Molecular Medicine 5: 471-476, 1999

Molecular Medicine

In vivo γδ T Cell Priming to Mycobacterial Antigens by Primary *Mycobacterium tuberculosis* Infection and Exposure to Nonpeptidic Ligands

Fabrizio Poccia,[1,2] Miroslav Malkovsky,[1] Aaron Pollak,[1] Vittorio Colizzi,[2] Guido Sireci,[3] Alfredo Salerno,[3] and Francesco Dieli[3]

[1]Department of Medical Microbiology and Immunology, University of Wisconsin Medical School, University of Wisconsin Comprehensive Cancer Center, and Wisconsin Regional Primate Research Center, Madison, Wisconsin, U.S.A.
[2]International Center for AIDS and Other Emerging Infections, L. Spallanzani Institute and Department of Biology, University of Rome "Tor Vergata," Rome, Italy
[3]Institute of General Pathology, University of Palermo, Palermo, Italy

Accepted May 25, 1999.

Abstract

Background: The recognition of phosphorylated nonpeptidic microbial metabolites by Vγ9Vδ2 T cells does not appear to require the presence of MHC molecules or antigen processing, permitting rapid responses against microbial pathogens. These may constitute an important area of natural anti-infectious immunity. To provide evidence of their involvement in immune reactivities against mycobacteria, we measured the responsiveness of peripheral blood Vγ9Vδ2 T cells in children with primary *Mycobacterium tuberculosis* (MTB) infections.
Materials and Methods: Peripheral blood mononuclear cells from 22 children with MTB infections and 16 positivity of tuberculin (PPD)-negative healthy children were exposed to nonpeptidic antigens in vitro and the reactivity of the Vγ9Vδ2 T cell subset with these antigens was determined using proliferation and cytokine assays. Also, responses of γδ T cells from rhesus monkeys stimulated with phosphoantigens in vivo were measured.
Results: The Vγ9Vδ2 T cell responses were highly increased in infected children in comparison with age-matched controls. This augmented Vγ9Vδ2 T cell reactivity subsided after successful antibiotic chemotherapy, suggesting that persistent exposure to mycobacterial antigens is required for the maintenance of γδ T cell activation in vivo. The in vivo reactivity of Vγ9Vδ2 T cells to phosphoantigens was also analyzed in a rhesus monkey model system. Intravenous injections of phosphoantigens induced an activated state of simian Vγ9Vδ2 T cells which decreased after 2 months, i.e., with a time course similar to that seen in MTB-infected children.
Conclusions: The increased reactivity of Vγ9Vδ2 T cells to phosphoantigens appears to be dependent on constant antigenic exposure. Consequently, the assessment of Vγ9Vδ2 responses may be useful for monitoring the efficacy of antimycobacterial therapies.

Introduction

Tuberculosis in children is usually due to a primary infection (1). Anti-*Mycobacterium tuberculosis* (MTB) immunity depends on the interaction of antigen-specific $CD4^+$ $\alpha\beta^+$ T lymphocytes with macrophages (2,3). However, several studies indicate that γδ T lymphocytes also play an important role in MTB immunosurveillance (4,5). In *Homo sapiens*, most γδ T cells express the Vγ9Vδ2 rearrangements (6,7). Vγ9Vδ2 T cells from healthy donors recognize nonpeptidic, tumor-associated or microbial phosphoantigens (8–11) and, similar to natural killer (NK) cells, display the inhibitory receptors for

Address correspondence and reprint requests to: Dr. M. Malkovsky, Department of Medical Microbiology and Immunology, University of Wisconsin Medical School, 1300 University Avenue, Madison, WI 53706, U.S.A. Phone and fax: 608-263-6316; E-mail: mmalkovs@facstaff.wisc.edu

major histocompatibility (MHC) class I molecules (12–17). These inhibitory receptors may control their reactivities toward conserved self-antigens and exogenous mycobacterial ligands (12). The phosphoantigenic recognition requires neither antigen uptake/processing nor classical polymorphic or nonpolymorphic MHC molecules, allowing for a rapid response to microbial immune challenge (18). This recognition is severely impaired in some patients with chronic viral (19,20) or bacterial (21) infections. Here we report our analyses of γδ T cell reactivities to phosphoantigens ex vivo in primary MTB-infected children and in vivo in rhesus monkeys (*Macaca mulatta*).

Materials and Methods

Cell Preparation and Stimulation

Peripheral blood mononuclear cells (PBMC) were isolated from 22 children with MTB infections (13 males, 9 females; 5.2 ± 3.3 years of age, range 1–12 years). Fourteen patients suffered from pulmonary MTB, four had MTB meningitis, three had lymphatic MTB, and one had renal MTB. The diagnoses of MTB infections were established by the presence of clinical symptoms, by the positivity of tuberculin (PPD) skin test, and by chest radiography. In some cases (i.e., MTB meningitis and renal MTB), positive cultures of microorganisms and/or MTB detection by polymerase chain reaction (PCR) further supported the clinical diagnosis. PPD-negative healthy children (9 males, 7 females; 6.2 ± 2.5 years of age, range 3–12 years) served as controls. Informed consent was obtained for each patient and control subject. In addition, PBMC were isolated from 12 healthy rhesus monkeys (5–13 years old). Mononuclear cells were cultured at 10^6 cells/ml in a complete culture medium [RPMI-1640, 10% v/v heat-inactivated fetal bovine serum (FBS), 2 mM L-glutamine, 100 IU/ml penicillin, and 100 μg/ml streptomycin]. Long-term cultures (10–14 days) were supplemented with 100 U/ml of recombinant interleukin-2 (IL-2) (Boehringer Mannheim, Mannheim, Germany), whereas short-term cultures (4 days) were performed in the presence of 5 U/ml of IL-2. Vγ9Vδ2 T cells were stimulated with the following: 0.5 mM ribose-1-phosphate (Rib-1-P, Sigma, St. Louis, MO), 0.5 mM xylose-1-phosphate (Xyl-1-P, Sigma), 0.5 mM dimethylallyl-pyrophosphate (DMAPP, Sigma), 50 μM monoethyl-pyrophopsphate [MEP, kindly provided by Drs. Y. Tanaka and B.R. Bloom (9)], 100 μM diphosphoglyceric acid (DPG, Sigma), 100 μM isopentenyl-pyrophosphate (IPP, Sigma), and the mycobacterial TUBAg1 sample diluted 1/1000 v/v [approximately at a final concentration of 1 nM, kindly provided by Dr. J.J. Fournié (8)]. After 1 week of culture, 50% of culture medium was replaced by fresh medium. The expansion of Vγ9Vδ2 T cells was followed by cytometric analysis as previously described (14,19), following double staining of the stimulated cells with anti-CD3 or anti-CD2 [phycoerythrin (PE)] and Vδ2 [fluorescein isothiocyanate (FITC)] MAb. The absolute number of Vδ2 T cell in each culture was calculated as follows: (% of Vδ2 T cells among total cells) × (total cell count)/100. The Vδ2 expansion index was then calculated by dividing the absolute number of Vδ2 T cells in specifically stimulated cultures by the absolute number of Vδ2 T cells before the initiation of culture (14,19). Thus, an expansion index higher than 1 represents a specific expansion of the Vδ2 T cell population. The Mann-Whitney U test was used and p values of <0.05 were considered significant.

Monoclonal Antibodies and FACS Analysis

The anti-CD3 (IgG1, clone SK7, Becton-Dickinson, Mountain View, CA) was coupled with PE. The B6.1 MAb (IgG_1, Pharmingen, San Diego, CA), which recognizes the Vδ2 region of the γδTCR, was unlabeled or coupled with FITC. The following anti-human MAbs cross-reactive with rhesus monkey antigens were used: anti-CD2-PE (Antigenix America, New York) and anti-Vδ2-FITC (T Cell Diagnostic, Wabum, MA). The anti-interferon γ (IFN-γ) antibodies (clone 4S.B3, IgG_1) were purchased from Pharmingen. Control MAb (IgG1 or IgG2a) for cell surface labeling were purchased from Becton Dickinson. Analysis of surface and intracellular antigen expression was done as described previously (14). For each sample, 2×10^4 double-stained viable lymphocytes were gated following size (FSC) and granularity (SSC) criteria and analyzed with the Lysis II Software Program (Becton-Dickinson).

Intravenous Injection of DPG in Rhesus Monkeys

All monkeys received a single injection of 100 mg/kg DPG (the maximum dose tolerated by rodents). A slight increase in body temperature was observed in all injected monkeys for 2 hr and two monkeys out of eight developed fever, but recovered within a few hours. (The experi-

Table 1. Vγ9/Vδ2 responses to different phosphoantigens in PPD skin test–positive children, age-matched PPD-negative controls, and healthy rhesus monkeys

PBMC	(*n*)	Vγ9/Vδ2 expansion index after in vitro culture with						
		Rib-1-P	Xyl-1-P	DMAPP	TUBAg	IPP	DPG	MEP
Healthy PPD$^-$ children	16	7 ± 6	2 ± 1	5 ± 2	6 ± 2	6 ± 1	n.d.	n.d.
MTB-infected PPD$^+$ children[a]	22	18 ± 7	30 ± 14	54 ± 24	50 ± 14	61 ± 20	n.d.	n.d.
Healthy rhesus monkeys	12	n.d.	n.d.	n.d.	8 ± 2	8 ± 3	6 ± 2	9 ± 3

PBMC were stimulated for 10–14 days with nonpeptidic antigens as described in Materials and Methods. The expansion index was calculated as the absolute number of Vδ2 T cells in stimulated cultures divided by the absolute number of Vδ2 T cells in the initial cultures prior to the antigenic exposure. n.d., not determined.

[a] $p < 0.01$ when compared to the healthy PPD$^-$ group.

mental protocol was approved by the Animal Research Committee of the University of Wisconsin.)

T Cell Proliferation Assay

PBMC from healthy donors (10^7/ml) were resuspended in complete medium and stimulated with 100 μM IPP and 5 U/ml IL-2. Cells were cultured for 4 days at 37°C in flat-bottomed microtiter wells, and T cell proliferation was measured following a 6-hr pulse with [^{3}H]-thymidine [0.5 μCi/well, Amersham, Bucks, U.K. (22)]. Cultures were harvested (Skatron Instruments, Lier, Norway) and the number of cpm was determined using a β-counter (Packard Gamma 5500).

TNF-α and IFN-γ Detection

Tumor necrosis factor α (TNF-α) release in the supernatants of IPP-stimulated PBMC was measured by ELISA (Amersham) after 24 hr. The intracellular staining of Vδ2 T cells producing IFN-γ was analyzed after 6 hr of culture with IPP as previously described (14). Monensin (10 μM, Sigma) was added during the last 4 hr of culture to block intracellular transport and allow cytokine accumulation. The stimulated cells were washed in phosphate-buffered saline (PBS), 1% bovine serum albumin (BSA), and 0.1% sodium azide and stained with the anti-Vδ2-FITC MAb for 15 min at 4°C. The cells were fixed and permeabilized in PBS 1% paraformaldehyde for 10 min at 4°C, and incubated for 30 min at room

Table 2. Frequency of γδ T lymphocytes in the peripheral blood of healthy PPD skin test–positive children, healthy PPD skin test-negative children, and PPD skin test-positive children with primary tuberculosis

Children Category	Proportion of CD3$^+$ Cells (Mean ± Standard Error)[a]		
	γδ$^+$	Vγ9Vδ2$^+$	Vδ2/CD45RO$^+$
Healthy PPD$^+$ (n = 46)	5.6 ± 0.5%	4.3 ± 0.4%	3.0 ± 0.4%
Healthy PPD$^-$ (n = 17)	5.3 ± 0.6%	4.0 ± 0.5%	3.2 ± 0.3%
TB patients PPD$^+$ (n = 27)	5.2 ± 0.6%	4.1 ± 0.4%	3.2 ± 0.4%

[a] The absolute numbers of γδ T cells (mean ± standard error) in healthy PPD$^+$: 84 ± 36 γδ$^+$ cells/μl (n = 46); healthy PPD$^-$: 79 ± 33 γδ$^+$ cells/μl (n = 17); TB patients PPD$^+$: 78 ± 39 γδ$^+$ cells/μl (n = 27).

temperature in the dark with the anti-cytokine MAb diluted in PBS with 1% BSA and 0.05% saponin. Finally, they were washed twice in PBS (1% BSA, 0.01% saponin) and analyzed using a FACScan (Becton Dickinson). The controls for nonspecific staining included cells stained with isotype-matched monoclonal antibodies. The proportions of cytokine-producing Vδ2 T cells were determined by FACScan analyses.

Results

To determine whether primary MTB-infected children possess Vγ9Vδ2 T cells exhibiting their constitutive reactivities (8–11), PBMC from 22 patients and 16 age-matched controls were stimulated with different phosphoantigens. These studies assessed the ability of Vγ9Vδ2 cells to expand in 14-day cultures with different synthetic [such as ribose-1-phosphate (Rib-1-P), xylose-1-phosphate (Xyl-1-P), dimethylallyl-pyrophosphate (DMAPP), monoethyl-pyrophosphate (MEP), diphosphoglyceric acid (DPG), and isopentenyl-pyrophosphate (IPP)] (9,10), or natural [TUBAg-1 (8)] phosphoantigens. The Vγ9Vδ2 T cell responses were highly increased in MTB-infected children in comparison to age-matched controls (Table 1). The Vγ9Vδ2 T cell subset in tuberculin-positive MTB-infected children responded well to all antigens used. The strongest responses were detected using IPP, with the mean expansion index of 61. In contrast, the IPP-induced expansion of Vγ9Vδ2 T cells from healthy, tuberculin-negative children was approximately 10 times lower, with the mean expansion index of 6. The absolute and relative numbers of γδ T cells, Vγ9Vδ2 T cells, and Vδ2/CD45RO cells measured in these children as well as in some additional young TB patients and controls were comparable (Table 2). The γδ T cell response was further studied in 15 tuberculin-positive children 3 to 9 months after chemotherapy (23). The increased responsiveness of Vγ9Vδ2 cells sharply declined close to the levels detected in healthy tuberculin-negative children (Fig. 1). This suggests that persistent mycobacterial exposure was required for the presence of hyperactivity against phosphoantigens. In contrast, responses of αβ T lymphocytes to tuberculin (PPD) and to the immunodominant 38 kDa protein of MTB (24) were increased after chemotherapy (data not shown).

To investigate the possibility of phosphoantigen-priming of the Vγ9Vδ2 T cell subset in vivo, rhesus monkeys whose Vγ9Vδ2 T lymphocytes react in vitro to IPP, MEP, and DPG (Table 1) received intravenous injections of DPG. Periph-

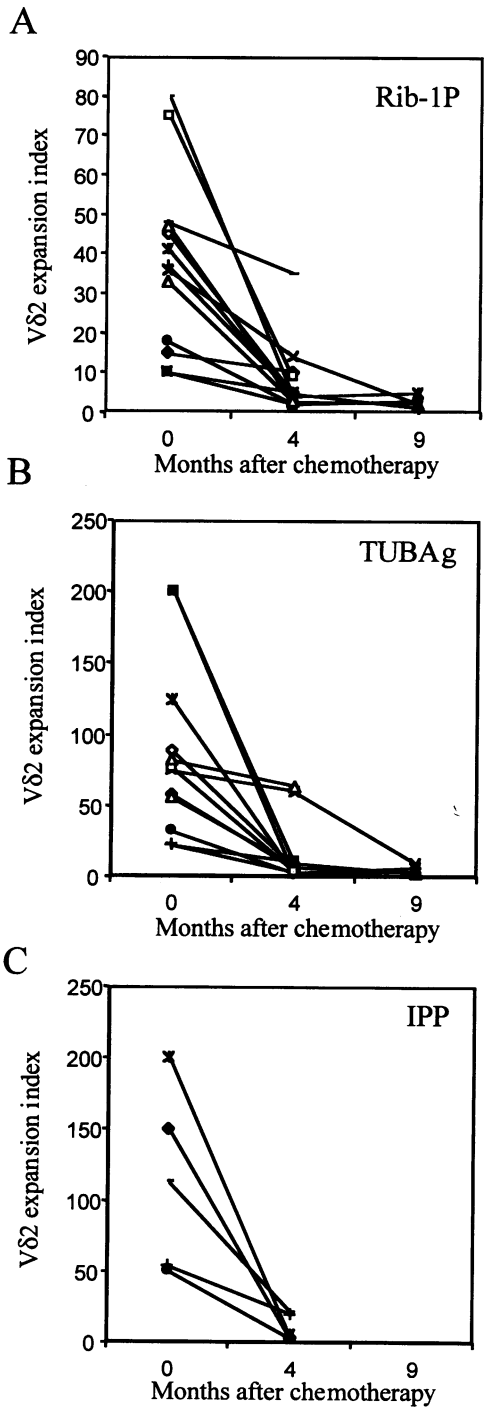

Fig. 1. Successful chemotherapies of *M. tuberculosis* infections are accompanied by reduced γδ T cell reactivities to nonpeptidic antigens. (A) Vδ2 expansion index to Rib-1P, (B) Vδ2 expansion index to TUBAg, (C) Vδ2 expansion index to IPP. Each symbol corresponds to one individual.

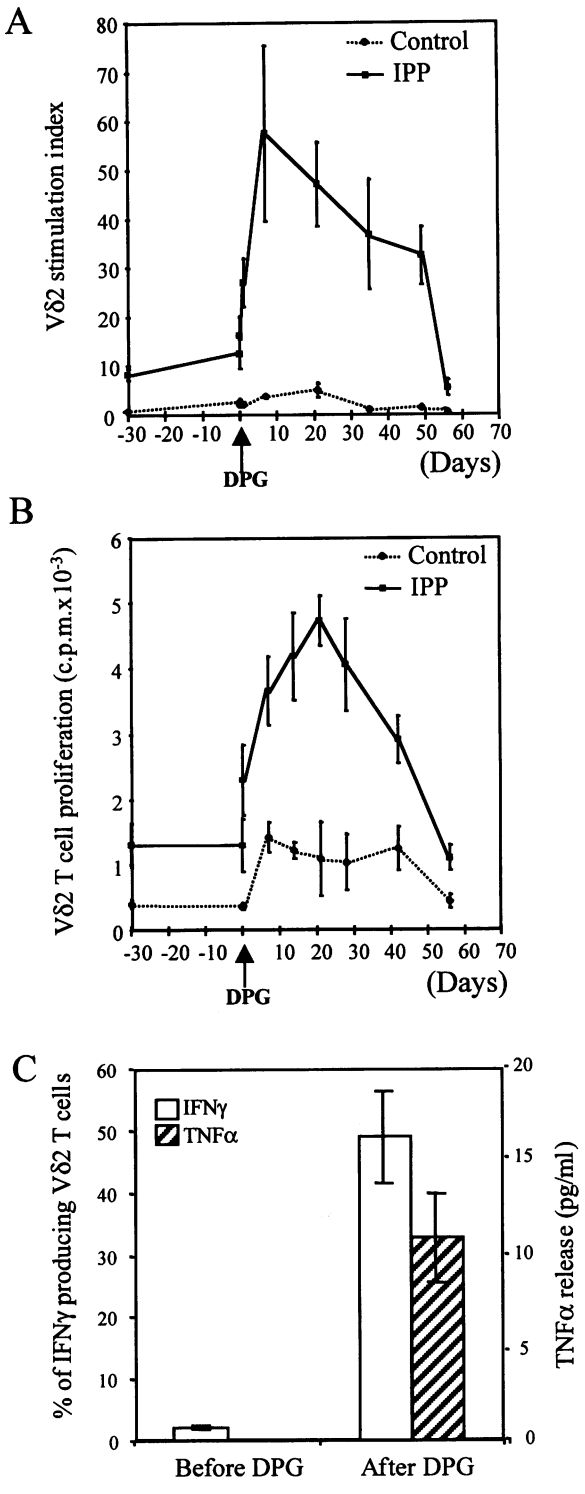

Fig. 2. Intravenous injection of DPG induces an increased reactivity of γδ T cells. The Vγ9Vδ2 T cell expansion was monitored by measuring DNA synthesis after 5-day stimulation (A) or by flow cytometry after 10 days (B). Previous experiments (data not shown) have indicated that among the peripheral blood leukocytes proliferating in the IPP response, the Vγ9Vδ2 T cell subset forms a dominant population, whereas other proliferating leukocytes (which respond by DNA synthesis to Vγ9Vδ2 T cell–produced cytokines) constitute a minority. Cytokine production (C) was monitored by ELISA (TNF-α) or by flow cytometry (IFN-γ) before and 1 month after the injection of DPG in vivo. The production of TNF-α was detectable by the ELISA assay only after the administration of DPG.

eral blood samples were collected at different time intervals and analyzed for functional reactivities in vitro. The DPG treatment resulted in a substantial up-regulation of both proliferative and cytokine (IFN-γ) responses to isopentenyl pyrophosphate in immunized animals (Fig. 2). IFN-γ-producing Vδ2 T cells increased from 2.2% prior to the in vivo treatment to 48.9% 1 month after the treatment. TNF-α-producing Vδ2 T cells were not detectable before, but were clearly present after, the treatment (Fig. 2).

Discussion

Historically, most vaccination strategies have utilized protein antigens. However, there are many lymphocytes that do not seem to recognize primarily peptide antigens bound to MHC molecules (8–12). Our data indicate that in vivo exposures to nonpeptidic antigens substantially augment the immunological reactivity of genetically unrestricted γδ T cells. When activated, these cells exert a powerful antibacterial activity (12–14) and release IFN-γ and TNF-α (14). These properties may be useful in the design and development of novel vaccines and/or adjuvants. The long-lasting memory response of αβ T lymphocytes is clinically applicable for evaluating previous exposures to MTB, but provides unsatisfactory information about the presence or absence of productive infection. In contrast, the γδ T cell hyperactivity against phosphoantigens requires persistent antigenic exposure. Thus, the assessment of Vγ9Vδ2 T cell responses may be a useful tool to monitor active MTB infections and potential failures of antimycobacterial therapies.

The recent elegant studies of Hoft et al. (25) provide evidence that the *Mycobacterium bovis Bacillus* Calmette-Guérin (BCG) vaccine augments human γδ T cell responsiveness to mycobacteria. This study is fully compatible with our observations of human and simian γδ T cells primed in vivo by either TB disease or intravenously ad-

ministered phosphoantigen. Therefore, both studies strongly suggest that γδ T cells in vivo may (*a*) allow for rapid and potent responses against the invading pathogen and (*b*) serve as targets for new TB vaccines.

Acknowledgments

This work was supported by grants from the NIH (AI42712 and RR00167) and AIDS/Tuberculosis Projects of the Italian Institute of Health "Istituto Superiore della Sanità." The authors are indebted to Jacque Mitchen for his expert help with animal experiments. This work is publication no. 39-017 of the Wisconsin Regional Primate Research Center.

References

1. Miller JWF. (1981) *Tuberculosis in Children.* Churchill Livingstone, Edinburgh.
2. North RJ. (1974) T cell dependence of macrophage activation and mobilization during infection with *Mycobacterium tuberculosis. Infect. Immun.* **10:** 66–71.
3. Kaufmann SHE. (1989) In vitro analysis of the cellular mechanisms involved in immunity to tuberculosis. *Rev. Infect. Dis.* **11:** 48–53.
4. Ladel CH, Blum C, Dreher A, Reifenberg K, Kaufmann SHE. (1995) Protective role of γ/δ and α/β T cells in tuberculosis. *Eur. J. Immunol.* **25:** 2877–2881.
5. Kabelitz D, Bender A, Shondelmaier S, Schoel B, Kaufmann SHE. (1991) The primary response of human γ/δ T cells to *Mycobacterium tuberculosis* is restricted to Vγ9-bearing cells. *J. Exp. Med.* **173:** 1331–1339.
6. Parker CM, Groh V, Band H, et al. (1990) Evidence for extrathymic changes in the T cell receptor gamma/delta repertoire. *J. Exp. Med.* **171:** 1597–1612.
7. Brenner MB, McLean J, Scheft H, et al. (1987) Two forms of the T-cell receptor gamma protein found on peripheral blood cytotoxic T lymphocytes. *Nature* **325:** 689–694.
8. Constant P, Davodeau F, Peyrat MA, et al. (1994) Stimulation of γδ T cells by nonpeptidic mycobacterial ligands. *Science* **264:** 267–270.
9. Tanaka I, Morita CT, Tanaka Y, Nieves E, Brenner MB, Bloom BR. (1995) Natural and synthetic non-peptide antigens recognized by human γδ T cells. *Nature* **375:** 155–158.
10. Burk MR, Mori L, De Libero G. (1995) Human Vγ9-Vδ2 cells are stimulated in a cross-reactive fashion by a variety of phosphorylated metabolites. *Eur. J. Immunol.* **25:** 2052–2058.
11. Fisch P, Malkovsky M, Kovats S, et al. (1990) Recognition by human Vγ9/Vδ2 T cells of a GroEL homolog on Daudi Burkitt's lymphoma cells. *Science* **250:** 1269–1273.
12. Poccia F, Gougeon ML, Bonneville M, et al. (1998) Innate T-cell immunity to nonpeptidic antigens. *Immunol. Today* **19:** 253–256.
13. Poccia F, Malkovsky M, Gougeon ML, et al. (1997) γδ T cell activation or anergy during infections: the role of nonpeptidic TCR ligands and HLA class I molecules. *J. Leukoc. Biol.* **62:** 1–5.
14. Poccia F, Cipriani B, Vendetti S, et al. (1997) CD94/NKG2 inhibitory receptor complex modulates both anti-viral and anti-tumoral responses of polyclonal phosphoantigen-reactive Vγ9Vδ2 T lymphocytes. *J. Immunol.* **159:** 6009–6017.
15. Battistini L, Borsellino G, Sawicki G, et al. (1997) Phenotypic and cytokine analysis of human peripheral blood γδ T cells expressing natural killer receptors. *J. Immunol.* **159:** 3723–3730.
16. Halary F, Peyrat M-A, Champagne E, et al. (1997) Control of self-reactive cytotoxic T lymphocytes expressing γδ T cell receptors by natural killer inhibitory receptors. *Eur. J. Immunol.* **27:** 2812–2821.
17. Carena I, Shamshiev A, Donda A, Colonna M, De Libero G. (1997) Major histocompatibility complex class I molecules modulate activation threshold and early signalling of T cell antigen receptor–γ/δ stimulated by nonpeptidic ligands. *J. Exp. Med.* **186:** 1769–1774.
18. Lang F, Peyrat MA, Constant P, et al. (1995) Early activation of human Vγ9Vδ2 T cell broad cytotoxicity and TNF production by nonpeptidic mycobacterial ligands. *J. Immunol.* **154:** 5986–5994.
19. Poccia F, Boullier S, Lecoeur H, et al. (1996) Peripheral Vγ9/Vδ2 T cell deletion and anergy to nonpeptidic mycobacterial antigens in asymptomatic HIV-1 infected persons. *J. Immunol.* **157:** 4449–4461.
20. Wallace M, Scharko AM, Pauza CD, et al. (1997) Functional γδ T-lymphocyte defect associated with human immunodeficiency virus infections. *Mol. Med.* **3:** 60–71.
21. Li B, Rossman MD, Imir T, et al. (1996) Disease-specific changes in γδ T cell repertoire and function in patients with pulmonary tuberculosis. *J. Immunol.* **157:** 4222–4229.
22. Malkovsky M, Asherson GL, Stockinger B, Watkins MC. (1982) Nonspecific inhibitor released by T acceptor cells reduces the production of interleukin-2. *Nature* **300:** 652–655.
23. Bothamley GH. (1995) Serological diagnosis of tuberculosis. *Eur. Respir. J.* **8:** 676–688.
24. Kadival GV, Chaparas SD, Hussong D. (1987) Characterization of serologic and cell-mediated reactivity of a 38-kDa antigen isolated from *Mycobacterium tuberculosis. J. Immunol.* **139:** 2447–2451.
25. Hoft DF, Brown RM, Roodman ST. (1998) Bacille Calmette-Guérin vaccination enhances human γδ T cell responsiveness to mycobacteria suggestive of a memory-like phenotype. *J. Immunol.* **161:** 1045–1054.