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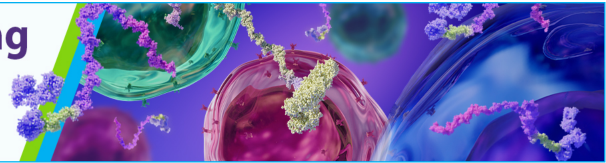

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CUTTING EDGE

Cutting Edge: Analysis of Human Vα24$^+$CD8$^+$ NK T Cells Activated by α-Galactosylceramide-Pulsed Monocyte-Derived Dendritic Cells

Tsuyoshi Takahashi,*† Shigeru Chiba,* Mie Nieda,¶ Takeshi Azuma,‡ Soichiro Ishihara,§ Yoichi Shibata,† Takeo Juji,¶ and Hisamaru Hirai[1]*

Human Vα24$^+$ NKT cells constitute a counterpart of mouse Vα14$^+$ NKT cells, both of which use an invariant TCR-α chain. The human Vα24$^+$ NKT cells as well as mouse Vα14$^+$ NKT cells are activated by glycolipids in a CD1d-restricted manner and produce many immunomodulatory cytokines, possibly affecting the immune balance. In mice, it has been considered from extensive investigations that Vα14$^+$ CD8$^+$ NKT cells that express invariant TCR do not exist. Here we introduce human Vα24$^+$CD8$^+$ NKT cells. These cells share important features of Vα24$^+$ NKT cells in common, but in contrast to CD4$^-$CD8$^-$ (double-negative) or CD4$^+$ Vα24$^+$ NKT cells, they do not produce IL-4. Our discovery may extend and deepen the research field of Vα24$^+$ NKT cells as well as help to understand the mechanism of the immune balance-related diseases. *The Journal of Immunology,* 2002, 168: 3140–3144.

Although mouse Vα14$^+$ NKT cells that express TCR and NKR, NK1.1 (NKR-P1C), may play some important roles in the immune system (1, 2), the details are unknown. These cells use an invariant TCR-α chain (Vα14-Jα281) that pairs preferentially with Vβ8, Vβ7, or Vβ2 (3–5). The human homolog of mouse Vα14$^+$ NKT cells appears to be Vα24$^+$ NKT cells, because many features identified in the former are conserved in the latter. The Vα24$^+$ NKT cells express CD161 (NKR-P1A) as an NKR and the TCR of the Vα24-JαQ invariant TCR-α chain that pairs preferentially with Vβ11 (human Vα24 and Vβ11 are homologs of mouse Vα14 and Vβ8, respectively) (6, 7). Phenotypically, CD4$^-$CD8$^-$ double-negative (DN)[2] and CD4$^+$ NKT cells have been reported in both mice and humans (8–10). In mice, it has been considered from extensive investigations that Vα14$^+$CD8$^+$ NKT cells that express invariant TCR do not exist (5, 8, 11, 12). The function of human Vα24$^+$ NKT cells is similar to that of mouse Vα14$^+$ NKT cells in many respects. Both are activated by synthetic glycolipids such as α-galactosylceramide (α-GalCer) in a CD1d-restricted and an invariant TCR-mediated manner (13–16). They exhibit cytotoxic activity against tumor cells (10, 17, 18), which may be important for natural anticancer immunity. On TCR stimulation, they produce a large amount of IFN-γ and IL-4, whereas the level of IL-4 production is much higher in CD4$^+$ NKT cells than in DN NKT cells (9, 10, 19–21).

In this report, we describe a novel subpopulation of human Vα24$^+$ NKT cells with a CD4$^-$CD8$^+$ phenotype (CD8$\alpha\beta$ single-positive, hereafter referred to as Vα24$^+$CD8$^+$ NKT), which have features similar to those of other Vα24$^+$ NKT subpopulations such as the usage of invariant TCR, CD1d-restricted activation, and cytotoxicity against tumor cells. Interestingly, the cytokine production patterns are distinctly different among them. Vα24$^+$CD8$^+$ NKT cells do not produce IL-4, whereas DN and CD4$^+$ Vα24$^+$ NKT cells do. Vα24$^+$CD4$^+$ NKT cells produce more IL-10 and IL-13 than do DN and CD8$^+$ Vα24$^+$ NKT cells. These differences in cytokine production patterns potentially influence the immune balance such as the determination of the Th1-Th2 profile and activation or suppression of immune response. Relative and absolute numbers of Vα24$^+$ NKT cells belonging to each subpopulation may be important in immune regulation.

Materials and Methods

Abs and reagents

The following Abs were purchased from Immunotech (Marseilles, France): IgG1 (679.1 Mc7); IgG2b (MOPC-195); anti-CD3 (UCHT-1); anti-CD4 (SFCI12T4D11(T4)); anti-CD8β (2ST8.5H7); anti-CD161 (191B8); anti-Vα24 (C15); and anti-Vβ11 (C21). Anti-CD1d (55.3.1) (22) was a gift from Dr. S. Porcelli (Albert Einstein College of Medicine, Bronx, NY). α-GalCer was obtained from Kirin (Gunma, Japan). rhGM-CSF and rhIL-4 were purchased from CellGenix (Freiburg, Germany), and rhIL-2 was obtained from Shionogi (Osaka, Japan).

Cell populations

Vα24$^+$ NKT cells were established as follows. Monocytes from the healthy human donors were each cultured in AIM medium (Life Technologies, Gaithersburg, MD) supplemented with 10% FCS with rhIL-4 (500 U/ml) and rhGM-CSF (500 U/ml) for 5 days, and used as monocyte-derived dendritic cells (Mo-DCs). Lymphocytes from the same donor were cultured with irradiated (50 Gy)-Mo-DCs which were pulsed for 12 h with α-GalCer (100 ng/ml). After 7 days, Vα24$^+$ cells were established by positive (Vα24) magnetic bead sorting (MACS; Miltenyi Biotec, Bergisch Gladbach, Germany) and maintained in the presence of rhIL-2 (40 U/ml). The cells were restimulated every 7 days. When the cells were expanded, Vα24$^+$Vβ11$^+$CD4$^-$CD8$^-$, Vα24$^+$Vβ11$^+$CD4$^+$, and Vα24$^+$Vβ11$^+$CD8$^+$

Departments of *Hematology and Oncology, †Transfusion Medicine, ‡Urology, and §Surgery, Graduate School of Medicine, University of Tokyo, and ¶Department of Research, The Japanese Red Central Blood Center, Tokyo, Japan

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[1] Address correspondence and reprint requests to Dr. Hisamaru Hirai, Department of Hematology and Oncology, Faculty of Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113, Japan. E-mail address: hhirai-tky@umin.u-tokyo.ac.jp

[2] Abbreviations used in this paper: DN, double-negative; α-GalCer, α-galactosylceramide; rh, recombinant human; Mo-DC, monocyte-derived dendritic cell; HD, healthy donor.

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subpopulations were sorted using the FACSVantage apparatus (BD Biosciences, San Jose, CA) and maintained in the presence of rhIL-2 (40 u/ml) and by restimulation every 7–10 days.

Phenotypic assay

Cells were analyzed by flow cytometry using a FACSVantage apparatus. Immunofluorescence staining using specific mAb was performed according to standard procedures.

Molecular analysis of TCR-α transcripts

Total RNA was extracted from 1 × 10^6 NKT or T cells according to the manufacturer's protocol (ISOGEN; Wako, Osaka, Japan) and reverse transcribed using the outer primer of the constant region of TCR-α. Then the transcribed cDNA was subjected to the first-round PCR amplification using the outer primer of the variable region of TCR Vα24 in a thermal cycler (RoboCycler; Stratagene, La Jolla, CA). The second PCR was performed using the inner primer pair. The PCR products were electrophoresed, transferred to a nylon membrane, hybridized with ^{32}P-labeled JαQ probe, and autoradiographed. The details of PCR primers and JαQ probe sequences and PCR conditions were described elsewhere (13). For the detection of Vα-Jα sequence, the DNA band was excised from the gel, and DNA was extracted according to the manufacturer's protocol (QIAquick Gel Extraction Kit; Qiagen, Hilden, Germany). Their nucleotide sequences were then determined (ABI PRISM 3100 Genetic Analyzer; PE Applied Biosystems, Foster City, CA) by a dideoxy chain termination method according to the manufacturer's protocol, with a DNA sequencing kit (PE Applied Biosystems).

Proliferative response to α-GalCer-pulsed Mo-DCs and their inhibition by anti-CD1d Ab

Proliferative response was assayed as follows. The bulk of 5 × 10^4 NKT cells as the responders and 5 × 10^4 irradiated-allogeneic Mo-DCs (all allogeneic monocytes were derived from the same donor) as the stimulators in 200 μl of culture medium were seeded onto flat-bottom microtiter wells with or without α-GalCer (100 ng/ml). The culture was incubated for 48 h. For the final 6 h of incubation, 1 μCi [^{3}H]TdR was added to each well, and the incorporation of [^{3}H]TdR was determined by liquid scintillation counting. To determine whether the response is CD1d restricted, 55.3.1 (anti-CD1d mAb) or IgG2b as a control was added to the wells (5 μg/ml).

Cytotoxicity assays

^{51}Cr release assay was performed as follows. ^{51}Cr-labeled ($Na_2{}^{51}CrO_3$) (Amersham, Arlington Heights, IL) U937 or K562 cell lines (5 × 10^3) as the target cells and various numbers of effector cells in 200 μl of culture medium were seeded onto round-bottom microtiter wells. The culture was incubated for 4 h, and 100 μl of supernatant were collected from each well. The percentage of specific ^{51}Cr release was calculated as [(cpm experimental release − cpm spontaneous release)/(cpm maximal release − cpm spontaneous release)] × 100.

Cytokine production

For the cytokine production assay, 5 × 10^4 NKT and 5 × 10^4 α-GalCer-pulsed Mo-DCs (all allogeneic monocytes were derived from the same donor) were suspended in 200 μl of culture medium in 96-well plates. After 18 h, the supernatants were collected from each well and assayed for the concentrations of IFN-γ, IL-4, IL-10, and IL-13 by ELISA according to the manufacturer's protocol (AN′ALYZA; Genzyme, Cambridge, MA).

Results and Discussion

Phenotypic and molecular characterization of lymphocytes activated by α-GalCer-pulsed Mo-DCs

Vα24^+ NKT cells have been reported to be activated by stimulation with α-GalCer-pulsed Mo-DCs in a CD1d-restricted manner (14–16). We generated Mo-DCs from healthy donors using IL-4 and GM-CSF in vitro, which were then pulsed with α-GalCer. The α-GalCer-pulsed Mo-DCs were cocultured with autolymphocytes to generate Vα24^+ NKT cells. On the basis of the responses of Vα24^+-sorted lymphocytes to the stimulation, we identified

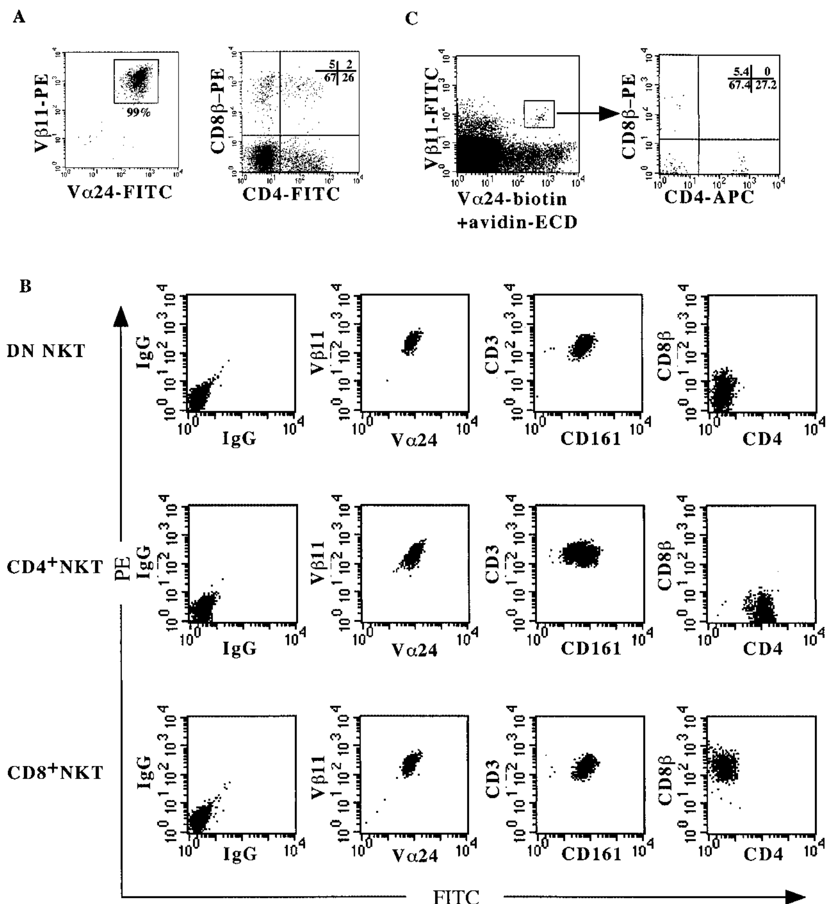

FIGURE 1. Phenotypic analysis of Vα24^+DN NKT, Vα24^+CD4^+ NKT, and Vα24^+CD8^+ NKT cells. The α-GalCer-pulsed Mo-DCs were cocultured with autolymphocytes and selected for expression of Vα24. The cells were then restimulated with α-GalCer-pulsed Mo-DCs and IL-2 for two cycles and then sorted into separate populations based on the expression of CD4 or CD8. The cells were stained by two-color flow cytometry using the indicated mAbs. Phenotypes of Vα24^+ NKT cells were indicated before (*A*) and after (*B*) sorting based on CD4/CD8 expression. The numbers in the dot plots indicate the percentage of each population. Shown are the dot plots obtained from the first donor (HD1). Similar results were obtained from the second donor (HD2) (data not shown). Next the CD4/CD8 phenotype of Vα24^+ NKT cells was examined in peripheral blood. Freshly isolated PBL from 10 healthy donors were analyzed by four-color flow cytometry analysis using Vβ11-FITC/CD8β-PE/Vα24-biotin-avidin-PE-Texas Red (ECD)/CD4-allophycocyanin (APC) stain. The cells were gated for lymphoid cells by light scatter characteristics, and expression of CD4/CD8 was examined within Vα24^+Vβ11^+ cells. Representative dot plots are shown (*C*). The numbers in the dot plot indicate the percentage of each subpopulation among Vα24^+Vβ11^+ cells.

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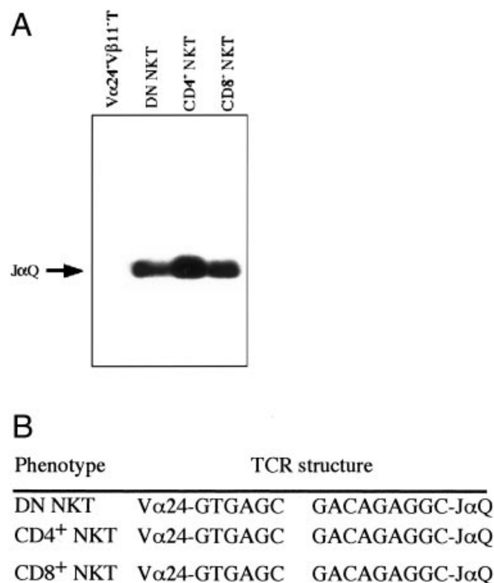

Phenotype	TCR structure	
DN NKT	Vα24-GTGAGC	GACAGAGGC-JαQ
CD4+ NKT	Vα24-GTGAGC	GACAGAGGC-JαQ
CD8+ NKT	Vα24-GTGAGC	GACAGAGGC-JαQ

FIGURE 2. Invariant TCR-α chain expression of Vα24+ NKT cells. Samples were from the first donor (HD1). In all samples, PCR was performed using Vα24-specific 5′ and Cα-specific 3′ primers. The PCR products were electrophoresed on 2% agarose gel, and fractionated products were transferred to a nylon membrane and then hybridized with a ^{32}P-labeled JαQ probe. All NKT cells except Vα24+Vβ11− T cells used the JαQ sequence (*A*). For detection of the Vα-Jα sequence, the nucleotide sequences of the PCR products were determined (*B*). The Vα-Jα sequence of Vα24+Vβ11− T cells could not be determined by this method.

CD8αβ single-positive Vα24+CD8+ NKT cells as well as DN and CD4+ Vα24+ NKT cells from two healthy donors (Fig. 1*A*). According to CD4/CD8 phenotype, each subpopulation was sorted. Each Vα24+ NKT subpopulation used Vβ11 for its TCR Vβ repertoire and expressed NK receptor, CD161 (NKR-P1A) (Fig. 1*B*). It did not express other NK receptors, CD16, CD56, or CD94 (data not shown). To confirm whether Vα24+CD8+ NKT cells use JαQ as an invariant TCR-α chain, we then performed RT-PCR with the Vα24 and Cα primers and analyzed the fragment encompassing the V-J junction, using >99% pure NKT subpopulations. A Vα24+Vβ11− T cell population was sorted and used as a control. When the PCR products were fractionated and hybridized with an oligonucleotide probe specific for JαQ on a filter, the expression of JαQ was detected in Vα24+CD8+ NKT cells as well as in DN and CD4+ Vα24+ NKT cells, but not in Vα24+Vβ11− T cells from the same donor (Fig. 2*A*). For further confirmation, we sequenced the PCR product of each subpopulation by a direct sequence method. Vα24+CD8+ NKT cells as well as DN and CD4+ Vα24+ NKT cells were confirmed to use the Vα24-JαQ invariant chain without N region diversity (Fig. 2*B*). Next we examined the frequency of CD8+ Vα24+ NKT cells in fresh PBL from 10 healthy donors (age 32.8 ± 6.4 years (mean ± SD)) to identify their physiological counterpart in the peripheral blood. We performed four-color analysis using Vβ11-FITC/CD8β-PE/Vα24-biotin-avidin-PE-Texas Red (ECD)/CD4-allophycocyanin (APC) stain. The frequency of Vα24+Vβ11+ cells in the CD3+ cells was 0.050 ± 0.030%. The percentage of CD4/CD8 phenotype in Vα24+Vβ11+ cells was: CD4−CD8−, 71.2 ± 11.8; CD4+CD8−, 24.8 ± 12.0; CD4−CD8+, 2.7 ± 1.8; CD4+CD8+, 0.3 ± 0.6. Although the frequency of CD8β+ Vα24+ NKT cells was <5%, this subpopulation clearly existed in fresh PBL (Fig. 1*C*).

A CD1d-restricted response of Vα24+CD8+ NKT cells to α-GalCer-pulsed Mo-DCs

Next, we examined the response of Vα24+CD8+ NKT cells to α-GalCer-pulsed Mo-DCs in a CD1d-restricted manner. The response of Vα24+CD8+ NKT cells as well as DN and CD4+ Vα24+ NKT cells to Mo-DCs was enhanced by α-GalCer and inhibited by an anti-CD1d mAb, indicating that Vα24+CD8+ NKT cells recognize a glycolipid, α-GalCer, in a CD1d-restricted manner similar to other Vα24+ NKT subpopulations (Fig. 3). It is still unknown whether there exists a murine homolog of human Vα24+CD8+ NKT cells, i.e., Vα14+CD8+ NKT cells in mice, although there have been reports that the forced expression of CD8 in transgenic mice resulted in deletion of this Vα14+ NKT population (5, 8) and that mouse CD8+NK1.1+ NKT cells do not use invariant Vα14 TCR nor are they restricted in CD1d (11, 12). Thus, CD8+ NKT subpopulations that express invariant TCR may exist in human Vα24+ NKT populations but not in mouse Vα14+ NKT populations.

Cytotoxic activity of Vα24+CD8+ NKT cells

It has been reported that human Vα24+ NKT cells as well as mouse Vα14+ NKT cells exhibit cytotoxicity against tumors. We thus examined the cytotoxic activity of human Vα24+CD8+ NKT cells. Vα24+CD8+ NKT cells as well as DN and CD4+ Vα24+ NKT cells exhibited cytotoxic activities against U937 cells (percent cytotoxicity, 20–40; E:T 10:1). However, none of them showed a cytotoxic activity against an erythroleukemic, NK-sensitive cell line, K562 (percent cytotoxicity, <10; E:T 10:1; data not shown).

Cytokine secretion of Vα24+ NKT cells

Because human and mouse DN and CD4+ NKT cells have been reported to produce a large amount of various cytokines such as IFN-γ and IL-4 when stimulated (9, 10, 19–21), we examined the cytokine production pattern of each subpopulation of Vα24+ NKT cells after stimulation with α-GalCer-pulsed Mo-DCs. All subpopulations produced a large amount of IFN-γ (Fig. 4*A*). Regarding IL-4 production, both DN and CD4+ Vα24+ NKT cells produced a large amount of IL-4, and Vα24+CD4+ NKT cells tended to produce more IL-4 than did Vα24+DN NKT cells. Thus, in contrast to Vα24+DN NKT cells, Vα24+CD4+ NKT cells had more Th2 bias. However, Vα24+CD8+ NKT cells produced a small amount of IL-4 (Fig. 4*B*). Regarding IL-10 and IL-13 production, Vα24+CD4+ NKT cells produced a large amount of

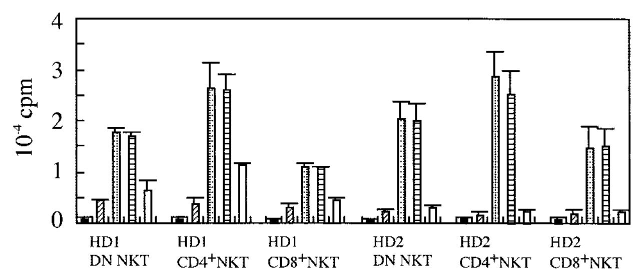

FIGURE 3. All subpopulations of human Vα24+ NKT cells respond to synthetic α-GalCer in a CD1d-restricted manner. All subpopulations of Vα24+ NKT cells from healthy donor 1 (HD1) and healthy donor 2 (HD2) nonstimulated (■), and stimulated with Mo-DC (▨), Mo-DC plus α-GalCer (▩), Mo-DC plus α-GalCer plus control Ig (▤), and Mo-DC plus α-GalCer plus αCD1d (□). Cells were incubated under each condition for 48 h, and [^{3}H]TdR was added to each well during the final 6 h of incubation. Results are the means ± SD of triplicate values. Representative results of three independent experiments.

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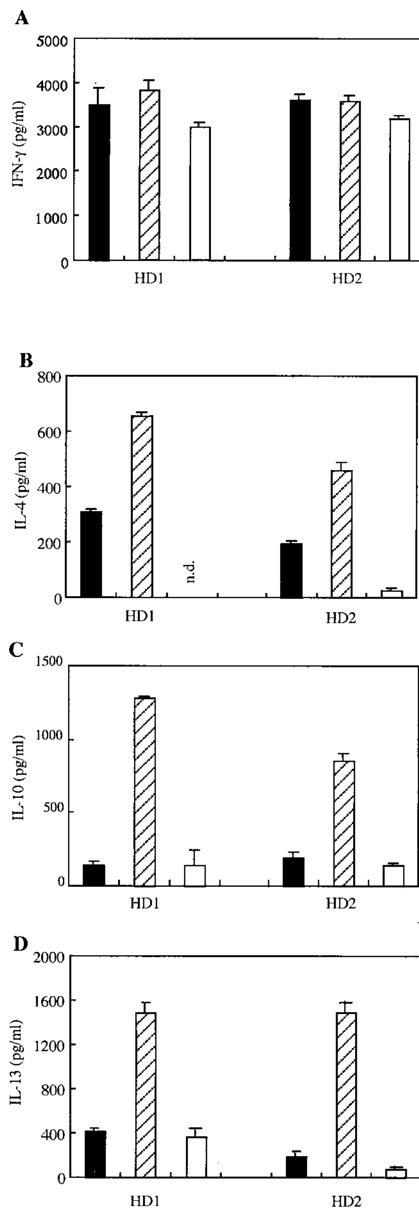

FIGURE 4. Assay for cytokine production. A total of 5×10^4 Vα24$^+$ NKT cells and α-GalCer-pulsed allogeneic Mo-DCs (all allogeneic Mo-DCs were derived from same donor) were cocultured for 48-h. The supernatant was collected from each well and assayed for the production of IFN-γ (*A*), IL-4 (*B*), IL-10 (*C*) or IL-13 (*D*) by ELISA. DN NKT (■), CD4$^+$ NKT (▨), CD8$^+$ NKT (□). Representative results of three independent experiments ± SD.

these cytokines on stimulation, but both DN and CD8$^+$Vα24$^+$ NKT cells produced a small amount of these cytokines (Fig. 4, *C* and *D*). These results indicated that the cytokine production pattern might be distinctly different among Vα24$^+$ NKT subpopulations and thus that each subpopulation could have a different effect on immunological response, although it is possible that repeated αGalCer/cytokine stimulation might influence the function such as cytokine production patterns. Recently, the immunoregulatory role of NKT cells has been well documented. NKT cell-derived IL-10 was essential for the differentiation of Ag-specific regulatory T cells in systemic tolerance (23). Vα24$^+$CD4$^+$ NKT cell-derived IL-13 prevented effective CTL-mediated tumor eradication (24). Furthermore, the relationship between autoimmune/allergy diseases and NKT cells has been recently reported in mouse and human (25–28). Therefore, relative and absolute numbers of Vα24$^+$ NKT cells belonging to each subpopulation may be important in physiological and pathological regulation of immune system. The relationship between changes in the ratio of each Vα24$^+$ NKT subpopulation and the disease status such as autoimmune disease, allergy, malignancy, infection, and transplantation must be studied. In contrast, when considering Vα24$^+$ NKT cells as a tool for cell therapy, we should choose the adequate subpopulation of NKT cells depending on its purpose in combination with selecting APC and/or ligands.

In conclusion, we report a novel Vα24$^+$ NKT subpopulation, Vα24$^+$CD8$^+$ NKT cells and indicate the distinct difference in the cytokine production patterns among Vα24$^+$ NKT subpopulations.

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