Selective Depression of Interferon-γ and Granulysin Production with Increase of Proliferative Response by Vγ9/Vδ2 T Cells in Children with Tuberculosis

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Vγ9/Vδ2 T cells can contribute to protective immune response against *Mycobacterium tuberculosis,* although the extent to which and mechanisms by which they could actually protect against human tuberculosis remain unclear. We have previously reported that Vγ9/Vδ2 T cells from tuberculin purified protein derivative (PPD)–positive children, either healthy or affected by different clinical forms of tuberculosis, strongly proliferate to different phosphoantigens in vitro, whereas Vγ9/Vδ2 T cells from PPD-negative healthy subjects proliferate very poorly. We report here that Vγ9/Vδ2 T cells from tuberculous children have an increased proliferative activity, but decreased interferon (IFN)–γ production and granulysin expression. After successful chemotherapy, the Vγ9/Vδ2 T cell proliferative response strongly decreased, whereas IFN-γ and granulysin production consistently increased. Disease-associated changes in Vγ9/Vδ2 T cell effector functions in patients with tuberculosis are consistent with the possibility that these T cells may play a protective role in immune response against *M. tuberculosis* infection.

The vast majority of individuals develop protective immunity after exposure to *Mycobacterium tuberculosis* and successfully contain the primary infection. It is commonly accepted that acquired resistance to *M. tuberculosis* primarily depends on the interaction of antigen-specific CD4 T cells and infected macrophages. In addition, both αβ CD8 and Vγ9/Vδ2 T cells can contribute to protective immune responses against *M. tuberculosis* by virtue of their ability to kill both infected macrophages and the intracellular pathogen [1].

Vγ9/Vδ2 T cells account for the majority (~75%) of all circulating γδ cells, which directly recognize nonpeptide ligands without presentation by major histocompatibility complex molecules. The nonpeptide ligands include natural phosphoesters derived from mycobacteria and, to a lesser extent, several ubiquitous metabolites, such as alkylamines from plant extracts, xylosyl- or ribosyl-1-phosphate, 2,3-diphosphoglycerate, and several synthetic aminobisphosphonates [2].

Several studies in humans have shown that both the relative percentage and absolute numbers of peripheral blood γδ cells do not change during tuberculosis (TB) infection [3–5], although most of the cited studies only included adult patients. We have previously found that Vγ9/Vδ2 T cells from purified protein derivative (PPD)–positive children, either healthy or affected by different clinical forms of TB, strongly proliferate to different phosphoantigens in vitro, whereas Vγ9/Vδ2 T cells from PPD-negative healthy subjects proliferate very poorly [6]. Because Vγ9/Vδ2 T cells are able to kill *M. tuberculosis* [7, 8], we have further assessed proliferative response and effector functions (interferon [IFN]–γ production and granulysin expression) in children with TB and healthy control subjects. None of the children in these tests groups had prior bacillus Calmette-Guérin (BCG) vaccination and it is plausible that the majority, or a major percentage, of TB cases were caused by primary infection, which refers to a disease that occurs in a person with no prior immunity [9]. Thus, our study gives a picture of Vγ9/Vδ2 T cell effector functions soon after tuberculous infection and in the absence of chemotherapeutic treatment.

Subjects, Materials, and Methods

Subject population. All the groups consisted of children from the same geographical area and of similar socioeconomic background. None of the children had been vaccinated with BCG during infancy. None of the case patients or control subjects had evidence of human immunodeficiency virus infection or were being treated with steroids or antitubercular drugs at the time of diagnosis and first sampling. The baseline characteristics of patients with TB and

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control children are shown in table 1. Diagnosis of TB was established by the presence of clinical symptoms of TB, chest radiography, positivity of the PPD skin test, and symptomatic improvement after chemotherapy. Positive culture of *M. tuberculosis* or *M. tuberculosis* detection by polymerase chain reaction was obtained in 5 of 7 children with TB meningitis, 1 child with pleural TB, and 1 child with renal TB further supported the diagnosis. Although most of the patients with TB infection probably represent primary TB, the possibility that some cases represent reactivation cannot be excluded. The PPD-psitive healthy children included in this study were not household contacts of individuals known to have TB. PPD skin test was considered positive when the induration diameter was >5 mm at 72 h after injection of 1 U of PPD (Statens SerumInstitut).

Cell preparation, proliferation, and production of IFN-γ. Peripheral blood mononuclear cells (PBMC) were isolated from heparinized blood by Ficoll-Hypaque (Pharmacia Biotech) and were cultured at 10^6 cells/mL in complete RPMI 1640 medium (Gibco) supplemented with 10% heat-inactivated fetal calf serum (FCS; Gibco), 2 m*M* L-glutamine, 20 n*M* HEPES, and 100 U/mL penicillin/streptomycin. PBMC were cultured for 12 days at 37° C in the presence of 5% CO_2 and 100 m*M* isopentenyl pyrophosphate (IPP; Sigma Chemical). Twenty units per milliliter of human recombinant interleukin-2 were added at day 3 and day 6 of culture. The expansion of Vγ9/Vδ2 T cells at the end of culture was determined by cytometric analysis using double staining (15 min at 4°C) with Quantum Red–conjugated anti-CD3 monoclonal antibody (MAb) (UCHT-1; Sigma) and fluorescein isothiocyanate (FITC)–conjugated anti-Vδ2 MAb (IMMU389; Immunotech). Isotype-matched control MAbs were used in all the experiments. After washing, the cells were suspended in PBS containing 1% FCS and were analyzed on a FACScan flow cytometer (Becton Dickinson Biosciences). The Vδ2 expansion factor (EF) was calculated as described elsewhere [6]. To assess IFN-γ production, PBMC were stimulated with IPP, as described above, supernatants were harvested at day 2 and day 7, and IFN-γ levels were assessed by 2-MAb sandwich ELISA, according to the manufacturer's recommendations (R&D Systems).

Intracellular staining for IFN-γ and granulysin. PBMC were stimulated with IPP, as described above, harvested at day 2 and day 7, and washed and stained for 15 min at 4°C with FITC-conjugated anti-Vδ2 MAb. After fixation and permeabilization, the cells were incubated for 1 h at 4°C with phycoerythrin (PE)–conjugated anti–IFN-γ MAb (Becton Dickinson Biosciences) [10]. For granulysin detection, cells were incubated with the clone of the anti-granulysin MAb (DH4; final concentration, 2 μg/mL) and PE-conjugated goat anti–mouse IgG (Sigma) [8]. Isotype-matched control MAb of irrelevant specificity were used in all the experiments. After washing, the cells were analyzed with a FACScan flow cytometer.

Statistical analysis. Differences among group means were evaluated by Mann-Whitney *U* test. $P < .05$ was considered to be significant.

Results

Proliferative response of Vγ9/Vδ2 T cells from children with TB and healthy control subjects. PBMC from children with TB and from healthy children were stimulated with IPP, and expansion of Vδ2 cells was determined. Prominent expansion of Vδ2 cells (figure 1) was observed in PBMC from healthy PPD-positive children (EF, 34.5), but expansion of Vδ2 cells in PBMC of PPD-positive patients with TB was greater (EF, 55.8), although differences did not attain statistical significance. In striking contrast, very low Vδ2 cell expansion was observed in PBMC from healthy PPD-negative children (EF, 6). PPD-negative patients with TB showed intermediate levels of Vδ2 cell expansion (EF, 22.7), which were significantly different from EF values detected in PPD-positive patients with TB and in PPD-negative healthy children.

IFN-γ response of Vγ9/Vδ2 T cells from children with TB and healthy control subjects. Despite their pronounced proliferative capacity, Vδ2 cells from patients with TB produced very low levels of IFN-γ after IPP stimulation, regardless of whether they were obtained from PPD-positive or PPD-negative pa-

Table 1. Baseline characteristics of patients with tuberculosis (TB) and control children.

	Patients with TB		Healthy control subjects	
Characteristic	PPD^+ (n = 43)	PPD^- (n = 15)	PPD^+ (n = 113)	PPD^- (n = 36)
Sex				
Male	28	10	75	21
Female	15	5	38	15
Age in years, mean ± SE	7.2 ± 3.7	6.4 ± 2.3	7.3 ± 4.3	6.9 ± 3.7
Age range, years	1–13	4–12	1–14	3–14
Vδ2 T cells/mL, mean ± SE	83 ± 41	71 ± 36	78 ± 36	81 ± 29
Clinical forms of tuberculosis				
Pulmonary	32	10		
Lymphoadenitis	4	0		
Meningitis	4	3		
Renal	1	1		
Pleural	1	1		
Pluropulmonary	1	0		

NOTE. PPD, purified protein derivative; +, positive; −, negative.

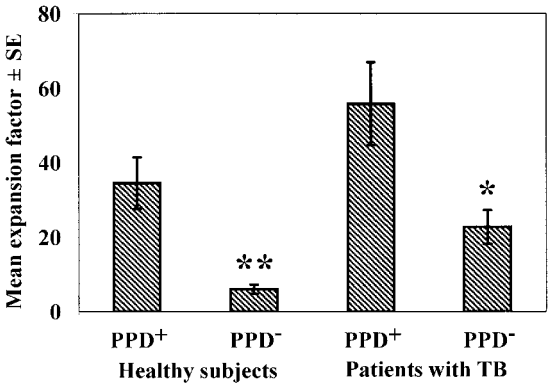

Figure 1. Proliferative response to isopentenyl pyrophosphate (IPP) by Vγ9/Vδ2 T cells from children with tuberculosis (TB) and healthy control subjects. Vγ9/Vδ2 T cells from purified protein derivative–positive (PPD^+) and PPD-negative (PPD^-) children with TB and healthy control subjects were stimulated with IPP in vitro, and their ability to proliferate was assessed. $*P < .05$, compared with all other groups; $**P < .01$, compared with PPD^+ patients with TB and PPD^+ healthy control subjects.

tients (figure 2*A*). Conversely, Vδ2 cells from healthy PPD-positive children produced high levels of IFN-γ, whereas Vδ2 cells from healthy PPD-negative children produced low levels of IFN-γ. To confirm that the difference observed between healthy control subjects and children with TB was not a consequence of prolonged in vitro stimulation, we retested IFN-γ production in a smaller group of the subjects (20 PPD-positive patients with TB, 10 PPD-negative patients with TB, 35 PPD-positive healthy control subjects, and 7 PPD-negative healthy control subjects) after in vitro culture with IPP for 2 days. Although IFN-γ levels detected at this early time point were higher than those detected at day 7 (figure 2*A*), children with TB produced very low levels of IFN-γ.

The IFN-γ ELISA data were confirmed by those obtained with intracellular staining for this cytokine performed in 20 PPD-positive patients with TB, 7 PPD-negative patients with TB, 10 PPD-negative patients with TB, 35 PPD-positive healthy control subjects, and 7 PPD-negative healthy control subjects. Figure 2*B* shows that, in PPD-positive healthy control subjects, 44% of Vδ2 cells stained positive for IFN-γ, but, in PPD-positive and PPD-negative patients with TB, ~10% of Vδ2 cells expressed IFN-γ. Similar results were obtained after stimulation of cells with IPP for 2 or 7 days.Figure 3 shows a typical 2-color fluorescence-activated cell sorter (FACS) analysis of Vδ2 and IFN-γ staining from 1 PPD-positive healthy control subject and 1 PPD-positive child with TB and clearly indicates that most of the IFN-γ was a product of Vδ2 cells.

Granulysin response of Vγ9/Vδ2 T cells from children with TB and healthy control subjects. We have previously reported that Vδ2 cells contain granulysin, which has crucial anti-mycobacterial activity in vitro [8].Figure 4 shows that, whereas in healthy PPD-positive children, 38% of Vδ2 cells expressed intracellular granulysin, in both PPD^+ and PPD-negative patients with TB ~10% of Vδ2 cells expressed granulysin. To confirm that the difference observed between healthy control subjects and children with TB was not a consequence of prolonged in vitro stimulation, we retested IFN-γ production in a smaller group of subjects (20 PPD^+ patients with TB, 10 PPD-negative patients with TB, 35 PPD^+ healthy control subjects, and 7 PPD-negative healthy control subjects) after in vitro culture of PBMC with IPP for 2 days. The results obtained confirm that granulysin expression in Vδ2 T cells from patients with TB was greatly reduced (figure 4). Figure 3 shows a typical 2-color FACS analysis of Vδ2 and granulysin staining from 1 PPD^+ healthy control subject and 1 PPD^+ child with TB.

Effect of chemotherapy on Vδ2 T cell functions. Vδ2 T cell responses were retested in 33 PPD^+ patients with TB every 3 months during chemotherapy. Vδ2 cell expansion in response to IPP strongly decreased during treatment (figure 5*A*). This was not due to a modification of Vδ2 cells, because their absolute number remained virtually unchanged after chemotherapy (data not shown). Conversely, both IFN-γ production (fig-

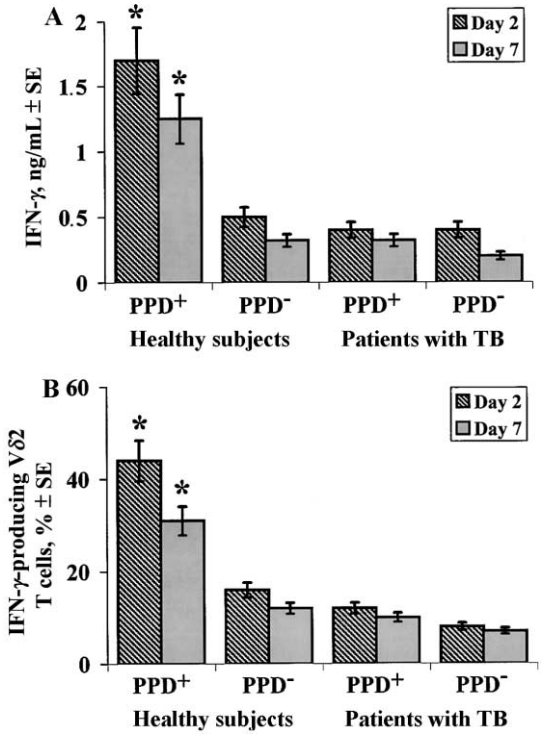

Figure 2. Interferon (IFN)–γ production in response to isopentenyl pyrophosphate (IPP) by Vγ9/Vδ2 T cells from children with tuberculosis (TB) and healthy control subjects. Vγ9/Vδ2 T cells from purified protein derivative–positive (PPD^+) and PPD-negative (PPD^-) children with TB and healthy control subjects were stimulated for 2 or 7 days with IPP in vitro, and their IFN-γ production was assessed by ELISA *(A)* or intracellular FACS analysis *(B)*. Data are mean ± SE (ng/mL) related to 2×10^3 Vδ2 T cells *(A)* or as percentage (±SE) of IFN-γ–producing Vδ2 T cells *(B)*. $*P < .05$, compared with all other groups.

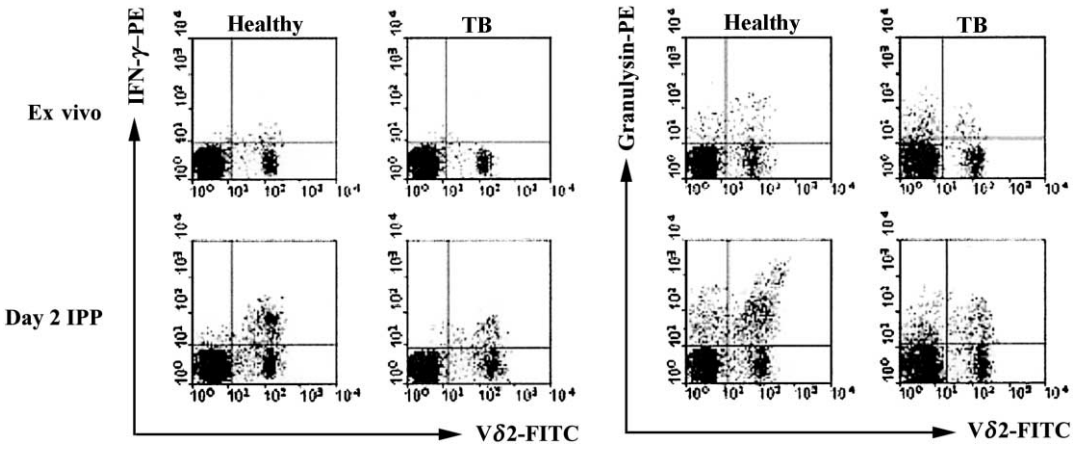

Figure 3. FACS analysis of interferon (IFN)–γ and granulysin expression by Vδ2 T cells. Peripheral blood mononuclear cells from 1 healthy subject (Healthy) and 1 patient with tuberculosis (TB) were stained immediately ex vivo or 2 days after in vitro stimulation with isopentenyl pyrophosphate (IPP) with anti-Vδ2 monoclonal antibody (MAb) and anti–IFN-γ or anti-granulysin MAb. Dot plots show the percentage of Vδ2 cells expressing IFN-γ or granulysin. FITC, fluorescein isothiocyanate. PE, phycoerythrin.

ure 5*B*) and the percentages of Vδ2 cells expressing granulysin (figure 5*C*) significantly increased after treatment and were close to values observed in PPD^+ healthy children. Thus, chemotherapy completely restores Vδ2 cell effector functions (IFN-γ production and granulysin expression) in children with TB.

Discussion

The results reported here demonstrate that Vγ9/Vδ2 T cells from children with TB have an increased ability to proliferate after stimulation with IPP but have strongly reduced effector functions, as shown by decreased IFN-γ production and granulysin expression. These data are consistent with previous observations indicating an increased proliferative activity of Vδ2 T cells from patients with TB [3–6] but reduced production of IFN-γ, compared with that of healthy tuberculin reactors [11, 12]. Recently, 2 different subsets of Vδ2 T cells have been described in humans on the basis of expression of CD27:$CD27^+$ cells that proliferate but produce low levels of IFN-γ and $CD27^-$ cells that show reciprocal properties [13]. Interestingly, adult patients with TB had a reduction of the Vδ2$CD27^-$ subset and, consequently, had increased proliferative response but reduced IFN-γ production after IPP stimulation [13]. Additionally, we report here that decrease of Vγ9/Vδ2 T cell effector functions involves not only IFN-γ production but also expression of granulysin, a molecule known to be responsible for the killing of *M. tuberculosis* [8, 14].

Granulysin, which is an homologous to the saposin-like protein family of lipid-binding proteins, exerts its cytolitic function and antibacterial activity via interaction with lipids that induce lesions on the surface of *M. tuberculosis* with a direct action on the mycobacterial glycolipid envelope [15, 16]. Interestingly, Ochoa et al. [17] found that granulysin-expressing T cells were detected in cutaneous leprosy lesions at a 6-fold greater frequency in patients with the localized tuberculoid form, compared with that in patients with the disseminated lepromatous form, of the disease. Thus, decreased production of granulysin might have profound implications in the development of mycobacterial diseases.

The reason for the loss of Vδ2 cell effector functions during TB is unknown. One possibility is that sustained in vivo mycobacterial stimulation of Vδ2 T cells causes their apoptosis [18, 19]. For example, high levels of bacteria (such as that those occur in patients with TB), resulting from the inability to contain and

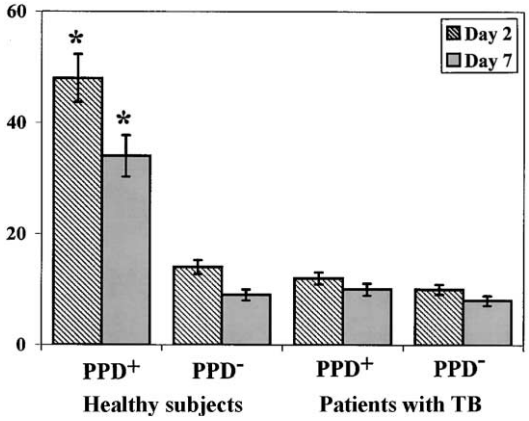

Figure 4. Granulysin expression in response to isopentenyl pyrophosphate (IPP) by Vγ9/Vδ2 T cells from children with tuberculosis (TB) and healthy control subjects. Vγ9/Vδ2 T cells from purified protein derivative–positive (PPD^+) and PPD-negative (PPD^-) children with TB and healthy control subjects were stimulated in vitro for 2 or 7 days with IPP, and granulysin expression was assessed by intracellular FACS analysis. Data are percentage (±SE) of granulysin-expressing Vδ2 T cells. $*P < .05$, compared with all other groups.

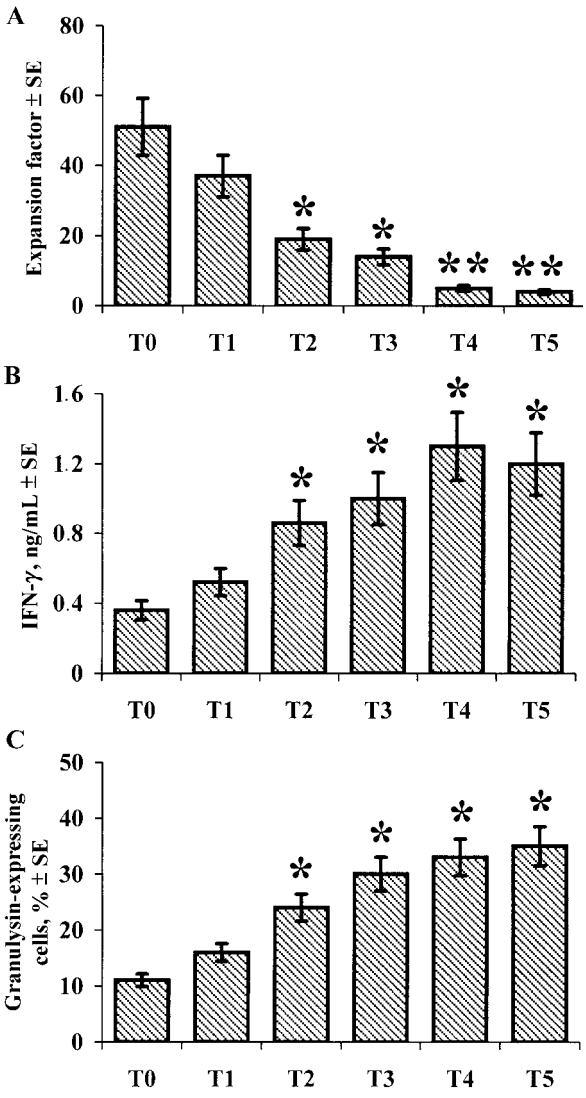

Figure 5. Vγ9/Vδ2 T cell responses before and after therapy. Vγ9/Vδ2 T cell proliferation *(A),* interferon (IFN)–γ production *(B),* and granulysin expression *(C)* were tested before therapy (T0) and every 3 months after therapy (T1–T5). $*P < .05$ and $**P < .01$, compared with values at T0.

prevent their spread, would presumably result in chronic stimulation of effector Vδ2 T cells by mycobacterial antigens and in their apoptosis, thus providing an explanation for why this population of γδ cells is lost in patients with active disease but recovers after drug therapy. Alternatively, it is possible that reduced IFN-γ and granulysin expression in children with TB, which recovers after disease improvement, could be the consequence of generalized illness.

Altogether, our finding of TB-associated changes in Vγ9/Vδ2 T cell effector functions demonstrate a correlation between the Vγ9/Vδ2 T cell response and manifestations of TB, which is consistent with the hypothesis that these T cells may play a protective role in immune response against *M. tuberculosis* infection. The finding that IFN-γ and granulysin production are restored by successful chemotherapy, which is suggested to induce the generation of a protective immune response, strongly supports this possibility.

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