mycobacterium tuberculosis*. Such natural infection is clinically inapparent in the majority of cases, but in the minority of cases, the outcome is the early development of primary or, later, postprimary disease following reactivation, which may be as long as 30 years after infection [3]. Chemotherapy of tuberculosis is highly effective, with a <5% relapse rate [4], and so any changes in the specificity and phenotype of T cell response occurring during chemotherapy may reflect the expression of protective immunity. Chemotherapy is known to result in an increase in peripheral CD4+ T cell counts [5], reduce soluble interleukin (IL)-2 receptor levels [6], change lipopolysaccharide (LPS)-induced monocyte cytokine secretion [7] and adrenal androgen production [8], increase serum antibody levels [9], and reverse skin test “anergy” [10]. The T cell response to distinct *M. tuberculosis* antigens during chemotherapy has not, however, been systematically investigated beyond our preliminary data on the 38-kDa antigen [11]. In this study, we have therefore compared the T cell proliferative responses of healthy BCG vaccine recipients (vaccinated controls, VC) with those of patients with a wide clinical spectrum of untreated postprimary disease. The compartmentalization of the T cell response in pleural disease and the effect of chemotherapy on the proliferative and cytokine response of a subset of patients with pulmonary or pleural disease was reassessed during chemotherapy.

Tuberculosis is characterized by a T cell response to a wide range of antigens [12]. In this study, we used six T cell–stimulatory peptides containing prominent epitopes from the 16-kDa, 19-kDa, and 38-kDa antigens, as well as a crude extract represented by purified protein derivative (PPD). The 16-kDa antigen representing a small heat-shock protein [13, 14] is predominant in the stationary growth phase of *M. tuberculosis* [15], and the T cell response to this antigen may be important in containing infection, given its potential role in mycobacterial nonreplicating persistence. Over 90% of tuberculosis-infected persons have a significant T cell proliferative and/or antibody response to the recombinant 16-kDa antigen (Wilkinson RJ, unpublished observations). Some 93% of sensitized healthy subjects respond to at least 1 of the 4 most stimulatory peptides from this molecule, implying HLA class II–permissive recognition [16], which is corroborated by peptide binding to more than one HLA class II molecule [17]. HLA-permissive T cell stimulatory peptides were also identified in the 19-kDa molecule [18, 19], a prominent
Table 1. Baseline characteristics of 59 patients with untreated tuberculosis and 46 bacille Calmette-Guérin (BCG)–vaccinated controls.

<table>
<thead>
<tr>
<th>Study category, group</th>
<th>n</th>
<th>No.</th>
<th>Sex</th>
<th>Average age (± SE)</th>
<th>Notes</th>
</tr>
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<tbody>
<tr>
<td>BCG-vaccinated healthy PPD skin test–positive controls</td>
<td>46</td>
<td>26</td>
<td>M</td>
<td>33.8 ± 1.8</td>
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<td></td>
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<tr>
<td>Untreated patients</td>
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<td>Lymphadenitis</td>
<td>7</td>
<td>6</td>
<td>M</td>
<td>33.7 ± 1.2</td>
<td>Sites: 6 cervical, 1 inguinal</td>
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<td></td>
<td>1</td>
<td>1</td>
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<tr>
<td>Localized extrapulmonary</td>
<td>9</td>
<td>7</td>
<td>M</td>
<td>41.9 ± 5.7</td>
<td>Sites: genitourinary (2), psos (2), skin and subcutaneous tissue of thigh (1), splenic cyst (1), peritoneal (1), pancreas (1), osteomyelitis in left wrist (1)</td>
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<td></td>
<td>2</td>
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<tr>
<td>Pulmonary</td>
<td>33</td>
<td>22</td>
<td>M</td>
<td>28.1 ± 2.2</td>
<td>All culture-positive. 75% unilobar disease; 78% postprimary pattern</td>
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<td></td>
<td>11</td>
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<tr>
<td>Pleural</td>
<td>10</td>
<td>6</td>
<td>M</td>
<td>37.8 ± 5.2</td>
<td>Postprimary pattern: all massive effusions with radiologic estimate of 1–2.5 liters of fluid present</td>
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<td>Subset analyses</td>
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<td>Effect of chemotherapy, pulmonary (untreated, during treatment, end of treatment)</td>
<td>15</td>
<td>10</td>
<td>M</td>
<td>32.7 ± 4.3</td>
<td>All culture-positive. 86% unilobar disease; 73% postprimary pattern</td>
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<td>Compartmentalization, pleural</td>
<td>6</td>
<td>4</td>
<td>M</td>
<td>43.0 ± 7.0</td>
<td>Clinical features as above; 3 were retested during chemotherapy</td>
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<td>Phenotype of PPD-responsive T cells</td>
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<tr>
<td>Pulmonary</td>
<td>7</td>
<td>6</td>
<td>M</td>
<td>25.9 ± 1.7</td>
<td>Tested before and after mean of 39 ± 8 days of chemotherapy</td>
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<td>2</td>
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<td></td>
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<tr>
<td>Healthy vaccinated controls</td>
<td>13</td>
<td>8</td>
<td>M</td>
<td>32.7 ± 2.9</td>
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NOTE. PPD, purified protein derivative.

target of both the antibody and cellular response in humans [20–22]. The 38-kDa protein is a potent stimulus of both the T cell and B cell response in humans [12, 23–25]. Vaccination with a plasmid DNA or recombinant vaccinia virus expressing the 19- or 38-kDa antigens protects mice against experimental infection [26, 27]. The response to peptide 38p350-369 (38G) has been of particular interest in view of the previously reported selective “anergy” (i.e., decreased peripheral blood mononuclear cell [PBMC] proliferative response) in patients with pulmonary tuberculosis [28].

Materials and Methods

Subjects. Patients were recruited from the Department of Infection and Tropical Medicine at Northwick Park Hospital. No patient had clinical or laboratory features suggestive of human immunodeficiency virus infection and, on anonymous random testing, 0 of 44 were positive by ELISA for human immunodeficiency virus type 1. Only PBMC from biopsy- or culture-proven patients were used. Patients were compared with BCG-vaccinated healthy controls. PPD positivity was defined as a skin reaction of >5 mm induration to 1 tuberculin unit or a Heaf grade ≥1. The basic characteristics of subjects (and subsets) used in the various studies are shown in table 1. Standard triple or quadruple antituberculous chemotherapy was used according to the regimen 2RHZE/6RH (rifampicin, isoniazid, pyrazinamide, ethambutol). No patient in the chemotherapy studies was infected with a drug-resistant isolate.

Synthetic peptides and antigens. Synthesis by Fmoc technology was employed using trialkoxy-diphenyl-methylester resin and Castro’s reagent pyBOP (benzatriazole-1-7-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate) for coupling. After cleavage with trifluoroacetic acid and deprotection, the peptides were purified by reverse-phase high-performance liquid chromatography (HPLC) in 0.1% trifluoroacetic acid/acetonitrile followed by gel filtration through Sephadex G15 (Pharmacia, Uppsala, Sweden) in 25% aqueous acetic acid. Homogeneity was confirmed by reverse-phase HPLC and mass spectroscopy and, in the case of some peptides, by amino acid composition. Peptides were used in T cell proliferation assays at 50 μg/mL. On completion of the study, the peptides were reanalyzed by reverse-phase HPLC to ensure that no degradation had taken place during the study period. PPD was obtained from Evans Medical (Liverpool, UK) and was used in assays at 10 U/mL. This is a clinical reagent and is endotoxin-free. All proliferation and cytokine assays included control wells containing the mitogen concanavalin A at 5 μg/mL.

Cell isolation and proliferation assay. Whole citrated blood was diluted 1/2 in PBS (Life Technologies Gibco BRL, Paisley, UK), and the PBMC were separated on a ficoll gradient (Pharmacia). Pleural fluid cells (PFC) free of contaminating red blood cells were not passed over ficoll. All cells were then washed twice in PBS and plated in triplicate wells at 1.5 × 10^5/well in RPMI 1640 plus 5% AB human serum in 96 U-well plates (Nunc, Roskilde, Denmark) for 6 days (PFC, 4–5 days). DNA synthesis was assayed by [ ^3 H]thymidine (Amersham, Amersham, UK) incorporation during the last 8 h of the culture. The lymphocyte
Results

**Cell proliferation in untreated patients and controls.** The T cell response of 105 persons in total was studied. A decreased frequency of proliferative response to all tested peptides was observed in all patient groups compared with the VC group (figure 1). A trend was seen from higher to lower reactivity for the peptides of the 16-kDa antigen and, to a lesser extent, in response to 19p61-80 and 38p65-83: VCbs had the highest values, followed by untreated patients with lymphadenitis (LN), localized extrapulmonary infection (EP), pulmonary infection (UP), and pleural tuberculosis (PL) (in descending order) (P < .03 for the trend in every case). In the case of the 19p61-80, the trend was not as uniform, as no EP patient reacted to this peptide. No trend in the patient group was discernible in response to 19p141-159 or 38p350-369, although the response of the largest group (UP) was depressed compared with that of VC (P = .08 and 0.01, respectively). The frequency of response to PPD was not significantly different between groups, although the mean SIs did show a similar ranking (VC ≥ UP = LN = EP ≥ UP = PL = 5.81 ± 1.9).

**Effect of chemotherapy on the lymphocyte proliferation of UP patients.** We investigated whether chemotherapy could reverse the depressed proliferative response in UP patients (table 1). We tested 15 patients before and after a mean of 76 ± 18 days of chemotherapy; 7 patients were tested a third time after the completion of chemotherapy at 243 ± 26 days. The net effect of chemotherapy was to increase the median response to all the tested peptides (figure 2). Individual cases showed variable spreading patterns of antipeptide reactivity, with occasional decreases as well as increases. There were marked differences in the extent of reversal, depending on the peptide. The 2 peptides of 16 kDa were associated with a sustained increase in reactivity to the end of chemotherapy. PPD and the 2 peptides of 19 kDa showed an increase followed by a slight decrease. In response to 38p65-83, there was an increase followed by a decrease such that the SIs at the end of chemotherapy were actually lower than before therapy, and little change in net response to 38p350-369. The most significant increases in stimulation during therapy were in response to PPD (P < .01), 16p21-40 (P = .02), and 19p61-80 (P = .04).

**Comparison of the proliferative response of PBMC and cells from PFC.** We investigated the possibility that the particularly depressed PBMC response of patients with untreated pleural tuberculosis (figure 1) was due to sequestration of responding lymphocytes in the PFC. Simultaneous testing of the T cell repertoire of PBMC and PFC in 6 patients was done (figure 3); PBMC from 3 patients (nos. 4–6) were subsequently retested during therapy. For 1 patient (no. 6), we were able to sample the pleural fluid twice. For 3 patients, (nos. 2–4), the repertoire of the PFC was wider than that of PBMC. In patient 4, treatment resulted in a widening of the PBMC repertoire not only to peptides previously recognized in PFC but also to other peptides previously unrecognized in either compartment. In contrast, patients 1 and 5 had a restricted repertoire in both the PFC and PBMC. Following treatment, patient 5 shows the type of spreading pattern of antipeptide reactivity in the PBMC response that characterized the pulmonary patients, as dis-
Figure 1. Frequency of positive proliferative response to peptides of the 16-, 19-, and 38-kDa antigens. Positive response is defined by stimulation index (SI, counts per minute [cpm] in presence of antigen/cpm in absence of antigen) $\geq 3$. Vaccinated controls (VCs) were compared with various types of untreated tuberculosis patients. Trend towards lower reactivity from VCs through lymph node (LN), localized extrapulmonary (EP), pulmonary (UP), and pleural (PL) disease is significant ($P < .05$) for peptides 16p21-40, 16p111-130, 19p61-80, and 38p65-83 but not for 19p141-159 and 38p350-369; difference between VCs and UP groups with respect to 38p350-369 is significant ($P = .011$, Fisher's exact test of probability). Median SIs for each peptide and purified protein derivative (PPD) (in order presented above) in each group were as follows: VC = 6.8, 6.5, 4.2, 3.3, 5.8, 2.5, and 50.3; LN = 3.9, 2.7, 1.8, 1.2, 3.3, 1.4, and 30.0; EP = 3.0, 3.0, 1.3, 1.6, 2.7, 1.5, and 8.7; UP = 2.1, 2.7, 1.5, 1.7, 2.3, 1.5, and 20.0; PL = 2.1, 1.5, 2.0, 1.6, 1.9, 1.6, and 5.8.

cussed above. In an exceptional instance (patient no. 6), when the PFC were sampled twice, the initial repertoire was different in PBMC and PFC, and after 23 days of treatment, the repertoire in both compartments widened but without overlap.

**IFN-γ and IL-10 production by PPD-stimulated PBMC.** IFN-γ and IL-10 secretion was tested in 8 and 7 tuberculosis patients, respectively, before and after 39 ± 8 days (mean ± SE) of treatment (figure 4). This response was compared with that of 13 (10 for IL-10) VCs. Striking individual variation in IFN-γ levels before and after chemotherapy lowered statistically significant differences (figure 4): 6 of 8 patients had an increase during therapy, but 2 initially high responding patients had a slight decline. The unstimulated production of IL-10 in patients was high before (126.8 ± 68.0 pg/mL) and after (301.2 ± 72.8 pg/mL) therapy compared with that of VCs (19.2 ± 43.6 pg/mL, $P = .66$ and .09, respectively). PPD-stimulated IL-10 production above the unstimulated level showed a pronounced decline following chemotherapy in 4 of 7 patients ($P = .028$). In 5 patients tested before and during therapy, no IL-4 was detected by either ELISA or RT-PCR, and levels of IL-12 p70 were found to be negligibly low by ELISA (data not shown).

**Discussion**

The depression of proliferative responses of PBMC to mycobacterial antigens in patients with active tuberculous disease has been documented in a number of studies by others [30–32]. Our main interest has been to investigate whether the degree of this depression is associated with the specificity of CD4+ T cells using permissively recognized 20-mer peptides from *M. tuberculosis* complex antigens [16, 18, 28]. CD8+ responses to peptides in human tuberculosis have been difficult to detect even using peptides of optimal length conforming to known class I motifs [33]. While we have not formally excluded them, we therefore consider that the 20-mer peptides used in this study, which are neither taken up nor processed,
were unlikely to have elicited the proliferation of CD8⁺ T cells. The results revealed marked differences in the frequency of peptide recognition depending upon the phenotype of disease ranging from 71% (16p21-40 in LN disease) to 0% (peptide 19p61-80 in EP disease) (figure 1). Even more striking were the differences in the extent to which the depressed proliferative response reversed, depending on the peptide, being the greatest and sustained for peptides of the 16-kDa antigen, transient for those of the 19-kDa antigen, and least for those of the 38-kDa antigen (figure 2). It is possible that a bias in peptide recognition could have arisen due to a more favorable HLA-binding profile of particular peptides for the class II alleles expressed by the patients and controls studied. All subjects in this study were HLA class II typed at DRB1* and DQB1* by the PCR-sequence–specific primers method (data not shown). The allele frequencies obtained were compared with the HLA binding profile of the peptides ([17]; Jurcevic S, Wilkinson RJ, unpublished observations), and no evidence of such bias was apparent.

Ideally, a group of non–BCG-vaccinated primary-infected controls who had remained healthy for several years should have been studied in addition to those who had received BCG vaccination, as classification on the latter basis reflects at best an incomplete immune phenotype. However, it is possible that important differences exist between the distinct antigens in their contribution to protective immunity. The relatively high frequency of response to the two peptides of the 16-kDa antigen is associated with the contained, less severe disease phenotypes (LN and EP) and also with the best recovery of response following chemotherapy. High intracytoplasmic expression of this protein is induced in stressed organisms [13, 15, 34], and it is possible that chemotherapy represents such a stress prior to bacterial killing, leading to reexpression and enhanced immunogenicity of this antigen in treated patients. However, the quantitative levels of expression of this antigen might not provide the full explanation, given that antibody levels are known to decrease rapidly in fully treated patients but to increase in poorly compliant patients [21]. By comparison, the 19-kDa and 38-kDa glycoproteins are secreted by actively dividing bacilli [35] and, therefore, the level of secretion of both proteins might be expected to decrease rapidly during chemotherapy. This interpretation is consistent with the transient increase in T cell reactivity but does not explain the depressed response in untreated patients (figure 1). The results indicate that this decreased response may be partially contributed to by sequestration of cells at sites of active disease (figure 3). Another possibility is that T cells responding to the peptides are not expanded by natural infection, due to failure of infected antigen-presenting cells to present antigen. Pertinent in this respect are the observations that live mycobacteria growing in human macrophages are sequestered from recognition by CD4⁺ T cells [36] and inhibit MHC class II presentation [37].

The observed high degree of decreased PBMC response in patients with pleural disease correlates with a previous finding of high antibody levels in patients with tuberculous effusions [9] and reflects the generally perceived reciprocal relationship between levels of T cell and humoral immunity. The patients we studied had extensive postprimary pleural tuberculosis,
which is known to involve a selective concentration of CD4+ cells in the effusion [38] and could conversely lead to a reduction in circulating CD4+ T cells in peripheral blood, as found in patients with active pulmonary tuberculosis [5]. We considered that recirculation of activated cells from effusions following treatment could increase the PBMC response during chemotherapy. Evidence in favor of this hypothesis was obtained in 3 of 6 cases, whose T cell repertoire was found to be clearly wider in PFC than in PBMC. However, in other cases the two repertoires showed only minor overlap and, additionally, a spread of PBMC recognition during treatment to peptides not originally recognized by T cells of the disease compartment was seen. Therefore, the determinant spreading during treatment occurs not simply as a result of recirculation of cells from the disease compartment, and other mechanisms, such as the production of anti-inflammatory cytokines or altered antigen presentation or recognition, may also play a role.

It is widely believed that IFN-γ is protective in tuberculosis [39–41]. In this study, we did not find a significant diminution in PPD-induced IFN-γ production in patients with pulmonary tuberculosis compared with VCs, confirming previous findings [22]. In 6 patients with an initially low IFN-γ response, there was an increase following therapy. In a similar study, household contacts of tuberculosis patients had higher secretion than untreated patients [42]. This apparent contradiction is probably explained by the difference in control groups, as household contacts (unlike our controls) would have a high probability of recent exposure.

Small numbers of IL-4–secreting cells in patients with tuberculosis were reported using the highly sensitive ELISSPOT method [22]. Using the same technique, El Ghazali et al. estimated the numbers of PPD-responsive IL-4–producing cells in peripheral blood to be on the order of 1/100,000 [43]. In this study, in a smaller number of patients, we were unable to obtain positive IL-4 values by either ELISA or RT-PCR. Others have reported IL-4 gene expression in PBMC without detecting secretion of IL-4 by ELISA [44, 45]. However, our present data support the view that IL-4 production in human tuberculosis is not pronounced [46–48] and that other cytokines probably

**Figure 3.** Peripheral blood mononuclear cells (PBMC) or pleural fluid cells (PFC) were cultured for 96–120 h in presence of various peptides and purified protein derivative (PPD). Bold indicates positive response (stimulation index ≥3) in PBMC; shaded box = positive response in PFC. NT, peptide not tested.
Figure 4. Effect of chemotherapy on the interferon (IFN)-γ and interleukin (IL)-10 response to purified protein derivative (PPD). Cytokine content of supernatant was measured following culture of $5 \times 10^6$ PBMC for 24 h in presence or absence of PPD. Eight patients (7 pulmonary, 1 pleural) were tested before and after mean of 39 ± 8 days of chemotherapy and compared with 13 (10 in case of IL-10) healthy bacille Calmette-Guérin–vaccinated controls. Presented PPD-stimulated cytokine levels are values following the subtraction of unstimulated levels, which were negligible for IFN-γ but variable for IL-10 with the following medians (pg/mL): vaccinated controls: 19.2 (0–261), untreated patients, 126.8 (0–594); treated patients, 301.2 (11–516). Although there was trend towards increase in IFN-γ, this was not statistically significant (although decrease in IL-10 accompanying therapy was $P = .028$, Mann-Whitney U test).

An important practical point of this work is that studies on T cell responsiveness need to differentiate treated from untreated patients. Thus the lack of posttreatment recovery of response to 38p350-369 explains the previously reported peptide-specific anergy [28]. In conclusion, the decreased responsiveness of PBMC in patients with tuberculosis seems to be contributed to by a number of factors. Sequestration of cells at the site of the disease and a shift in cytokine production by PBMC, IL-10 down-regulates M. tuberculosis–induced Th1 responses and CTLA-4 expression [49] and can depress the proliferative response of patients and controls [32, 42]. A prominent role in the depressed proliferative response has also been attributed to the monocyte production of transforming growth factor-β [42], which enhances macrophage ability to produce IL-10 [50], but the possibility that IL-10 and transforming growth factor-β may act synergistically in their effects on T cells has not been investigated.

We did not detect any bioactive IL-12 p70 by ELISA, perhaps because IL-12 production can be better stimulated by live or dead M. tuberculosis [51, 52], and this secretion requires their phagocytosis [53]. PPD is a potent lymphocyte activating agent, and our negative data perhaps suggest that lymphocyte-dependent IL-12 production as a consequence of ligation of CD40 [54] may not be quantitatively important in tuberculosis. An important practical point of this work is that studies on T cell responsiveness need to differentiate treated from untreated patients. Thus the lack of posttreatment recovery of response to 38p350-369 explains the previously reported peptide-specific anergy [28]. In conclusion, the decreased responsiveness of PBMC in patients with tuberculosis seems to be contributed to by a number of factors. Sequestration of cells at the site of the disease and a shift in cytokine production by PBMC, particularly of IL-10, are undoubtedly involved. Some of these differences resolve during chemotherapy, and the cytokine production by patients tends towards that of PPD-positive controls, though some differences in peptide recognition remain. A number of mycobacterial antigens are candidates for prophylactic vaccination [27, 55–57]. Our data contribute to the better understanding of the fine specificity of the T cell response and could be valuable in the design and further evaluation of such protective subunits.

Acknowledgments

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References