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Human memory Vγ9Vδ2 T cells require homeostatic cytokines for proliferation and differentiation

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A B S T R A C T

We have analysed four subsets of human Vγ9Vδ2 T cells for their capacity to proliferate and differentiate in response to antigen or homeostatic cytokines. Antigen-stimulated cells acquired a central memory (T_{CM}) or effector memory (T_{EM}) phenotype, while IL-15-stimulated cells maintained their phenotype, with the exception of T_{CM} cells, which expressed CD27 and CD45RA in various combinations. These results show that human Vγ9Vδ2 memory T cells have different proliferation and differentiation potentials and that terminally differentiated (T_{EMRA}) cells result from the T_{CM} subset upon homeostatic proliferation in the absence of antigen.

Key words: Vγ9Vδ2 T cells, IL-15, cytokine receptors, Isopentenyl pyrophosphate.

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Vγ9Vδ2 T lymphocytes, similar to CD4 and CD8 T cells, are heterogeneous and comprise different populations that can be distinguished on the basis of surface marker expression and effector functions, such as cytokine secretion and cytotoxicity.

Naive (T_{naive}) $CD45RA^+CD27^+$ and central memory (T_{CM}) $CD45RA^-CD27^+$ cells express lymph node homing receptors, abound in lymph nodes and lack immediate effector functions. Conversely, effector memory (T_{EM}) $CD45RA^-CD27^-$ and terminally differentiated (T_{EMRA}) $CD45RA^+CD27^-$ cells express receptors for migration to inflamed tissues, are poorly represented in the lymph nodes while abounding at sites of inflammation, where they display immediate effector functions (cytokine production and cytotoxicity, respectively).[1]

Little is known about homeostasis of human Vγ9Vδ2 T lymphocytes and how the functional heterogeneity of the memory pool is maintained.[2-4] There is growing evidence that cytokines that bind to receptors containing the common γ chain are involved in T cell maintenance and homeostasis. Especially, IL-15, secreted by several cell types as dendritic cells (DC), plays an essential role in T cell homeostasis as demonstrated by Lanzavecchia *et al.* for human CD8 T cells.[5-8]

We considered the possibility that the phenotype and function of distinct subsets of Vγ9Vδ2 T lymphocytes may be influenced by homeostatic mechanisms.

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-Vδ2-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-Vγ9 (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).

Vγ9Vδ2 T cells were isolated by positive selection with magnetic beads (MACS; Miltenyi, Bergisch Gladbach, Germany). The cells obtained were more than 98% $V\gamma9V\delta2^+$ $CD3^+$. T_{naive}, T_{CM}, T_{EM} and T_{EMRA} $V\gamma9V\delta2^+$ 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).

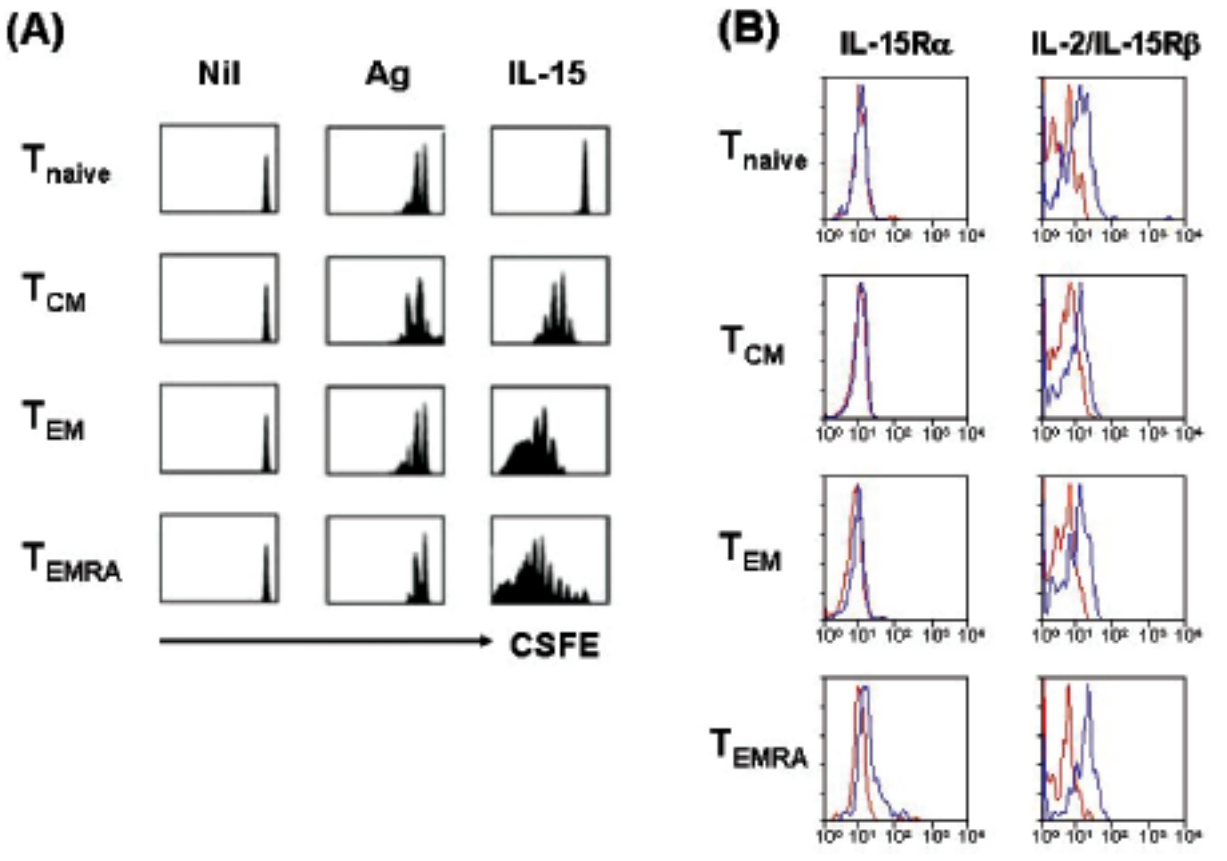

Figure 1. Proliferation of different subsets of Vγ9Vδ2 T cells following antigen or IL 15 stimulation and their cytokine receptors expression. (A) T_{naive}, T_{CM}, T_{EM} and T_{EMRA} subsets were isolated from peripheral blood, labelled with CFSE and compared for their capacity to proliferate in response to antigen (phosphoantigen, IPP) or IL-15. Cell division was measured after 7 days. One representative experiment out of five is shown. (B) The subsets of T cells were isolated from peripheral blood and stained with antibodies to the indicated cytokine receptors (blue lines). The red lines indicate staining with isotype-matched control antibodies. One experiment out of seven is shown.

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} Vγ9Vδ2$^+$ T lymphocytes, sorted as described above, were labelled with CFSE and cultured for 7 days at 37°C, in the presence of 5% CO_2, at 10^6 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.[2] Alternatively, sorted Vγ9Vδ2 subsets 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 (4×10^3 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% CO_2. 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 (10^4) or Daudi lymphoma cells (10^4) were incubated for 5 h at 37°C in 96-well round-bottom plates with Vγ9Vδ2$^+$ 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

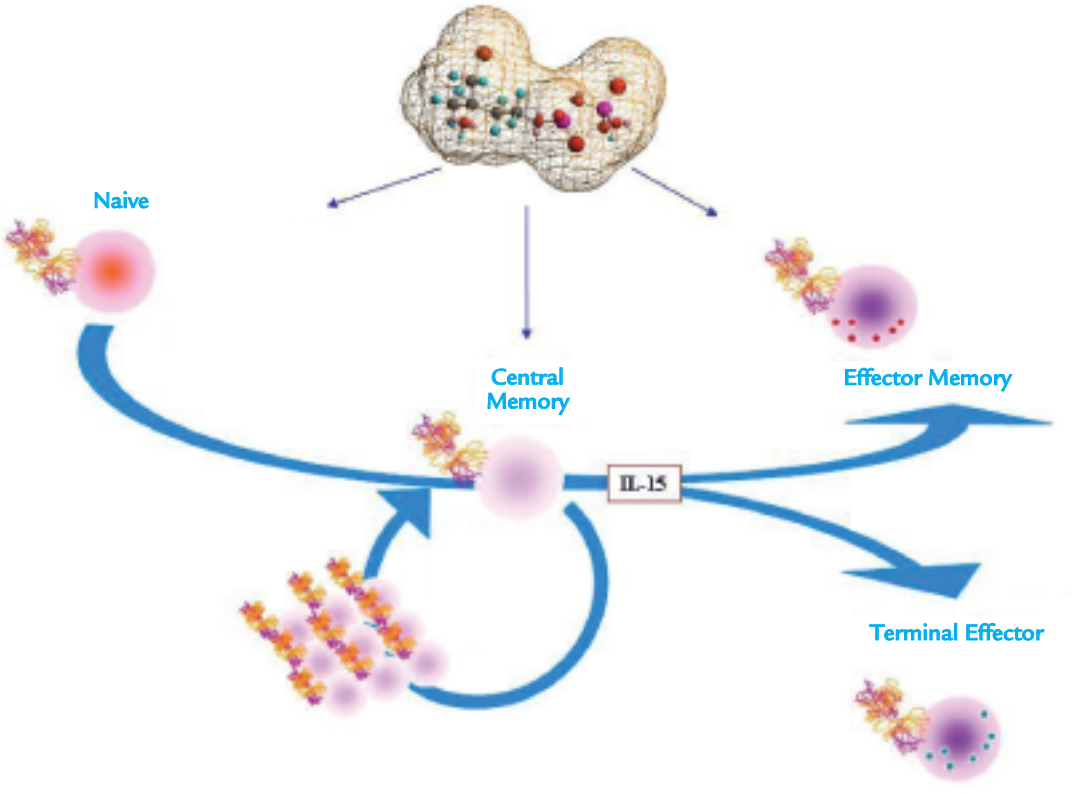

Figure 2. Differentiation of central memory Vγ9Vδ2 T cell subset following IL-15 stimulation.

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_{naive}, 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_{naive} 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_{naive} and T_{CM} cells and slightly decreased in T_{EM} and T_{EMRA} cells (*data not shown*), IL-15R and IL-2/15R chain expression were low on T_{naive} 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 we 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_{naive} 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.[9-13]

To evaluate the potential differentiation of Vγ9Vδ2 T lymphocytes, sorted T_{naive}, T_{CM}, T_{EM} and T_{EMRA} cells were

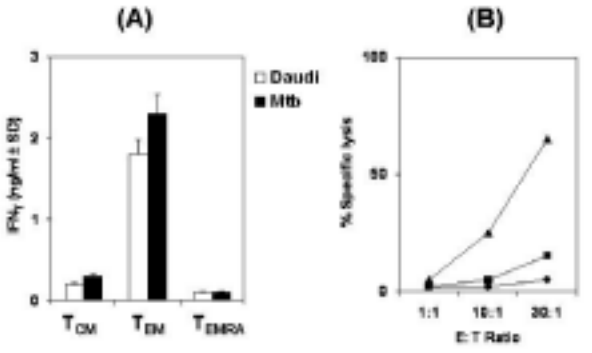

Figure 3. IFN-γ production (A) and cytotoxicity (B) of Vγ9Vδ2 T_{EM} and T_{EMRA} cells generated by IL-15-stimulated T_{CM} cells.

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_{CM} and T_{EM} 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_{CM} and T_{EM} 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_{CM} 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[14] and Mtb-infected macrophages (Figure 3) and are also able to consistently reduce the viability of intracellular Mtb (*data not shown*).[15]

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.

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