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Cutting Edge: CD1d Restriction and Th1/Th2/Th17 Cytokine Secretion by Human Vδ3 T Cells

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Human γδ T cells expressing the Vδ3 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 Vδ3 T cells. Vδ3 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. Vδ3 T cells were sorted and expanded by mitogen stimulation in the presence of IL-2. Expanded Vδ3 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, Vδ3 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 (Vα24Jα18 in humans and Vα14Jα18 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 Vα7.2-Jα33 TCR in humans (Vα19-Jα33 in mice) and recognize microbial vitamin B metabolites presented by MR1 (3). Recently, a second CD1d-restricted T cell population, invariant Vα10-Jα50 TCR α-chains with a distinct glycolipid Ag specificity, was described in mice (4). The most abundant innate T cells in humans are γδ T cells, of which there are two major subsets. Vγ9Vδ2 T cells recognize pyrophosphate intermediates of isoprenoid synthesis in certain bacteria (5), and Vδ1 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 Vγ9Vδ2 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-Vδ1 and non-Vγ9Vδ2 γδ T cells in humans express the Vδ3 TCR chain. The ligand specificities of Vδ3 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 Vδ3 T cells from human peripheral blood and developed a method for their expansion ex vivo. We show that Vδ3 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, Vδ3 T cells include CD1d-restricted T cells with functional similarities, but distinct Ag specificities, to those of iNKT cells, properties that place Vδ3 T cells as candidate targets for therapeutic immunomodulation.

Materials and Methods

Enumeration and phenotyping of Vδ3 T cells

PBMCs were isolated from healthy donors. Vδ3 T cells were enumerated and phenotyped by staining PBMCs with an anti-Vδ3 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 Vα24Jα18 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 Vδ3 T cell and iNKT cell lines

Vδ3 T cells were enriched from PBMCs by sorting of Vδ3$^+$CD3$^+$ cells using a MoFlo XDP Cell Sorter (Beckman Coulter). A total of 1000 Vδ3 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 μg/ml streptomycin, and 2.5 μg/ml amphotericin B Fungizone. Cells were stimulated with 1 μg/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. Vδ3 cells were expanded for 2–4 wk in the presence of IL-2. Purity and phenotype of Vδ3 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 Vδ3 T cell lines

Expanded Vδ3 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 Vδ3 (clones 42.1 and P11.5B) or isotype control Ab (10 μg/ml). As positive controls, cells were stimulated with 10 ng/ml PMA and 1 μg/ml ionomycin. Vδ3 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 Vδ3 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 Vδ3 T cells or 1 μg/ml LPS in the absence or presence of blocking mAbs against CD1d, Vδ3, CD40, or CD40L or isotype-control Ab (10 μg/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 Vδ3 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.

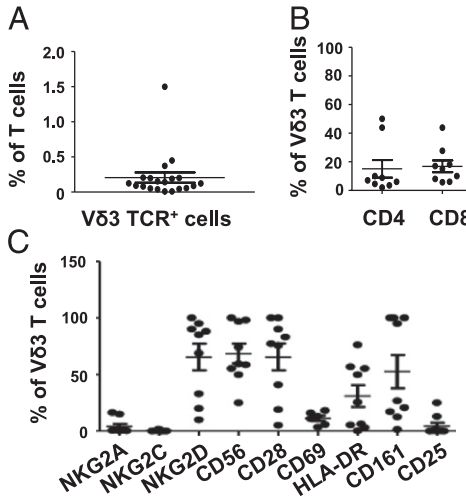

FIGURE 1. Frequencies and phenotypes of human Vδ3 T cells. (**A**) Scatterplot showing percentages of freshly isolated circulating CD3$^+$ cells from 20 healthy donors that express the Vδ3 TCR chain, as detected by flow cytometry. (**B**) Percentages of circulating Vδ3 T cells from nine healthy donors that express CD4, CD8, or neither (DN). (**C**) Percentages of circulating Vδ3 T cells from nine healthy donors that express NKG2A, NKG2C, NKG2D CD56, CD28, CD69, HLA-DR, CD161, and CD25. Horizontal lines represents the means.

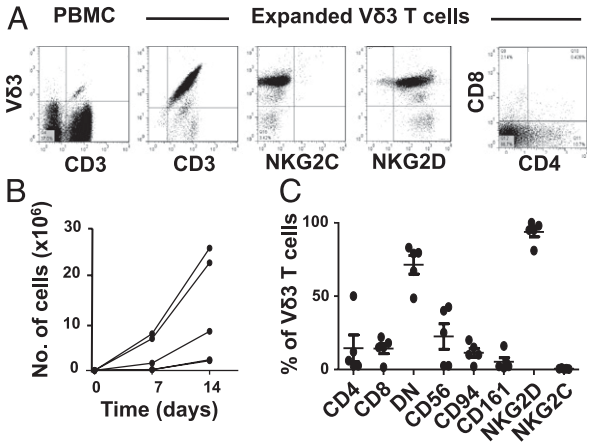

FIGURE 2. Ex vivo expansion of human Vδ3 T cells. (**A**) Flow cytometric dot plots showing the expression of CD3 and the Vδ3 TCR by fresh PBMCs (*left panel*) and by sorted and expanded Vδ3 T cells (*second panel*) and the expression of NKG2C (*third panel*), NKG2D (*fourth panel*), and CD4 and CD8 (*right panel*) by expanded Vδ3 T cells. (**B**) Kinetics of mitogen-stimulated Vδ3 T cell expansion starting with 1000 sorted Vδ3 T cells. (**C**) Percentages of Vδ3 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 Vδ3 T cells

Flow cytometric analysis of CD3 and Vδ3 TCR expression by PBMCs from 20 healthy donors revealed that Vδ3 T cells account for 0.2 ± 0.3% (mean ± SEM) of peripheral T cells (Fig. 1A). This compares with ~0.05% for human iNKT cells (2, 21), ~3% for Vγ9Vδ2 T cells (5, 11), and up to 10% for MAIT cells (3) in blood. Phenotypic analysis showed that fresh Vδ3 T cells can express CD4 or CD8, but the majority (69 ± 19%) were double negative for CD4 and CD8 (DN)

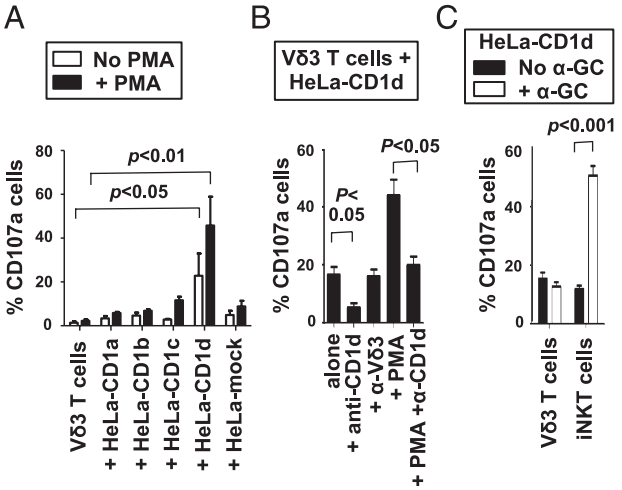

FIGURE 3. Vδ3 T cells recognize CD1d. (**A**) Mean percentages of Vδ3 T cells expanded from five donors that externalize CD107a after culturing for 4 h in medium alone, with HeLa cells expressing CD1a, CD1b, CD1c, or CD1d, or with mock-transfected HeLa cells in the absence and presence of PMA. (**B**) Mean percentages of expanded Vδ3 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 Vδ3 (α-Vδ3). (**C**) Mean percentages of Vδ3 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.

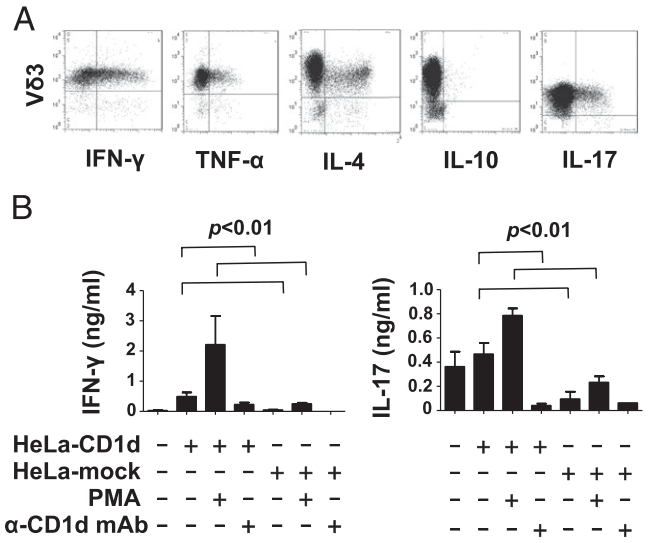

FIGURE 4. Vδ3 T cells produce multiple cytokines upon stimulation with PMA and ionomycin or CD1d$^+$ cells. (**A**) Flow cytometric dot plots showing expression of IFN-γ, TNF-α, IL-4, IL-10, or IL-17 by expanded Vδ3 T cell lines after stimulation for 4 h with PMA and ionomycin. Results are representative of data using expanded Vδ3 T cells from six donors. (**B**) Mean levels of IFN-γ and IL-17 released by expanded Vδ3 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, Vγ9Vδ2 T cells, and MAIT cells, and they have distinct functional activities (3, 11, 21). Like other innate T cells, most fresh Vδ3 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 Vδ3 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 Vδ3 T cells in vitro

Because of their low frequencies in peripheral blood, Vδ3 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 Vδ3 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 Vδ3 T cells from as few as 1000 cells in 14 d and with purities >95% (Fig. 2A, 2B). Phenotypic analysis of Vδ3 T cell lines from five donors showed that the CD4/CD8/DN distributions of expanded Vδ3 T cells were similar to those of fresh Vδ3 T cells. Expanded Vδ3 T cells also retained expression of NKG2D, were negative for NKG2C, and had lower frequencies of CD56 and CD161 expression than did fresh Vδ3 T cells (Fig. 2A, 2C). Thus, highly pure Vδ3 T cells can be readily expanded by mitogen stimulation in vitro.

Vδ3 T cells recognize CD1d

A significant fraction of human T cells, including γδ T cells, recognize autoantigens presented by CD1a, CD1b, CD1c, or CD1d (4, 6–8, 22–24). We investigated whether expanded Vδ3 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, Vδ3 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-Vδ3 mAb did not prevent Vδ3 T cell activation. Future studies are required to determine whether this mAb can block or stimulate Vδ3 T cell activation.

Many CD1d-restricted T cells do not express the Vα24Jα18 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 Vδ3 and Vα24Jα18 TCR chains by PBMCs, expanded Vδ3 T cells, or expanded iNKT cells and found that these TCR chains were never coexpressed by the same cells (data not shown), indicating that Vδ3 T cells are not iNKT cells. Vδ3 T cell responses to CD1d were not augmented by adding α-GC, as was seen when iNKT cells were used (Fig. 3C), indicating that Vδ3 and iNKT cells have distinct Ag specificities. Thus, some (if

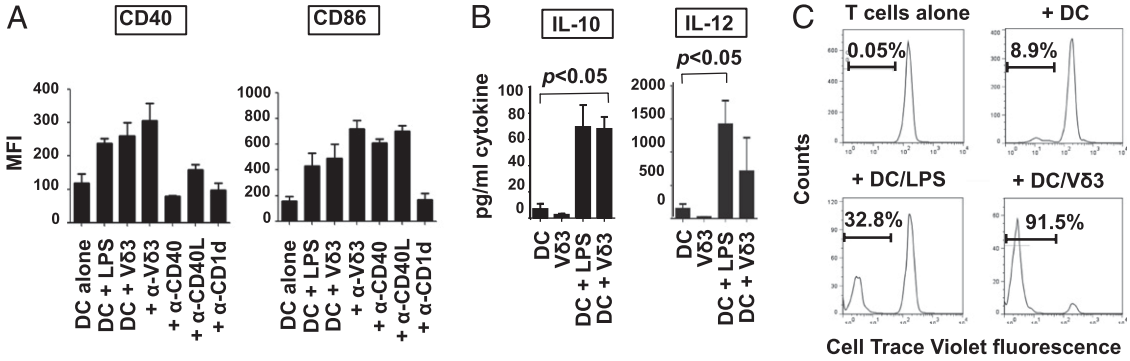

FIGURE 5. Vδ3 T cells induce DC maturation. (**A**) Mean fluorescence intensities (MFI) of expression of CD40 and CD86 by monocyte-derived DCs after culturing them for 3 d in medium alone, with LPS, or with equal numbers of Vδ3 T cells in the absence and presence of blocking mAbs against CD1d, Vδ3, CD40, or CD40L. Results are means of five different DC–Vδ3 T cell combinations. Error bars represent SEM. (**B**) Levels of IL-10 and IL-12 released by DCs or Vδ3 T cells cultured alone for 2 d, DCs cultured with LPS, and DCs cultured with Vδ3 T cells. Results are means of three experiments using different DCs and Vδ3 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 Vδ3 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) Vδ3 T cells fit the definition of type 2 NKT cells. CD1d-restricted activation by other glycolipids, including sulphatide and cardiolipin, was reported for human Vδ1 T cells (7, 8) and murine γδ T cells (25).

Cytokine production by Vδ3 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 Vγ9Vδ2 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 Vδ3 T cells stimulated with HeLa cells expressing CD1d or PMA and ionomycin. Fig. 4A shows that some Vδ3 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 Vδ3 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, Vδ3 T cells can regulate adaptive immune responses via production of multiple Th-polarizing cytokines.

DC maturation by Vδ3 T cells

iNKT cells and Vγ9Vδ2 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 Vδ3 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 Vδ3 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 Vδ3 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 Vδ3 T cell–mediated DC maturation required CD1d but not CD40–CD40L interactions. Vδ3 T cells also induced IL-10 and IL-12 production by DCs (Fig. 5B), and Vδ3 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) Vδ3 T cells promote maturation of DCs into APCs capable of activating naive T cells.

Concluding remarks

We identified Vδ3 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 Vδ3 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, Vδ3 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 Vδ3 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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