Human memory $\gamma^9\delta^2$ T cells require homeostatic cytokines for proliferation and differentiation

**ABSTRACT**

We have analysed four subsets of human $\gamma^9\delta^2$ T cells for their capacity to proliferate and differentiate in response to antigen or homeostatic cytokines. Antigen-stimulated cells acquired a central memory (TCM) or effector memory (TEM) phenotype, while IL-15-stimulated cells maintained their phenotype, with the exception of TCM cells, which expressed CD27 and CD45RA in various combinations. These results show that human $\gamma^9\delta^2$ memory T cells have different proliferation and differentiation potentials and that terminally differentiated (TEMRA) cells result from the TCM subset upon homeostatic proliferation in the absence of antigen.

**Key words:** $\gamma^9\delta^2$ T cells, IL-15, cytokine receptors, Isopentenyl pyrophosphate.

**Materials and Methods**

**FACS staining and sorting**

PBMC were isolated from heparinized blood of healthy donors by ficoll-hypaque (Pharmacia Biotech, Uppsala, Sweden). PBMC were incubated with the following antibodies in different combinations: anti-$\delta^2$-FITC (Coulter, Miami, FL), anti-CD27-PE (BD PharMingen, San Diego, CA), anti-CD45RA-PE-Cy5 (Coulter), anti-CD45RO-PE-Cy5 (Coulter), anti-CD3-PE (Sigma, St. Louis, MO), and unconjugated anti-$\gamma^9$ (BD PharMingen), anti-CD69 (BD PharMingen), anti-CD25 (BD PharMingen), anti-CD122 (IL-2/IL-15R; BD PharMingen), anti-CD132 (BD PharMingen), anti-IL-7R (R&D, Minneapolis, MN), anti-IL-15R (R&D). Bcl-2 expression was assessed by anti-Bcl-2 mAb (BD PharMingen) after fixation with paraformaldehyde and permeabilization with saponin. Data were acquired on a FACSCalibur (BD Biosciences) and analyzed using CellQuest software (BD Immunocytometry Systems, San Jose, CA).

$\gamma^9\delta^2$ T cells were isolated by positive selection with magnetic beads (MACS; Miltenyi, Bergisch Gladbach, Germany). The cells obtained were more than 98% $\gamma^9\delta^2$ T cells. T naive, TCM, TEM and TEMRA. $\gamma^9\delta^2$ T lymphocytes were then purified to more than 99% by cell sorting, using anti-CD45RA and anti-CD27 antibodies. Cell sorting was performed on a FACSVantage (BD Biosciences).
Cell culture

The medium used was complete RPMI 1640 medium (Gibco, Grand Island, NY) supplemented with 10% heat-inactivated FCS (Gibco), 2 mM L-glutamine, 20 nM Hepes and 100 U/mL penicillin/streptomycin. T naive, T CM, T EM and T EMRA γδ T lymphocytes, sorted as described above, were labelled with CFSE and cultured for 7 days at 37°C, in the presence of 5% CO₂, at 10⁶ cells/mL in 96-well flat-bottom plates (0.2 mL/well), with different concentrations of IPP (Sigma) and 20 U/mL human recombinant IL-2, added at the 3rd day of culture. Alternatively, sorted Vγ9Vδ2 sub-sets were cultured with recombinant IL-2 (20 U/mL, final concentration), IL-7 or IL-15 (both used at 25 ng/mL final concentration).

Infection of macrophages with Mtb

The myelomonocytic THP-1 target cells (4×10⁴ cells/well) were incubated with phorbol 12-myristate 13-acetate (PMA; Sigma) at a final concentration of 10 ng/mL in 96-well round-bottom plates for 24 h at 37°C in the presence of 5% CO₂. Nonadherent cells were removed and the macrophages were infected with Mtb strain H37Ra overnight at a multiplicity of infection of 10:1. After extensive washing, macrophages were detached and the efficiency of infection was determined by staining a sample portion with auramine-rhodamine acid-fast staining. Approximately 85% of the cells were infected with Mtb, with an average of three bacteria per cell.

Cytokine production and cytotoxicity assay

Mtb-infected macrophages (10⁴) or Daudi lymphoma cells (10⁴) were incubated for 5 h at 37°C in 96-well round-bottom plates with Vγ9Vδ2 γδ T cells at E:T ratios of 30:1, 10:1 and 1:1. Assays were performed in triplicate for each E:T ratio. Cytotoxicity was analyzed using a nonradioactive colorimetric cytotoxicity assay (CytoTox 96; Promega).

IFN γ levels in the 24-h culture supernatants were
assessed by two-mAb sandwich ELISA assay following the manufacturer’s recommendations (R&D Systems).

**Results and Discussion**

We studied the proliferation and differentiation of Vγ9Vδ2 T cell subsets in response to antigen or homeostatic cytokines. Purified T naïve, T CM, T EM and T EMRA subsets of Vγ9Vδ2 T lymphocytes were labelled with CFSE and compared for their capacity to proliferate in response to phosphoantigen or to homeostatic cytokines. We recorded that T naïve and T CM expanded consistently upon antigenic stimulation provided by IPP + IL-2, while T EM cells and especially T EMRA cells performed few divisions (if any at all) and were recovered in lower numbers (data not shown). Although T EM and particularly T EMRA Vγ9Vδ2 cells proliferate weakly, antigenic stimulation elicits effector responses such as IFNγ production and cytotoxicity, respectively.

Proliferation in response to IL-15 was low in T naïve cells, intermediate in T CM cells, and high in T EM and T EMRA cells (Figure 1A).

According with their high cytokine responsiveness, T CM and T EM cells were got back in higher numbers after stimulation with IL-15, as compared to naive T cells. Cytokine responsiveness correlated with the expression of the relevant cytokine receptors (Figure 1B). Thus, whereas IL-7R chain expression was high on T naïve and T CM cells and slightly decreased in T EM and T EMRA cells (data not shown), IL-15R and IL-2R/15R chain expression were low on T naïve cells, intermediate on T CM cells, and high on T EM and T EMRA cells.

In contrast, T EMRA cells cannot accumulate, despite the fact that they expressed high levels of IL-15R, and most of them underwent cell division. The inability of cytokine-stimulated T EMRA cells to accumulate was linked with a high amount of cell death and low Bcl-2 expression, letting us think that the intrinsic high propensity to cell death compromises the accumulation of T EMRA cells in response to homeostatic cytokines, despite their high cytokine responsiveness (data not shown).

Altogether, these results show that whereas antigen-dependent expansion, cell viability and Bcl-2 expression are progressively lost from T naïve and T CM to T EM and T EMRA cells, IL-15R expression and cytokine responsiveness have a reciprocal pattern and are progressively acquired with differentiation.2–13

To evaluate the potential differentiation of Vγ9Vδ2 T lymphocytes, sorted T naïve, T CM, T EM and T EMRA cells were

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Figure 2. Differentiation of central memory Vγ9Vδ2 T cell subset following IL-15 stimulation.
labelled with CFSE and stimulated with either phosphoantigen or IL-15. On day 7, CD27 and CD45RA expression were analyzed on cells that had performed the same number of divisions. Antigenic stimulation of T naive cells resulted in the generation of T CM cells, while antigenic stimulation of T EM and T EMRA cells induces the generation of a homogeneous population of the latter cells. Antigen-stimulated T EMRA cells maintained their phenotype, but their number consistently decreased over the 7-day culture period. In no case did antigen stimulation of T naive, T EM and T EMRA cells give rise to T EMRA cells (data not shown).

In contrast, whereas T naive, T EM and T EMRA cells proliferating in response to IL-15 largely maintained their phenotype, T EM cells gave rise to T CM cells and to T EM and T EMRA cells. Thus, some cytokine-stimulated T CM cells maintained their phenotype, whereas others generated cells with a T EM or a T EMRA phenotype, as reported in Figure 2.

Altogether, these results suggest that cytokine-stimulated T CM cells, in the absence of antigen, can self-renew and generate different types of effector cells, including T EMRA cells.

Vγ9Vδ2 T EMRA cells generated by IL-15 stimulated T CM cells exert potent cytotoxicity against Daudi cells

and Mtb-infected macrophages (Figure 3) and are also able to consistently reduce the viability of intracellular Mtb (data not shown). Our results indicate that Vγ9Vδ2 T lymphocyte subsets have different proliferative capacities in vitro and that T CM cells have the unique ability to differentiate in an antigen-independent fashion into T EM and T EMRA Vγ9Vδ2 T lymphocytes.

Future studies will now aim at elucidating the conditions that favour the selective generation of human Vγ9Vδ2 T EMRA lymphocytes, due to the need of generating cytotoxic effectors for anticancer immunotherapy purposes.

References


Figure 3. IFN-γ production (A) and cytotoxicity (B) of Vγ9Vδ2 T CM and T EMRA cells generated by IL-15-stimulated T CM cells.