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Cutting Edge: CD1d Restriction and Th1/Th2/Th17 Cytokine Secretion by Human V δ 3 T Cells

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Human $\gamma\delta$ T cells expressing the V δ 3 TCR make up a minor lymphocyte subset in blood but are enriched in liver and in patients with some chronic viral infections and leukemias. We analyzed the frequencies, phenotypes, restriction elements, and functions of fresh and expanded peripheral blood V δ 3 T cells. V δ 3 T cells accounted for ~0.2% of circulating T cells, included CD4⁺, CD8⁺, and CD4[−]CD8[−] subsets, and variably expressed CD56, CD161, HLA-DR, and NKG2D but neither NKG2A nor NKG2C. V δ 3 T cells were sorted and expanded by mitogen stimulation in the presence of IL-2. Expanded V δ 3 T cells recognized CD1d but not CD1a, CD1b, or CD1c. Upon activation, they killed CD1d⁺ target cells, released Th1, Th2, and Th17 cytokines, and induced maturation of dendritic cells into APCs. Thus, V δ 3 T cells are glycolipid-reactive T cells with distinct Ag specificities but functional similarities to NKT cells. *The Journal of Immunology*, 2013, 191: 30–34.

In addition to conventional MHC-restricted T cells, a number of innate T cell populations that recognize nonpeptide Ags in an MHC-unrestricted manner have been described in mice and humans. Invariant NKT (iNKT) cells express a TCR composed of an invariant α -chain (V α 24J α 18 in humans and V α 14J α 18 in mice) that pairs with a limited number of β -chains and recognize glycolipid Ags presented by the MHC class I-like molecule CD1d (1, 2). Mucosal-associated invariant T (MAIT) cells express an invariant V α 7.2-J α 33 TCR in humans (V α 19-J α 33 in mice) and recognize microbial vitamin B metabolites presented by MR1 (3). Recently, a second CD1d-restricted T cell population, invariant V α 10-J α 50 TCR α -chains with a distinct glycolipid Ag specificity, was described in mice (4). The most abundant innate T cells in humans are $\gamma\delta$ T cells, of which

there are two major subsets. V γ 9V δ 2 T cells recognize pyrophosphate intermediates of isoprenoid synthesis in certain bacteria (5), and V δ 1 T cells can be activated by CD1c, CD1d, or the stress-inducible molecule MICA/B expressed by virus-infected and tumor cells (6–8).

Innate T cells can respond to ligand stimulation by rapidly and potently killing target cells, by releasing cytokines that polarize adaptive immune responses, and by transactivating NK cells, dendritic cells (DCs), and B cells (1–3, 5, 9–12). iNKT cells can prevent disease in animal models (1, 2). Human iNKT cells and V γ 9V δ 2 T cells display potent antitumor activity in vitro and are currently being tested as adjuvants for cellular immunotherapies in clinical trials for cancer (13, 14).

The majority of non-V δ 1 and non-V γ 9V δ 2 $\gamma\delta$ T cells in humans express the V δ 3 TCR chain. The ligand specificities of V δ 3 T cells are unknown, but these cells are reported to be expanded in peripheral blood of renal and stem cell transplant recipients with CMV activation (15–17), in patients with HIV infection (18) or B cell chronic lymphocytic leukemia (19), and in healthy livers (20). In this study, we enumerated and phenotyped V δ 3 T cells from human peripheral blood and developed a method for their expansion *ex vivo*. We show that V δ 3 T cells include cells that recognize CD1d and respond by killing CD1d⁺ target cells, releasing Th1, Th2, and Th17 cytokines and promoting maturation of DC into APCs. Thus, V δ 3 T cells include CD1d-restricted T cells with functional similarities, but distinct Ag specificities, to those of iNKT cells, properties that place V δ 3 T cells as candidate targets for therapeutic immunomodulation.

Materials and Methods

Enumeration and phenotyping of V δ 3 T cells

PBMCs were isolated from healthy donors. V δ 3 T cells were enumerated and phenotyped by staining PBMCs with an anti-V δ 3 TCR mAb (clone P11.5B; Beckman Coulter) and mAbs specific for CD3, CD3, CD4, CD8, CD25, CD28, CD56, CD69, CD161, HLA-DR, NKG2A, NKG2C, and NKG2D, as well as the V α 24J α 18 TCR expressed by iNKT cells. Cells were acquired

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Abbreviations used in this article: DC, dendritic cell; DN, double negative for CD4 and CD8; α -GC, α -galactosylceramide; iNKT, invariant NKT; MAIT, mucosal-associated invariant T.

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using a Cyan flow cytometer (Beckman Coulter) and analyzed using FlowJo (TreeStar) using fluorescence-minus-one controls.

Generation of V δ 3 T cell and iNKT cell lines

V δ 3 T cells were enriched from PBMCs by sorting of V δ 3⁺CD3⁺ cells using a MoFlo XDP Cell Sorter (Beckman Coulter). A total of 1000 V δ 3 T cells was cultured in RPMI 1640 medium containing 0.05 mM L-glutamine, 10% v/v HyClone FBS, 0.02 M HEPES buffer, 100 U/ml penicillin, 100 μ g/ml streptomycin, and 2.5 μ g/ml amphotericin B Fungizone. Cells were stimulated with 1 μ g/ml PHA-P and 250 U/ml IL-2 in the presence of excess (2×10^5) irradiated allogeneic PBMCs, prepared from two donors. After 24 and 48 h, medium was replaced with fresh medium containing 250 U/ml IL-2. V δ 3 cells were expanded for 2–4 wk in the presence of IL-2. Purity and phenotype of V δ 3 T cell lines were assessed by flow cytometry. iNKT cell lines were generated and characterized as described previously (21).

Analysis of CD1 recognition and effector function of V δ 3 T cell lines

Expanded V δ 3 T cells or iNKT cells were cocultured with equal numbers of mock-transfected HeLa cells or HeLa cell-expressing transfected CD1a, CD1b, CD1c, or CD1d in the absence or presence of the iNKT cell agonist glycolipid α -galactosylceramide (α -GC; 100 ng/ml), PMA (1 ng/ml), and blocking mAbs against CD1d and V δ 3 (clones 42.1 and P11.5B) or isotype control Ab (10 μ g/ml). As positive controls, cells were stimulated with 10 ng/ml PMA and 1 μ g/ml ionomycin. V δ 3 T cell activation was measured by flow cytometric analysis of cell surface expression of the marker of cytolytic degranulation, CD107a, and intracellular production of cytokines, as described (21). Levels of cytokines released into cell supernatants were measured by ELISA.

Analysis of adjuvant effects of V δ 3 T cell lines for DCs

Monocyte-derived DCs were generated by culturing magnetic bead-enriched CD14⁺ monocytes for 6 d with GM-CSF and IL-4 (11). Immature DCs were then cultured with equal numbers of expanded V δ 3 T cells or 1 μ g/ml LPS in the absence or presence of blocking mAbs against CD1d, V δ 3, CD40, or CD40L or isotype-control Ab (10 μ g/ml). The expression by the DCs of molecules commonly found on APCs (CD40, CD54, CD80, CD83, CCR7, and HLA-DR) was analyzed after 3 d by flow cytometry. Cytokine release (IL-10 and IL-12) was measured by ELISA. The capacity of DCs, cultured for 3 d in the absence or presence of V δ 3 T cells or LPS, to drive proliferation of naive alloreactive T cells was determined by labeling the T cells with CellTrace Violet (Invitrogen) and analyzing dye dilution after 6 d by flow cytometry.

Statistical analysis

Groups were compared using the Mann–Whitney U test or Student *t* test, as appropriate. The *p* values < 0.05 were considered statistically significant.

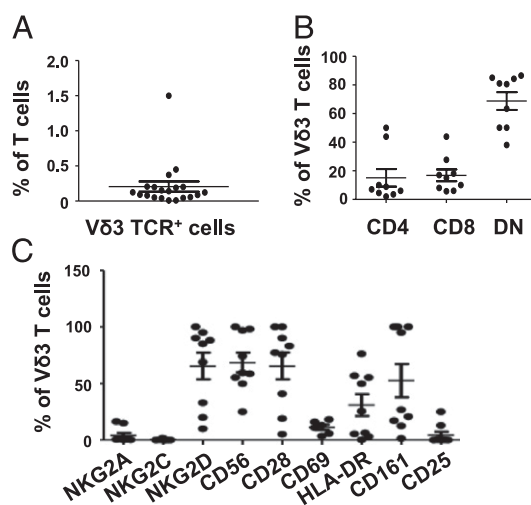


FIGURE 1. Frequencies and phenotypes of human V δ 3 T cells. (A) Scatterplot showing percentages of freshly isolated circulating CD3⁺ cells from 20 healthy donors that express the V δ 3 TCR chain, as detected by flow cytometry. (B) Percentages of circulating V δ 3 T cells from nine healthy donors that express CD4, CD8, or neither (DN). (C) Percentages of circulating V δ 3 T cells from nine healthy donors that express NKG2A, NKG2C, NKG2D, CD56, CD28, CD69, HLA-DR, CD161, and CD25. Horizontal lines represent the means.

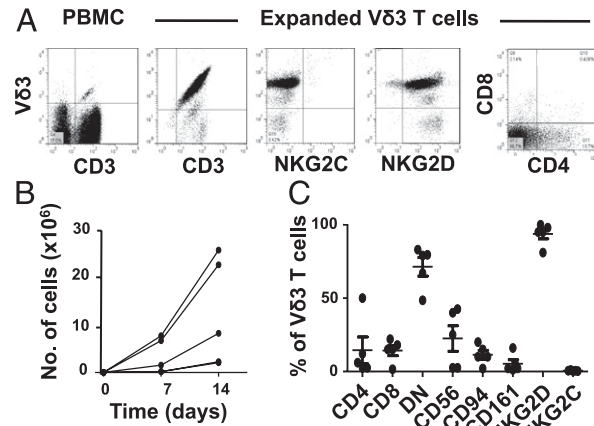


FIGURE 2. Ex vivo expansion of human V δ 3 T cells. (A) Flow cytometric dot plots showing the expression of CD3 and the V δ 3 TCR by fresh PBMCs (left panel) and by sorted and expanded V δ 3 T cells (second panel) and the expression of NKG2C (third panel), NKG2D (fourth panel), and CD4 and CD8 (right panel) by expanded V δ 3 T cells. (B) Kinetics of mitogen-stimulated V δ 3 T cell expansion starting with 1000 sorted V δ 3 T cells. (C) Percentages of V δ 3 T cells expanded from five healthy donors that express CD4, CD8, neither CD4 nor CD8 (DN), CD56, CD94, CD161, NKG2D, or NKG2C. Horizontal lines represent the means.

Results and Discussion

Frequency and phenotype of fresh V δ 3 T cells

Flow cytometric analysis of CD3 and V δ 3 TCR expression by PBMCs from 20 healthy donors revealed that V δ 3 T cells account for $0.2 \pm 0.3\%$ (mean $\pm$ SEM) of peripheral T cells (Fig. 1A). This compares with $\sim 0.05\%$ for human iNKT cells (2, 21), $\sim 3\%$ for V γ 9V δ 2 T cells (5, 11), and up to 10% for MAIT cells (3) in blood. Phenotypic analysis showed that fresh V δ 3 T cells can express CD4 or CD8, but the majority ($69 \pm 19\%$) were double negative for CD4 and CD8 (DN)

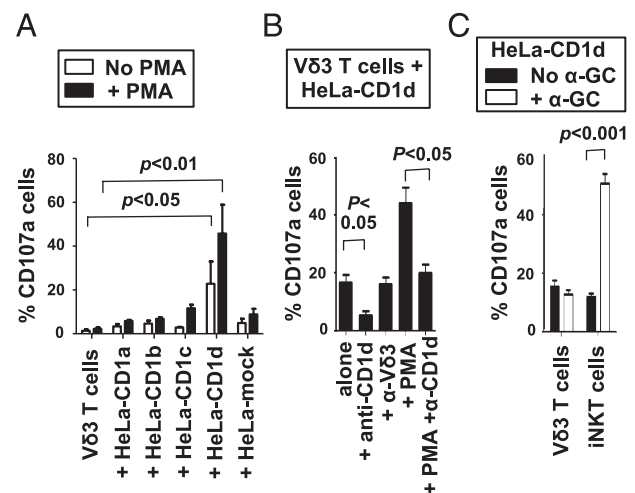


FIGURE 3. V δ 3 T cells recognize CD1d. (A) Mean percentages of V δ 3 T cells expanded from five donors that externalize CD107a after culturing for 4 h in medium alone, with HeLa cells expressing CD1a, CD1b, CD1c, or CD1d, or with mock-transfected HeLa cells in the absence and presence of PMA. (B) Mean percentages of expanded V δ 3 T cells from three donors that degranulate in response to HeLa-CD1d in the absence and presence of PMA and mAbs specific for CD1d (α -CD1d) or V δ 3 (α -V δ 3). (C) Mean percentages of V δ 3 T cells or iNKT cells from five donors that degranulate in response to HeLa-CD1d cells in the absence and presence of α -GC. Error bars represent SEM.

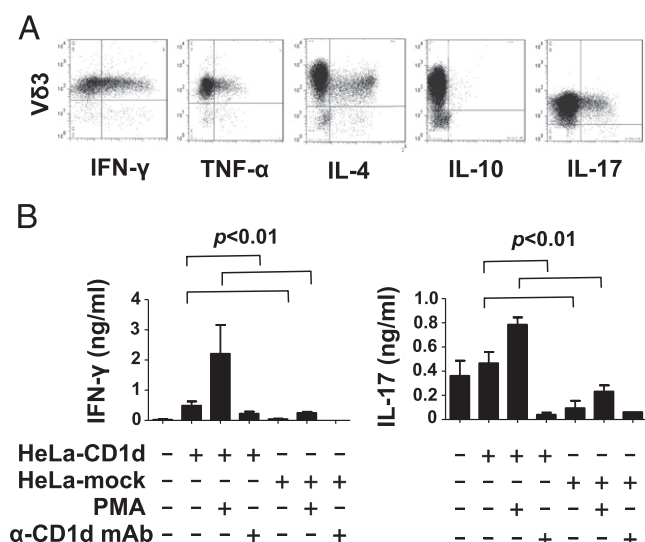


FIGURE 4. V δ 3 T cells produce multiple cytokines upon stimulation with PMA and ionomycin or CD1d⁺ cells. **(A)** Flow cytometric dot plots showing expression of IFN- γ , TNF- α , IL-4, IL-10, or IL-17 by expanded V δ 3 T cell lines after stimulation for 4 h with PMA and ionomycin. Results are representative of data using expanded V δ 3 T cells from six donors. **(B)** Mean levels of IFN- γ and IL-17 released by expanded V δ 3 T cells from four donors after stimulation for 24 h with mock-transfected HeLa cells or HeLa-CD1d cells in the absence or presence of PMA and blocking anti-CD1d mAb. No blocking was seen when isotype-matched control Ab was used (data not shown). Error bars represent SEM.

(Fig. 1B). Interestingly, CD4⁺, CD8⁺, and DN cell subsets are also found within iNKT cells, V γ 9V δ 2 T cells, and MAIT cells, and they have distinct functional activities (3, 11, 21). Like other innate T cells, most fresh V δ 3 T cells expressed the NK cell-associated receptors NKG2D, CD56, and CD161 but not NKG2A or NKG2C. Most displayed phenotypes of resting T cells, being CD28⁺ and CD25⁻CD69⁻, whereas HLA-DR was variably expressed (Fig. 1C). Therefore, human V δ 3 T cells are similar to other innate T cell populations in that they display phenotypic heterogeneity with regard to their expression of NK cell-associated receptors and CD4 and CD8.

Expansion of V δ 3 T cells in vitro

Because of their low frequencies in peripheral blood, V δ 3 T cells need to be expanded ex vivo to obtain sufficient

numbers for functional studies or for clinical use. We tried a number of T cell-expansion protocols and found that a single stimulation of sorted V δ 3 T cells with PHA in the presence of irradiated feeder cells, followed by culturing with IL-2, was optimal. This method yielded up to 25 million V δ 3 T cells from as few as 1000 cells in 14 d and with purities >95% (Fig. 2A, 2B). Phenotypic analysis of V δ 3 T cell lines from five donors showed that the CD4/CD8/DN distributions of expanded V δ 3 T cells were similar to those of fresh V δ 3 T cells. Expanded V δ 3 T cells also retained expression of NKG2D, were negative for NKG2C, and had lower frequencies of CD56 and CD161 expression than did fresh V δ 3 T cells (Fig. 2A, 2C). Thus, highly pure V δ 3 T cells can be readily expanded by mitogen stimulation in vitro.

V δ 3 T cells recognize CD1d

A significant fraction of human T cells, including $\gamma\delta$ T cells, recognize autoantigens presented by CD1a, CD1b, CD1c, or CD1d (4, 6–8, 22–24). We investigated whether expanded V δ 3 T cells could recognize and kill target cells expressing CD1 isotypes by coculturing them with mock-transfected HeLa cells or HeLa cells expressing transfected CD1a, CD1b, CD1c, or CD1d and measuring the expression of the degranulation marker CD107a. In the absence of added glycolipid, V δ 3 T cells degranulated in response to HeLa cells expressing CD1d but not CD1a, CD1b, or CD1c (Fig. 3A). The requirement for CD1d was confirmed in blocking experiments in which anti-CD1d mAb abrogated degranulation in response to CD1d alone but not CD1d + PMA (Fig. 3B). In contrast, treatment with an anti-V δ 3 mAb did not prevent V δ 3 T cell activation. Future studies are required to determine whether this mAb can block or stimulate V δ 3 T cell activation.

Many CD1d-restricted T cells do not express the V α 24J α 18 TCR found on iNKT cells or recognize the CD1d-binding glycolipid α -GC (4, 8, 24) and are termed type 2 NKT cells. We analyzed the coexpression of the V δ 3 and V α 24J α 18 TCR chains by PBMCs, expanded V δ 3 T cells, or expanded iNKT cells and found that these TCR chains were never coexpressed by the same cells (data not shown), indicating that V δ 3 T cells are not iNKT cells. V δ 3 T cell responses to CD1d were not augmented by adding α -GC, as was seen when iNKT cells were used (Fig. 3C), indicating that V δ 3 and iNKT cells have distinct Ag specificities. Thus, some (if

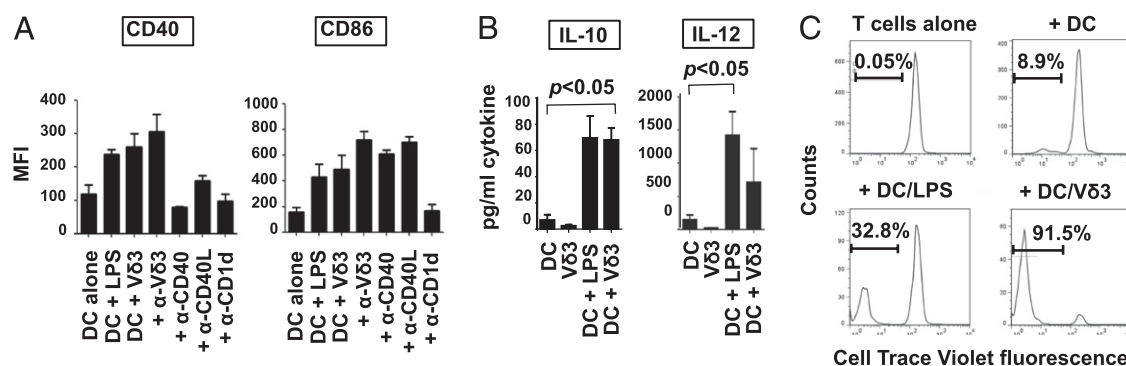


FIGURE 5. V δ 3 T cells induce DC maturation. **(A)** Mean fluorescence intensities (MFI) of expression of CD40 and CD86 by monocyte-derived DCs after culturing them for 3 d in medium alone, with LPS, or with equal numbers of V δ 3 T cells in the absence and presence of blocking mAbs against CD1d, V δ 3, CD40, or CD40L. Results are means of five different DC-V δ 3 T cell combinations. Error bars represent SEM. **(B)** Levels of IL-10 and IL-12 released by DCs or V δ 3 T cells cultured alone for 2 d, DCs cultured with LPS, and DCs cultured with V δ 3 T cells. Results are means of three experiments using different DCs and V δ 3 T cells. **(C)** Proliferation of naive allogeneic T cells in response to medium alone, immature DCs, or DCs matured for 24 h with LPS or V δ 3 T cells. T cells were labeled with CellTrace Violet before adding to the DCs. Results show representative flow cytometry graphs (from four experiments) showing CellTrace dye dilution after 6 d.

not all) V δ 3 T cells fit the definition of type 2 NKT cells. CD1d-restricted activation by other glycolipids, including sulphatide and cardiolipin, was reported for human V δ 1 T cells (7, 8) and murine $\gamma\delta$ T cells (25).

Cytokine production by V δ 3 T cells

A notable feature of innate T cells is their capacity to rapidly secrete large amounts of Th cell–polarizing cytokines that can skew adaptive immune responses. iNKT cells can secrete Th1, Th2, Th17, and regulatory T cell cytokines, sometimes simultaneously (1, 2, 21), whereas V γ 9V δ 2 T cells most readily produce Th1 cytokines but can be induced, under certain conditions, to produce Th2 and Th17 cytokines (5, 11), and MAIT cells can produce Th1 and Th17 cytokines (3). We examined intracellular production of IFN- γ , TNF- α , IL-4, IL-10, IL-13, and IL-17 by expanded V δ 3 T cells stimulated with HeLa cells expressing CD1d or PMA and ionomycin. Fig. 4A shows that some V δ 3 T cells produced IFN- γ , TNF- α , IL-4, or IL-17, but not IL-10, indicating that like iNKT cells, they can promote Th1, Th2, and Th17 responses. CD1d-dependent release of IFN- γ and IL-17 by V δ 3 T cells into cell supernatants was also shown by ELISA (Fig. 4B) and found to be blocked by treatment with anti-CD1d, but not isotype-control, Ab. Thus, like other innate T cells, V δ 3 T cells can regulate adaptive immune responses via production of multiple Th-polarizing cytokines.

DC maturation by V δ 3 T cells

iNKT cells and V γ 9V δ 2 T cells can drive the differentiation of immature DCs into APCs (1, 9, 11), and this property led to their testing as adjuvants in DC-based immunotherapies (13, 14). We tested whether V δ 3 T cells can similarly induce DC maturation in vitro. Immature monocyte-derived DCs were cocultured for 3 d alone or with equal numbers of expanded V δ 3 T cells from four donors or LPS, and the expression of the APC markers CD40, CD54, CD80, CD83, CCR7, and HLA-DR by DCs was analyzed by flow cytometry. We found that V δ 3 T cells upregulated CD40, CD83, CD86, and HLA-DR expression by DCs to levels comparable to LPS-stimulated DCs. CD80, CCR7, and CD54 were not upregulated (Fig. 5A shows data for CD40 and CD86). Inclusion of blocking mAbs showed that V δ 3 T cell–mediated DC maturation required CD1d but not CD40–CD40L interactions. V δ 3 T cells also induced IL-10 and IL-12 production by DCs (Fig. 5B), and V δ 3 T cell–matured DCs induced increased proliferation of naive alloreactive T cells compared with immature DCs (Fig. 5C). These findings argue that some (if not all) V δ 3 T cells promote maturation of DCs into APCs capable of activating naive T cells.

Concluding remarks

We identified V δ 3 TCR $^+$ T cells as a novel population of human CD1d-restricted T cells whose glycolipid specificities are distinct from those of iNKT cells. Like iNKT cells, activated V δ 3 T cells kill CD1d $^+$ cells, release Th1, Th2, and Th17 cytokines, and promote maturation of DCs into APCs. Future studies are required to find out whether, like iNKT cells, V δ 3 T cells can be targeted for tumor immunotherapy; however, two studies (16, 26) showed that they can kill tumor intestinal epithelial cells but not healthy epithelial cells. Because CD4 $^+$, CD8 $^+$, and DN subsets of iNKT cells have distinct cytokine profiles (21), it is likely that a functional comparison

of CD4 $^+$, CD8 $^+$, and DN V δ 3 T cell subsets will be required to identify the optimal antitumor cells.

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Disclosures

The authors have no financial conflicts of interest.

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