

**OPEN ACCESS**

**Repository of the Max Delbrück Center for Molecular Medicine (MDC)  
in the Helmholtz Association**

<http://edoc.mdc-berlin.de/6792>

## **Differentiation of effector/memory V $\alpha$ 2 T cells and migratory routes in lymph nodes or inflammatory sites**

---

Dieli, F. and Poccia, F. and Lipp, M. and Sireci, G. and Caccamo, N. and Di Sano, C. and Salerno, A.

This is a copy of the final article, which was first published online on 04 August 2003 and in final edited form in:

Journal of Experimental Medicine  
2003 AUG 04 ; 198(3): 391-397  
doi: [10.1084/jem.20030235](https://doi.org/10.1084/jem.20030235)

Publisher: [Rockefeller University Press](https://www.rockefeller.edu/)

## Differentiation of Effector/Memory V $\delta$ 2 T Cells and Migratory Routes in Lymph Nodes or Inflammatory Sites

Francesco Dieli,<sup>1</sup> Fabrizio Poccia,<sup>2</sup> Martin Lipp,<sup>3</sup> Guido Sireci,<sup>1</sup>  
Nadia Caccamo,<sup>1</sup> Caterina Di Sano,<sup>4</sup> and Alfredo Salerno<sup>1,4</sup>

<sup>1</sup>Department of Biopathology, University of Palermo, 90134 Palermo, Italy

<sup>2</sup>Laboratory of Immunology, National Institute for Infectious Diseases "L. Spallanzani," 00149 Rome, Italy

<sup>3</sup>Max-Delbrück Center for Molecular Medicine, 13122 Berlin-Busch, Germany

<sup>4</sup>Institute of Biomedicine and Molecular Immunology, National Research Council, 90134 Palermo, Italy

### Abstract

V $\delta$ 2 T lymphocytes recognize nonpeptidic antigens without presentation by MHC molecules and mount both immediate effector functions and memory responses after microbial infection. However, how V $\delta$ 2 T cells mediate different facets of a memory response remains unknown. Here, we show that the expression of CD45RA and CD27 antigens defines four subsets of human V $\delta$ 2 T cells with distinctive compartmentalization routes. Naive CD45RA<sup>+</sup>CD27<sup>+</sup> and memory CD45RA<sup>-</sup>CD27<sup>+</sup> cells express lymph node homing receptors, abound in lymph nodes, and lack immediate effector functions. Conversely, memory CD45RA<sup>-</sup>CD27<sup>-</sup> and terminally differentiated CD45RA<sup>+</sup>CD27<sup>-</sup> cells, which express receptors for homing to inflamed tissues, are poorly represented in the lymph nodes while abounding at sites of inflammation, and display immediate effector functions. These observations and additional *in vitro* experiments indicate a lineage differentiation pattern for human V $\delta$ 2 T cells that generates naive cells circulating in lymph nodes, effector/memory cells patrolling the blood, and terminally differentiated effector cells residing in inflamed tissues.

Key words:  $\gamma\delta$  cells • effector functions • chemokine receptors • functional subsets • phosphoantigens

### Introduction

$\gamma\delta$  T cells are a minor T cell population with a unique pattern of antigen recognition. Several types of antigen specificities have been reported for human and mice  $\gamma\delta$  cells; they include allo-MHC molecules, peptide-MHC complexes, stress-related proteins such as MICA/B and heat shock protein 60, and nonpeptidic molecules (for review see reference 1). The vast majority of circulating  $\gamma\delta$  T cells in humans express a TCR heterodimer comprised of V $\gamma$ 9 and V $\delta$ 2 chains that directly recognize nonpeptide ligands without presentation by MHC molecules (1). The nonpeptide ligands, referred to as phosphoantigens, comprise natural phosphoesters derived from mycobacteria (2–5), and to a lesser extent several ubiquitous metabolites such as alkylamines from plant extracts (6), xylosyl- or ribosyl-1-phosphate, 2,3-diphosphoglycerate (7), and several synthetic aminobisphosphonates (8). Common to all of these small compounds (molecular mass below 500 D) is

the presence of either a pyrophosphate or a primary amine located at the distal end of their organic C2–C5 skeleton.

With regard to peptide/MHC recognition by conventional  $\alpha\beta$  TCR, phosphoantigen recognition by V $\gamma$ 9V $\delta$ 2 TCR presents unusual features. First, V $\gamma$ 9V $\delta$ 2 cells circumvent the need for APCs to interact with phosphoantigens, as these ligands seem to bind directly to the reactive TCR, enabling isolated  $\gamma\delta$  cells to respond to exogenous soluble phosphoantigens (9, 10). Second and more strikingly, phosphoantigen recognition by any V $\gamma$ 9V $\delta$ 2 cells is simultaneously cross-reactive (different molecules are recognized) and ligand-discriminating (structurally related molecules are not recognized; reference 11).

V $\gamma$ 9V $\delta$ 2 cells are not the predominant  $\gamma\delta$  subset in peripheral blood at birth. Rather, their number increases from birth and peaks at  $\sim$ 7 yr of life in the absence of a parallel thymic wave, pointing to an environmental antigen-driven peripheral expansion (12). Accordingly, cord blood V $\gamma$ 9V $\delta$ 2 T cells express the CD45RA naive phenotype, whereas the majority of adult V $\gamma$ 9V $\delta$ 2 T cells express the CD45RO memory phenotype (12).

F. Dieli and F. Poccia contributed equally to this work.

Address correspondence to Francesco Dieli, Dept. of Biopathology, University of Palermo, Corso Tukory 211, Palermo 90134, Italy. Phone: 39-091-655-5916; Fax: 0039-091-655-5924; email: dieli@unipa.it

V $\gamma$ 9V $\delta$ 2 T cells have been shown to be capable of both immediate effector functions (such as cytokine production and cytotoxic activity; reference 1) as well as to mount a memory response upon microbial reinfection (13–15), suggestive of the existence of different functional subsets (16). Recently, two subsets of V $\delta$ 2 T cells have been described in humans, based on expression of CD27: CD27<sup>+</sup> cells proliferate but produce low IFN $\gamma$ , whereas CD27<sup>-</sup> cells show reciprocal properties (17). However, in contrast to human CD4 and CD8 T cells, description of V $\delta$ 2 naive, memory, and effector phenotypes is still rather fragmentary. In this work, we have used simultaneous staining with CD45RA and CD27 mAbs to separate functionally distinct subpopulations of human V $\delta$ 2 T cells.

## Materials and Methods

**Subjects.** PBMCs were obtained from the heparinized blood of 15 healthy volunteers upon informed consent. Mononuclear cells were taken from inguinal lymph nodes of six organ donors collected to perform HLA typing and cross-match analysis for organ transplantation. We selected lymph node specimens from donors who neither received drugs nor blood or plasma transfusion, and who had no signs of infection. Additionally, mononuclear cells were collected from one ascitic and two cerebrospinal fluids from three patients affected by tuberculous peritonitis and meningitis, respectively. None of these three patients had evidence of HIV infection, or was being treated with steroids or antitubercular drugs at the time of diagnosis and inflammatory fluids sampling.

**FACS<sup>®</sup> Staining and Sorting.** PBMCs were isolated from heparinized blood, lymph nodes, or inflammatory fluids, by Ficoll-Hypaque (Amersham Biosciences). For subset identification, PBMCs were incubated with the following antibodies in different combinations: anti-V $\delta$ 2 FITC (IMMU389; Coulter), anti-CD27 PE (M-T271; BD Biosciences), anti-CD45RA PE-Cy5 (2H4; Coulter), anti-CD45RO PE-Cy5 (UCHL-1; Coulter), anti-CD62L allophycocyanin (Dreg56; BD Biosciences), anti-CD3 PE (UCHT-1; Sigma-Aldrich), anti-CD4 PE (Q420; Sigma-Aldrich), anti-CD8 PE (UCHT-4; Sigma-Aldrich), and anti-V $\gamma$ 9 unconjugated (B3; BD Biosciences).

Staining with purified antibodies to human chemokine receptors CXCR3, CCR2, CCR6 (R&D Systems), CCR5 (BD Biosciences), and CCR7 (provided by M. Lipp) was followed by allophycocyanin-conjugated anti-mouse or anti-rat IgG antibodies (BD Biosciences). Data were acquired on a FACSCalibur<sup>™</sup> instrument (BD Biosciences) and analyzed using CELLQuest<sup>™</sup> software (BD Immunocytometry Systems).

Cell sorting was performed on a FACSVantage<sup>™</sup> (BD Biosciences).

**Proliferation, IFN $\gamma$  Production, and BLT-esterase Release Assays.** The medium used throughout was complete RPMI 1640 medium (GIBCO BRL) supplemented with 10% heat-inactivated FCS (GIBCO BRL), 2 mM L-glutamine, 20 nM HEPES, and 100 U/ml penicillin/streptomycin. Sorted V $\delta$ 2 cell subsets were cultured at 37°C in the presence of 5% CO<sub>2</sub>, at 10<sup>6</sup>/ml in 96-well flat-bottomed plates (0.2 ml/well), with different concentrations of isopentenyl pyrophosphate (IPP; \* Sigma-Aldrich).

\*Abbreviations used in this paper: BLT, N $\alpha$ -CBZ-L-lysine-thiobenzyl; IPP, isopentenyl pyrophosphate.

Proliferation was measured 72 h later by adding 1  $\mu$ Ci/well of [<sup>3</sup>H]thymidine (Amersham Biosciences) during the last 6 h of culture. Cells were harvested, and [<sup>3</sup>H]thymidine incorporation was measured with a liquid scintillation  $\beta$ -counter. Results are expressed as mean counts per minute of triplicate wells  $\pm$  SD. IFN $\gamma$  levels in the 24-h culture supernatants were assessed by two-mAbs sandwich ELISA assay following the manufacturer's recommendations (R&D Systems). N $\alpha$ -CBZ-L-lysine-thiobenzyl (BLT)-esterase levels in 24-h culture supernatants were determined by the BLT-esterase assay. In brief, 20  $\mu$ l of culture supernatant was incubated with 35  $\mu$ l 1 mM BLT (Sigma-Aldrich), 35  $\mu$ l 1 mM 5-5'-dithio-bis-(2-nitrobenzoic acid; Sigma-Aldrich), and 10  $\mu$ l 0.1% Triton X-100 (Sigma-Aldrich). After incubation for 30 min at 37°C, the absorbance at 405 nm was measured.

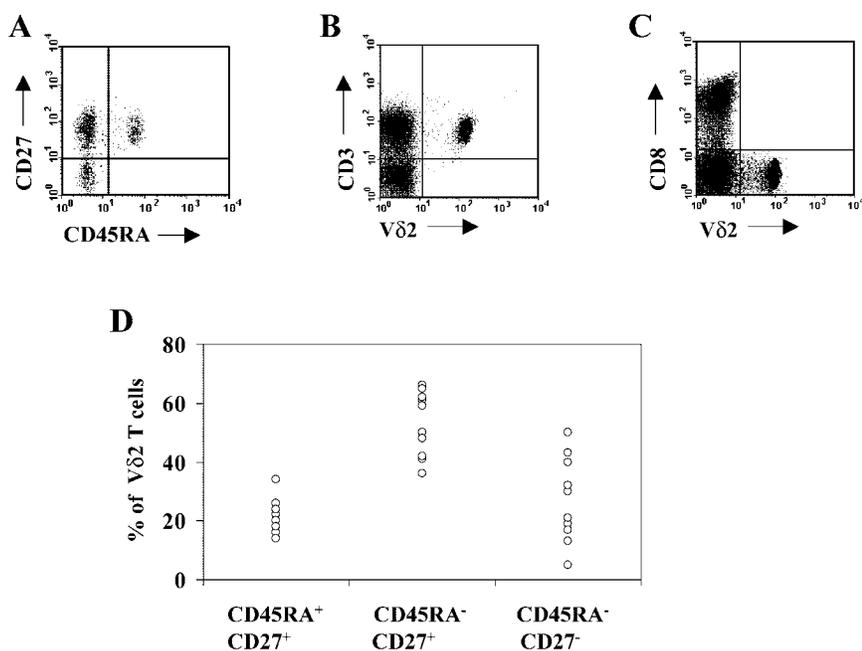
**Cell Cultures.** Sorted V $\delta$ 2 subsets were cultured for 12 d with IPP, as described in the *Proliferation, IFN $\gamma$  Production, and BLT-esterase Release Assays* section, except 20 U/ml human recombinant IL-2 was added at the third and the sixth day of culture. After the culture period, cells were recovered and surface marker expression assessed by FACS<sup>®</sup>. Additionally, the cells were restimulated with IPP as described in the *Proliferation, IFN $\gamma$  Production, and BLT-esterase Release Assays* section: proliferation was measured after 72 h by [<sup>3</sup>H]thymidine incorporation and IFN $\gamma$  levels in the 24-h culture supernatants assessed by two-mAbs sandwich ELISA assay. The length of telomeres was determined using a Teloquant kit (BD Biosciences).

## Results and Discussion

Staining of PBMCs with antibodies to CD45RA and CD27 identifies three subsets of V $\delta$ 2 T cells (Fig. 1 A): one subset with a naive CD45RA<sup>+</sup>CD27<sup>+</sup> phenotype and two subsets with a memory CD45RA<sup>-</sup>CD27<sup>+</sup> and CD45RA<sup>-</sup>CD27<sup>-</sup> phenotype.

In a population of healthy adult donors (Fig. 1 D,  $n = 15$ ), the distribution of the V $\delta$ 2 subsets, as defined by simultaneous CD45RA and CD27 staining was as follows: CD45RA<sup>+</sup>CD27<sup>+</sup> cells, 21.4  $\pm$  5.7 (range 14–34); CD45RA<sup>-</sup>CD27<sup>+</sup> cells, 52.1  $\pm$  10.1 (range 36–66); and CD45RA<sup>-</sup>CD27<sup>-</sup> cells, 26.5  $\pm$  15.3 (range 0–50). In all tested individuals, the V $\delta$ 2<sup>+</sup> T cells were CD3<sup>+</sup> and double (CD4 and CD8) negative, even if in two patients 1.3% and 0.8% of the V $\delta$ 2<sup>+</sup> cells stained positive for CD8. Fig. 1 (B and C) shows typical two-color FACS<sup>®</sup> analysis for V $\delta$ 2/CD3 and V $\delta$ 2/CD8 expression.

To further define the cell surface phenotype of these subsets and to address if they had a different migratory machinery, V $\delta$ 2 T cell subsets were sorted yielding >98% pure populations and analyzed with antibodies to different chemokine receptors and adhesion molecules. Data are reported in Table I. CD45RA<sup>+</sup>CD27<sup>+</sup> cells showed characteristics of naive cells because they were CD45RO<sup>-</sup> and expressed the lymph node homing receptors CCR7 and CD62L (18, 19), while lacking expression of receptors for inflammatory chemokines such as CCR2, CCR5, CCR6, and CXCR3 (20). CD45RA<sup>-</sup>CD27<sup>+</sup> cells share chemokine receptor expression with naive cells because they still express high levels of CD62L and CCR7, but ~25% of these cells also express CCR5 and CXCR3. Despite expression of the secondary lymphoid organs homing chemo-



**Figure 1.** Phenotypic characterization of Vδ2 T cell subsets. (A) Peripheral blood mononuclear cells were stained with antibodies to Vδ2, CD45RA, and CD27. Upon analysis on gated Vδ2<sup>+</sup> cells, three subsets were identified. (B and C) Two-color FACS<sup>®</sup> analysis of peripheral blood mononuclear cells stained with antibodies to Vδ2, CD3, and CD8. (D) Upon analysis in a healthy population, the proportion of cells within different subsets was variable among individuals, the variability being more pronounced in the CD45RA<sup>-</sup>CD27<sup>-</sup> subset.

kine receptors CD62L and CCR7, CD45RA<sup>-</sup>CD27<sup>+</sup> cells had the CD45RO<sup>+</sup> memory phenotype, thus resembling central memory αβ T cells (21). CD45RA<sup>-</sup>CD27<sup>-</sup> cells showed an effector phenotype; they had down-regulated both CD62L and CCR7, but expressed high levels of receptors for inflammatory chemokines such as CCR2, CCR5, CCR6, and CXCR3, and were CD45RO<sup>+</sup>.

Altogether, these results identify three subsets of human peripheral blood Vδ2 carrying distinct homing receptors: one naive CD45RA<sup>+</sup>CD27<sup>+</sup> and one memory CD45RA<sup>-</sup>CD27<sup>+</sup> subset, preferentially expressing the lymph node homing receptors CD62L and CCR7, and one effector CD45RA<sup>-</sup>CD27<sup>-</sup> subset preferentially expressing receptors for inflammatory chemokines.

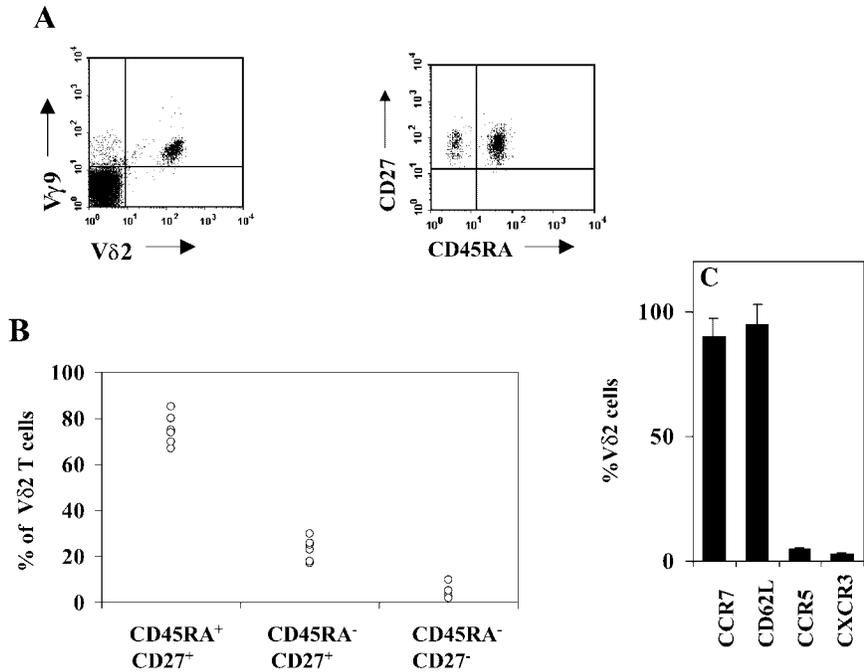
**Table I.** Chemokine Receptor, CD45RO and CD62L Expression on Subsets of Vδ2<sup>+</sup> T Cells

	CD45RA <sup>+</sup> CD27 <sup>+</sup>	CD45RA <sup>-</sup> CD27 <sup>+</sup>	CD45RA <sup>-</sup> CD27 <sup>-</sup>
CXCR3	6 ± 2 (14)	20 ± 3 (48)	95 ± 11 (144)
CCR2	5 ± 1 (18)	13 ± 1 (21)	95 ± 9 (38)
CCR5	8 ± 2 (28)	25 ± 2 (54)	80 ± 12 (156)
CCR6	5 ± 1 (12)	10 ± 2 (14)	15 ± 2 (27)
CCR7	75 ± 10 (64)	63 ± 7 (53)	0 (0)
CD62L	95 ± 11 (73)	86 ± 10 (66)	3 ± 2 (4)
CD45RO	1 ± 1 (5)	96 ± 8 (125)	97 ± 12 (164)

Three subsets of peripheral blood Vδ2<sup>+</sup> cells were sorted by expression of CD45RA and CD27. The sorted cells were stained and analyzed for the expression of chemokine receptors, CD45RO and CD62L. Values indicate the percentage of positive cells ± SD of three different experiments. Values in brackets indicate the mean fluorescence intensity (MFI) mean value.

To confirm the migratory properties of the different Vδ2 subsets, as deduced by chemokine receptor expression, we studied their distribution in lymph nodes and at sites of inflammation. While in peripheral blood, cells with the memory CD45RA<sup>-</sup>CD27<sup>+</sup> phenotype represent the most abundant Vδ2 subset; in lymph nodes, Vδ2 cells with a naive phenotype (CD45RA<sup>+</sup>CD27<sup>+</sup>) are the predominant population (Fig. 2 A). About 25% of Vδ2 cells within lymph nodes had a memory CD45RA<sup>-</sup>CD27<sup>+</sup> phenotype and only a small percentage expressed the CD45RA<sup>-</sup>CD27<sup>-</sup> phenotype. Similar to that finding (Fig. 4 D; reference 1), the vast majority of Vδ2<sup>+</sup> cells in the lymph nodes also coexpressed the Vγ9 chain (Fig. 2 A), and we also detected a small proportion of Vγ9<sup>+</sup> cells that did not express Vδ2. The distribution of the three Vδ2 T cell subsets in lymph nodes from six different donors is shown in Fig. 2 B; whereas there was a certain variability in subsets distribution, it was less pronounced than that in PBMC. Further analysis of chemokine receptor expression by lymph nodes Vδ2 T cells (Fig. 2 C) shows that they express CCR7 and CD62L, but not CCR5 and CXCR3.

Collectively, these data indicate that lymphoid organs mainly contain Vδ2 cells with a naive or memory phenotype which express receptors for homing to secondary lymphoid organs. Therefore, we assessed Vδ2 subset distribution in one ascite sample (Fig. 3 A) and in two cerebrospinal fluids (Fig. 3, B and C), from three patients affected by tuberculous peritonitis and tuberculous meningitis, respectively. Fig. 3 shows that only two Vδ2 subsets were detected at inflammatory sites: one effector CD45RA<sup>-</sup>CD27<sup>-</sup> subset and one abundant subset with an unusual CD45RA<sup>+</sup>CD27<sup>-</sup> phenotype, which resembles a subset of terminally differentiated CD8 memory T cells (22, 23). The relative percentages of these two sub-

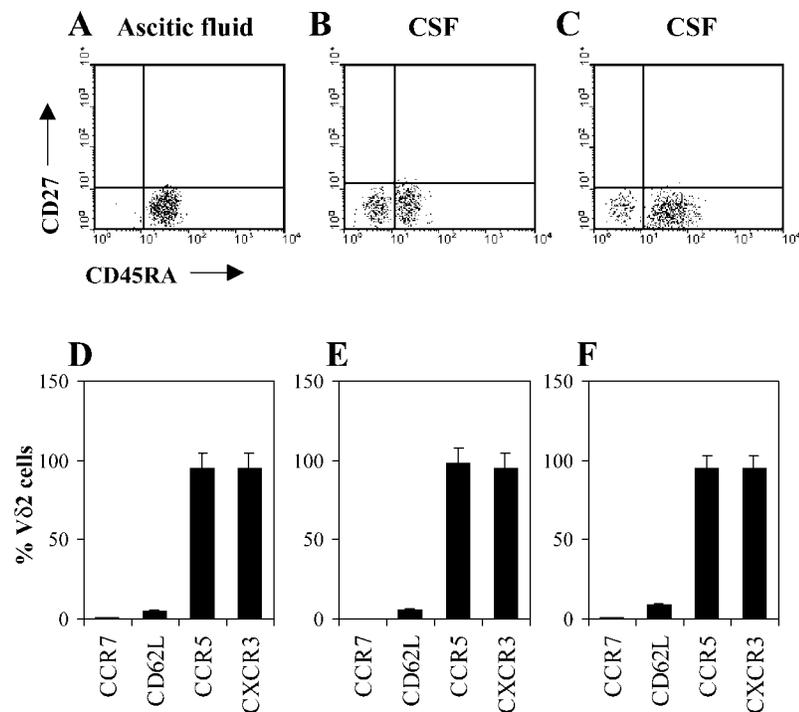


**Figure 2.** Distribution of Vδ2 T cell subsets in lymph nodes. (A) Mononuclear cells were taken from inguinal lymph nodes of organ donors. Cells were stained with antibodies to Vδ2, CD45RA, and CD27. As a control, cells were double stained with antibodies to Vδ2 and Vγ9. (B) Analysis of the relative proportion of the CD45RA<sup>+</sup>CD27<sup>+</sup>, CD45RA<sup>-</sup>CD27<sup>+</sup>, and CD45RA<sup>-</sup>CD27<sup>-</sup> subsets of Vδ2<sup>+</sup> T cells in the lymph nodes of six donors. (C) Vδ2 T cell subsets were sorted and analyzed for the expression of different chemokine receptors and CD62L. Results similar to those shown here were obtained in a total of six donors.

sets differed in the three tested inflammatory fluids, although the CD45RA<sup>+</sup>CD27<sup>-</sup> one was the only detectable Vγ9Vδ2 subset in the ascitic fluid (Fig. 3 A). Additionally, Vδ2 T cells collected from these inflammatory fluids expressed CCR5 and CXCR3 (Fig. 3, D-F), but were virtually negative for CCR7 and CD62L expression.

Different subsets of human Vγ9Vδ2 T cells with distinct homing receptors and localization routes might exert dif-

ferent immediate effector functions after short term antigenic stimulation (21). Therefore, we assessed proliferation, cytokine production, and cytotoxic activities of naive CD45RA<sup>+</sup>CD27<sup>+</sup>, memory-type CD45RA<sup>-</sup>CD27<sup>+</sup> and CD45RA<sup>-</sup>CD27<sup>-</sup> cells, and of the CD45RA<sup>+</sup>CD27<sup>-</sup> subset. Because this last subset was only detected in inflamed tissues, analysis was performed on Vγ9Vδ2 T cells sorted from inflammatory fluids. To this end, sorted subsets of Vγ9Vδ2 T cells were stimulated with IPP and prolifera-



**Figure 3.** Distribution of Vδ2 T cell subsets at sites of inflammation. Mononuclear cells were collected from one ascitic (A) and two cerebrospinal fluids (B and C) from three patients affected by tuberculous peritonitis and meningitis, respectively. Cells were stained with antibodies to Vδ2, CD45RA, and CD27. (D-F) Vδ2 T cell subsets were sorted and analyzed for the expression of different chemokine receptor and CD62L.

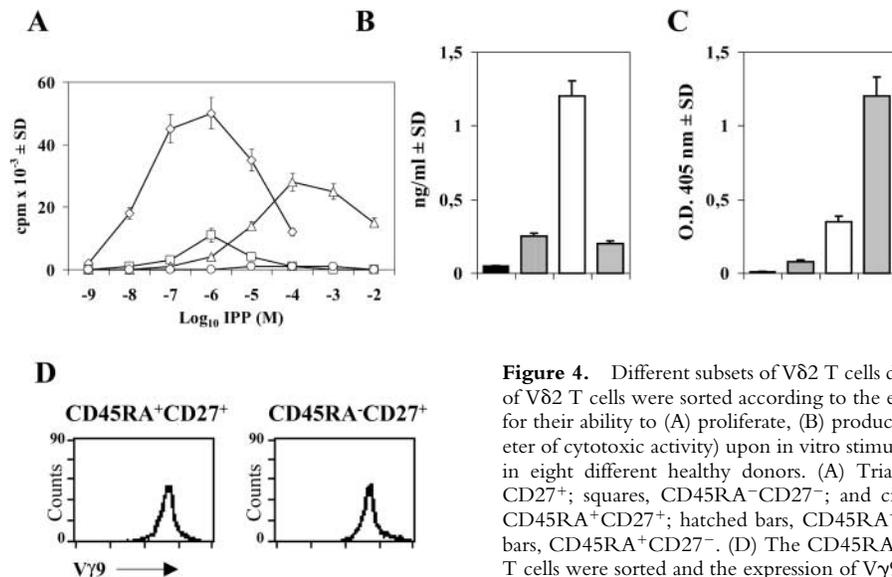
tion; IFN $\gamma$  production and release of BLT-esterase (as a parameter of cytotoxicity) were tested.

As shown in Fig. 4, naive CD45RA<sup>+</sup>CD27<sup>+</sup> cells were only able to proliferate in response to IPP in vitro (Fig. 4 A), whereas they failed to produce IFN $\gamma$  (Fig. 4 B) and release BLT-esterase (Fig. 4 C). Moreover, proliferative response of naive CD45RA<sup>+</sup>CD27<sup>+</sup> cells (Fig. 4 A) required stimulation with high IPP concentrations (generally 10<sup>-3</sup>–10<sup>-4</sup> M). Memory-type CD45RA<sup>-</sup>CD27<sup>+</sup> cells showed a significant proliferative activity in response to IPP in vitro, but peak proliferative response usually occurred at IPP concentrations lower than those required for proliferation of naive CD45RA<sup>+</sup>CD27<sup>+</sup> cells (i.e., 10<sup>-6</sup>–10<sup>-7</sup> M). The different proliferative response of naive CD45RA<sup>+</sup>CD27<sup>+</sup> and memory-type CD45RA<sup>-</sup>CD27<sup>+</sup> cells to different concentrations of IPP did not seem to be due to an enrichment of IPP-nonreactive cells in the former, as both subsets expressed the V $\gamma$ 9 chain at a similar extent (Fig. 4 D). Moreover, similar differential sensitivity of naive CD45RA<sup>+</sup>CD27<sup>+</sup> and memory-type CD45RA<sup>-</sup>CD27<sup>+</sup> cells was observed when plastic-bound anti-CD3 monoclonal antibody was used as a stimulant in the proliferation assay (unpublished data). Also, similarly to CD45RA<sup>+</sup>CD27<sup>+</sup> naive cells, CD45RA<sup>-</sup>CD27<sup>+</sup> cells produced very low amounts of IFN $\gamma$  (Fig. 4 B) and virtually failed to release BLT-esterase (Fig. 4 C). Conversely, the memory-type CD45RA<sup>-</sup>CD27<sup>-</sup> subset showed very low capacity to proliferate upon in vitro stimulation by IPP (Fig. 4 A), but secreted very high amounts of IFN $\gamma$  (Fig. 4 B) and discrete amounts of BLT-esterase (Fig. 4 C). Finally, CD45RA<sup>+</sup>CD27<sup>-</sup> cells taken from inflammatory sites failed to proliferate (Fig. 4 A), produced low amounts of IFN $\gamma$  (Fig. 4 B), but released substantial amounts of BLT-esterase upon IPP stimulation in vitro (Fig. 4 C). Additionally, these CD45RA<sup>+</sup>CD27<sup>-</sup> cells had prominent expression of intracellular perforin and granzysin (unpublished data), supporting the notion that

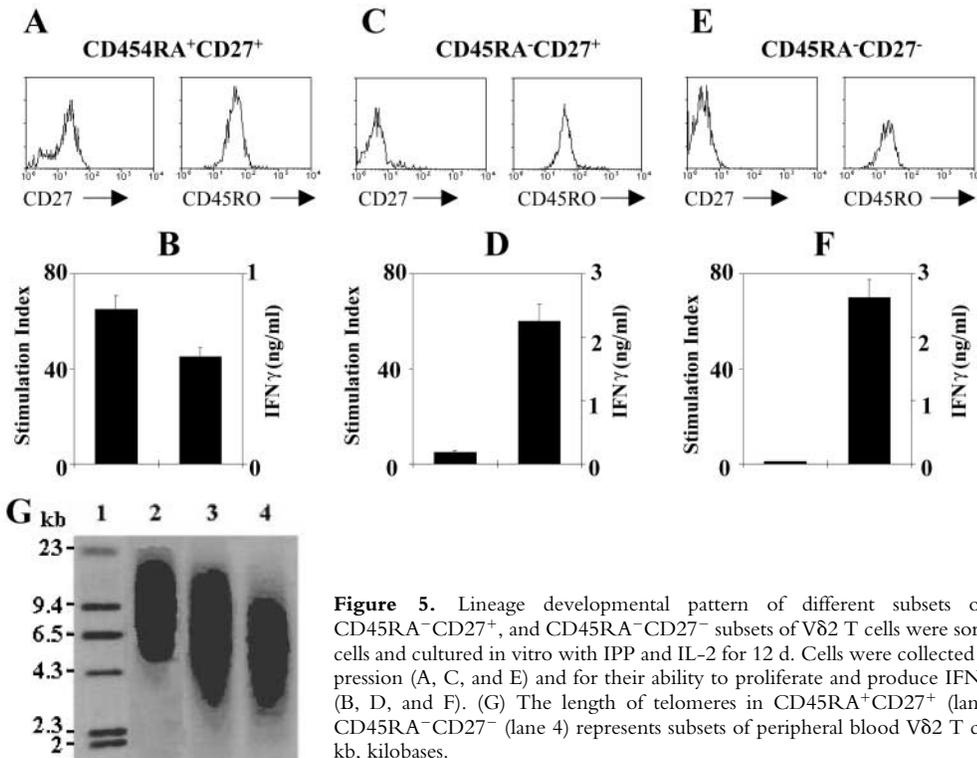
they are differentiated to exert prevalent cytotoxic activities.

To determine the lineage differentiation pattern of these four subsets of human V $\delta$ 2 T cells, we analyzed the differentiation of purified CD45RA<sup>+</sup>CD27<sup>+</sup>, CD45RA<sup>-</sup>CD27<sup>+</sup>, and CD45RA<sup>-</sup>CD27<sup>-</sup> subsets upon in vitro culture with IPP in the presence of IL-2 for 12 d, using a long term culture system described previously to determine differentiation of CD4 T cells (21). As shown in Fig. 5 A, when CD45RA<sup>+</sup>CD27<sup>+</sup> naive V $\delta$ 2 T cells were stimulated with IPP, all cells lost CD45RA and became CD45RO<sup>+</sup>, but most of them (~70%) retained CD27 expression. From a functional point of view, these cells had strong proliferative capacity and low IFN $\gamma$  production (Fig. 5 B). When memory-type CD45RA<sup>-</sup>CD27<sup>+</sup> cells were stimulated under the same conditions, almost all cells recovered after a 12-d culture had lost CD27 expression (Fig. 5 C), and upon subsequent restimulation in vitro with IPP, produced high levels of IFN $\gamma$  (Fig. 5 D). Finally, CD45RA<sup>-</sup>CD27<sup>-</sup> cells retained this phenotype after a 12-d culture (Fig. 5 E) and produced IFN $\gamma$  upon in vitro stimulation with IPP (Fig. 5 F). Therefore, these data suggest that, at least upon in vitro culture with antigen, V $\gamma$ 9V $\delta$ 2 T lymphocytes differentiate through the following lineage pattern: CD45RA<sup>+</sup>CD27<sup>+</sup>  $\rightarrow$  CD45RA<sup>-</sup>CD27<sup>+</sup>  $\rightarrow$  CD45RA<sup>-</sup>CD27<sup>-</sup>  $\rightarrow$  CD45RA<sup>+</sup>CD27<sup>-</sup>. This possibility is in part supported by the analysis of telomere length, which decreases as a consequence of cell division (24), in different V $\delta$ 2 T cell subsets. Fig. 5 G shows that the length of telomeres progressively decreases during the transition from CD45RA<sup>+</sup>CD27<sup>+</sup> to CD45RA<sup>-</sup>CD27<sup>+</sup> to CD45RA<sup>-</sup>CD27<sup>-</sup> phenotype, with the latter subset having the lowest telomere length, suggesting that it had undergone a high number of divisions.

In this paper, we have defined discrete subsets of human V $\delta$ 2 T cells based on expression patterns of CD45RA and



**Figure 4.** Different subsets of V $\delta$ 2 T cells display different effector functions. The four subsets of V $\delta$ 2 T cells were sorted according to the expression of CD45RA and CD27 and were tested for their ability to (A) proliferate, (B) produce IFN $\gamma$ , and (C) release BLT-esterase (as a parameter of cytotoxic activity) upon in vitro stimulation with IPP. Comparable results were obtained in eight different healthy donors. (A) Triangles, CD45RA<sup>+</sup>CD27<sup>+</sup>; diamonds, CD45RA<sup>-</sup>CD27<sup>+</sup>; squares, CD45RA<sup>+</sup>CD27<sup>-</sup>; and circles, CD45RA<sup>-</sup>CD27<sup>-</sup>. (B and C) Black bars, CD45RA<sup>+</sup>CD27<sup>+</sup>; hatched bars, CD45RA<sup>-</sup>CD27<sup>+</sup>; open bars, CD45RA<sup>-</sup>CD27<sup>-</sup>; and gray bars, CD45RA<sup>+</sup>CD27<sup>-</sup>. (D) The CD45RA<sup>+</sup>CD27<sup>+</sup> and CD45RA<sup>-</sup>CD27<sup>+</sup> subsets of V $\delta$ 2<sup>+</sup> T cells were sorted and the expression of V $\gamma$ 9 was assessed by FACS<sup>®</sup> staining.



**Figure 5.** Lineage developmental pattern of different subsets of V $\delta$ 2 T cells. CD45RA<sup>+</sup>CD27<sup>+</sup>, CD45RA<sup>-</sup>CD27<sup>+</sup>, and CD45RA<sup>-</sup>CD27<sup>-</sup> subsets of V $\delta$ 2 T cells were sorted from peripheral blood mononuclear cells and cultured in vitro with IPP and IL-2 for 12 d. Cells were collected and tested for CD45RO and CD27 expression (A, C, and E) and for their ability to proliferate and produce IFN $\gamma$  upon restimulation with IPP in vitro (B, D, and F). (G) The length of telomeres in CD45RA<sup>+</sup>CD27<sup>+</sup> (lane 2), CD45RA<sup>-</sup>CD27<sup>+</sup> (lane 3), and CD45RA<sup>-</sup>CD27<sup>-</sup> (lane 4) represents subsets of peripheral blood V $\delta$ 2 T cells. Lane 1, molecular weight markers. kb, kilobases.

CD27; chemokine receptors and adhesion molecules; and homing and functional properties. However, we must point out that we have actually no formal evidence for the last step of the proposed differentiation pathway to occur, as we consistently failed to recover CD45RA<sup>+</sup>CD27<sup>-</sup> cells in IPP stimulated cultures of either CD45RA<sup>-</sup>CD27<sup>+</sup> or CD45RA<sup>-</sup>CD27<sup>-</sup> cells. Additionally, because of the very low numbers and recovery of CD45RA<sup>+</sup>CD27<sup>-</sup> V $\delta$ 2 T cells from inflammatory fluids, we could not perform any further functional analysis on this subset. Moreover, the frequency of these cells in the peripheral blood of healthy donors appears to be extremely low (<0.1%; unpublished data) and therefore not accessible for assays that depend on discrete quantities of purified cells. Finally, the possibility should be considered that transition from CD45RA<sup>-</sup>CD27<sup>-</sup> to CD45RA<sup>+</sup>CD27<sup>-</sup> cells may take place in peripheral compartments, such as inflamed tissues, due to the presence of high amounts of antigen or the local production of cytokines, or both.

Based on works in different experimental mouse models, which have reported early accumulation of  $\gamma\delta$  cells at sites of infection and inflammation (for review see reference 25),  $\gamma\delta$  T cells have been long considered as a readily available pool of cells that migrate preferentially to sites of inflammation and virtually lack long-term memory. However, analyses in humans have suggested that V $\delta$ 2 T cells may provide memory-type secondary responses (13, 14), and recent results in macaques have provided strong evidence for both primary and recall V $\delta$ 2 T cell response to

*Mycobacterium tuberculosis* (15, 16), indicating that they contribute to both innate and adaptive immune response against microbial infection.

Data provided in this paper offer a plausible explanation to this apparent paradox: the CD45RA<sup>-</sup>CD27<sup>+</sup> memory subset homing to the lymph node and lacking effector functions may represent an antigen-primed V $\delta$ 2 population trafficking to the lymph nodes and upon encounter with antigen generates a new wave of effector cells. On the other hand, CD45RA<sup>-</sup>CD27<sup>-</sup> cells represent a readily available pool of antigen-primed V $\delta$ 2 T cells which enter the peripheral tissues, where they can eventually further differentiate to CD45RA<sup>+</sup>CD27<sup>-</sup> cells, and by their ability to produce cytokines and exert cytotoxicity can contribute to the containment of invading microbial pathogens. Due to the protective role played by V $\delta$ 2 T cells in immune responses against intracellular microbial pathogens, such as *M. tuberculosis* (15, 26), our results might be of help in understanding the dynamics and expansion of V $\delta$ 2 T cells in disease states such as tuberculosis and AIDS, characterized by a dramatic loss of V $\delta$ 2 effector functions (17, 27, 28).

This work was supported by European Community grants QLK2-1999-00367 (to F. Dieli and A. Salerno) and QLK2-CT-1999-01093 (to F. Poccia), and by a grant from the Ministry for Education, University, and Research (PRIN 2001 to F. Dieli).

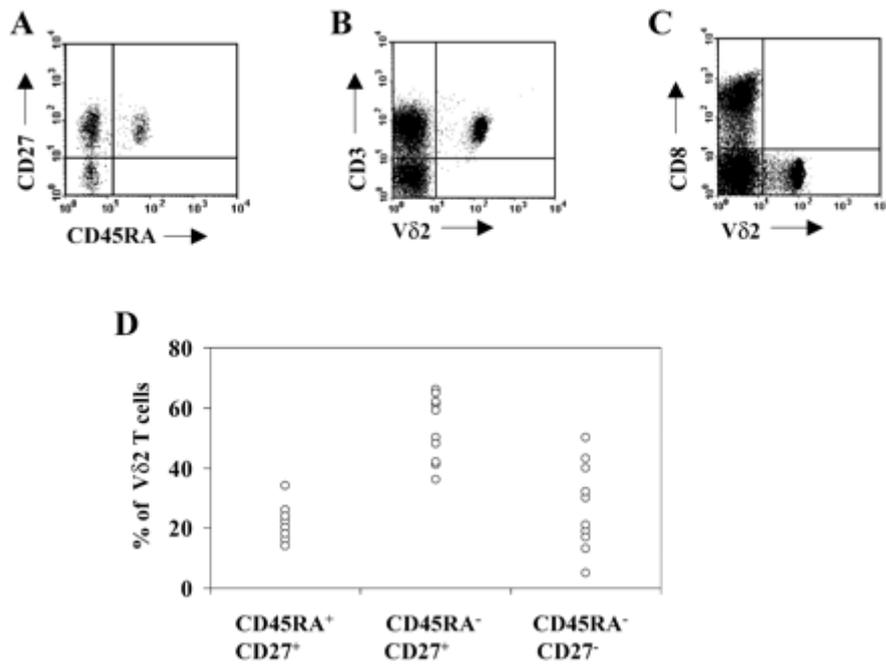
Submitted: 11 February 2003

Revised: 10 June 2003

Accepted: 10 June 2003

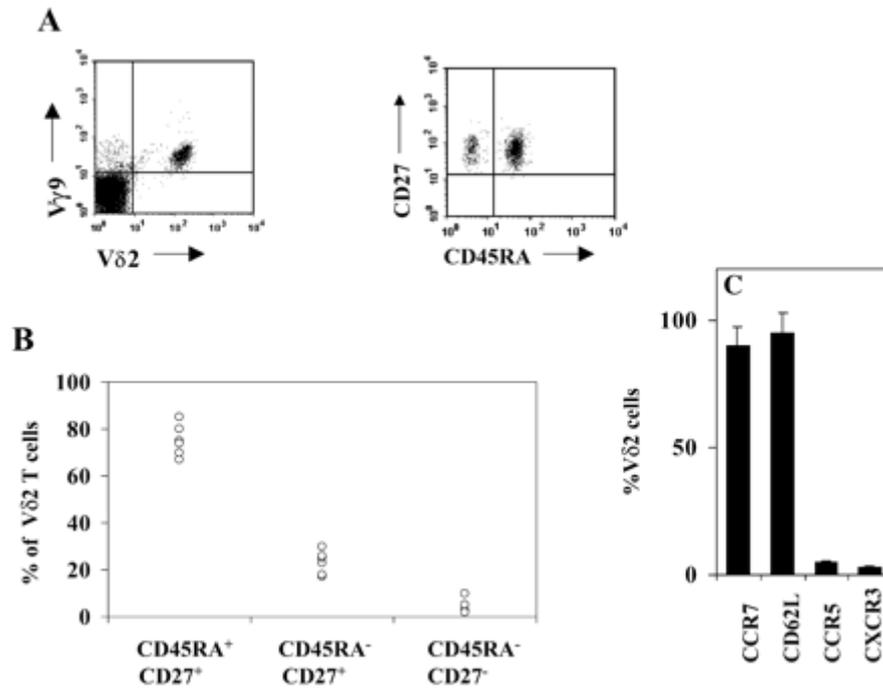
## References

1. Morita, C.T., R. Mariuzza, and M.B. Brenner. 2000. Antigen recognition by human  $\gamma\delta$  T cells: pattern recognition by the adaptive immune system. *Springer Semin. Immunopathol.* 22:191–217.
2. Constant, P., F. Davodeau, M.A. Peyrat, Y. Poquet, G. Puzo, M. Bonneville, and J.J. Fournié. 1994. Stimulation of human  $\gamma\delta$  T cells by nonpeptidic mycobacterial ligands. *Science.* 264:267–270.
3. Tanaka, Y., S. Sano, E. Nieves, G. De Libero, D. Rosa, R.L. Modlin, M.B. Brenner, B.R. Bloom, and C.T. Morita. 1994. Nonpeptide ligands for human  $\gamma\delta$  T cells. *Proc. Natl. Acad. Sci. USA.* 91:8175–8179.
4. Tanaka, Y., C.T. Morita, E. Nieves, M.B. Brenner, and B.R. Bloom. 1995. Natural and synthetic non-peptide antigens recognized by human  $\gamma\delta$  T cells. *Nature.* 375:155–158.
5. Belmant, C., E. Espinosa, F. Halary, I. Apostolou, H. Sicard, M.A. Payrat, A. Vercellone, P. Kourilsky, G. Gachelin, R. Poupot, et al. 1999. 3-Formyl-1-butyl pyrophosphate A novel mycobacterial metabolite-activating human  $\gamma\delta$  T cells. *J. Biol. Chem.* 274:32079–32084.
6. Bukowsky, J.F., C.T. Morita, and M.B. Brenner. 1999. Human  $\gamma\delta$  T cells recognize alkylamines derived from microbes, edible plants, and tea: implications for innate immunity. *Immunity.* 11:57–65.
7. Burk, M.R., L. Mori, and G. De Libero. 1995. Human V $\gamma$ 9-V $\delta$ 2 cells are stimulated in a cross-reactive fashion by a variety of phosphorylated metabolites. *Eur. J. Immunol.* 25:2052–2058.
8. Kunzmann, V., E. Bauer, and M. Wilhelm. 1999. Gamma/delta T-cell stimulation by pamidronate. *N. Engl. J. Med.* 340:737–738.
9. Lang, F., M.A. Peyrat, P. Constant, F. Davodeau, J. David-Ameline, Y. Poquet, H. Vie, J.J. Fournié, and M. Bonneville. 1995. Early activation of human V $\gamma$ 9V $\delta$ 2 T cell broad cytotoxicity and TNF production by nonpeptidic mycobacterial ligands. *J. Immunol.* 154:5986–5994.
10. Morita, C.T., E.M. Beckman, J.F. Bukowsky, Y. Tanaka, H. Band, B.R. Bloom, D.E. Golan, and M.B. Brenner. 1995. Direct presentation of nonpeptide prenyl pyrophosphate antigens to human  $\gamma\delta$  T cells. *Immunity.* 3:495–507.
11. Sireci, G., E. Espinosa, C. Di Sano, F. Dieli, J.J. Fournié, and A. Salerno. 2001. Differential activation of human  $\gamma\delta$  cells by nonpeptide phosphoantigens. *Eur. J. Immunol.* 31:1628–1635.
12. Parker, C.M., V. Groh, H. Band, S.A. Porcelli, C.T. Morita, M. Fabbi, D. Glass, J.L. Strominger, and M.B. Brenner. 1990. Evidence for extrathymic changes in the T cell receptor  $\gamma/\delta$  repertoire. *J. Exp. Med.* 171:1597–1612.
13. Hoft, D.F., R.M. Brown, and S.T. Roodman. 1998. Bacille Calmette-Guerin vaccination enhances human  $\gamma\delta$  T cell responsiveness to mycobacteria suggestive of a memory-like phenotype. *J. Immunol.* 161:1045–1054.
14. Dieli, F., G. Sireci, C. Di Sano, A. Romano, L. Titone, P. Di Carlo, J. Ivanyi, J.J. Fournié, and A. Salerno. 2000. Ligand-specific  $\alpha\beta$  and  $\gamma\delta$  T cell responses in childhood tuberculosis. *J. Infect. Dis.* 181:294–301.
15. Shen, Y., D. Zhou, L. Qiu, X. Lai, M. Simon, L. Shen, Z. Kou, Q. Wang, L. Jiang, J. Estep, et al. 2002. Adaptive immune response of V $\gamma$ 2V $\delta$ 2<sup>+</sup> T cells during mycobacterial infections. *Science.* 295:2255–2258.
16. Chen, Z.W., and N.L. Letvin. 2003. Adaptive immune response of V $\gamma$ 2V $\delta$ 2 T cells: a new paradigm. *Trends Immunol.* 24:213–219.
17. Gioia, C., C. Agrati, R. Casetti, C. Cairo, G. Borsellino, L. Battistini, G. Mancino, D. Goletti, V. Colizzi, L.P. Pupillo, and F. Poccia. 2002. Lack of CD27<sup>-</sup>CD45RA<sup>-</sup>V $\gamma$ 9V $\delta$ 2<sup>+</sup> T cell effectors in immunocompromised hosts and during active pulmonary tuberculosis. *J. Immunol.* 168:1484–1489.
18. Gunn, M.D., K. Tangemann, C. Tam, J.G. Cyster, S.D. Rosen, and L.T. Williams. 1998. A chemokine expressed in lymphoid high endothelial venules promotes the adhesion and chemotaxis of naive T lymphocytes. *Proc. Natl. Acad. Sci. USA.* 95:258–263.
19. Forster, R., A. Schubel, D. Breitfeld, E. Kremmer, I. Renner-Muller, E. Wolf, and M. Lipp. 1999. CCR7 coordinates the primary immune response by establishing functional microenvironments in secondary lymphoid organs. *Cell.* 99:23–33.
20. Baggiolini, M. 1998. Chemokines and leukocyte traffic. *Nature.* 392:565–568.
21. Sallusto, F., S. Lenig, R. Forster, M. Lipp, and A. Lanzavecchia. 1999. Two subsets of memory T lymphocytes with distinct homing potentials and effector functions. *Nature.* 401:708–712.
22. Michie, C.A., A. McLean, C. Alcock, and P.C. Beverley. 1992. Lifespan of human lymphocyte subsets defined by CD45 isoforms. *Nature.* 360:264–265.
23. Hamann, D., P.A. Baars, M.H. Rep, B. Hooibrink, S.R. Kerkhoff-Garde, M.R. Klein, and R.A. van Lier. 1997. Phenotypic and functional separation of memory and effector human CD8<sup>+</sup> T cells. *J. Exp. Med.* 186:1407–1418.
24. Weng, N.P., K.S. Hathcock, and R.J. Hodes. 1998. Regulation of telomere length and telomerase in T and B cells: a mechanism for maintaining replicative potential. *Immunity.* 9:151–157.
25. Salerno, A., and F. Dieli. 1998. Role of  $\gamma\delta$  T lymphocytes in immune response in humans and mice. *Crit. Rev. Immunol.* 18:327–357.
26. Dieli, F., M. Troye-Blomberg, J. Ivanyi, J.J. Fournié, M. Bonneville, M.A. Peyrat, G. Sireci, and A. Salerno. 2000. V $\gamma$ 9/V $\delta$ 2 T lymphocytes reduce the viability of intracellular *Mycobacterium tuberculosis*. *Eur. J. Immunol.* 30:1512–1519.
27. Li, B., M.D. Rossman, T. Imir, A.F. Oner-Eyuboglu, C.W. Lee, R. Biancanello, and S.R. Carding. 1996. Disease-specific changes in  $\gamma\delta$  T cell repertoire and function in patients with pulmonary tuberculosis. *J. Immunol.* 157:4222–4229.
28. Dieli, F., G. Sireci, N. Caccamo, C. Di Sano, L. Titone, A. Romano, P. Di Carlo, A. Barera, A. Accardo-Palumbo, A.M. Krensky, and A. Salerno. 2002. Selective depression of interferon- $\gamma$  and granulysin production with increase of proliferative response by V $\gamma$ 9/V $\delta$ 2 T cells in children with tuberculosis. *J. Infect. Dis.* 186:1835–1839.



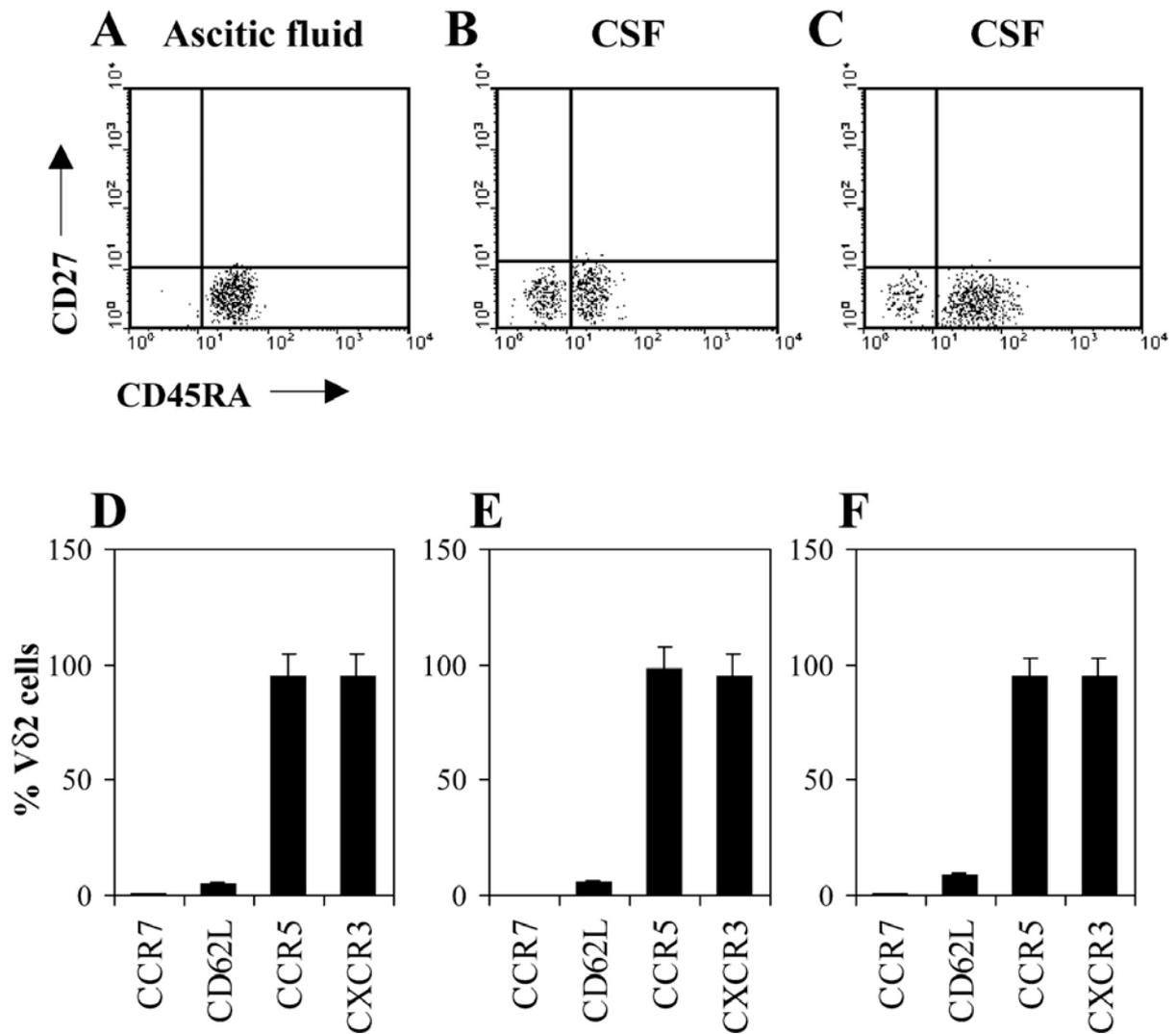
**Figure 1.**

Phenotypic characterization of Vδ2 T cell subsets. (A) Peripheral blood mononuclear cells were stained with antibodies to Vδ2, CD45RA, and CD27. Upon analysis on gated Vδ2<sup>+</sup> cells, three subsets were identified. (B and C) Two-color FACS<sup>®</sup> analysis of peripheral blood mononuclear cells stained with antibodies to Vδ2, CD3, and CD8. (D) Upon analysis in a healthy population, the proportion of cells within different subsets was variable among individuals, the variability being more pronounced in the CD45RA<sup>-</sup>CD27<sup>-</sup> subset.



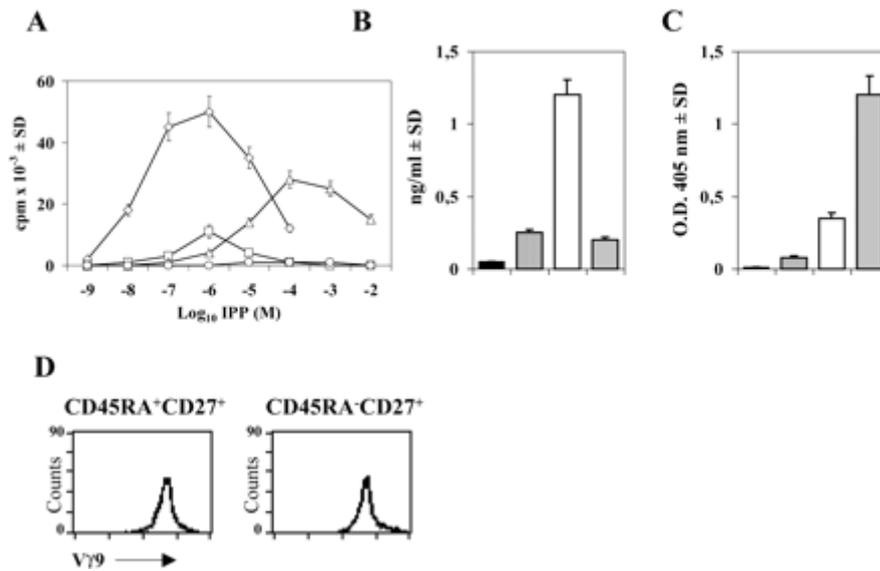
**Figure 2.**

Distribution of Vδ2 T cell subsets in lymph nodes. (A) Mononuclear cells were taken from inguinal lymph nodes of organ donors. Cells were stained with antibodies to Vδ2, CD45RA, and CD27. As a control, cells were double stained with antibodies to Vδ2 and Vγ9. (B) Analysis of the relative proportion of the CD45RA<sup>+</sup>CD27<sup>+</sup>, CD45RA<sup>-</sup>CD27<sup>+</sup>, and CD45RA<sup>-</sup>CD27<sup>-</sup> subsets of Vδ2<sup>+</sup> T cells in the lymph nodes of six donors. (C) Vδ2 T cell subsets were sorted and analyzed for the expression of different chemokine receptors and CD62L. Results similar to those shown here were obtained in a total of six donors.



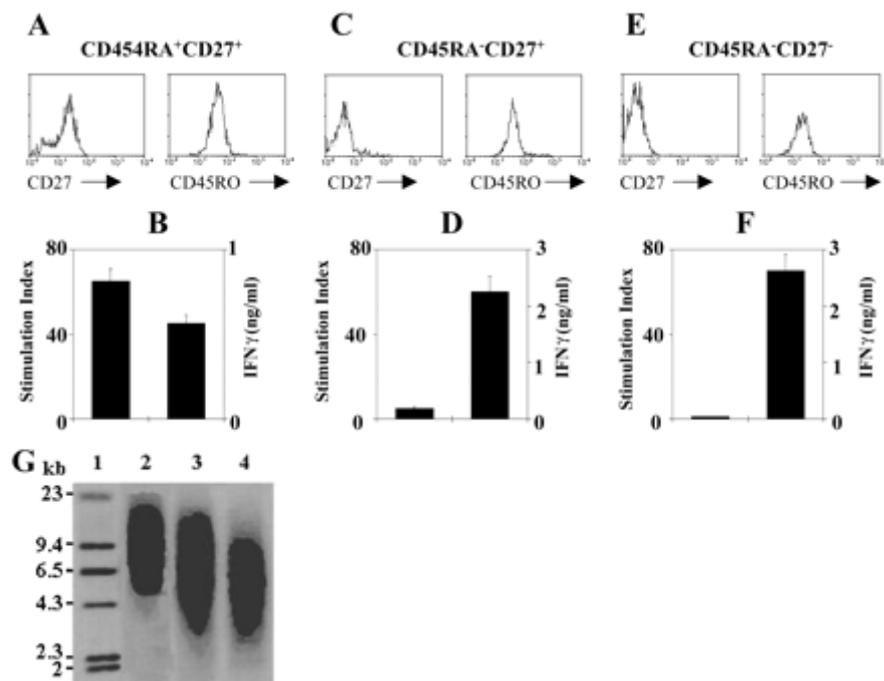
**Figure 3.**

Distribution of V $\delta$ 2 T cell subsets at sites of inflammation. Mononuclear cells were collected from one ascitic (A) and two cerebrospinal fluids (B and C) from three patients affected by tuberculous peritonitis and meningitis, respectively. Cells were stained with antibodies to V $\delta$ 2, CD45RA, and CD27. (D–F) V $\delta$ 2 T cell subsets were sorted and analyzed for the expression of different chemokine receptor and CD62L



**Figure 4.**

Different subsets of V $\delta$ 2 T cells display different effector functions. The four subsets of V $\delta$ 2 T cells were sorted according to the expression of CD45RA and CD27 and were tested for their ability to (A) proliferate, (B) produce IFN $\gamma$ , and (C) release BLT-esterase (as a parameter of cytotoxic activity) upon in vitro stimulation with IPP. Comparable results were obtained in eight different healthy donors. (A) Triangles, CD45RA<sup>+</sup>CD27<sup>+</sup>; diamonds, CD45RA<sup>-</sup>CD27<sup>+</sup>; squares, CD45RA<sup>-</sup>CD27<sup>-</sup>; and circles, CD45RA<sup>+</sup>CD27<sup>-</sup>. (B and C) Black bars, CD45RA<sup>+</sup>CD27<sup>+</sup>; hatched bars, CD45RA<sup>-</sup>CD27<sup>+</sup>; open bars, CD45RA<sup>-</sup>CD27<sup>-</sup>; and gray bars, CD45RA<sup>+</sup>CD27<sup>-</sup>. (D) The CD45RA<sup>+</sup>CD27<sup>+</sup> and CD45RA<sup>-</sup>CD27<sup>+</sup> subsets of V $\delta$ 2<sup>+</sup> T cells were sorted and the expression of V $\gamma$ 9 was assessed by FACS<sup>®</sup> staining.



**Figure 5.**

Lineage developmental pattern of different subsets of V $\delta$ 2 T cells. CD45RA<sup>+</sup>CD27<sup>+</sup>, CD45RA<sup>-</sup>CD27<sup>+</sup>, and CD45RA<sup>-</sup>CD27<sup>-</sup> subsets of V $\delta$ 2 T cells were sorted from peripheral blood mononuclear cells and cultured in vitro with IPP and IL-2 for 12 d. Cells were collected and tested for CD45RO and CD27 expression (A, C, and E) and for their ability to proliferate and produce IFN $\gamma$  upon restimulation with IPP in vitro (B, D, and F). (G) The length of telomeres in CD45RA<sup>+</sup>CD27<sup>+</sup> (lane 2), CD45RA<sup>-</sup>CD27<sup>+</sup> (lane 3), and CD45RA<sup>-</sup>CD27<sup>-</sup> (lane 4) represents subsets of peripheral blood V $\delta$ 2 T cells. Lane 1, molecular weight markers. kb, kilobases.

JEM

**Table I.**Chemokine Receptor, CD45RO and CD62L Expression on Subsets of V $\delta$ 2<sup>+</sup> T Cells

	CD45RA <sup>+</sup> CD27 <sup>+</sup>	CD45RA <sup>-</sup> CD27 <sup>+</sup>	CD45RA <sup>-</sup> CD27 <sup>-</sup>
CXCR3	6 ± 2 (14)	20 ± 3 (48)	95 ± 11 (144)
CCR2	5 ± 1 (18)	13 ± 1 (21)	95 ± 9 (38)
CCR5	8 ± 2 (28)	25 ± 2 (54)	80 ± 12 (156)
CCR6	5 ± 1 (12)	10 ± 2 (14)	15 ± 2 (27)
CCR7	75 ± 10 (64)	63 ± 7 (53)	0 (0)
CD62L	95 ± 11 (73)	86 ± 10 (66)	3 ± 2 (4)
CD45RO	1 ± 1 (5)	96 ± 8 (125)	97 ± 12 (164)

- Three subsets of peripheral blood V $\delta$ 2<sup>+</sup> cells were sorted by expression of CD45RA and CD27. The sorted cells were stained and analyzed for the expression of chemokine receptors, CD45RO and CD62L. Values indicate the percentage of positive cells ± SD of three different experiments. Values in brackets indicate the mean fluorescence intensity (MFI) mean value.