Brief Definitive Report

Distinct Functional Lineages of Human Vα24 Natural Killer T Cells

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Abstract

CD1d-restricted autoreactive natural killer (NK)T cells have been reported to regulate a range of disease conditions, including type I diabetes and immune rejection of cancer, through the secretion of either T helper (Th)2 or Th1 cytokines. However, mechanisms underlying Th2 versus Th1 cytokine secretion by these cells are not well understood. Since most healthy subjects express <1 NKT cell per 1,000 peripheral blood lymphocytes (PBLs), we devised a new method based on the combined used of T cell receptor (TCR)-specific reagents α-galactosylceramide (αGalCer) loaded CD1d-tetramers and anti-Vα24 monoclonal antibody, to specifically identify and characterize these rare cells in fresh PBLs. We report here that $CD4^+$ and $CD4^-CD8^-$ (double negative [DN]) NKT cell subsets represent functionally distinct lineages with marked differences in their profile of cytokine secretion and pattern of expression of chemokine receptors, integrins, and NK receptors. $CD4^+$ NKT cells were the exclusive producers of interleukin (IL)-4 and IL-13 upon primary stimulation, whereas DN NKT cells had a strict Th1 profile and prominently expressed several NK lineage receptors. These findings may explain how NKT cells could promote Th2 responses in some conditions and Th1 in others, and should be taken into consideration for intervention in relevant diseases.

Key words: CD1 • NKT cells • cytokine • IDDM • T cell development

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Introduction

NKT cells are a conserved subpopulation of αβ T cells, which are restricted by the antigen-presenting molecule CD1d, and appear to regulate several disease processes ranging from tumor rejection to autoimmune diseases (for a review, see references 1 and 2). They express a conserved canonical TCR (Vα14Jα18-Vβ8 in mouse and Vα24Jα18-Vβ11 in human) that is thought to recognize a self-antigen mimicked by the glycolipid αGalCer. Since NKT cells are present at high frequency in various mouse tissues and in human liver, they seem to participate in the innate, rather than the adaptive arm of the immune response and resemble other innate lymphocytes such as B-1 B cells and γδ T cells which express canonical antigen receptors responding to cell stress and tissue damage (3, 4). Importantly, CD1d is mainly expressed on dendritic cells (DCs), macrophage, and B cells, implying that NKT cells primarily interact with APCs rather than tissue cells.

The secretion of Th1 and Th2 cytokines by NKT cells is thought to underlie their regulatory properties. For example, they can suppress type I diabetes in NOD mouse through the secretion of IL-4 and IL-10 (5, 6) and their defects in both NOD mice and humans with IDDM may contribute to pathogenesis (7, 8). Conversely, they naturally suppress methylcholantrene-induced carcinogenesis through IFN-γ (9). In another report, secretion of the Th2 cytokine IL-13 was found to inhibit the immune rejection of a tumor graft (10). Collectively, these findings suggest that regulated expression of Th1 or Th2 cytokines by NKT cells, rather than mere changes in its frequency (7, 11, 12), might control the outcome of some disease conditions.

How could the Th1- versus Th2-promoting functions of NKT cells be selectively recruited? It has been suggested that altered αGalCer ligands with shorter sphingosine chain could selectively activate Th2 functions (13), whereas NK1.1 signaling could favor Th1 response (14). However, the possibility that subsets of NKT cells might specialize in Th1 versus Th2 functions has not been thoroughly investigated.

One obstacle to the identification of NKT cell subsets has been that, until recently, NKT cells could not be unambiguously identified. The generation of CD1d-αGalCer tetramers specific for both mouse and human canonical TCR makes it possible to identify NKT cells based on their specificity rather

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than their phenotype (15–17). CD1d tetramers have already revealed several important findings, including a subset of CD1d-restricted murine NKT cells that do not express the NK1.1 marker and differ from the NK1.1$^+$ cells with respect to their pattern of integrins (15). However, detailed examination of human NKT cells has not been performed.

An additional challenge to the study of fresh human PBLs is the very low frequency of canonical NKT cells, often well below common background level staining of 0.1%. Here, we have used a combination of CD1d-αGalCer tetramers and anti-Vα24 mAb, which specifically identifies the canonical NKT cells even at the very low frequencies found in human PBLs, to investigate human NKT cell subsets. We have dissected the phenotype of these cells into CD4 and double negative (DN) populations, and found that they systematically differed in many functionally relevant ways with respect to Th cytokine profile, pattern of chemokine receptors, and integrin expression, and array of NK receptors displayed on the cell surface. These findings suggest that human CD4 and DN Vα24 NKT cells represent functionally separate lineages that may promote different Th responses.

Materials and Methods

Antibodies. Fluorochrome or biotin conjugates of antibodies against Vα24, Vβ11, CD4, CD25, CD28, CD56, CD94, NKG2A (Beckman Coulter); CCR1, CCR2, CXCR6 (R&D Systems); CCR4, CCR5, CCR6, CCR7, CXCR3, CXCR4, CD45RA, CD45RB, CD45RO, CD49a, CD49b, CD49d, CD49e, CD49f, CD69, CD152, CD154, CD158a, CD158b, CD161, IL-4, IL-13, TNF-α, and IFN-γ (BD Biosciences) were used.

Flow Cytometric Analysis. PBLs were obtained from whole blood of healthy donors by centrifugation over Ficoll (Amersham Pharmacia Biotech) gradient. Cells were then washed three times with PBS before surface staining. Staining with CD1d-αGalCer tetramers was as follows. Cells were incubated with 1 μg/ml unlabeled streptavidin (Pierce Chemical Co.) for 15 min at room temperature, followed by incubation with CD1d-αGC tetramers (15) for 1 h at room temperature. Other mAbs such as CD4 and Vα24 were then added for a further 30 min incubation on ice. Cells were then washed with staining buffer (PBS, 0.1% BSA; Sigma-Aldrich) and 0.01% sodium azide (Sigma-Aldrich) and analyzed by flow cytometry using FACSort™ and CELLQuest™ software (Becton Dickinson). For studies involving CD152 or intracellular cytokines, cells were first stained with tetramers, then permeabilized with Cytofix/Cytoperm™ (BD Biosciences), and washed with Perm/Wash™ buffer (BD Biosciences). Appropriate mAbs were then added for 30 min before two further washes with Perm/Wash™ buffer.

Stimulation of Cytokine Production. PBLs were cultured for 12 h at a concentration of 5 × 10^6 cells per milliliter in RPMI 1640 supplemented with 10% FCS (Biofluids) in the presence of 1 ng/ml of phorbol-myristate-acetate (PMA), 1 μM of ionomycin, and 5 μg/ml of Brefeldin A (all from Sigma-Aldrich).

Results

Specific Identification of Human NKT Cells Using CD1d-αGalCer Tetramers and Anti-Vα24 mAbs. To accurately identify potential subsets of the rare circulating human NKT cells above background staining levels, we combined the use of two TCR-specific reagents, a mAb to Vα24 and CD1d-αGalCer tetramers. We found that, although CD1d-αGalCer tetramer staining is completely inhibited by prior incubation with the anti-Vα24 mAb (15), the reverse reaction order allowed significant binding of anti-Vα24, presumably because the tetramers require contiguous clusters of TCR to bind, leaving a significant amount of unbound TCR available for bright Vα24 staining. Fig. 1 A shows that the frequency of Vα24/CD1d-αGalCer double positive canonical NKT cells in the fresh PBLs of healthy volunteers is between 0.01 and 0.1%. Importantly, tetramer staining alone invariably included ~0.01–0.05% nonVα24 cells, which are noncanonical cells that presumably reflect background staining (Fig. 1 B, top left quadrants in left dot plots). Thus, whereas the population defined by conventional tetramer staining alone included a significant proportion of nonNKT cells in most healthy individuals, especially in those with low NKT cell frequency, the double staining combination allowed complete specificity. This is shown by the 100% expression of Vβ11 among the Vα24/CD1d-αGalCer double-positive cells (Fig. 1 B, right dot plots). In the sample expressing 0.01% NKT cells, 100 out of 100 gated cells were Vβ11$^+$, indicating that this technique specifically identified all of the 100 canonical NKT cells present among 1 million PBL.

CD4 and DN Subsets Have Different Cytokine Secretion Profiles. A fraction of Vα24 NKT cells expresses CD4 while the remaining is CD8β-negative and is called DN. While the proportion of the CD4 and DN varied considerably between individuals, on average, they were roughly equal (50%) in a group of 10 healthy individuals (Fig. 1 B, and data not shown). Surprisingly, despite reports that the corresponding mouse subsets exhibit similar cytokine secretion properties (18), systematic differences were found in 10/10 individual human subjects examined. Thus, upon ex vivo stimulation with the combination of ionomycin and

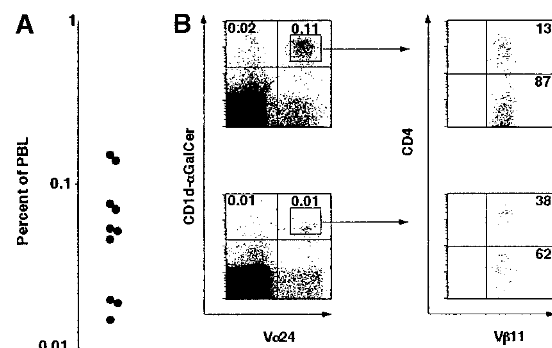

Figure 1. Identification of human NKT cells. (A) NKT cells present among the PBL of 10 healthy adults were enumerated after double staining with CD1d-αGC tetramers followed by anti-Vα24. Each dot represents an individual sample. (B) PBL from individuals expressing high (top panels) or low (bottom panels) NKT cell numbers were stained with CD1d-αGC tetramers followed by anti-Vα24, -Vβ11, and CD4 mAb. Two-color combinations are displayed to illustrate correlations between different subsets. Right panels are gated on Vα24/CD1d-αGC double-positive cells.

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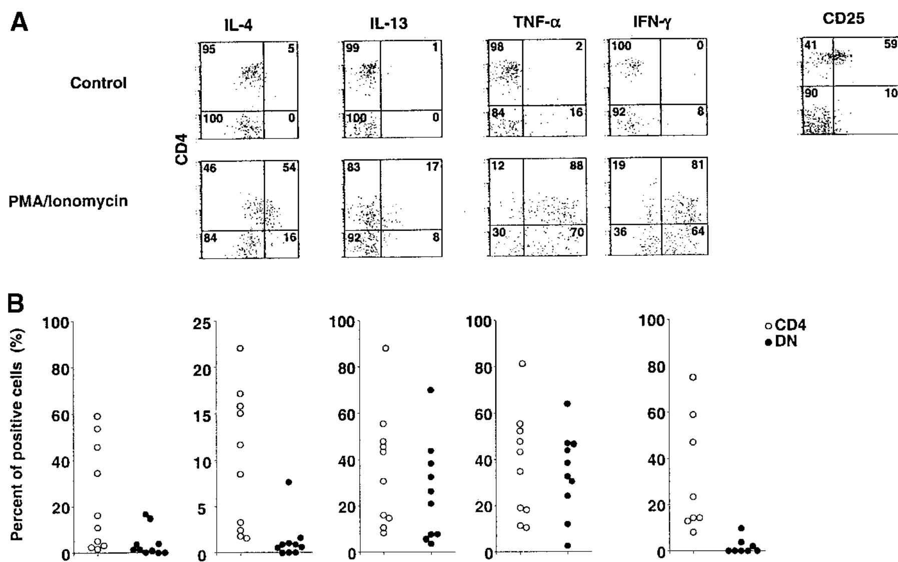

Figure 2. Different cytokine and cytokine receptor profiles of CD4 and DN NKT cells. Fresh PBLs were rested or stimulated with PMA/ionomycin for 12 h before staining with CD1d-αGalCer tetramers followed by permeabilization and staining with mAbs to Vα24, CD4, and cytokines or cytokine receptors. (A) FACS® dot plots from a representative individual are shown, after gating on CD1d-αGalCer/Vα24 double-positive cells. Numbers in the quadrants indicate percentages of cells within the CD4 or DN subsets (top and bottom quadrants, respectively). (B) Summary of cytokine expression from 10 individual subjects. White circles represent the CD4 subset while black circles represent the DN subset. Significant differences (Student's paired *t* test) were found for IL-4 ($P < 0.01$) and IL-13 ($P < 0.01$). Staining of CD25 (IL-2Rα) was performed without membrane permeabilization.

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PMA, nearly all the IL-4 and IL-13 stained by intracellular FACS® were present in CD4$^+$ cells (Fig. 2). In contrast, both the CD4 and DN subsets produced abundant Th1 cytokines such as TNF-α and IFN-γ. Interestingly, the IL-2Rα chain (CD25) was exclusively expressed by CD4$^+$ cells (10–80% positive), indicating that CD4$^+$ NKT cells represent a fraction of human CD4$^+$CD25$^+$ regulatory cells.

CD4 and DN Subsets Have Different Patterns of Chemokine Receptors and Integrins. When we examined a broad range of chemokine receptors and integrins (Fig. 3), significant differences were systematically found between CD4 and DN cells with respect to the expression of CCR5, CCR6, CXCR6, and CD49a. Other chemokine receptors and integrins were similarly expressed in both subsets, including CCR1 and CCR2 (<2%), CXCR3 and CXCR4 (2–100%), CCR4 (100%), and CCR7 (<2%) (data not shown). Likewise, both CD4 and DN NKT cells expressed abundant CD49d, CD49e, and CD49f but limited CD49b (data not shown).

NK lineage receptors are mainly expressed by the DN subset. One of the hallmarks of mouse NKT cells is the expression of receptors of the NK lineage, which regulate cellular activation by fine tuning TCR signaling. Using a battery of NK receptor-specific reagents, we have found systematic differences between CD4 and DN NKT cells (Fig. 4). Thus, CD161, which costimulates TCR activation

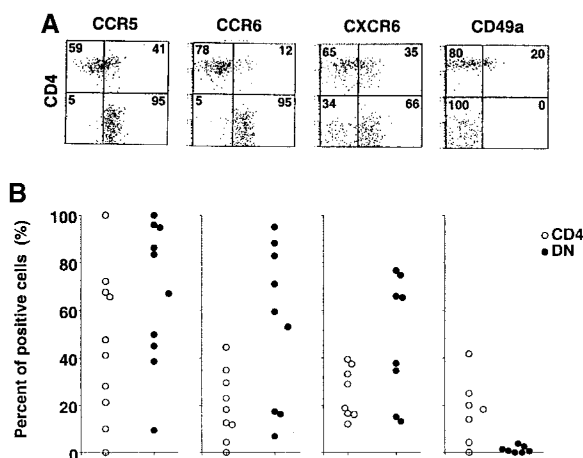

Figure 3. Different chemokine receptor and integrin pattern of CD4 and DN NKT subsets. The CD1d-αGalCer/Vα24 double-positive PBLs were gated for analysis of CD4 and chemokine receptors or CD49a. (A) FACS® dot plots of a representative PBL sample. Numbers in the quadrants indicate percentages of cells within the CD4 or DN subsets (top and bottom quadrants, respectively). (B) Summary of chemokine receptor and CD49a expression from 10 individual subjects. White circles represent the CD4 subset, while black circles represent the DN subset. Significant differences (Student's paired *t* test) were found for CCR5 ($P < 0.05$), CCR6 ($P < 0.01$), CXCR6 ($P < 0.05$), and CD49a ($P < 0.05$).

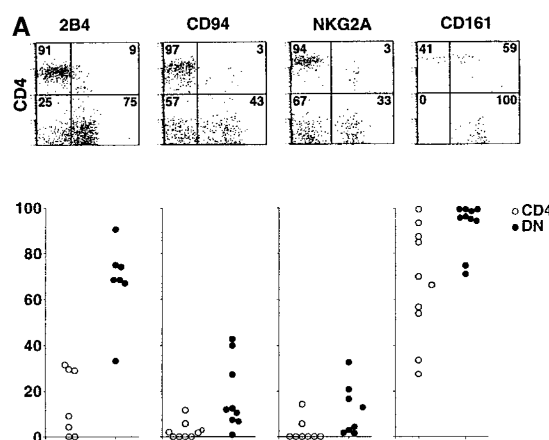

Figure 4. Different patterns of NK receptor expression by CD4 and DN NKT cells. The CD1d-αGalCer/Vα24 double-positive PBLs were gated for analysis of CD4 and 2B4, CD94, NKG2A, or CD161. (A) FACS® dot plots of a representative PBL sample. Numbers in the quadrants indicate percentages of cells within the CD4 or DN subsets (top and bottom quadrants, respectively). (B) Summary of NK receptor expression from 10 individual subjects. White circles represent the CD4 subset, while black circles represent the DN subset. Significant differences (Student's paired *t* test) were found for 2B4 ($P < 0.001$), CD94 ($P < 0.05$), NKG2A ($P < 0.05$), and CD161 ($P < 0.01$).

(14), was expressed at a higher density and at greater frequency by DN NKT cells. Likewise, 2B4, CD94, and NKG2A were nearly exclusively expressed by DN NKT cells. On the other hand, CD56 was highly expressed by both subsets, whereas the KIR CD158a and CD158b were generally not expressed (data not shown).

Other Surface Receptors Expressed by Fresh NKT Cell Subsets. There were no systematic differences for costimulatory receptors such as CD28, which was generally expressed by most CD4 and DN NKT cells. In contrast, cytotoxic T lymphocyte antigen 4 and CD40L were expressed by very small fractions of NKT cells (data not shown). Most CD4 and DN NKT cells expressed CD45RO and CD45RB, but not CD45RA (data not shown). CD69 was equally expressed by $<$50% of both CD4 and DN NKT cells.

Discussion

By combining the use of TCR-specific reagents such as CD1d-αGalCer tetramer and anti-Vα24 mAb, we could specifically identify all canonical Vα24 NKT cells, even at the very low frequencies found among the PBLs of most healthy individuals. Indeed, we demonstrated that the double staining method allowed correct and specific identification of every NKT cell among 1 million PBLs, a 100–1,000-fold improvement over conventional tetramer tracking methods, which is likely to apply to other T cell subsets. Using this methodology, we were able to characterize functionally distinct subsets and show that they segregate with the CD4 and DN subsets of NKT cells.

While both CD4 and DN NKT cells could produce Th1 cytokines, the release of Th2 cytokines such as IL-4 and IL-13 was the exclusive property of CD4 T cells. CD4 and DN NKT cells also exhibited systematic differences in their pattern of chemokine receptors and integrins, suggesting different migratory properties. Finally, the expression of several NK receptors was restricted to the DN lineage. Altogether, these findings reveal that the CD4 and DN subsets represent distinct lineages with markedly different functional properties.

The restricted production of Th2 cytokines by fresh CD4 NKT cells apparently conflicts with a prior study showing that DN NKT cell clones derived from healthy individuals produced Th2 cytokines, whereas those of IDDM patients were Th1 (7). It is possible that the culture system modified their primary cytokine profile, as repeated stimulation was reported to downmodulate Th1 cytokines and upregulate Th2 (19). Our study suggests that a reexamination of the functional status of NKT cell subsets in IDDM patients is warranted to verify whether the conclusions derived from the in vitro studies will apply to fresh NKT cell subsets.

This study of human Vα24 NKT cells also points to a surprising difference between the mouse and the human system. Indeed, the mouse CD4 and DN NKT cells do not clearly differ with respect to Th1 versus Th2 cytokine secretion or NK receptor expression (18). Further studies are required to elucidate the mechanisms underlying Th2 versus Th1 regulation in mouse. It is possible that other subsets or alternative mechanisms, such as altered glycolipid ligand (13) or NK receptor signaling (14), could account for the control of Th function in these conditions.

In conclusion, our study of fresh human Vα24 NKT revealed the existence of two functionally distinct lineages of CD4 and DN cells. These lineages might be differentially altered or recruited in various disease conditions, providing a potential mechanism explaining how NKT cells might promote opposite Th1 or Th2 responses. A detailed understanding of this regulation will be critical to design future strategies to manipulate the immune response through NKT cell activation.

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