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## **Distinct Cytokine Profiles of Neonatal Natural Killer T Cells after Expansion with Subsets of Dendritic Cells**

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### **Abstract**

Natural killer T (NKT) cells are a highly conserved subset of T cells that have been shown to play a critical role in suppressing T helper cell type 1-mediated autoimmune diseases and graft versus host disease in an interleukin (IL)-4-dependent manner. Thus, it is important to understand how the development of IL-4- versus interferon (IFN)- $\gamma$ -producing NKT cells is regulated. Here, we show that NKT cells from adult blood and those from cord blood undergo massive expansion in cell numbers (500–70,000-fold) during a 4-wk culture with IL-2, IL-7, phytohemagglutinin, anti-CD3, and anti-CD28 mAbs. Unlike adult NKT cells that preferentially produce both IL-4 and IFN- $\gamma$ , neonatal NKT cells preferentially produce IL-4 after polyclonal activation. Addition of type 2 dendritic cells (DC2) enhances the development of neonatal NKT cells into IL-4<sup>+</sup>IFN- $\gamma$ <sup>-</sup> NKT2 cells, whereas addition of type 1 dendritic cells (DC1) induces polarization towards IL-4<sup>-</sup>IFN- $\gamma$ <sup>+</sup> NKT1 cells. Adult NKT cells display limited plasticity for polarization induced by DC1 or DC2. Thus, newly generated NKT cells may possess the potent ability to develop into IL-4<sup>+</sup>IFN- $\gamma$ <sup>-</sup> NKT2 cells in response to appropriate stimuli and may thereafter acquire the tendency to produce both IL-4 and IFN- $\gamma$ .

Key words: cord blood • interleukin 4 • interferon  $\gamma$  • autoimmune diseases • graft versus host disease

### **Introduction**

NKT cells are a conserved subset of T cells that express a highly limited TCR repertoire, composed of an invariant TCR  $\alpha$  chain (V $\alpha$ 24-J $\alpha$ Q in humans) and a biased set of TCR  $\beta$  chains (predominantly V $\beta$ 11 in humans) (1). These TCRs recognize a nonpolymorphic, MHC class I-like molecule, CD1d, which presents glycolipids (2). NKT cells have a conspicuous ability to rapidly produce large amounts of IL-4 and IFN- $\gamma$  upon TCR triggering (1), suggesting a regulatory function at the early stage of immune responses.

Development of various autoimmune diseases in mice (3, 4) and humans (5, 6) was found to be associated with a decrease in the number of NKT cells. NKT cells have been shown to prevent diabetes in nonobese diabetic mice (7)

and graft versus host disease (GVHD) after bone marrow transplantation (8) in an IL-4-dependent manner. These findings indicate that NKT cells are an important regulatory cell type that prevents a variety of deleterious immune responses. Thus, it is important to understand how the development of IL-4- versus IFN- $\gamma$ -producing NKT cells is regulated. Furthermore, obtaining a large number of IL-4-producing NKT cells may be instrumental in controlling certain autoimmune diseases and GVHD. To this end, two questions need to be addressed. First, because the frequency of NKT cells in human blood is extremely low (9–11), how can we expand NKT cell numbers? Second, because most NKT cells produce both IL-4 and IFN- $\gamma$ , how can we promote NKT cells to produce only IL-4 but not IFN- $\gamma$ ?

In humans, small numbers of NKT cells exist in umbilical cord blood and adult blood (9–11). Recent studies have shown that not only NKT cells in adult blood (adult NKT cells) but also those in cord blood (neonatal NKT cells) have an activated/memory phenotype (10, 11), suggesting

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that human NKT cells are stimulated even before birth, probably by endogenous ligands. However, in contrast to adult NKT cells, neonatal NKT cells do not produce IL-4 or IFN- $\gamma$  upon primary stimulation (11). This suggests that neonatal NKT cells are functionally immature at birth and need to be primed to become fully functional. Thus, the nature of initial stimuli may strongly influence the direction of neonatal NKT cell differentiation, i.e., toward IL-4-IFN- $\gamma$ <sup>+</sup> NKT1 versus IL-4<sup>+</sup>IFN- $\gamma$ <sup>-</sup> NKT2 cells.

Recent studies have shown that distinct types of dendritic cells (DCs) are capable of directing Th differentiation toward a Th1 or Th2 type (12). In humans, monocyte-derived DCs (designated DC1) produce a large amount of IL-12 after stimulation with maturation-inducing factors such as CD40 ligand (L) and induce Th1 differentiation of naive CD4<sup>+</sup> T cells (13). In contrast, type 2 dendritic cells (DC2), which develop from CD4<sup>+</sup>CD3<sup>-</sup>CD11c<sup>-</sup> plasmacytoid cells in the presence of IL-3 and CD40L (13) or TNF- $\alpha$  (14), do not produce a significant amount of IL-12 and preferentially induce Th2 differentiation. Recent studies have shown that CD1d-expressing DCs stimulate mouse (2) and human (15) NKT cells. Thus, the type of DCs may also influence NKT cell differentiation into NKT1 or NKT2 cells.

Here, we studied how to obtain a large number of IL-4-producing NKT2 cells in vitro. To this end, we cultured NKT cells from cord blood or adult blood with anti-CD3 and anti-CD28 mAbs, DC1, or DC2 in the presence of IL-2, IL-7, and PHA. We show that neonatal NKT cells undergo massive proliferation and strong polarization toward NKT2 cells during 4-wk culture with IL-2, IL-7, PHA, anti-CD3, and anti-CD28 mAbs. Addition of DC2 enhances the differentiation of neonatal NKT cells into NKT2 cells.

## Materials and Methods

**Isolation of NKT Cells from Cord Blood or Adult Blood.** Cord blood and buffy coat from healthy donors were purchased from Advanced Bioscience Technologies and Stanford Blood Center, respectively. B cells and monocytes were depleted from mononuclear cells using anti-CD19 and anti-CD14 mAbs and magnetic beads coated with goat anti-mouse IgG (Dynabeads M-450). The cells were frozen until needed. They were stained with FITC-conjugated anti-V $\alpha$ 24 (C15) and PE-conjugated anti-V $\beta$ 11 mAbs (C21; Immunotech). V $\alpha$ 24<sup>+</sup>V $\beta$ 11<sup>-</sup> conventional T cells and V $\alpha$ 24<sup>+</sup>V $\beta$ 11<sup>+</sup> NKT cells were purified by cell sorting.

**Generation of DC1 and DC2.** Monocytes and CD4<sup>+</sup>CD11c<sup>-</sup>lin<sup>-</sup> DC2 precursors were isolated from human peripheral blood as described previously (16). The cells were cultured in RPMI 1640 (BioWhittaker) supplemented with 10% FCS (BioWhittaker), 2 mM L-glutamine, 10 mM HEPES, 1 mM sodium pyruvate, 55  $\mu$ M 2-mercaptoethanol, penicillin G, and streptomycin (all from Life Technologies). Monocytes were cultured in the presence of 50 ng/ml GM-CSF (Schering-Plough) and 200 U/ml IL-4 (Schering-Plough) for 6 d. The resulting immature DCs were washed and cultured for 2 d with human CD40L-transfected L cells (16) (irradiated at 5,500 rads), to obtain mature DC1. CD4<sup>+</sup>CD11c<sup>-</sup>lin<sup>-</sup> DC2 precursors were cultured with 10

ng/ml IL-3 (R&D Systems) and irradiated CD40L-transfected L cells for 6 d to obtain mature DC2.

**Sequencing of TCR V $\alpha$ 24 mRNA.** cDNAs of TCR- $\alpha$  transcripts were amplified by PCR. The primers were V $\alpha$ 24 sense, 5'-GATATACAGCAACTCTGGATGCA-3' and C $\alpha$  antisense, 5'-TGAAGTCCATAGACCTCATGTC-3'. PCR products were subcloned using a TA Cloning<sup>®</sup> Kit (Invitrogen), and the nucleotide sequences were determined by automated sequencing using an Applied Biosystems 373 sequencer.

**In Vitro Expansion of NKT Cells and Coculture with DCs.** V $\alpha$ 24<sup>+</sup>V $\beta$ 11<sup>+</sup> NKT cells were stimulated with allogeneic DC1, allogeneic DC2, or plate-bound anti-CD3 mAb SPV-T3b (10  $\mu$ g/ml; reference 16) and 1  $\mu$ g/ml soluble anti-CD28 mAb L293.1 (Becton Dickinson) in Yssel's medium (Irvine Scientific) containing 10% FCS, 100 U/ml IL-2 (Schering-Plough), 50 ng/ml IL-7 (R&D Systems), and 0.5  $\mu$ g/ml PHA (Wellcome Diagnostics). NKT cells from cord blood were cultured in Terasaki plates (300–1,500 T cells per well depending on available cell numbers), and those from adult blood were cultured in 96-well round-bottomed culture plates (1,500–16,000 T cells per well). DC1 and DC2 were irradiated (3,000 rads) and cultured with NKT cells at a 1:2 DCs/T cells. The proliferating cells were supplemented with medium containing IL-2 and IL-7 but not PHA. After 14 d of stimulation, the cells were restimulated for another 14 d under the same condition as the first stimulation. The resulting cells were used for cytokine assays. The purity of V $\alpha$ 24<sup>+</sup>V $\beta$ 11<sup>+</sup> cells was >95% at the time of restimulation for cytokine assays.

**Analysis of Cytokine Production by ELISA or Flow Cytometry.** 10<sup>6</sup> NKT cells per milliliter were restimulated with plate-bound anti-CD3 and 1  $\mu$ g/ml soluble anti-CD28 mAbs for 24 h. IFN- $\gamma$  and IL-4 in supernatants were measured with a Quantikine ELISA kit (R&D Systems). Intracellular cytokines produced by NKT cells were analyzed by flow cytometry as described (13).

## Results

**Both Adult and Neonatal NKT Cells Undergo Massive Expansion in Cultures with IL-2, IL-7, PHA, Anti-CD3, and Anti-CD28 mAbs.** NKT cells were isolated from cord blood and adult blood by cell sorting, based on the double expression of V $\alpha$ 24 and V $\beta$ 11 (17). The frequency of V $\alpha$ 24<sup>+</sup>V $\beta$ 11<sup>+</sup> NKT cells in cord blood was 0.031  $\pm$  0.023% (SD) of total lymphocytes (range 0.001–0.083%,  $n$  = 11), which tended to be lower than the frequency in adult blood (0.094  $\pm$  0.129% of total lymphocytes, range 0.007–0.540%,  $n$  = 16). Previous studies showed that IL-2 and IL-7 are important for NKT cell proliferation (18, 19). Therefore, 1,500–16,000 adult NKT cells or 300–1,500 neonatal NKT cells were cultured with IL-2, IL-7, PHA, anti-CD3, and anti-CD28 mAbs. After 2 wk of culture, between 0.08 and 6.2  $\times$  10<sup>6</sup> NKT cells were generated, representing a 30–7,600-fold expansion (Table I). After two more weeks of culture in the same condition, 1.2–27  $\times$  10<sup>6</sup> NKT cells were obtained, representing a total 500–70,000-fold expansion. As compared with the expansion during the first 2 wk (30–7,600-fold), the expansion during the second 2 wk was modest (2–16-fold), possibly due to clonal exhaustion caused by continuous stimulation. These results indicate that although only small numbers of

**Table I.** Adult and Neonatal NKT Cell Expansion after Cultures with Anti-CD3 and Anti-CD28 mAbs

|              |        | Day 0       |             | Day 14        |             | Day 28        |  |
|--------------|--------|-------------|-------------|---------------|-------------|---------------|--|
|              |        | Cell number | Cell number | Fold increase | Cell number | Fold increase |  |
| Adult NKT    | Exp. 1 | 16          | 2,200       | 140           | 7,700       | 480           |  |
|              | Exp. 2 | 16          | 500         | 31            | NA          | NA            |  |
|              | Exp. 3 | 1.5         | 78          | 52            | 1,210       | 810           |  |
| Neonatal NKT | Exp. 1 | 1.5         | 6,200       | 4,100         | NA          | NA            |  |
|              | Exp. 2 | 0.4         | 3,030       | 7,600         | 27,000      | 67,500        |  |
|              | Exp. 3 | 0.3         | 920         | 3,100         | 1,910       | 6,400         |  |

V $\alpha$ 24<sup>+</sup>V $\beta$ 11<sup>+</sup> NKT cells were purified by cell sorting and expanded with plate-bound anti-CD3 and soluble anti-CD28 mAbs in the presence of PHA, IL-2, and IL-7 for two rounds (14 d each). Cell numbers were counted on days 0, 14, and 28, and are shown at  $\times 10^{-3}$ . Fold increase: compared to the starting cell numbers.

Exp., experiment.  
NA, not available.

NKT cells are isolated from adult and cord blood, NKT cells can be efficiently expanded in cultures.

More than 95% of cultured NKT cells expressed V $\alpha$ 24<sup>+</sup>V $\beta$ 11<sup>+</sup> cells after the 4 wk of culture by FACS<sup>®</sup> analyses (data not shown). In addition, sequence analyses of TCR  $\alpha$  chains of these cultured cells showed the invariant V $\alpha$ 24-J $\alpha$ Q TCR without N sequences, which is identical to that reported for NKT cells (reference 20 and data not shown). In contrast, cDNA sequences of TCR  $\alpha$  chains of V $\alpha$ 24<sup>+</sup>V $\beta$ 11<sup>-</sup> T cells were polyclonal at the V-J junctions and the J regions (data not shown). These data indicate that the expanded cells after 4 wk of culture are NKT cells but not contaminating cells.

*Neonatal but Not Adult NKT Cells Preferentially Become IL-4<sup>+</sup>IFN- $\gamma$ <sup>-</sup> NKT2 Cells after Polyclonal Stimulation.* To determine the potential of cytokine production by the expanded NKT cells, the cells were restimulated with anti-CD3 and anti-CD28 mAbs for either 5 (intracellular cytokine staining) or 24 h (cytokine analyses by ELISA). Adult NKT cells produced considerable amounts of both IL-4 (268–885 pg/ml) and IFN- $\gamma$  (2,914–3,366 pg/ml; Table II). Intracellular cytokine staining showed that the adult NKT cells mainly differentiated into IL-4<sup>-</sup>IFN- $\gamma$ <sup>+</sup> NKT1 cells (23%) and IL-4<sup>+</sup>IFN- $\gamma$ <sup>+</sup> NKT0 (16%; Fig. 1). Only 8% of the cells differentiated into IL-4<sup>+</sup>IFN- $\gamma$ <sup>-</sup> NKT2 cells. This confirms the previous finding that NKT cells produce both IL-4 and IFN- $\gamma$  upon activation through TCRs (1).

Interestingly, neonatal NKT cells produced two to six times more IL-4 (548–5,559 pg/ml) but two to four times less IFN- $\gamma$  (650–1,889 pg/ml) than adult NKT cells (Table II). These differences are reflected in higher IL-4/IFN- $\gamma$  ratios from neonatal NKT cells than those from adult NKT cells (Table II). Intracellular cytokine staining showed that the predominant population of neonatal NKT cells became IL-4<sup>+</sup>IFN- $\gamma$ <sup>-</sup> NKT2 cells (31%; Fig. 1). Only 5 and 4% of the NKT cells differentiated into IL-4<sup>+</sup>IFN- $\gamma$ <sup>+</sup> NKT0 and IL-4<sup>-</sup>IFN- $\gamma$ <sup>+</sup> NKT1 cells, respectively. These data indi-

cate that neonatal NKT cells and adult NKT cells display strikingly different patterns of cytokine production; neonatal and adult NKT cells preferentially develop into IL-4<sup>+</sup>IFN- $\gamma$ <sup>-</sup> NKT2 cells and IL-4<sup>+</sup>IFN- $\gamma$ <sup>+</sup> NKT0 cells, respectively, in response to TCR engagement.

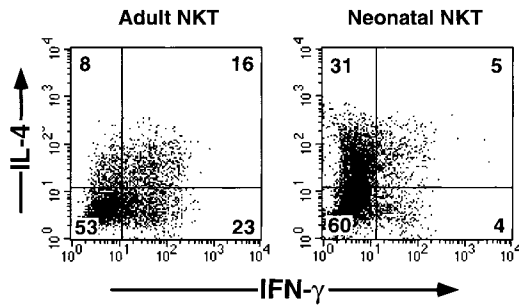
*DC2 Promote Neonatal NKT Cells To Become IL-4<sup>+</sup>IFN- $\gamma$ <sup>-</sup> NKT2 Cells.* Because neonatal NKT cells preferentially develop into IL-4<sup>+</sup>IFN- $\gamma$ <sup>-</sup> NKT2 cells after polyclonal stimulation, we asked whether addition of Th2-inducing DCs could further promote neonatal NKT cell development into the NKT2 type. Our previous study showed that while CD40L-activated monocyte-derived

**Table II.** IFN- $\gamma$  and IL-4 Production by NKT Cells Stimulated with Anti-CD3 and Anti-CD28 mAbs

|                 |        | IFN- $\gamma$ | IL-4  | IL-4/IFN- $\gamma$ |
|-----------------|--------|---------------|-------|--------------------|
|                 |        | pg/ml         | pg/ml | ratio              |
| Adult blood NKT | Exp. 1 | 3,321         | 484   | 0.15               |
|                 | Exp. 2 | 3,366         | 885   | 0.26               |
|                 | Exp. 3 | 2,914         | 268   | 0.09               |
| Cord blood NKT  | Exp. 1 | 1,671         | 1,069 | 0.64               |
|                 | Exp. 2 | 1,889         | 5,559 | 2.94               |
|                 | Exp. 3 | 650           | 548   | 0.84               |

V $\alpha$ 24 $\beta$ 11<sup>+</sup> NKT cells were purified by cell sorting, expanded with plate-bound anti-CD3 and soluble anti-CD28 mAbs in the presence of PHA, IL-2, and IL-7 for two rounds (14 d each), and restimulated with immobilized anti-CD3 and soluble anti-CD28 mAbs at  $\times 10^6$  cells per milliliter for 24 h. IFN- $\gamma$  and IL-4 in the supernatants were measured by ELISA.

Exp., experiment.

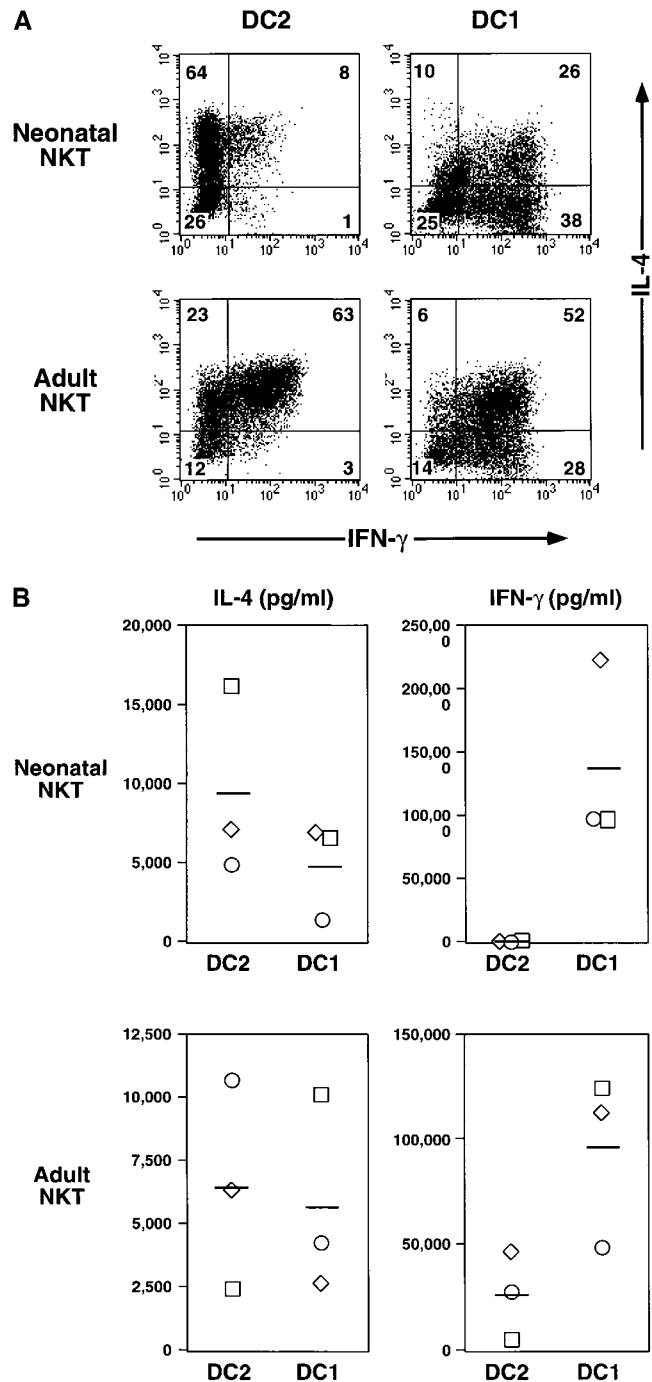


**Figure 1.** Intracellular staining for IL-4 and IFN- $\gamma$  in NKT cells from adult or cord blood stimulated with anti-CD3 and anti-CD28 mAbs.  $V\alpha 24^+V\beta 11^+$  NKT cells were purified by cell sorting, expanded with anti-CD3 and anti-CD28 mAbs in the presence of PHA, IL-2, and IL-7 for two rounds (14 d each), and restimulated with immobilized anti-CD3 mAb and soluble anti-CD28 mAb at  $10^6$  cells per milliliter ml for 5 h. Brefeldin A was added at 2.5 h for intracellular cytokine staining. Percentages in each quadrant are indicated on the plots. The data shown are from one experiment representative of three experiments.

DC1 preferentially induce Th1 differentiation, CD40L-activated CD11c<sup>-</sup> plasmacytoid cell-derived DC2 preferentially induce Th2 differentiation of naive CD4<sup>+</sup> T cells (13). Thus, DC2 were added to the NKT cell cultures together with PHA, IL-2, and IL-7 at the beginning of the culture and at the end of 2 wk of culture. After a total of 4 wk of culture, NKT cells were restimulated with anti-CD3 and anti-CD28 mAbs for either 5 (for intracellular cytokine staining) or 24 h (for cytokine analyses by ELISA). In parallel, the effect of DC1 on neonatal NKT cell differentiation was analyzed.

Addition of DC2 resulted in a twofold increase in the percentage of IL-4<sup>+</sup>IFN- $\gamma$ <sup>-</sup> NKT2 cells (64%; Fig. 2 A) compared with the polyclonal stimulation with anti-CD3 and anti-CD28 mAbs (31%; Fig. 1). Only 8 and 1% of the NKT cells cultured with DC2 became IL-4<sup>+</sup>IFN- $\gamma$ <sup>+</sup> NKT0 cells and IL-4<sup>-</sup>IFN- $\gamma$ <sup>+</sup> NKT1 cells, respectively (Fig. 2 A). In contrast, addition of DC1 preferentially induced neonatal NKT cells to become IL-4<sup>-</sup>IFN- $\gamma$ <sup>+</sup> NKT1 cells (38%), and to a lesser extent IL-4<sup>+</sup>IFN- $\gamma$ <sup>+</sup> NKT0 cells (26%) and IL-4<sup>+</sup>IFN- $\gamma$ <sup>-</sup> NKT2 cells (10%; Fig. 2 A). Neonatal NKT cells cultured with DC2 produced more IL-4 (4,900–16,200 pg/ml) and similar amounts of IFN- $\gamma$  (230–1,450 pg/ml; Fig. 2 B) compared with those stimulated with anti-CD3 and anti-CD28 mAbs (548–5,559 pg/ml IL-4 and 650–1,889 pg/ml IFN- $\gamma$ ; Table I). Neonatal NKT cells cultured with DC1 produced much more IFN- $\gamma$  (97,000–222,000 pg/ml) and less IL-4 (1,400–6,900 pg/ml) than those cultured with DC2 (230–1,450 pg/ml IFN- $\gamma$  and 4,900–16,200 pg/ml IL-4; Fig. 2 B). These results suggest that DC2 further promote development of neonatal NKT cells into IL-4<sup>+</sup>IFN- $\gamma$ <sup>-</sup> NKT2 cells. In addition, a large fraction of neonatal NKT cells can be polarized into IL-4<sup>+</sup>IFN- $\gamma$ <sup>+</sup> NKT1 cells by DC1.

**Adult NKT Cells Have Limited Plasticity for Polarization Induced by DC1 or DC2.** Next, we examined whether the addition of DC2 would induce a large number of IL-4<sup>+</sup>IFN- $\gamma$ <sup>-</sup> NKT2 cells from adult NKT cells, which pro-



**Figure 2.** IL-4 and IFN- $\gamma$  production by NKT cells from cord blood or adult blood cultured with DC1 or DC2.  $V\alpha 24^+V\beta 11^+$  NKT cells were purified by cell sorting, expanded with DC1 or DC2 in the presence of PHA, IL-2, and IL-7 for two rounds (14 d each), and restimulated as in Fig. 1. (A) Intracellular staining for IL-4 and IFN- $\gamma$ . Percentages in each quadrant are indicated on the plots. The data shown are from one experiment representative of three experiments. (B) ELISA. Data from three experiments are shown by scattergram.

duce both IL-4 and IFN- $\gamma$  after polyclonal TCR stimulation (Table II, and Fig. 1). Although DC2 induced an increase in the percentage of IL-4<sup>+</sup>IFN- $\gamma$ <sup>-</sup> NKT2 cells (23%; Fig. 2 A) compared with polyclonal TCR stimula-

tion (8%; Fig. 1), the predominant population of adult NKT cells cultured with DC2 became IL-4<sup>+</sup>IFN- $\gamma$ <sup>+</sup> NKT0 cells (63%; Fig. 2 A). Accordingly, adult NKT cells cultured with DC2 produced large amounts of IL-4 (2,400–10,700 pg/ml) as well as IFN- $\gamma$  (5,000–46,000 pg/ml; Fig. 2 B). Similarly, adult NKT cells cultured with DC1 preferentially developed into IL-4<sup>+</sup>IFN- $\gamma$ <sup>+</sup> NKT0 cells (52%) and to a lesser extent into IL-4<sup>-</sup>IFN- $\gamma$ <sup>+</sup> NKT1 cells (28%; Fig. 2 A). Accordingly, adult NKT cells cultured with DC1 produced a large amount of IFN- $\gamma$  (48,000–124,000 pg/ml) and IL-4 (2,600–10,100 pg/ml; Fig. 2 B). These results indicate that the predominant population of adult NKT cells has an NKT0 phenotype and poor plasticity for polarization to NKT1 or NKT2 cells.

## Discussion

Accumulating findings indicate that IL-4-producing NKT cells are likely to play an important role in controlling various Th1-mediated pathological conditions such as type 1 diabetes (7) and GVHD (8). Thus, it is important to understand how the development of IL-4- versus IFN- $\gamma$ -producing NKT cells is regulated. Furthermore, expanding the population of IL-4-producing NKT cells may serve to reduce the activity of these diseases. Here we demonstrated that the rare human NKT cells isolated from adult or cord blood have great potential to expand in cultures with IL-2, IL-7, PHA, anti-CD3, and anti-CD28 mAbs. Although adult NKT cells displayed an IL-4<sup>+</sup>IFN- $\gamma$ <sup>+</sup> NKT0 phenotype, a large population of neonatal NKT cells became IL-4<sup>+</sup>IFN- $\gamma$ <sup>-</sup> NKT2 cells in this culture condition. Moreover, neonatal NKT cells could be further polarized into IL-4<sup>+</sup>IFN- $\gamma$ <sup>-</sup> NKT2 cells in cultures with DC2, whereas adult NKT cells showed poor plasticity for polarization. These results suggest that newly generated and functionally immature NKT cells, such as the ones in cord blood rather than the already primed mature NKT cells, such as the those in adult blood, may have greater potential to develop into IL-4-producing NKT2 cells and thus to inhibit Th1-mediated pathological conditions.

It has been shown that adult but not neonatal NKT cells have oligoclonally expanded and have obtained primary effector functions (11). In addition, mouse NKT cell numbers increase after birth (1). Here we showed that adult blood tends to contain more NKT cells than cord blood. These findings suggest that NKT cells may continue to be stimulated by self- and/or environmental antigens presented on CD1d, leading to chronic activation and expansion after birth. This, together with our finding that adult NKT cells produce more IFN- $\gamma$  and display less plasticity for polarization than neonatal NKT cells, suggests that NKT cells may progressively acquire the bias to produce IFN- $\gamma$  by chronic stimulation and may thus trigger the onset of autoimmune diabetes in predisposed individuals at certain ages (6).

It remains to be determined whether neonatal NKT cells have an intrinsic tendency to differentiate into IL-4<sup>+</sup>IFN- $\gamma$ <sup>-</sup> NKT2 cells or if the culture conditions used in this study selectively expanded the cells that had acquired the ability

to produce this pattern of cytokines. Nevertheless, the clear differences in cytokine profiles between neonatal and adult NKT cells cultured in comparable conditions indicate greater flexibility of neonatal NKT cells in changing their cytokine profiles as a population level. This property of neonatal NKT cells may be related to their functional immaturity, as demonstrated by the inability to produce IL-4 or IFN- $\gamma$  upon primary stimulation (11), and is reminiscent of a property of conventional naive CD4<sup>+</sup> T cells, which need to be primed to acquire the ability to produce Th1 or Th2 cytokines.

The invariant V $\alpha$ 24<sup>+</sup> TCR on human NKT cells specifically recognizes CD1d (21). CD40L-activated DC1 expressed a low level of CD1d as reported (15), and anti-CD1d mAb diminished IFN- $\gamma$  production by NKT cells cultured with DC1 (data not shown). However, CD40L-activated DC2 did not express a detectable level of CD1d, and anti-CD1d mAb did not diminish IFN- $\gamma$  production by NKT cells cultured with DC2 (data not shown). Thus, DC1 stimulate NKT cells through CD1d, whereas DC2 promote NKT2 cell differentiation in a TCR-independent manner, as DCs modulate functions of B cells (22) and NK cells (23) independently of the interaction between MHC class molecules and TCRs. In addition, compared with culture with anti-CD3 and anti-CD28 mAbs, culture with DC1 or DC2 overall increased the absolute level of cytokines from NKT cells, except for IFN- $\gamma$  from neonatal NKT cells cultured with DC2. This enhancement of cytokine production is probably due to combined effects of various costimulatory molecules and cytokines derived from DCs.

In summary, this study demonstrates the remarkable capacity of cord blood NKT cells to preferentially develop into IL-4<sup>+</sup>IFN- $\gamma$ <sup>-</sup> NKT2 cells. Furthermore, the cytokine-producing capacity of NKT cells can be modulated by the distinct subsets of DCs, DC1 and DC2. Developing efficient methods to isolate and expand NKT cells, particularly the IL-4<sup>+</sup>IFN- $\gamma$ <sup>-</sup> NKT2 population, may offer the possibility of using them in cell therapies for autoimmune diseases and GVHD.

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