Ligand-Specific αβ and γδ T Cell Responses in Childhood Tuberculosis

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The αβ and γδ T cell responses were analyzed in the peripheral blood of children affected by active tuberculosis (TB) and in healthy children who tested positive (PPD^+) or negative (PPD^-) for purified protein derivative. PPD^+ healthy and diseased children responded equally well to PPD in vitro. In contrast, only 18% of PPD^+ TB patients responded to peptide p38G derived from the 38-kDa protein of *Mycobacterium tuberculosis*. Analysis of the whole γδ T cell population and of its Vγ9/Vδ2 subset showed similar frequencies in PPD^+ children with TB and in healthy PPD^+ and PPD^- children. Vγ9/Vδ2 cells from children with TB responded to 5 different phosphoantigens similarly to those from healthy PPD^+ children, but healthy PPD^- children responded very poorly. Chemotherapy had contrasting effects on the tested lymphocyte population, represented by increase of αβ and decline of Vγ9/Vδ2 T cell responses. T cell responses in childhood TB may be similar to those in adult TB.

Tuberculosis (TB) remains the leading cause of mortality among human infectious diseases in the world [1]. After infection with *Mycobacterium tuberculosis* (Mtb), only a small proportion of individuals develops the disease, while most remain healthy. It has been known for some time that acquired resistance to Mtb infection depends on the interaction of antigen-specific T cells and activated macrophages [2, 3]. Consequently, T cell–deficient animals [4] and immunocompromised individuals are highly susceptible to TB [5]. It is believed that Mtb-specific T cells release cytokines that activate tuberculocidal and/or tuberculostatic alveolar macrophages [6, 7], promote macrophage development and activation, and promote granuloma formation [8]. Most or all of these activities are thought to be contained primarily within the αβ T cell population.

Recently, several studies have suggested that γδ T cells may play an important role in the immune response to Mtb. In mice, there is a large increase in the number of γδ T cells that accumulate in the lungs after intranasal challenge with PPD [9], and, in some experimental conditions, mice lacking γδ T cells because of δ gene targeting suffer a more severe form of TB and fail to control the infection [10, but see 11]. In normal healthy individuals, γδ T cells contain the highest frequency of Mtb-reactive T cells in the peripheral blood [12–16], and the predominant subset of Mtb-reactive γδ T cells express a TCR encoded by Vγ9 and Vδ2 gene segments [13, 17–20]. Several Mtb-γδ T cell antigens have been chemically defined and include nonproteic and/or nucleotide molecules containing critical phosphate moieties [21–24]. Although the results of these studies strongly suggest that γδ T cells play a role in the immune response to Mtb, the limited number of studies of these cells that have been done in human TB produced contradictory results. Whereas some studies have reported an increase in γδ T cells in the peripheral blood of TB patients [25, 26], others have shown that γδ T cell numbers remain constant in both the peripheral blood and the granulomatous lymph node lesions of TB patients [27–30]. Finally, a more recent report has shown a dramatic reduction in Vγ9/Vδ2 T cells in TB patients, which were refractory to in vitro stimulation by Mtb antigens [31]. These contrasting results may be a consequence of analyzing γδ T cells from patients at different stages of disease progression.

All studies cited earlier involved adult patients. There is no corresponding knowledge about the immune repertoire in childhood, except for one serological study [32]. In this paper we have analyzed the γδ and αβ T cell responses in the peripheral blood of children affected by TB and compared these responses to those detected in healthy children who tested positive (PPD^+) or negative (PPD^-) for purified protein derivative. None of the children in these test groups had prior bacille Calmette-Guérin (BCG) vaccination and it is plausible that the majority (or a major fraction) of TB cases were due to primary infection, disease that occurs in a person with no prior immunity [33]. Thus our study gives a picture of γδ and αβ populations soon

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after tuberculous infection and in the absence of chemotherapeutic treatment.

Materials and Methods

Patient population. All the groups consisted of children from the same geographic area and of similar socioeconomic background. None of the children had been vaccinated in infancy with BCG. None of the case or control patients had evidence of human immunodeficiency virus (HIV) infection, or was being treated with steroids or antitubercular drugs at the time of first sampling. Peripheral blood was obtained from 27 children with tuberculous infections (17 boys, 10 girls; age range 1–13 years, average age ± SE, 7.1 ± 3.3) from the Children's Hospital G. Di Cristina, Palermo, Italy. Although most of the cases are likely to represent primary TB, the possibility that some cases represent reactivation cannot be excluded. Seventeen children were affected by pulmonary TB, 4 were affected by TB meningitis, 4 were affected by lymphatic TB, 1 was affected by renal TB, and 1 was affected by pleural TB. Diagnosis of TB was established by the presence of clinical symptoms of TB, by the positivity of the tuberculin (PPD) skin test, and by chest radiography. In some circumstances (i.e., meningitis, pleural TB, and renal TB) positive culture of Mtb and/or Mtb detection by polymerase chain reaction further supported the diagnosis. All patients included in the study had a positive PPD skin test.

Healthy children who were used as normal control patients were also recruited from the Children's Hospital G. Di Cristina, Palermo, Italy and were divided into 2 groups according to the results of the PPD skin test. The PPD^+ group consisted of 46 children (30 boys, 16 girls; age range 1–14 years; average age ± SE, 7.1 ± 4.1), and the PPD^- group consisted of 17 children (10 boys, 7 girls, age range 3–14 years; average age ± SE, 7.2 ± 3.5). The PPD^+ children were not household contacts of known TB case patients.

Tuberculin (PPD) skin tests were considered positive when the induration diameter was larger than 5 mm at 72 h since injection of 1 U of PPD (Statens Seruminstitut, Copenhagen, Denmark).

Synthetic peptide. The carboxy-terminal (350–369) 20mer peptide of the 38-kDa protein of *M. tuberculosis* (p38G) of the sequence DQVHFQPLPPAVVSKDSALI was prepared by using solid-phase/Fmoc chemistry as described in detail elsewhere [34]. The peptide was of 90% purity and its homogeneity was confirmed by analytical reverse-phase high-performance liquid chromatography (HPLC), mass spectroscopy, and amino acid composition analysis.

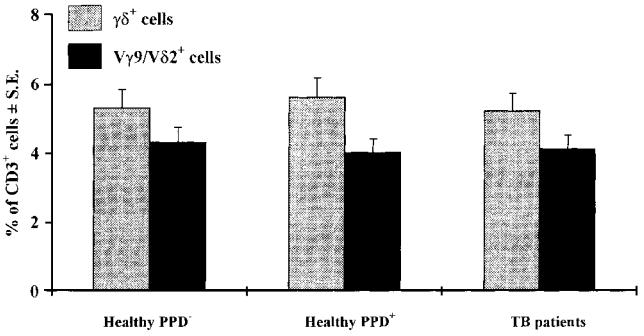

Figure 1. Frequency of γδ T lymphocytes in peripheral blood of healthy children and those with tuberculosis (TB) who tested positive (PPD^+) or negative (PPD^-) for purified protein derivative.

Purification of mycobacterial TUBAg1. TUBAg1 was obtained from *Mycobacterium fortuitum*-secreted antigens. Briefly, 2.5 L of *M. fortuitum* biovar *fortuitum* was grown for 6 weeks as velum on Sauton's medium. Culture medium was collected, filtered, and partitioned twice between chloroform and water. The water phase was concentrated, loaded onto DEAE, and eluted with increasing concentrations of ammonium acetate. The 0.2 *M* salt fraction was collected, dried 3 times, and separated on HPLC C18 in 0.1 *M* ammonium acetate. The fractions were collected and tested for bioactivity for the G12 clone as described elsewhere [24]. The first active peak, eluting after 1.3 V was collected, dried, and reseparated by mobile phase ion pair (MPIC–HPLC) by using the modified Di Pierro's method, as already described [35]. TUBAg1 was further characterized by its reverse-phase time on high pH anion exchange chromatography (HPAEC) and its sensitivity to treatment by alkaline phosphatase (data not shown).

Purified TUBAg1 stock concentration was estimated at around 1 μ*M* from HPAEC conductimetric quantification by using a method described elsewhere [36]. Bioactivity of purified TUBAg1 was titrated as usual [24] and this material was used at a 1 : 1000 dilution, giving a 1-n*M* final concentration in assays.

αβ T lymphocyte proliferation. Peripheral blood mononuclear cells (PBMC) were isolated from heparinized blood by centrifugation on Ficoll-Hypaque (Pharmacia, Uppsala, Sweden). Cells

Table 1. Proliferative response to purified protein derivative (PPD) and synthetic peptide p38G in the peripheral blood of healthy PPD^- or PPD^+ children and children with tuberculosis (TB).

	Healthy PPD^- (n = 17)	Healthy PPD^+ (n = 46)	TB children PPD^+ (n = 27)
Medium	560 ± 78 (60–1131)	800 ± 100 (126–2954)	640 ± 80 (67–1561)
PPD	585 ± 121 (159–2120)	15,920 ± 1760 (2421–63,348)	16,422 ± 2960 (1424–62,805)
	0	44 (96%)[a]	23 (86%)[a]
cp38G	590 ± 78 (125–1529)	5915 ± 950 (344–34,316)	1090 ± 209 (159–5049)[b]
	0	35 (77%)[a]	5 (18%)[a,b]
PHA	21,210 ± 4350 (3190–63,096)	24,930 ± 2332 (6209–73,588)	25,800 ± 3980 (4732–83,987)

NOTE. Results are expressed as counts per minute (cpm) ± SE. The range of cpm values is shown in parentheses. *n,* no. of individuals tested; PHA, phytohemagglutinin.

[a] Proportion of responder individuals (SI > 3 and mean cpm value >1000)

[b] $P < .001$ when compared with the healthy PPD^+ group.

Table 2. Vγ9/Vδ2 expansion factor to several different phosphoantigens in vitro.

Children category	Vγ9/Vδ2 EF ± SE after in vitro culture with				
	Rib-1-P	Xyl-1-P	IPP	DMAPP	TUBAg
Healthy PPD^- ($n = 17$)[a]	2 ± 1	3 ± 2	5 ± 1	5 ± 2	6 ± 3
Healthy PPD^+ ($n = 46$)	18 ± 8	19 ± 5	35 ± 9	32 ± 18	57 ± 12
TB children PPD^+ ($n = 27$)	15 ± 7	24 ± 14	40 ± 12	32 ± 9	51 ± 14

NOTE. DMAPP, dimethylallylpyrophosphate; EF, expansion factor; IPP, isopentenylpyrophosphate; PPD, purified protein derivative; Rib-1-P, ribose-1 phosphoantigen; TUBAg, phosphoantigen secreted from *Mycobacterium fortuitum;* Xyl-1-P; xylose-1 phosphoantigen.

[a] $P < .01$ when compared with the healthy subjects who tested positive (PPD^+) and with the children with tuberculosis (TB). Differences between the healthy PPD^+ group and the TB patient group were all $P > .05$.

were cultured in RPMI 1640 medium (Gibco, Grand Island, NY) supplemented with 10% heat-inactivated human AB serum, 2 m*M* l-glutamine, 20 m*M* hepes, and 100 U/mL penicillin/streptomycin. To assess αβ T cell proliferation, PBMC were cultured in triplicate at 2×10^5/well with PPD (Statens Seruminstitut, Copenhagen, Denmark) at the final concentration of 10 μg/mL, phytohemagglutinin (PHA) (Sigma, St. Louis, MO) at the final concentration of 1 μg/mL, and p38G at the final concentration of 50 μg/mL. Proliferation was set up in a final volume of 0.2 mL in 96 flat-bottomed microtiter plates (Nunc, Copenhagen, Denmark), at 37°C in the presence of 5% CO_2. Five days later, 1 μCi/well [^{3}H]thymidine (Amersham, Buckinghamshire, UK) was added and the cultures were harvested 18 h later. Results are expressed as Stimulation Indices (SIs), that is, mean counts per minute in the presence of antigen divided by mean counts per minute in the absence of antigen.

Monoclonal antibodies and flow cytometry. Monoclonal antibodies (mAbs) specific for human surface antigens were used as follows: anti-CD3-Quantum Red (UCHT-1, Sigma), anti-TCR αβ-PE (T10B9, PharMingen, San Diego, CA), anti-TCR γδ-PE (B1.1, PharMingen), and anti-TCR Vδ2-FITC (IMMU389) and anti-Vγ9 (IMMU360), both from Immunotech (Marseille, France, through Delta Biologicals, Rome, Italy).

PBMC (10^6 in 100 μL PBS with 1% heat-inactivated fetal calf serum and 0.02% Na-azide) were incubated at 4°C for 30 min with the anti-Vγ9 mAb, washed, and then incubated with phycoerythrin (PE)–conjugated secondary mAb. After further washing, Quantum Red-conjugated anti-CD3 and FITC-conjugated anti-Vδ2 mAbs were simultaneously added. Alternatively, the cells were simultaneously labeled with PE-conjugated anti-αβ and FITC-conjugated anti-Vδ2 mAbs. Fluorochrome-conjugated isotype-matched mAbs were used as negative controls. After washing, the cells were suspended in PBS with 1% FCS and analyzed on a FACScan flow cytometer (Becton Dickinson, Mountain View, CA), by using forward scatter/side scatter gating to select the lymphocyte population for analysis.

Expansion of Vγ9/Vδ2 T cells. PBMC were isolated from heparinized blood by centrifugation on Ficoll-Hypaque and cultured at 5×10^5 cells/0.5 mL in medium RPMI 1640 supplemented with 10% heat-inactivated human AB serum, 2 m*M* l-glutamine, 20 m*M* hepes, and 100 U/mL penicillin/streptomycin. PBMC were incubated with the following phosphoantigens: TUBAg1 (1 : 1000 v/v final concentration, which corresponds to a 1-n*M* final concentration), ribose-1-P (Rib-1-P, Sigma, 0.5 m*M* final concentration), xylose-1-P (Xyl-1-P, Sigma, 0.5 m*M* final concentration), dimethylallylpyrophosphate (DMAPP, Sigma, 0.5 m*M* final concentration), and isopentenylpyrophosphate (IPP, Sigma, 0.5 m*M* final concentration). After 72 h, cultures were supplemented with a 0.5-mL medium containing 20 U/mL recombinant human interleukin (rhIL)–2, and every 72 h a 0.5-mL medium was replaced with a 0.5-mL fresh medium containing 20 U/mL rhIL-2 [37]. After 14 days, cells were washed 3 times in medium, and expansion of Vγ9/Vδ2 T cells was assessed by FACS as described earlier. The absolute number of Vδ2 T cells in each culture was calculated according to the following formula: %Vδ2 $^+$ cells before culture × total cell count/100. The Vδ2 expansion factor (EF) was then calculated by dividing the absolute number of Vδ2 cells in specifically stimulated cultures by the absolute number of Vδ2 cells cultured in the absence of any antigen.

Statistical analysis. The Mann–Whitney U-test was used to compare responses in different groups. Values of $P < .05$ were chosen for rejection of the null hypothesis.

Results

Lymphocyte proliferation to PPD and peptide p38G. We examined the in vitro PBMC response of 27 PPD^+ children affected by TB, 46 healthy PPD^+ children, and 17 healthy PPD^- children to PPD and p38G in vitro. The results are presented in table 1. The response to PPD, expressed as mean counts per minute, was similar in PPD^+ healthy children and in PPD^+ children with TB. In contrast, a significant difference was found in the response to peptide p38G, whereby PPD^+ children with TB had a very low proliferation when compared with healthy PPD^+ children ($P < .001$). All children who were tested responded equally well to PHA at all test points (i.e., before and during chemotherapy, see table 1 and data not shown).

The response frequencies, defined by a stimulation index of >3 to PPD, were 96% for healthy PPD^+ children, 86% for children with TB, and 0% for healthy PPD^- children (table 1). A strikingly different finding was observed when the p38G response frequencies were analyzed: whereas 77% of the healthy PPD^+ children responded to this peptide, only 18% of the children with TB responded to p38G and this difference was highly

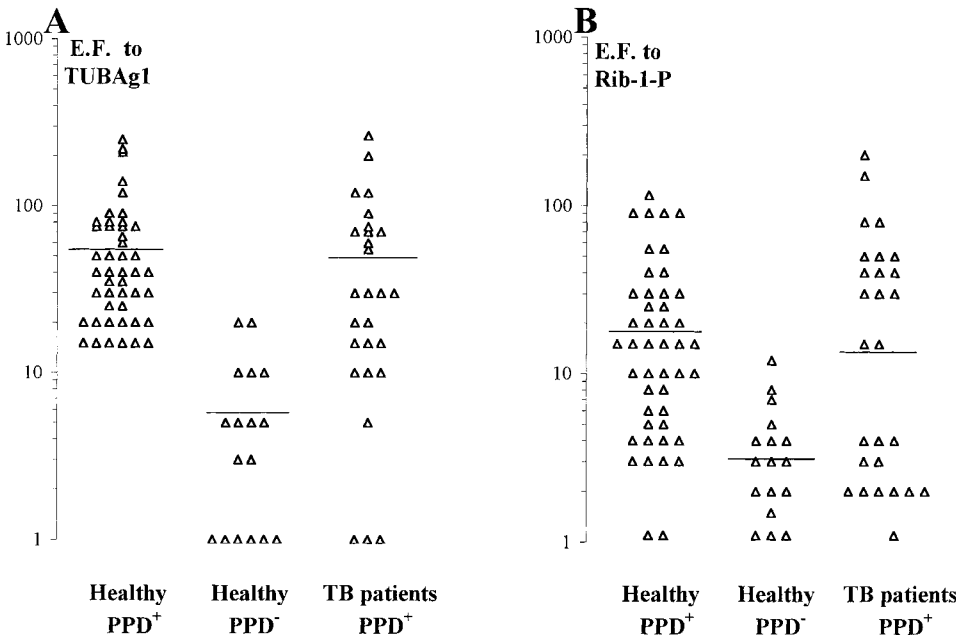

Figure 2. Expansion of Vγ9/Vδ2 cells in peripheral blood of healthy children and those with tuberculosis (TB) who tested positive (PPD^+) or negative (PPD^-) for purified protein derivative. Peripheral blood mononuclear cells (PBMC) (5×10^5 cells/well) were cultured in 24-well plates with different phosphoantigens and interleukin-2 for 14 days and analyzed by flow cytometry. Values represent the expansion factor (EF) calculated as described in Materials and Methods.

significant ($P < .001$). Notably, of the 5 children who responded, 3 had the lymphatic form of TB; therefore the anergy in the other clinical forms is even more profound. These results demonstrate a peptide (p38G)-specific decreased response in vitro in children with TB that is similar to that described previously in adult pulmonary TB [34].

Distribution of Vγ9/Vδ2 T lymphocytes in the peripheral blood of children with TB. Two- and three-color FACS analysis was done to assess the percentage of the γδ T lymphocytes and the Vγ9/Vδ2 subset in the peripheral blood of children with TB, as well as in healthy PPD^+ and PPD^- children (figure 1). The percentages of total γδ and Vδ2 T lymphocytes within peripheral blood $CD3^+$ T lymphocytes were virtually identical in the 3 tested groups: total γδ cells represented about 5% and the Vδ2 subset represented 4% of the CD3 population. The absolute number of total γδ T cells present in the blood of PPD^+ TB patients (78 ± 39 cells/μL) was similar to that of healthy PPD^+ (84 ± 36 cells/μL) and healthy PPD^- children (79 ± 33 cells/μL). Further analysis confirmed that all of the Vδ2 cells coexpressed the Vγ9 chain (data not shown).

From these results, we conclude that the absolute numbers and the percentages of total $\gamma\delta^+$ and $V\delta2^+$ T lymphocytes remain constant in the peripheral blood of children affected by TB when compared with healthy PPD^+ and PPD^- control patients.

Vγ9/Vδ2 T lymphocyte response to mycobacterial and synthetic antigens. To evaluate the functional responsiveness of Vγ9/Vδ2 T lymphocytes to stimulation by mycobacterial and synthetic antigens, PBMC from children affected by TB and from healthy PPD^+ and PPD^- children were cultured with 5 different molecules known to selectively stimulate Vγ9/Vδ2 T cells, and the EF was determined as described in Materials and Methods. Prominent expansion of Vγ9/Vδ2 cells was observed in PBMC from healthy PPD^+ children (table 2). There was a certain hierarchy in the expansion of Vγ9/Vδ2 toward the different antigens, with the highest EF obtained with TUBAg, the lowest obtained with Rib-1-P and Xyl-1-P, and an intermediate EF obtained with IPP and DMAPP. In striking contrast, very low Vγ9/Vδ2 expansion was observed in PBMC from the vast majority of healthy PPD^- children, and the EF detected with all 5 molecules was usually 5–8-fold lower than that detected in healthy PPD^+ children. The pronounced expansion of Vγ9/Vδ2 cells in PBMC from TB patients showed no significant difference from that seen in healthy PPD^+ children in response to all tested phosphoantigens. In no case did the EF of PPD^- subjects reach the mean EF value of the PPD^+ healthy subjects. Figure 2 shows a typical cytofluorometric analysis of expansion of Vδ2 cells from 1 healthy PPD^+ child and 1 healthy PPD^- child on stimulation with TUBAg. Figure 3 shows that there was a great variability in the expansion of Vγ9/Vδ2 cells within each single group of children (i.e., healthy PPD^-, healthy PPD^+, and TB patients PPD^+).

Effect of chemotherapy on αβ and γδ T cell responses. The αβ and γδ responses were retested in 16 PPD^+ children, halfway

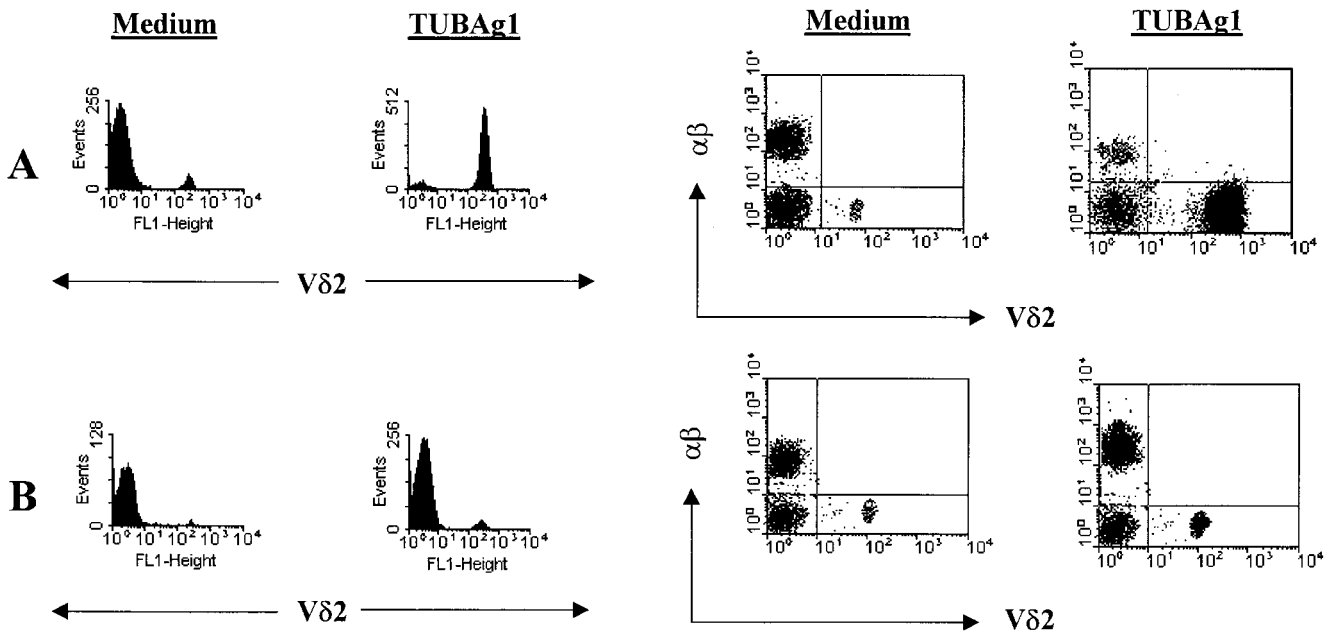

Figure 3. Cytofluorimetric analysis of Vδ2 cells in peripheral blood mononuclear cells (PBMC) from 1 healthy subject who tested positive (PPD^+) and 1 who tested negative (PPD^-) for purified protein derivative after in vitro culture with phosphoantigen secreted from *Mycobacterium fortuitum* (TUBAg) or medium alone. PBMC (5×10^5 cells/well) were cultured in 24-well plates with medium alone or with TUBAg and interleukin-2 for 14 days and analyzed by flow cytometry. Histograms show the percentage of cells expressing Vδ2, and dot plots show the percentage of αβ and Vδ2 cells. *A,* Results obtained from 1 healthy PPD^+ child. *B,* Results obtained from 1 healthy PPD^- child.

(~4 months) through chemotherapy. The stimulation indices reflecting αβ T cell responses increased after treatment in response to PPD in 14 of 16 children, and responses to p38G consistently increased in 16 of 16 children (figure 4*A,* 4*B*). In striking contrast, the γδ T cell response to Rib-1-P and TUBAg strongly decreased during treatment (figure 4*C,* 4*D*). This was not a result of modification of Vγ9/Vδ2 cells, because their percentages remained unchanged after chemotherapy (4.0 ± 0.5 before therapy vs. 3.8 ± 0.4 after therapy). Similarly, the absolute number of circulating γδ cells also was not substantially modified by chemotherapy (78 ± 39 cells/μL vs. 71 ± 25 cells/μL in TB patients and 84 ± 36 cells/μL versus 77 ± 30 cells/μL in healthy PPD^+ children). However, no significant correlation was detected between the increase in the PPD and p38G responses and the decrease in the γδ responses to Rib-1-P and TUBAg (data not shown).

Discussion

The majority of individuals, on exposure to Mtb, develop protective immunity and successfully contain the primary infection. Numerous studies in murine models have shown that T cells play a crucial role in establishing the protective immune response to Mtb. The key process has been attributed to the interaction of CD4 and CD8 αβ T cells with macrophages in controlling mycobacterial growth, but recent studies in humans as well as in mice suggest that γδ T cells may play an important, yet poorly defined, role in the initial immune response to Mtb [10–16, 38].

The present study was designed to examine the αβ and γδ T cell responses in child TB. Because the tested population was not previously vaccinated with BCG, it is important to consider that the observed data in both PPD^+ healthy and active TB case patients can be probably be attributed to infection with pathogenic Mtb. The proliferative responses of PBMC to PPD and p38G in vitro were used as a parameter of αβ T cell activity. The response to PPD showed no difference between healthy PPD^+ children and TB patients. In contrast, the majority (77%) of healthy PPD^+ children responded to p38G in vitro, whereas responses to this peptide were detected in only 5 of 27 children with TB (18%). These results agree with the findings of a previous report that described the selective anergy to p38G in adult TB patients [34]. In that study, anergy to p38G occurred in patients with pulmonary (90% nonresponders) and nonlymphatic extrapulmonary (75% nonresponders) TB, whereas patients with lymphatic TB responded to p38G as well as healthy PPD^+ control subjects did [34]. It is noteworthy that 3 of the 5 children with TB who were responsive to p38G were affected by lymphatic TB. Furthermore, both PPD and p38G responses increased after chemotherapy. The cause of diminished αβ T cell response in untreated active TB is probably multifactorial, with compartmentalization at the site of disease [39, 40], pro-

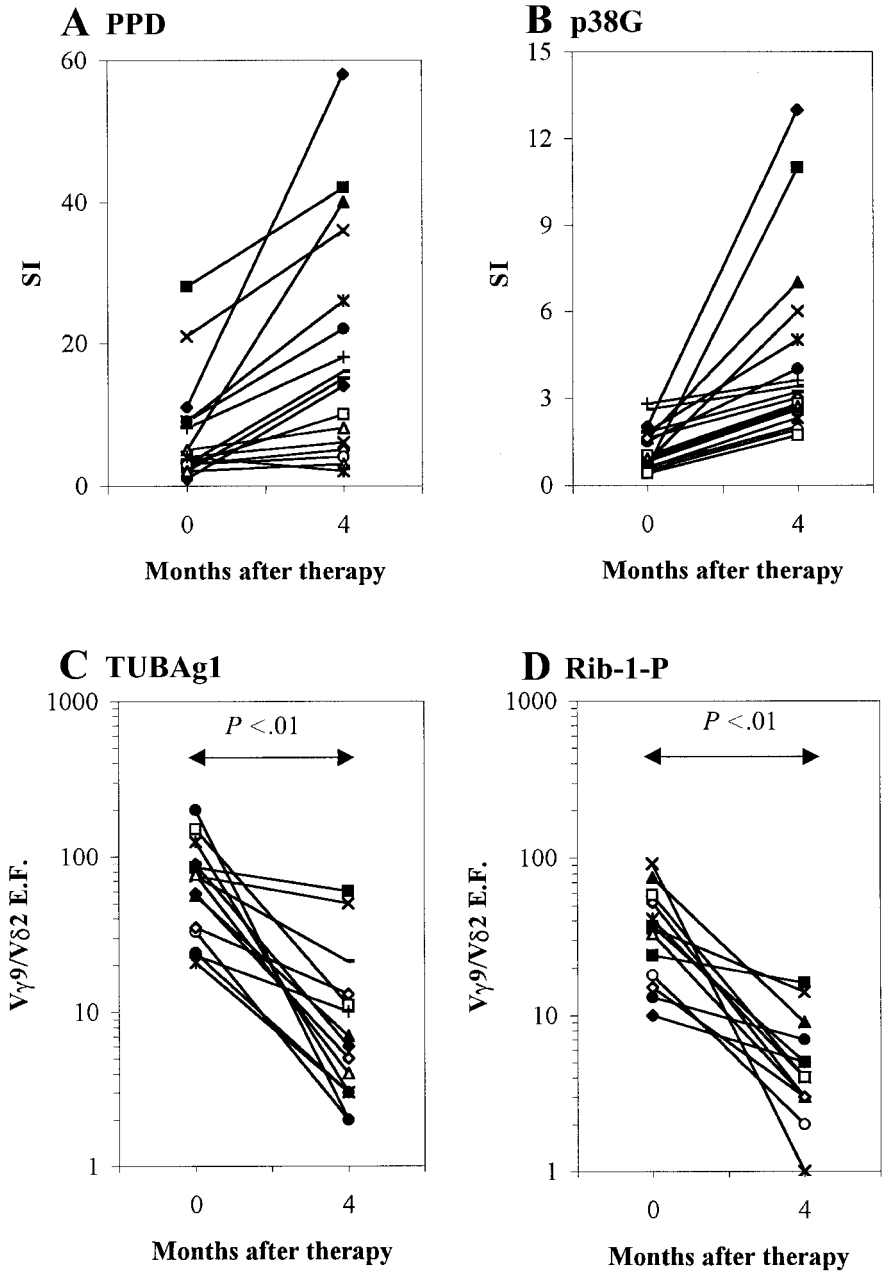

Figure 4. Changes in cellular responses of children with tuberculosis (TB) after 4 months of chemotherapy. Proliferative response to purified protein derivative (PPD) *(A)* and p38G *(B)*, and expansion of Vγ9/Vδ2 cells to phosphoantigen secreted from *Mycobacterium fortuitum* (TUBAg) *(C)* and ribose-1 phosphoantigen (Rib-1-P) *(D)*.

duction of suppressive cytokines such as interleukin (IL)–10 or TGF-β [41], and defective antigen presentation [42] all contributing.

Consistent with previous analysis of γδ T lymphocytes in TB patients [27–30], the frequency of the whole γδ T cell population, as well as that of the Vγ9/Vδ2 subset, in the blood of children with TB was similar to the values obtained for both PPD^+ and PPD^- healthy children. These results agree with the reported lack of modification in the percentage of γδ cells [27–30], but not with those reporting their increased frequency in the peripheral blood of adult TB patients [25, 26]. However, it should be noted that, in one of those studies, particularly low levels of peripheral γδ cells were reported in control subjects (i.e., 1.7 ± 0.9%) [25], and the other study concerned patients affected by tuberculous pleuritis [26] rather than the pulmonary form of TB.

Vγ9/Vδ2 T cells are not predominant in human peripheral blood at birth. Rather, their number increases from birth and peaks at about 7 years. The expansion of Vγ9/Vδ2 T cells during the first few years of life may be the result of peripheral antigen-driven expansion, because it occurs in the absence of a parallel thymic wave [43]. Primary in vitro culture of PBMC with a crude sonicate of Mtb results in a preferential expansion of Vγ9/Vδ2 T cells, not only in individuals with no prior exposure to Mtb, but also in children and in newborns (umbilical cord blood), suggesting that the Vγ9/Vδ2 T cell response to Mtb does not require prior exposure to this microorganism [reviewed in 44].

We found that Vγ9/Vδ2 cells from children with TB responded to 5 different phosphoantigens known to selectively expand this T cell subset, to an extent similar to that detected in healthy PPD^+ children. Notably, the expansion of Vγ9/Vδ2 T cells consistently decreased after therapy. This confirms our initial observations in a smaller group of children affected by pulmonary TB [45]. It would be of interest to investigate whether this reversal could be used to measure the efficacy of chemotherapy and even of chemoprophylaxis. Another point of interest is that Vγ9/Vδ2 expansion in vitro may be induced by a number of different Mtb antigens in vivo. In fact, there is considerable junctional heterogeneity among Vγ9/Vδ2 cells [46, 47], and some TCR combining sites could confer stronger reactivity than others.

The reduction of Vγ9/Vδ2 cell expansion observed after drug treatment is attributable to the reduction in the viable bacterial load. Because γδ cells apparently lack long-lasting memory, their expansion by phosphoantigens in vitro will sharply decline, whereas primed αβ T cells that do have a long-lasting memory will retain their ability to proliferate in response to PPD or relevant peptides. The γδ T cells apparently require priming in vivo to expand in the presence of mycobacterial phosphoantigens in vitro. This contention agrees with a previous study that reported larger in vitro Mtb-stimulated expansion of γδ T cells in PPD^+ subjects (tuberculin reactors, tuberculous pleuritis) than that in healthy PPD^- subjects [29]. Furthermore, a significant increase of the Vγ9/Vδ2 T cell response to IPP in vitro after BCG vaccination also suggests that Vγ9/Vδ2 T cells have a memory-like response [48].

In contrast with our data, a selective loss of the Vδ2 subset was reported in the peripheral blood and lungs of adult TB patients, and the remaining Vδ2 cells were anergic in response to Mtb antigens [31]. However, that study did inform whether the testing was done before or during chemotherapy, a treatment that had a striking effect on γδ T cell response in this study. Furthermore, these studies used only the indiscriminatory whole Mtb for stimulation of γδ cells. Mtb activates γδ cells aided by IL-2 released from $CD4^+$ $\alpha\beta^+$ cells [20]; therefore, the levels of γδ cell expansion will reflect the extent of αβ cell activation. Hence, the apparent anergy to Mtb of Vγ9 cells in HIV-infected individuals is due to CD4 Th1 cell deficiency and is completely restored by addition of exogenous IL-2 [49]. The natural, mycobacterial (TUBAg) and synthetic phosphoantigens used in our study do not activate $CD4^+$ $\alpha\beta^+$ cells. Therefore, our results give a more reliable direct measure of Vγ9/Vδ2 T cell response. However, as expansion of Vγ9/Vδ2 cells was done from whole PBMC, we cannot exclude the possibility that other cells contributed to their activation. In this context, a key role might be played by macrophages and macrophage-derived IL-12, which is important for γδ T cell activation [50]. Because macrophages from healthy or TB PPD^+ children produce more IL-12 than do macrophages from healthy PPD^- subjects ([51] and our unpublished observation), the increased IL-12 production might support Vγ9/Vδ2 T cell activation in vivo and contribute to their expansion by phosphoantigens in vitro.

Our results provide new information about αβ and γδ T cell responses during primary Mtb infection and active TB in children. Further definition of the cytokine profiles and effector functions of the Vγ9/Vδ2 cells is necessary to improve our understanding of their potential role in host resistance against mycobacterial disease. Although the selective anergy of αβ T cell proliferation to PPD and to p38G would at least to some extent discriminate between self-healing infection and active disease, the response of Vγ9/Vδ2 T cells to phosphoantigens may sensitively monitor the response to chemotherapy. Considering the presently very poor laboratory support for the diagnosis and management of childhood TB, further studies with large numbers of subjects are required to define the clinical value of such assays.

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