



[haematologica reports]  
2006;2(3):10-13

## A subset of V $\gamma$ 9V $\delta$ 2 T cells help B cells for antibody production

CACCAMO N  
MERAIGLIA S  
LA MENDOLA C  
BUCCHERI S  
D'ASARO M  
DIELI F  
SALERNO A

Dipartimento di Biopatologia e  
Metodologie Biomediche, Università di Palermo, Italy

A B S T R A C T

V $\gamma$ 9V $\delta$ 2 T lymphocytes recognize nonpeptidic antigens and mount effector functions in cellular immune responses against microorganisms and tumors, but little is known about their role in antibody-mediated immune responses. Here we report on the identification of a unique subset of V $\gamma$ 9V $\delta$ 2 T cells which express the costimulatory molecules ICOS and CD40L, secrete IL-2, IL-4 and IL-10 and help B cells for antibody production. These properties portray CXCR5+ V $\gamma$ 9V $\delta$ 2 T cells as a distinct memory T cell subset with B cell helper function.

Key words: V $\gamma$ 9V $\delta$ 2 T cells; CXCR5; B-cell help; antibody production; HIV infection.

Correspondence:  
Francesco Dieli,  
Dipartimento di Biopatologia e  
Metodologie Biomediche  
Università di Palermo,  
Corso Tukory 211,  
90134 Palermo, Italy.  
Phone: +39-091-6555916.  
Fax: +39-091-6555924.  
E-mail: dieli@unipa.it

V $\gamma$ 9V $\delta$ 2 T cells are a minor T cell population with a unique pattern of antigen recognition. They are heterogeneous and comprise distinct populations that can be distinguished on the basis of surface markers expression, effector functions and migratory properties.<sup>1</sup> Based on their effector properties, V $\gamma$ 9V $\delta$ 2 T lymphocytes are supposed to play an important role in cellular immune responses against intracellular microorganisms and tumors.<sup>2</sup> However, whether or not V $\gamma$ 9V $\delta$ 2 T lymphocytes also participate in antibody-mediated immune responses remains unclear. Earlier studies in mice<sup>3</sup> and humans<sup>4</sup> have shown that  $\gamma\delta$  T cells help B cells for antibody production, but the subset responsible for this activity was not identified.

We show here that expression of CXCR5 defines a unique subset of peripheral blood T<sub>CM</sub> V $\gamma$ 9V $\delta$ 2 cells which upon antigen stimulation express the costimulatory molecules ICOS and CD40L, secrete IL-4 and IL-10 and provide potent B-cell help for antibody production *in vitro*.

### Materials and Methods

#### FACS staining and sorting

PBMC were isolated from heparinized blood or inflamed tonsils by Ficoll-Hypaque (Pharmacia Biotech, Uppsala, Sweden). The following conjugated antibodies were used in different combinations: anti-V $\delta$ 2 (Coulter, Miami, FL), anti-V $\gamma$ 9 (Coulter), anti-CD27 (BD Pharmingen, San Diego, CA), anti-CD45RA (Coulter), anti-CD45RO (Coul-

ter), anti-CD3 (Sigma, St. Louis, MO), anti-CD25 (BD Pharmingen), anti-CD62L (BD Pharmingen), anti-CCR7 (a gift of Dr. M. Lipp, Max-Delbrück-Center for Molecular Medicine, Berlin, Germany), anti-HLA DR monomorphic (a gift of Prof. V. Horejsi, Institute of Molecular Genetics, Academy of Science of the Czech Republic, Prague), anti-CCR5 (BD Pharmingen), anti-CXCR3 (BD Pharmingen), anti-ICOS (a generous gift of Dr. R.A. Kroccek), anti-CD40L (BD Pharmingen) and anti-CXCR5 (R&D Systems, Minneapolis, MN, USA).

Data were acquired on a FACSCalibur (BD Biosciences) and analysed using CellQuest software (BD Immunocytometry Systems, San José, CA). Cell sorting was performed on a FACS Vantage (BD Biosciences).

#### Cytokine production

The medium used throughout was complete RPMI 1640 medium (Gibco, Grand Island, NY) supplemented with 10% heat-inactivated fetal calf serum (FCS, Gibco), 2 mM L-glutamine, 20 nM Hepes and 100 U/ml penicillin/streptomycin. Sorted V $\delta$ 2 cell subsets were labelled with CFSE as above described and cultured at 37° C, in the presence of 5% CO<sub>2</sub>, at 10<sup>5</sup>/mL in 96-well flat-bottomed plates (0.2 mL/well), with different concentrations of bromohydrinpyrophosphate (BrHPP, a gift of Dr. J.J. Fournié) in the presence of irradiated (5,000 Rads from a caesium source) allogeneic dendritic cells. IFN- $\gamma$ , TNF- $\alpha$ , IL-4 and IL-10 levels in the 24-hr culture supernatants were assessed by two-mAbs sandwich ELISA

assay following manufacturer's recommendations (R&D Systems).

The chemotactic ability of CXCR5<sup>+</sup> V $\gamma$ 9V $\delta$ 2 cells was assayed using a double-chamber system with 3- $\mu$  pores (Transwell Costar, Cambridge, MA), according to literature.<sup>5,6</sup> Briefly, 10<sup>5</sup> sorted CXCR5<sup>+</sup> V $\gamma$ 9V $\delta$ 2 cells, were added to the upper chamber and CXCL13 (recombinant human CXCL13, BCA-1, R&D Systems, Minneapolis, MN, 3  $\mu$ M final concentration) to the lower chamber and incubated at 37° for 2 hr in a 5% CO<sub>2</sub> humidified incubator. Assays were performed in triplicate. Afterwards, the membrane was removed, washed on the upper side with PBS, fixed, and stained. Migrated cells were counted microscopically at 1,000 magnification in five randomly selected fields per well. Percentage migration was calculated by measuring the counts recovered from the lower chamber and comparing them to the total input counts; results represent the mean  $\pm$  SD of three independent experiments.

### Antibody production *in vitro*

V $\gamma$ 9V $\delta$ 2 T cell help in antibody production was studied as follows. Different subsets of peripheral blood V $\gamma$ 9V $\delta$ 2 T cells were sorted by FACS and co-cultured with sorted tonsillar B cells in 96-well plates at 10<sup>5</sup> cells/well each of T and B cells in the presence or absence of BrHPP for 10 days. IgM, IgG, and IgA levels in the culture supernatants were determined by ELISA.

## Results and Discussion

Expression of the chemokine receptor CXCR5 defines a population of CD4 T helper cells which localizes to B cell follicles and support the production of immunoglobulins.<sup>5</sup>

CXCR5 expression was studied on peripheral blood and tonsil V $\gamma$ 9V $\delta$ 2 T cells. Within PBMC, about 15% of total V $\gamma$ 9V $\delta$ 2 T cells are CXCR5<sup>+</sup>, while CXCR5<sup>+</sup> V $\gamma$ 9V $\delta$ 2 T cells are highly enriched in inflamed tonsils where they account for about half the size of the V $\gamma$ 9V $\delta$ 2 T cell population. As shown in Table 1, the vast majority of CXCR5<sup>+</sup> V $\gamma$ 9V $\delta$ 2 cells does not express CD45RA, but express CD27, CD45RO, CCR7 and CD62L, thus identifying them as a subpopulation of T central memory (T<sub>CM</sub>) V $\gamma$ 9V $\delta$ 2 cells. Peripheral blood CXCR5<sup>+</sup> V $\gamma$ 9V $\delta$ 2 T cells do not express the activation markers CD25 and HLA-DR and also lack expression of the costimulatory molecules CD40L and ICOS. In tonsils, CXCR5<sup>+</sup> V $\gamma$ 9V $\delta$ 2 T cells had a T<sub>CM</sub> phenotype like their peripheral blood counterpart, but most of them express several activation markers and costimulatory molecules (CD25, HLA-DR, CD40L and ICOS), suggesting that they are engaged in immune responses occurring in tonsils.<sup>7,8</sup> Moreover, expression of CCR7, which causes homing to the T cell areas of

**Table 1. Surface markers expression on CXCR5<sup>+</sup> V $\gamma$ 9V $\delta$ 2<sup>+</sup> T cells in peripheral blood and inflamed tonsils.**

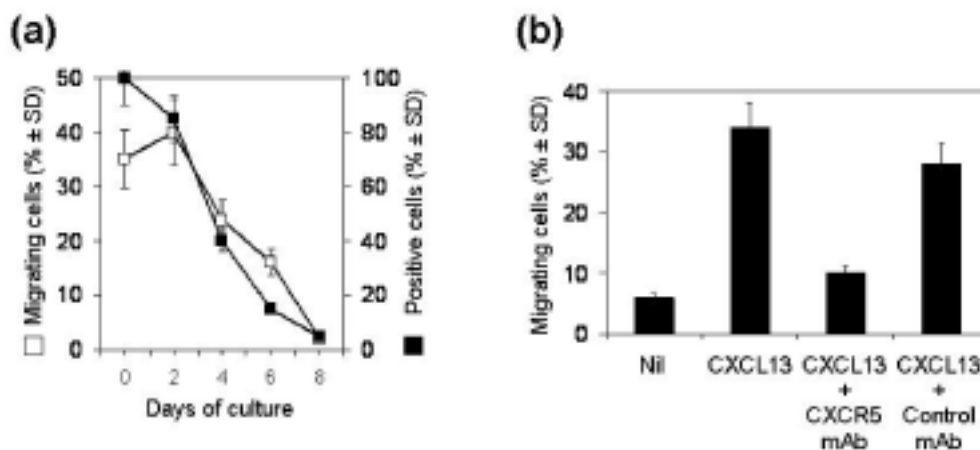
	Peripheral blood (n = 15)	Tonsils (n = 6)
CD45RA	1 $\pm$ 2 (4)	2 $\pm$ 3 (5)
CD45RO	98 $\pm$ 7 (85)	95 $\pm$ 9 (38)
CD25	1 $\pm$ 0.4 (2)	65 $\pm$ 7 (44)
HLA-DR	0.5 $\pm$ 0.1 (4)	58 $\pm$ 6 (34)
CD40L	2.4 $\pm$ 0.4 (4)	78 $\pm$ 9 (84)
ICOS	1.5 $\pm$ 0.2 (3)	98 $\pm$ 6 (115)
CD62L	95 $\pm$ 4 (73)	33 $\pm$ 2 (30)
CCR7	97 $\pm$ 3.9 (86)	28 $\pm$ 5 (25)
CCR5	1 $\pm$ 0.1 (5)	15 $\pm$ 2 (18)
CXCR3	2.1 $\pm$ 0.6 (7)	9.5 $\pm$ 3.1 (11)

Peripheral blood and tonsil mononuclear cells were stained with mAbs to V $\delta$ 2, CXCR5 and other cell surface markers, and were analysed by FACS. Values indicate the percentage $\pm$ SE of CXCR5<sup>+</sup> V $\delta$ 2<sup>+</sup> cells expressing the indicated cell surface markers. Values in brackets indicate the Mean Fluorescence Intensity mean value.

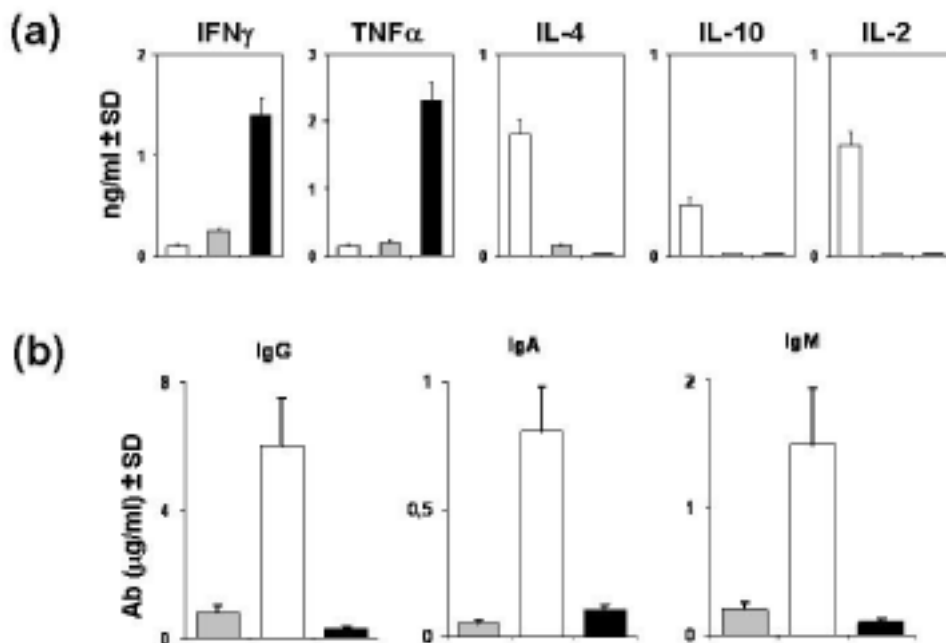
secondary lymphoid tissues is found on the vast majority of peripheral blood CXCR5<sup>+</sup> V $\gamma$ 9V $\delta$ 2 T cells but is markedly reduced on tonsillar CXCR5<sup>+</sup> V $\gamma$ 9V $\delta$ 2 T cells, suggesting the possibility of a ligand-induced CCR7 downmodulation. Similarly, CD62L expression is reduced on tonsillar CXCR5<sup>+</sup> V $\gamma$ 9V $\delta$ 2 T cells, implying that the majority of tonsillar CXCR5<sup>+</sup> V $\gamma$ 9V $\delta$ 2 T cells have recently immigrated from circulation. The chemokine receptors CXCR3 and CCR5 were weakly expressed on tonsillar CXCR5<sup>+</sup> V $\gamma$ 9V $\delta$ 2 T cells, but were not detected on peripheral blood CXCR5<sup>+</sup> V $\gamma$ 9V $\delta$ 2 T cells (Table 1). This finding is in agreement with our previous results<sup>1</sup> demonstrating that CXCR3 and CCR5 are expressed by TEM and TEMRA, but not by TCM V $\gamma$ 9V $\delta$ 2 T cells.

Freshly isolated tonsillar CXCR5<sup>+</sup> V $\gamma$ 9V $\delta$ 2 T cells migrated readily in response to CXCL13/BCA-1 (Figure 1a). Responsiveness to BCA-1 was enhanced when the cells were cultured *in vitro* with BrHPP, but by day 3 of culture migration in response to BCA-1 consistently decreased; this effect was paralleled by CXCR5 receptor expression, which uniformly decreased upon *in vitro* culture with BrHPP (Figure 1a). Of note, migration of tonsillar CXCR5<sup>+</sup> V $\gamma$ 9V $\delta$ 2 T cells in response to CXCL13/BCA-1 was consistently inhibited by anti-CXCR5 mAb (Figure 1b).

We then studied the pattern of cytokine production in CXCR5<sup>+</sup> and CXCR5<sup>-</sup> V $\gamma$ 9V $\delta$ 2 T<sub>CM</sub> and V $\gamma$ 9V $\delta$ 2 T<sub>EM</sub> cells, after a 24 hrs stimulation period with BrHPP and irradiated dendritic cells *in vitro*. As shown in Figure 2a, CXCR5<sup>-</sup> V $\gamma$ 9V $\delta$ 2 T<sub>CM</sub> produced very few amounts of cytokines, whereas V $\gamma$ 9V $\delta$ 2 T<sub>EM</sub> cells produced significant amounts of IFN- $\gamma$  and TNF- $\alpha$ , but neither IL-4 nor IL-10, thus confirming our previous results (1). However, antigen-stimulated CXCR5<sup>+</sup> V $\gamma$ 9V $\delta$ 2 T<sub>CM</sub> cells had a different cytokine profile as they produced IL-2, IL-4



**Figure 1.** Modulation of migration to CXCL13/BCA-1 and CXCR5 expression during culture of tonsillar CXCR5<sup>+</sup> Vγ9Vδ2 T cells. Freshly isolated tonsillar CXCR5<sup>+</sup> Vγ9Vδ2 T cells were cultured with BrHPP for up to 8 days and examined for *in vitro* migration to CXCL13/BCA-1 and CXCR5 expression (a). In (b), migration of tonsillar CXCR5<sup>+</sup> Vγ9Vδ2 T cells to CXCL13/BCA-1 was carried out in the presence of anti-CXCR5 or isotype-matched control mAbs. Data are representative of three independent experiments.



**Figure 2.** Cytokine production and B-cell helper activity of CXCR5<sup>+</sup> Vγ9Vδ2 T cells. (a) CXCR5<sup>+</sup> and CXCR5<sup>-</sup> T<sub>CM</sub> and T<sub>EM</sub> subsets of Vγ9Vδ2 T lymphocytes were cultured with BrHPP. Cytokine levels were assessed by ELISA. (b) Tonsillar B cells were cultured alone (Nil) or in the presence of CXCR5<sup>+</sup> and CXCR5<sup>-</sup> T<sub>CM</sub> and T<sub>EM</sub> Vγ9Vδ2 T lymphocytes, in the presence of BrHPP. Ten days later, total IgG, IgA and IgM levels in culture supernatants were assessed by ELISA. One out of 5 different experiments is shown. CXCR5<sup>+</sup> (white columns) and CXCR5<sup>-</sup> (grey filled columns) T<sub>CM</sub> and T<sub>EM</sub> (black filled columns) subsets of Vγ9Vδ2 T lymphocytes are shown.

and, to a lower extent, IL-10, but neither IFN-γ nor TNF-α.

As CXCR5<sup>+</sup> Vγ9Vδ2 T cells express costimulatory molecules, produce IL-4 and IL-10 and localize to B cell follicles, we tested whether or not these cells were able

to support B cells to secrete immunoglobulins. Peripheral blood derived CXCR5<sup>+</sup> and CXCR5<sup>-</sup> Vγ9Vδ2 T<sub>CM</sub> and Vγ9Vδ2 T<sub>EM</sub> cells were sorted and cultured with CD19<sup>+</sup> B cells isolated from the tonsil of the same donor, in the presence or absence of BrHPP. Figure 2b shows one

typical experiment out of five. B cells produced comparable very low amounts of IgA, IgG and IgM when cultured for 10 days without V $\gamma$ 9V $\delta$ 2 T cells or with the CXCR5<sup>-</sup> fractions (i.e. CXCR5<sup>-</sup> V $\gamma$ 9V $\delta$ 2 T<sub>CM</sub> and V $\gamma$ 9V $\delta$ 2 T<sub>EM</sub> cells). In contrast, co-culture of B cells with CXCR5<sup>+</sup> V $\gamma$ 9V $\delta$ 2 T<sub>CM</sub> and BrHPP resulted in an 18-fold increase in the production of IgG, 8-fold increase in the production of IgA and 7-fold increase in the production of IgM.

In conclusion, data reported in this paper allow us to identify a unique subset of peripheral blood T<sub>CM</sub> V $\gamma$ 9V $\delta$ 2 cells defined by expression of CXCR5, which express the costimulatory molecules ICOS and CD40L, secrete IL-2, IL-4 and IL-10, localize to B cell follicles and provide potent B-cell help for antibody production *in vitro*. Thus, CXCR5<sup>+</sup> V $\gamma$ 9V $\delta$ 2 T<sub>CM</sub> cells may influence humoral immune responses early during microbial infections, before full development of acquired responses mediated by CD4 T cells, which depends on a series of time-consuming steps, including Ag uptake and processing by tissue DCs, their relocation to draining lymph nodes, and T-cell priming and effector cell development.

## References

1. Dieli, F., Poccia, F., Lipp, M., Sireci, G., Caccamo, N., Di Sano, C. and Salerno, A. Differentiation of effector/memory V $\delta$ 2 T cells and migratory routes in lymph nodes or inflammatory sites. *J Exp Med* 2003;198:391-7.
2. Bonneville M, Fournié, J.J. Sensing cell stress and transformation through V $\gamma$ 9V $\delta$ 2 T cell-mediated recognition of the isoprenoid pathway metabolites. *Microbes Infect* 2005; 7:503-9.
3. Pao W, Wen L, Smith AL, Gulbranson-Judge A, Zheng, B., Kelsoe, G., et al.  $\delta$ T cell help of B cells is induced by repeated parasitic infection, in the absence of other T cells. *Curr Biol* 1996; 6:1317-25.
4. Brandes M, Willimann K, Lang AB, Nam KH, Jin C, Brenner MB, et al. Flexible migration program regulates  $\gamma\delta$  T-cell involvement in humoral immunity. *Blood* 2003; 102:3693-701.
5. Breitfeld D, Ohl L, Kremmer E, Ellwart J, Sallusto F, Lipp M, et al. Follicular B helper T cells express CXC chemokine receptor 5, localize to B cell follicles, and support immunoglobulin production. *J Exp Med* 2000; 192:1545-52.
6. Förster R, Mattis EA, Kremmer E, Wolf E, Brem G, Lipp M. A putative chemokine receptor, BLR1, directs B cell migration to defined lymphoid organs and specific anatomic compartments of the spleen. *Cell* 1996; 87:1037-47.
7. Hutloff A, Dittrich AM, Beier KC, Eljaschewitsch B, Kraft R, Anagnostopoulos I, Kroczeck RA. ICOS is an inducible T-cell co-stimulator structurally and functionally related to CD28. *Nature* 1999; 397:263-6.
8. Grewal IS, Flavell RA. CD40 and CD154 in cell-mediated immunity. *Annu Rev Immunol* 1998; 16:111-35.