Interleukin-18 regulates T helper 1 or 2 immune responses of human cord blood CD4+ Vα24+Vß11+ natural killer T cells

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Abstract. Natural killer T (NKT) cells, exhibiting both T-cell and NK-cell markers, are known to regulate immune responses by secreting T-helper (Th) 1 and Th2 cytokines. We analyzed NKT cells in cord blood (CB) for phenotypical and functional characteristics and regulatory mechanisms that control Th1 and Th2 determination. Human CB Vα24+Vß11+ NKT cells were predominantly the CD4+ single positive (SP) phenotype (approximately 96%), in contrast to adult peripheral blood Vα24+Vß11+ NKT cells which are composed of a dominant population of the CD4-CD8- double negative (DN) phenotype and a minor population of the CD4+ SP phenotype. The CB CD4+ Vα24+ NKT cells, following stimulation with the primary culture, gained the capacity to secrete interferon (IFN)-γ, a Th1 cytokine, and interleukin (IL)-13, a Th2 cytokine. The combination of IL-18 and IL-12 induced IFN-γ production in CB CD4+ Vα24+ NKT cells, while IL-18 in combination with IL-2 induced IL-13 production in these cells. Thus, IL-18 regulates the determination of the Th1 or Th2 immune response by human CD4+ Vα24+ NKT cells through different cytokine combinations.

Introduction

Natural killer T (NKT) cells have been implicated in various immune responses, including bacterial infection, tumor rejection, immune tolerance and the regulation of autoimmune diseases (1,2). NKT cells recognize glycolipids [α-galactosylceramide (α-GalCer)] presented by the MHC-like molecule CD1d. Although murine CD1d-restricted NKT cells express an invariant T-cell receptor (TCR) consisting of Vα14/Jα18 paired with Vß8, Vß7 or Vß2 (Vα14+ NKT cells), human CD1d-restricted NKT cells express a TCR consisting of a Vα24/JαQ chain paired with a Vß11 chain (Vα24+ NKT cells) (1,2). In human adult peripheral blood (PB), CD4-CD8- double negative (DN) cells consist predominantly of Vα24+ NKT cells (2-4) which have been reported to be decreased in patients with autoimmune diseases (5) and type 1 diabetes (6). NKT cells potentially have the ability to produce both T helper (Th) 1 cytokines, such as interferon (IFN)-γ, and Th2 cytokines, such as interleukin (IL)-4 and IL-13 (1). The former is responsible for inducing the inflammatory process while the latter is responsible for the anti-inflammatory or allergic process (7).

Human umbilical cord blood (CB) is known to contain NKT cells with an activated/memory phenotype, usually observed in adult peripheral blood NKT cells (8,9), although the cellular immune system of the newborn infant is generally known to be immature and hypo-responsive in comparison to the adult system (10). Functionally, cord blood NKT cells do not produce cytokines upon primary stimulation (8), but produce Th1 or Th2 cytokines upon secondary activation (11).

IL-18 is a multifunctional cytokine which augments both innate and acquired immunity and potentiates the Th1 and Th2 reaction (12). IL-18 in combination with IL-12 strongly augments the IFN-γ production by T cells (12,13). A high level of IL-18Rα expression correlates with the ability of IL-18 to induce IFN-γ production (14). The synergistic effect of IL-12 and IL-18 occurs in the absence of TCR ligation and this may represent an alternative pathway for the production of IFN-γ during the inflammatory response (12). IL-18 also has the paradoxical capacity to promote the Th2 response under the different cytokine milieu. IL-18 in combination with IL-2 induces the production of Th2 cytokines, such as IL-4 and IL-13, in the T cells (12). In mice, the administration of IL-18 caused the production of Th2 cytokines by Vα14+ NKT cells (15). However, the effect of IL-18 on human Vα24+ NKT cells has not yet been investigated.

In the present study, we analyzed phenotypical and functional characteristics of CB Vα24+ NKT cells and the regulatory mechanism that determines the Th1 and Th2 profile in these cells. We showed that IL-18 in combination with IL-12

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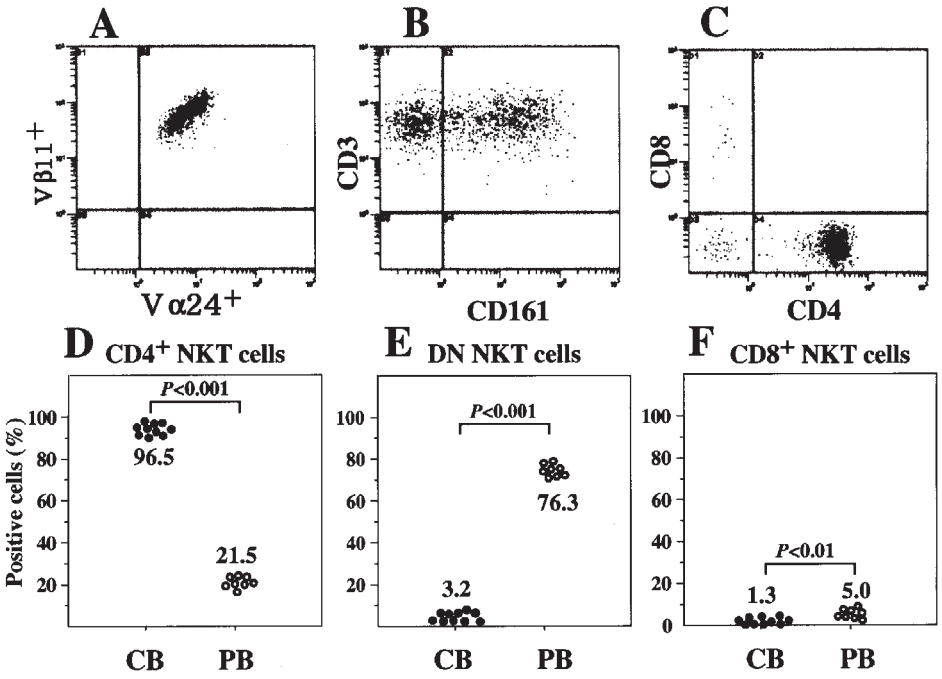

Figure 1. Analysis of surface phenotype of Vα24+Vß11+ NKT cells in cord blood and adult blood. Vα24+ cells were enriched from fresh cord blood and adult peripheral blood by immunomagnetic bead separation, and further subjected to flow cytometrical analysis. (A) Vα24+Vß11+ cells were gated in the lymphocyte fraction focused on forward scatter and side scatter. (B) CD3 and CD161 expression of Vα24+Vß11+ gated NKT cells. (C) CD4 and CD8 expression of the CB Vα24+Vß11+ NKT cells. The data are representative of 10 independent experiments (A-C). Proportion of CD4+ (D), DN (E) or CD8+ (F) NKT cells in cord blood (CB) (n=10) and adult peripheral blood (PB) (n=8) Vα24+Vß11+ NKT cells. The numbers indicate the mean percentages of positive cells of each phenotype in Vα24+Vß11+ NKT cells.

was able to induce the CB CD4+ Vα24+ NKT cells to produce Th1 cytokine, while IL-18 in combination with IL-2 was able to induce Th2 cytokine production in these cells. Therefore, IL-18 regulated the Th1 or Th2 determination by the CB CD4+ Vα24+ NKT cells.

Materials and methods

Cell preparation and culture. Umbilical cord blood (CB) was obtained from normal, full-term deliveries and adult peripheral blood was obtained by venous puncture after receiving informed consent. Mononuclear cells (MC) were separated by density gradient centrifugation using Ficoll-Paque (Amersham Pharmacia, Uppsala, Sweden). Vα24+ cells were obtained by treatment with a phycoerythrin (PE)-conjugated-anti-Vα24 antibody (Immunotech, Marseille Cedex, France) and anti-PE-magnetic beads (Miltenyi Biotec, CA, USA) and by sorting with the automated magnetic cell sorter (autoMACS) system (Miltenyi Biotec). Vα24+ cells were cultured for 14 days in RPMI-1640 medium (Sigma Chemical Co., St. Louis, MO, USA) containing 10% fetal calf serum (Gibco BRL, Grand Island, NY, USA) in the presence of 10 ng/ml human recombinant IL-2 (Pepro Tech Inc., Rocky Hill, NJ) and 10 ng/ml α-galactosylceramide [(α-GalCer) KRN 7000; kindly provided by Kirin Brewery Co. Ltd., Tokyo, Japan] (16).

Flow cytometry. Vα24+ cells freshly prepared or cultured in the presence of α-GalCer and IL-2 were stained with various combinations of monoclonal antibodies (Ab), and subjected to a four-color immunofluorescence analysis using an EPICS XL flow cytometer (Beckman Coulter, Miami, FL, USA). Fluorescein isothiocyanate (FITC)-conjugated anti-Vß11 Ab (Immunotech), PE- or biotin-conjugated anti-Vα24 Ab, phycoerythrin-Texas Red (ECD)-conjugated-anti-CD8 Ab (Beckman Coulter), PC5 conjugated anti-CD3 or CD4 Ab and PE-conjugated-anti-CD161 (Immunotech), and PE-conjugated anti-IL-12Rß1 and -IL-18Rα Ab (R&D Systems, Minneapolis, MN) were used. The intracellular cytokines were stained with PE-conjugated anti-IL-4 Ab and FITC-conjugated anti-IFN-γ Ab (Immunotech), after the cells were fixed and permeabilized using an Intra Prep permeabilization kit (Beckman Coulter). Ionomycin (Sigma) and Phorbol-12-myristate-13-acetate (PMA) (Calbiochem, Germany) were used for the augmentation of cytokine production. Brefeldin A (Sigma) was added for 4 h before the end of incubation. Appropriate isotype-matched control Abs were used in all experiments.

Assay for cytokines produced by NKT cells. CD4+ Vα24+ NKT cells cultured in the presence of IL-2 and α-GalCer were washed twice and incubated in RPMI-1640 medium in the presence of different combinations of IL-2 (10 ng/ml), IL-12 (10 ng/ml) (Pepro Tech) and IL-18 (10 ng/ml) (Hayashibara Biochemical Laboratories, Inc., Okayama, Japan) for 7 days in 96-well flat-bottom tissue culture plates. The culture supernatants were harvested and assayed for IFN-γ and IL-4 using commercially available ELISA kits (Biosource, Camarillo, CA, USA).

Statistical analysis. The data are presented as the mean ± standard deviation (SD). Student's t-test was used to compare two groups. P values <0.05 were considered to be statistically significant.

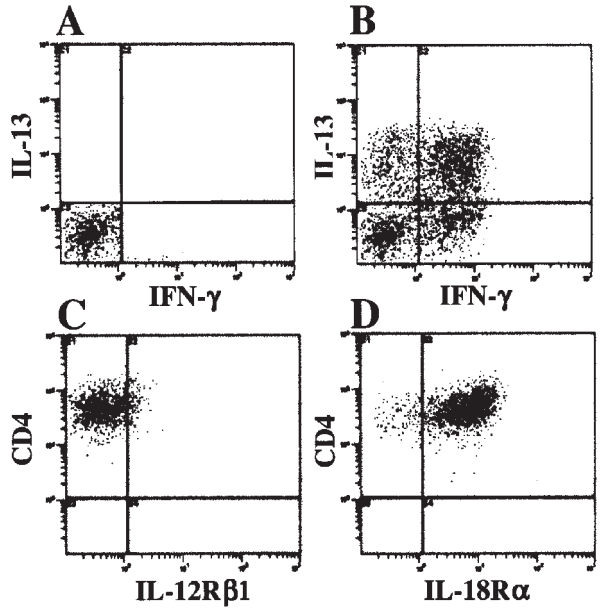

Figure 2. Cytokine production and IL-12Rß1/IL-18Rα expression in CB CD4+ Vα24+ NKT cells. Immunomagnetic bead-separated Vα24+ cells were cultured with α-GalCer and IL-2 for 14 days, which resulted in the preferential expansion of CD4+ Vα24+Vß11+ NKT cells with >95% purity. Intracellular staining for IL-13 and IFN-γ in fresh (A) and cultured CB CD4+ Vα24+Vß11+ NKT cells (B) after stimulation by ionomysin/PMA for 4 h. Expression of IL-12Rß1 (C) and IL-18Rα (D) on cultured CD4+ Vα24+Vß11+ NKT cells. The data are representative of 4 independent experiments (A-D).

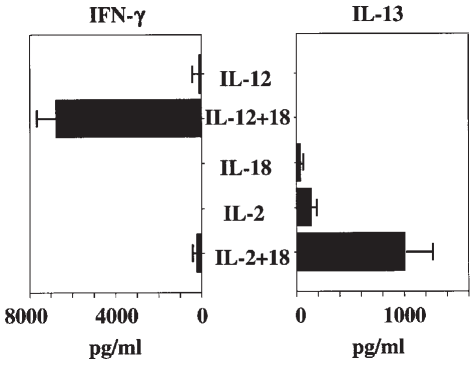

Figure 3. IFN-γ/IL-13 secretion by CD4+ Vα24+ NKT cells. Cultured CD4+ Vα24+Vß11+ NKT cells were washed and incubated in the presence of IL-2 or IL-12 with or without IL-18. The culture supernatants were harvested 7 days later and analyzed by ELISA. The data are shown as the mean ± SD from 5 separate experiments.

Results

Phenotypical analysis of cord blood NKT cells. We first phenotypically analyzed the Vα24+Vß11+ NKT cells that were freshly prepared from the cord blood (CB) and the adult peripheral blood (PB). We enriched the Vα24+ cells using immunomagnetic beads, and identified the Vα24+Vß11+ cells by flow cytometry (Fig. 1A). Seventy percent of the CB CD3+ Vα24+Vß11+ cells expressed a natural killer marker, CD161 (Fig. 1B). Freshly prepared CB Vα24+Vß11+ NKT cells were mostly the CD4+ single positive (SP) phenotype (Fig. 1C). As shown in Fig. 1D, 96.5±1.6% of the Vα24+Vß11+ NKT cells were positive for CD4, in sharp contrast to the finding that only 21.5±10.1% of the adult PB NKT cells were positive for CD4 ($P<0.001$). In addition, only 3.2±1.3% of the CB Vα24+Vß11+ NKT cells were the DN phenotype (Fig. 1E). In contrast, 76.3±10.7% of the adult PB Vα24+Vß11+ NKT cells were the DN phenotype ($P<0.001$). We observed that 1.3±1.0% of the CB NKT cells and 5.0±0.9% of the adult PB NKT cells were positive for CD8 (Fig. 1F).

Cytokine production and receptor expression by CB NKT cells. We examined the cytokine production in CB NKT cells by intracellular staining. Freshly prepared CB NKT cells did not produce IFN-γ or IL-13 upon primary stimulation (Fig. 2A) (8). Culturing CB Vα24+ cells with IL-2 and α-GalCer for 14 days yielded >95% CD4+ Vα24+ Vß11+ NKT cells. The CD4+ Vα24+ NKT cells produced both IFN-γ and IL-13 upon treatment with PMA/ionomycin (Fig. 2B), indicating that CB CD4+ Vα24+ NKT cells have the capacity to produce both Th1 and Th2 cytokines. We next examined the IL-18 receptor expression on CB NKT cells. Freshly prepared CB CD4+ Vα24+ NKT cells expressed negligible levels of the IL-12 receptor (R) ß1 and low levels of IL-18Rα (data not shown). Culturing CB CD4+ Vα24+ NKT cells with IL-2 and α-GalCer for 14 days up-regulated the IL-12Rß1 and IL-18Rα expression (Fig. 2C and D).

IL-18 regulates Th1 and Th2 responses of NKT cells. We examined the effect of IL-18 on the cytokine production in human CD4+ Vα24+ NKT cells in the presence of different combinations of IL-12, IL-2 and IL-18, as shown in Fig. 3. When CD4+ Vα24+ NKT cells were incubated with IL-12 alone, low levels of IFN-γ were produced. Combined stimulation with IL-18 and IL-12 induced markedly high levels of IFN-γ. However, IL-13 was not produced by the stimulation with IL-12 alone or IL-12+IL-18. In contrast, IL-18 or IL-2 alone produced low levels of IL-13, and a combination of IL-18 and IL-2 induced markedly high levels of IL-13. These results show that IL-18 differentially regulates human CD4+ Vα24+ NKT cells to produce IFN-γ and IL-13 in collaboration with IL-12 and IL-2, respectively.

Discussion

The secretion of Th1 and Th2 cytokines by NKT cells underlies the immuno-regulatory properties of these cells. NKT cells can suppress type 1 diabetes in non-obese diabetic (NOD) mice through the secretion of IL-4 and IL-10 (17). Defects of NKT cells may contribute to the pathogenesis of type 1 diabetes in both NOD mice and humans (6). NKT cells are reported to suppress carcinogenesis through IFN-γ (18). Furthermore, the secretion of the Th2 cytokine IL-13 was found to inhibit the immune rejection of tumor grafts (19). These findings suggest that the regulated expression of Th1 and Th2 cytokines by NKT cells may control the outcome of some disease conditions. It is thus important to understand how the production of Th1 and Th2 cytokines by human NKT cells is regulated. In this study, we addressed this issue using human CB Vα24+Vß11+ NKT cells.

Although both murine and human NKT cells are restricted by CD1d, there are several phenotypical and functional

differences between murine and human NKT cells and even between human neonatal and adult NKT cells (1,20,21). Murine Vα14+ NKT cells primarily appear in the thymus as a result of TCR-α rearrangement in CD4 CD8 double-positive (DP) thymocytes. These cells are selected by CD1d in a thymic enviroment, and matured into CD4+ single positive (SP) and CD4 CD8 double-negative (DN) Vα14+ T cells (21). The ratio of mature CD4+ to DN NKT thymocytes is approximately 6:4 in the murine thymus, which is close to that in the peripheral blood and other tissues. Murine CD4+ and DN Vα14+ T cells are functionally identical and depend mainly on IL-15 for their survival and homeostatic expansion in the periphery (21). In humans, in contrast to mice, NKT cells are produced in the thymus with CD4+ dominance (>90%), and peripheral blood CD4+ NKT cells are maintained mainly by the thymic output with limited peripheral expansion (20). On the other hand, DN NKT cells undergo extensive peripheral expansion to form a major population in adult peripheral blood (20). In this study, we confirmed the essential phenotypical differences between NKT cells in cord blood and adult blood. DN NKT cells constituted the major population in adult PB Vα24+Vß11+ NKT cells, whereas CD4+ NKT cells were the majority in CB Vα24+ NKT cells (Fig. 1).

There have been a limited number of reports documenting the mechanism underlying the Th2 versus Th1 cytokine secretion by NKT cells. In mice, altered αGalCer ligands with shorter sphingosine chains activate the Th2 function (22), while NK1.1 signaling favors the Th1 response (23). Although both murine CD4+ and DN Vα14+ T cells are functionally identical, human Vα24+ NKT cells in the adult peripheral blood consist of two populations with distinct functions: CD4+ NKT cells that produce both IFN-γ and IL-4 and DN NKT cells that produce IFN-γ but not IL-4 (24). We showed that cord blood NKT cells were mainly composed of CD4+ Vα24+ NKT cells (Fig. 1) and able to produce both Th1 and Th2 cytokines through secondary but not primary stimulation (Fig. 2A and B). Using cord blood NKT cells, Kadowaki *et al* have demonstrated that type 1 myeloid dendritic cells (DC1) preferentially induce CB NKT cells to produce IFN-γ, while type 2 lymphoid DC (DC2) preferentially induce IL-4 production (11). In the present study, we showed that the determination toward Th1 or Th2 cytokine production by human CD4+ NKT cells was regulated by cytokine stimulation. We found that IL-18 was a key cytokine for Th1 or Th2 determination of human NKT cells. In human CD4+ Vα24+ NKT cells (Fig. 3), the combined stimulation of IL-18 with IL-12 promoted IFN-γ production, while the combined stimulation of IL-18 with IL-2 promoted IL-13 production. This regulation of the Th1/Th2 determination occurred in the absence of TCR engagement. Since the dendritic cells produce IL-12 and IL-18, and DC1 is a high producer of IL-12, our observation that IL-12 plus IL-18 promotes IFN-γ production may be consistent with the observation by Kadowaki *et al* that DC1 preferentially induces IFN-γ production in CB NKT cells (11).

In conclusion, the present study showed that IL-18 controls the Th1 and Th2 immune response of human CD4+ Vα24+ NKT cells through different cytokine combinations. Present results may provide an understanding of the regulation of the Th1 or Th2 determination of NKT cells which is a critical event for immuno-regulation by these cells.

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