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Enhancement of Human Cord Blood CD34+ Cell-Derived NK Cell Cytotoxicity by Dendritic Cells

Ying Yu,*† Masao Hagihara,2*‡ Kiyoshi Ando,*‡ Balgansuren Gansuvd,‡ Hideyuki Matsuzawa,* Takahide Tsuchiya,* Yoko Ueda,† Hiroyasu Inoue,*‡ Tomomitsu Hotta,‡ and Shunichi Kato*‡

NK cells and dendritic cells (DCs) are both important in the innate host defense. However, the role of DCs in NK cell-mediated cytotoxicity is unclear. In this study, we designed two culture systems in which human cord blood CD34+ cells from the same donor were induced to generate NK cells and DCs, respectively. Coculture of the NK cells with DCs resulted in significant enhancement of NK cell cytotoxicity and IFN-γ production. However, NK cell cytotoxicity and IFN-γ production were not increased when NK cells and DCs were grown together separated by a transwell membrane. Functional studies demonstrated that 1) concanamycin A, a selective inhibitor of perforin/granzyme B-based cytolytic pathways, blocked DC-stimulated NK cytotoxicity against K562 cells; and 2) neutralizing mAb against Fas ligand (FasL) significantly reduced DC-stimulated NK cytotoxicity against Fas-positive Jurkat cells. In addition, a marked increase of FasL mRNA and FasL protein expression was observed in DC-stimulated NK cells. The addition of neutralizing mAb against IL-18 and IL-12 significantly suppressed DC-stimulated NK cell cytotoxicity. Neutralizing IFN-γ Ab almost completely inhibited NK cell cytotoxicity against Jurkat cells. These observations suggest that DCs enhance NK cell cytotoxicity by up-regulating both perforin/granzyme B- and FasL/Fas-based pathways. Direct interaction between DCs and NK cells is necessary for DC-mediated enhancement of NK cell cytotoxicity. Furthermore, DC-derived IL-18 and IL-12 were involved in the up-regulation of NK cell cytotoxicity, and endogenous IFN-γ production plays an important role in Fas-mediated cytotoxicity. The Journal of Immunology, 2001, 166: 1590–1600.

Natural killer (NK) cells are important as the first line of host defense, and as one of the final effector cells in resistance to tumors, metastases, and viral infections (1, 2). The perforin/granzyme B- and the Fas ligand (FasL)/Fas-based cytolytic pathways play central roles in NK cell-mediated cytotoxicity (3). Several endogenous cytokines, such as IL-15, in conjunction with IFN-γ, IL-12, and IL-18, have been shown to stimulate NK cell cytotoxicity in the primary host defense against pathogens (4–6).

Dendritic cells (DCs) are the most potent APCs in the initiation of an immune response and adaptive immunity, and may be one of the earliest cell types exposed to pathogens (7–9). Expression of large numbers of different cytokines and costimulatory factors provides DCs with an ability to induce primary anti-tumor immune responses (9, 10).

Both NK cells and DCs appear to be involved in the initiation of an immune response, and DC-derived cytokines, such as IL-12 and IL-18, are essential in initiating the activation of NK cells in response to pathogens (11–13). Therefore, it was proposed that DCs might be involved in the differentiation and maturation of NK cells. Fernandez et al. (1999) reported that adaptively transferred or Flt3 ligand-expanded DCs enhanced NK cell-dependent antitumor effects in mice with MHC class I-negative tumors. They also suggested that cell-to-cell contact between DCs and resting NK cells resulted in a substantial increase in both NK cell cytotoxicity and IFN-γ production (14). However, the mechanism by which they do so has not been defined.

In this study, we used two culture systems with purified human cord blood CD34+ progenitors from the same donor. In one culture we generated a large number of NK cells and in the other culture DCs. Then, we compared the effects of DCs on NK cells by coculturing them directly or separated by a transwell membrane, and examined the roles of the FasL/Fas-based pathway and perforin/granzyme B-based pathway in modulating DC-stimulated NK cell cytotoxicity. The roles of endogenous IL-12 and IL-18 were investigated because DCs have been described as potential sources of IL-12 and IL-18 (10, 15). In addition, we evaluated the roles of endogenous IFN-γ production in DC-stimulated NK cell cytotoxicity.

Materials and Methods

Human umbilical cord blood (HUCB)

HUCB samples were obtained during normal full-term deliveries according to the Tokai University Committee on Clinical Investigation. The cells were stored at room temperature and processed within 24 h of collection.

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Purification of CD34+ cells from HUCB

Mononuclear cells were isolated from HUCB by Ficoll-Hypaque (gradient = 1.077 g/ml) density gradient centrifugation. CD34+ cells were isolated from mononuclear cells using magnetic-activated cell sorting (MACS) immunomagnetic separation system (Miltenyi Biotec, Glodach, Germany) as described previously (16). The purity of CD34+ cells was over 99% as determined by flow cytometry using FITC-conjugated anti-human CD34 mAb (ImmunoTech, Marseille, France).

Generation of HUCB CD34+ cell-derived NK cells

HUCB CD34+ cell-derived NK cells were prepared according to a modified protocol originally described by Carayol et al. (17). Briefly, CD34+ cells (2 × 10^6 cells per well) were incubated in six-well microplates (Iwaki Glass, Chiba, Japan) in 2 ml complete medium (α-MEM medium, Life Technologies, Grand Island, NY) supplemented with 10% heat-inactivated FBS (Cell-Banker; Nichirei, Tokyo, Japan), 100 U/ml penicillin, and 100 μg/ml streptomycin (Life Technologies)). Cytokines were supplemented as indicated with 30–50 ng/ml human recombinant (hr) stem cell factor (SCF), a gift from Agen, Thousand Oaks, CA) and 30–50 ng/ml hr-hG-CSF (Peprotech, London, U.K.). The plates were incubated for 4–5 wk at 37°C in a humidified atmosphere with 5% CO2. Half of the medium volume was replaced with fresh medium and cytokines once every week.

Generation of HUCB CD34+ cell-derived DCs

CD34+ cell-derived DCs were generated from the same donor according to a modified two-step protocol originally described by Arrighi et al. (18). Briefly, CD34+ cells were first incubated for 3 wk in six-well plates (2 × 10^6 cells/well) in 2 ml complete medium containing hrSCF (50 ng/ml), hr-GM-CSF (50 ng/ml, provided by Kirin Brewery, Gunmd, Japan) and Flt3-L (20 ng/ml; Peprotech). Then these cells were exposed to secondary condition medium containing 50 ng/ml hr-GM-CSF and 10 ng/ml IL-4 (BioSource International, Camarillo, CA) for 10 days and to the same medium with 10 ng/ml TNF-α (BioSource International) for an additional 2 days. Cultures were maintained in a humidified atmosphere at 37°C and 5% CO2. Half of the medium volume was replaced with fresh medium and cytokines once every week.

Flow cytometric immunophenotyping and cell morphology

The surface markers of the cells were analyzed by FACSCalibur flow cytometry using CellQuest software (Becton Dickinson, Mountain View, CA) as described before (16, 19). Briefly, aliquots of fresh cells or cultured cells were suspended in buffer containing 0.5% BSA and 2 mM EDTA in PBS (staining buffer) and stained for 20–30 min with specific FITC-, PE-conjugated mAbs. Unbound Abs were removed by two washes with staining buffer, and then the cells were resuspended in staining buffer and subjected to a two-color flow cytometric analysis. Cells labeled with FITC- and PE-conjugated mouse isotype-matched Abs (PharMingen, San Diego, CA) were used as controls. Living cells were identified on the basis of their physical characteristics (forward and side scatter, i.e., FSC and SSC, respectively).

FITC-conjugated Ab directed at CD2, CD3, CD7, CD16, CD56 (all obtained from Becton Dickinson, San Jose, CA), CD94 and CD161 (PharMingen), and PE-conjugated Ab directed at CD27 and CD56 (Immunootech), CD28 (Becton Dickinson), CD40 ligand (CD154; Medical and Biological Laboratories, Nagoya, Japan) were used to determine the phenotypes of NK cells.

For DC surface marker analysis, cultured cells were first incubated with rabbit immunoglobulin fraction (Dako, Glostrup, Denmark) to block the Fc receptor. Then FITC-conjugated Abs directed at CD1a (Dako), CD40, CD54, and HLA-DR (Becton Dickinson), HLA-class I (hybridoma purchased from American Type Culture Collection, Manassas, VA, and purified Abs were FITC-labeled by Interdepartmental Labs, Laboratory of Structure and Function Research, Tokai University) and PE-conjugated Ab directed at CD11c, CD14, CD58 (Becton Dickinson), CD80, CD83, and CD86 (Immunootech) were used to determine the phenotypes of DCs.

To observe cell morphology, cultured cells were mounted onto slides with a cytospin, and the cells on the glass were then stained with Wright-Giemsa. The morphology of these cells was observed under the optical microscope.

 Coculture systems

HUCB CD34+ cell-derived NK cells were resuspended at a concentration of 1 × 10^3/ml in 1.5 ml complete medium in 24-well microplates (Iwaki Glass). HUCB CD34+ cell-derived DCs were added at a concentration of 3 × 10^3/ml in 1 ml complete medium in the same wells. The ratio of NK cells to DCs was 5:1. Twenty-four-well microplates equipped with a transwell insert (Becton Dickinson Labware, Mountain View, CA) were used in preventing direct contact of NK cells and DCs in cocultures. DCs were plated in the lower wells, and NK cells were added to the upper wells in the same final concentration as described above. The soluble factors could diffuse freely through a microporous polycarbonate membrane (0.45 μm). NK cells or DCs alone were plated in 24-well microplates as controls. To maintain the activity and survival of NK cells, hrIL-15 was added to every culture medium at 10 ng/ml. After coculture for 48 h, cells were subjected to further examination.

NK cell cytotoxicity

NK cell cytotoxicity was measured in a standard 4-h 51Cr release assay. Briefly, cells from the NK cell/DC cocultures or single cultures incubated for 48 h were collected. Viable trypan blue-excluding cultured cells were counted, with the exact number of NK cells calculated by using actual percentage of CD56+ cells. The cells were then suspended in fresh complete medium at appropriate concentrations and used as effector cells.

K562, Jurkat, and Daudi cells were used as target cells. One million target cells were labeled with 25 μl Na2CrO4 (sodium chromate, 1 mCi/ml; Amersham International, Bucks, U.K.) by incubation for 1.5–2 h at 37°C in 5% CO2. Labeled cells were washed three times in complete medium and resuspended in complete RPMI 1640 medium at a concentration of 1 × 10^5 cells/ml. Mixtures of 100 μl of effector cells and 50 μl of 51Cr-labeled target cells (5 × 10^3 cells) were incubated for 4 h at 37°C in 5% CO2 in 96-well U-bottom culture plates (Nunc, Kastrup, Denmark) at various ratios of effector cells and target cells (ranging from 15:1 to 0.1:1). After centrifugation at 1500 rpm for 5 min, 100 μl of supernatant was harvested from each well, and 51Cr release was determined using a gamma scintillation counter (Packard Instrument Company, Downers Grove, IL). Spontaneous and maximum release were determined from aliquots of supernatant from wells in which only radiolabeled target cells were incubated in complete medium or in 1 N HCl. Spontaneous lysis ranged from 8 to 10% of the maximum release. The percent specific lysis was calculated by the following formula: percent specific lysis = (experimental 51Cr release − spontaneous 51Cr release)/maximum 51Cr release − spontaneous 51Cr release) × 100.

Intracellular flow cytometric analysis

Intracellular INF-γ staining was performed as previously described (20). Cells (2–5 × 10^5) from the NK cell/DC cocultures or NK single cultures were stimulated with 20 ng/ml PMA plus 10 μg/ml ionomycin in 1 ml of complete medium in the presence of the intracellular transporter inhibitor brefeldin A (10 μg/ml) for 4 h at 37°C. At the end of the incubation period, the cells were stained with PE-conjugated anti-CD56 mAb for 20 min at room temperature. Cells were then fixed and permeabilized with FACS permeabilizing solution (Becton Dickinson) for 10 min at room temperature. The permeabilized cells were incubated for 30 min at 4°C with FITC-conjugated IL-12 (1591; PharMingen) or mouse IgG2a, Becton Dickinson) or an appropriate negative control. At least 5000 cells per aliquot were analyzed by flow cytometry and gated on the presumptive lymphoid region to exclude DCs by size and granularity. Analysis was performed with Cell Quest software.

For intracellular perforin or FasL staining, 2–5 × 10^5 cells from the NK cell/DC cocultures, DC single cultures, or NK single cultures were first stained with PE-conjugated CD56 mAb or FITC-conjugated CD56 mAb followed by treatment with permeabilization buffer as described above. The fixed cells were incubated for 30 min at room temperature with FITC-conjugated murine anti-human perforin mAb (6C9, IgG2b; PharMingen) or biotinylated murine anti-human FasL mAb (NOK1, IgG2a; PharMingen). Unbound Abs were removed by two washes with staining buffer. To detect the FasL expression, the cells were stained with streptavidin PE (SA-PE; Becton Dickinson) in staining buffer for 30 min at room temperature. In addition, to better evaluate and compare the levels of FasL expressed on the cell surface and within the cell, nonpermeabilized cells were analyzed.

For intracellular IL-12 and IL-18 staining of DCs, 5 × 10^5 DCs were fixed and permeabilized as described above. The fixed cells were incubated for 30 min at 4°C with rabbit anti-human IL-12 mAb (C8.1, IgG1, specific against the p40 and the p70 subunits; BioSource International) or murine anti-human IL-18 mAb (125-2H, IgG1; Medical and Biological Laboratories) at 1.0 μg/ml. Control samples were treated with control murine IgG. After washing twice with staining buffer, the cells were resuspended in staining buffer and stained with goat anti-mouse Ig FITC (GAM; Becton Dickinson) for 30 min at room temperature. At least 10,000 cells per aliquot were analyzed by flow cytometry and gated on cells that expressed large size and high granularity.

Assessment of INF-γ in culture supernatants

Cell-free supernatants were collected from each culture and stored at −80°C until assayed. INF-γ concentrations in culture supernatants were

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determined following the manufacturer’s recommended procedure using the human IFN-γ ELISA kit (R&D Systems, Minneapolis, MN). The limit of detection was 1 ng/ml.

**RNA extraction and RT-PCR**

Total RNA was isolated from cultured cells by using the single-step method (21) with ISOGEN-LS reagent (Nippon Gene, Toyama, Japan) as per the manufacturer’s instructions. The amount of RNA was measured by spectrophotometric absorbance at 260 nm. For all RT-PCR experiments, each sample, containing 2 μg RNA were used to synthesize first-strand cDNA by using avian myeloblastosis virus (AMV) reverse transcriptase XL (RNA PCR Kit, AMV, Ver.2.1; Takara Shuzo, Otsu, Japan) in a reaction mixture of 20 μl (10 mmol/L Tris-HCl, 50 mmol/L KCl, 2.5 mmol/L MgCl₂, 10 mmol/L DTT, 1.25 mmol/L oligo(dt), 0.5 mmol/L dNTPs and 5 U AMV reverse transcriptase XL). Reverse transcription was performed for 60 min at 42°C. cDNA was amplified by using GeneAmp PCR system 2400 (Perkin-Elmer/Cetus, Norwalk, CT) in a 50-μl reaction mixture (2 μl of cDNA products, 10 mmol/L Tris-HCl, 50 mmol/L KCl, 1.5 mmol/L MgCl₂, 0.2 mmol/L dNTPs, 0.2 nmol/L sense and antisense primers, and 1.25 U Taq DNA polymerase (Takara TaqTM, Takara Shuzo)). The amplification protocol involved a denaturation step at 94°C for 5 min, followed by 30–35 cycles of 94°C for 30 s, 55°C for 1 min, and 72°C for 30 s, and finally an extension stage at 72°C for 10 min. The oligonucleotides used as PCR primers were synthesized based on published sequences. Among them, primers for perforin (22) were sense primer, 5′-ACAGG CACATCTGTGCTG-3′ and antisense primer, 5′-GAAGGAGGCGCTACATCTGGCTT-3′; primers for granzyme B (23) were sense primer, 5′-TGCGAGAAATGCAAGTGGC-3′ and antisense primer, 5′-GAGGGCATGCTATGTTCTGCT-3′; and primers for FasL (24) were sense primer, 5′-CAAGTCCTTTCAACCTAGAGG-3′ and antisense primer, 5′-GAGCTTCCTGAAATTCGCTTGGAG-3′. The expected size of amplified cDNAs for the cytolytic mediators were 459, 180, and 510 bp for perforin, granzyme B, and FasL, respectively. As a control for integrity of total RNA, primer specific for GAPDH, sense primer 5′-GATGACATCAA GAAAGCTGTTG-3′ and antisense primer 5′-GCGTGAAGCATA AATTCTGCT-3′ were used. The expected size of the amplified cDNA for GAPDH was 198 bp. PCR-amplified products were separated on 2% agarose gels and visualized with GelStar gel staining (FMC Bioproducts, Rockland, ME).

**Western blot analysis**

Cultured cells were gently washed twice with cold PBS. Whole cells were extracted at 5 × 10⁶ cells/ml in lysis buffer (20 mM Tris pH 8.0, 137 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 100 μg/ml PMSF, and 30 μg/ml aprotinin) for 30 min on ice. Lysates were sonicated and centrifuged at 15,000 rpm for 15 min, and the insoluble fraction was discarded. Aliquots (10 μl) were mixed and boiled in SDS-PAGE sample buffer for 3 min. The released proteins were fractionated by 15% SDS-PAGE (Bio-Rad, Richmond, CA) and then transferred to polyvinylidene difluoride (PVDF) membranes by electroblotting in a Mini Trans-Blot Cell transfer apparatus (Bio-Rad) under conditions recommended by the manufacturer. After incubating overnight at 4°C in blocking buffer (0.1% TWEEN 20 in PBS) containing 5% Bacto skim milk powder (Difco, Troy, MI), PVDF membranes were incubated with biotin-conjugated mouse anti-human FasL Ab (1:1000 dilution, NOK-1; PharMingen) followed by strong expression of CD11c, CD40, CD54, CD58, CD80, CD83, CD86, and CD122 (28). Some of these cells were positive for CD7, CD94, and CD161, and DC-stimulated NK cells were mainly dead cells and debris; this was confirmed by trypan blue staining (data not shown).

**Administration of concanamycin A (CMA) and anti-FasL mAb**

CMA was used to inhibit the perforin/granzyme B-based NK cell cytotoxicity (25). NK cells were pretreated with 10–100 mmol/L CMA (Sigma, St. Louis, MO) for 2 h. The same agents were also added to the cultures during the 4-h ³¹Cr release assay as described (26). In these controls, the experiment release was measured both in the presence and absence of the inhibitors to ensure that the reagents alone did not alter control release. NK cells were cocultured with each type of target cell for 4 h in the presence of the neutralizing mouse anti-human FasL mAb NOK-2 (PharMingen), range 0.2–2 μg/ml, to block Fas-FasL-dependent cytotoxicity (27). NK cell cytotoxicity was analyzed using the ³¹Cr release assay. Normal murine IgG from the pooled sera of nonimmunized mice or rabbit were used as isotype controls. After washing twice with PBS, the NK cell cytotoxicity assay was performed.

**Statistical analysis**

Data are presented as mean ± SEM (SE of mean). Statistical analysis was performed with SuperANOVA or Statview version 4.11 software (Abacus Concepts, Berkeley, CA) on a power Macintosh computer. One-way ANOVA followed by Fisher’s protected least significant difference (PLSD) test was used to test for significant differences between groups when appropriate.

**Results**

**Phenotype and morphology of HUCB CD34⁺ cell-derived NK cells and DCs, and DC-stimulated NK cells**

CD34⁺ cell-derived NK cells were harvested at week 4–5 from cultures initiated with CD34⁺ cells under the conditions described above. Active CD2⁺ CD3⁻ CD56⁺ NK cells (median 93%, range from 90 to 98%) were routinely produced with the majority of cells expressing CD16⁻ CD56⁺. The population of CD34⁺ cell-derived NK cells is different from peripheral NK cells that consist mostly of CD16⁺ CD56⁺ cells with only a minor population of CD16⁻ CD56⁺ cells (28). Some of these cells were positive for CD7, CD94, and CD161, and <10% of the cells expressed CD16 or CD40 ligand (Fig. 1A). Cell morphology showed large granules within the cytoplasm, demonstrating the typical NK cell morphology (Fig. 2a, a). Flow cytometric analysis for these harvested cells revealed that as well as the cells in the presumptive lymphoid region, there was a large proportion of non-NK cells. These cells were mainly dead cells and debris; this was confirmed by trypan blue staining (data not shown).

CD34⁺ cell-derived DCs were generated in the presence of SCF, GM-CSF, and Flt-3 ligand for 3 wk followed by supplementation with IL-4 for 10 days and to the same medium with TNF-α for an additional 2 days. Enriched CD1a⁺ DCs (median 87%, range from 83 to 92%) were routinely produced and characterized by strong expression of CD1c, CD40, CD54, CD58, CD80, CD83, CD86, and HLA-DR surface marker (Fig. 1B). Cell morphology showed a sheet-like membrane, demonstrating the typical DC morphology (Fig. 2b, b).

After coculture of CD34⁺ cell-derived NK cells with DCs, the phenotypes of NK cells were examined by flow cytometric analysis of forward- and side-scatter characteristics and expression of Ags associated with NK cells. No significant differences were observed in these NK cells cultured with or without DCs (data not shown). However, we did observe that many DCs not only expressed CD83, but also expressed the NK-cell specific marker CD56 (Fig. 2A), implying that some NK cells were bound to DCs. Further studies on cell morphology also showed that some NK cells bind closely to the membrane of DCs (Fig. 2B, c and d).
FIGURE 1. Phenotypes of HUCB CD34<sup>+</sup> cell-derived NK cells and DCs. CD34<sup>+</sup> cell-derived NK cells and DCs were generated as described in Materials and Methods, and the cell phenotypes were analyzed by flow cytometry. Results are representative from more than eight independent experiments. A. The expression of Ags associated with NK cells were analyzed by using PE-conjugated anti-CD2, -CD7, -CD28, -CD56, or -CD40 ligand mAbs, and FITC-conjugated anti-CD3, -CD16, -CD56, -CD94, or -CD161 mAbs. At least 10,000 cells per aliquot were analyzed and gated on the presumptive lymphoid region by size and granularity. B. The expression of Ags associated with DCs was analyzed by using PE-conjugated anti-CD11c, -CD14, -CD54, -CD80, -CD83, or -CD86 mAbs, and FITC-conjugated anti-CD1a, -CD40, -CD58, HLA class I, or HLA-DR mAbs. At least 10,000 cells that expressed large size and high granularity were gated and analyzed.

Effects of DCs on CD34<sup>+</sup> cell-derived NK cell cytotoxicity

To examine the effects of DCs on the CD34<sup>+</sup> cell-derived NK cell cytotoxicity, we cocultured NK cells with DCs at the ratio of 5:1 for 48 h. As shown in Fig. 3, A and B, compared with NK cell single cultures, direct contact coculture with DCs enhanced NK cell cytotoxicity against K562 and Jurkat cells. When NK cells were cocultured with DCs separately by a 0.45-micron membrane, no significant enhancement of NK cell cytotoxicity against K562 cells or Jurkat cells was observed. No NK cell cytotoxicity against NK-resistant Daudi cells was measured in any of the coculture systems examined (Fig. 3C). DC single cultures had no cytolytic activity against K562, Jurkat, or Daudi cells (data not shown).

Effects of DCs on IFN-γ production of CD34<sup>+</sup> cell-derived NK cells

IFN-γ is generally believed to be an important mediator of NK cell cytotoxicity. Therefore, intracytoplasmic IFN-γ molecule synthesis, at the single-cell level, was examined by flow cytometry. Compared with NK cell single cultures, direct contact coculture of NK cells and DCs significantly increased expression of IFN-γ in NK cells (Fig. 4A). This effect was also confirmed by IFN-γ ELISA (Fig. 4B). No significant increase in IFN-γ production was observed when NK cells and DCs were grown together separated by a transwell membrane.

Effects of CMA or anti-FasL mAb on DC-stimulated NK cell cytotoxicity

The enhancement of NK cell cytotoxicity was further documented by a study in the killing machinery. CMA, an inhibitor of the perforin/granzyme B-based cytolytic activity, was used (25). As shown in Fig. 5A, at the lower concentrations of CMA used (10 nmol/L), DC-stimulated NK cell cytotoxicity against K562 was almost completely blocked. However, the enhancement of NK cell cytotoxicity against Jurkat cells was only partially blocked (Fig. 5B).

FasL/Fas-based NK cell cytotoxicity was tested in the presence of neutralizing mAb against FasL. As shown in Fig. 6B, anti-FasL mAb markedly inhibited DC-stimulated NK cell cytotoxicity against Fas-positive Jurkat cells. However, anti-FasL mAb did not affect the DC-stimulated NK cell cytotoxicity against K562 cells (Fig. 6A).

These observations confirmed the enhancing effects of DCs on NK cell cytotoxicity. They also demonstrate that both FasL-based and perforin/granzyme B-based pathways are involved in these effects.

Effects of DCs on perforin/granzyme B mRNA and perforin protein expression of CD34<sup>+</sup> cell-derived NK cells

To identify the mediators involved in DC-stimulated NK cell cytotoxicity, we initially examined perforin and granzyme B mRNA expression by using RT-PCR analysis. Fig. 7A shows the results of RT-PCR analysis of perforin and granzyme B mRNA expression in DC-stimulated NK cells, single cultured NK cells, and single cultured DCs. CD34<sup>+</sup> cell-derived NK cells, cultured with or without DCs, expressed similar mRNA levels of perforin and granzyme B. In addition, perforin or granzyme B mRNA was not expressed in DCs. The results of intracellular staining for perforin also showed no significant increase in perforin protein expression in DC-stimulated NK cells (Fig. 7B).

Effects of DCs on FasL mRNA and protein expression of CD34<sup>+</sup> cell-derived NK cells

Next, we examined DC-stimulated NK cell lysates for the presence of FasL mRNA. Fig. 8A shows the results of RT-PCR analysis of FasL mRNA expression in DC-stimulated NK cells, single cultured NK cells, and single cultured DCs. There was significantly
greater expression of FasL mRNA in DC-stimulated NK cells. However, FasL mRNA expression was only weakly detected in single cultured NK cells, and was not expressed in DCs.

To clarify whether the constitutive transcription of FasL gene in stimulated NK cells was accompanied by translation and protein expression, we analyzed the expression of FasL protein using flow cytometry and Western blot analysis. Intracytoplasmic staining showed that the expression of FasL molecule was only detected when DCs directly stimulated NK cells (Fig. 8B). Similar results were obtained using cell surface staining (data not shown). These findings were confirmed by Western blot analysis (Fig. 8C).

**Expression of intracytoplasmic IL-18 and IL-12 on CD34<sup>+</sup> cell-derived DCs**

It is reported that functional IL-18 and IL-12 can be released by human DCs (11, 29). To determine whether IL-18 and IL-12 were also expressed in CD34<sup>+</sup>-derived DCs, intracytoplasmic IL-18 and IL-12 molecule synthesis in CD34<sup>+</sup>-cell-derived DCs was examined by intracellular flow cytometric analysis. Fig. 9A showed that ~30% of DCs expressed IL-12; however, a much larger proportion (88%) of DCs expressed IL-18 (Fig. 9B).

**Effects of anti-IL-18 mAb and anti-IL-12 mAb on DC-stimulated NK cell cytotoxicity**

To examine the involvement of DC-derived endogenous IL-18 and IL-12 in the DC-stimulated NK cell cytotoxicity, NK cells and DCs were directly cocultured in the presence of neutralizing mAb against IL-12 or IL-18. As represented in Fig. 10, A and B, addition of anti-IL-12 mAb (50 μg/ml) or anti-IL-18 mAb (10 μg/ml) alone resulted in the reduction of DC-stimulated NK cell cytotoxicity against K562 and Jurkat cells. Furthermore, the effects of combinations of both anti-IL-12 mAb and anti-IL-18 mAb were determined. At the lowest concentrations of mAbs examined, DC-stimulated NK cell cytotoxicity was not affected. At the highest
concentrations of mAbs, NK cell cytotoxicity against K562 or Jurkat cells was significantly reduced, and was less than that observed with either mAb alone; however, it was still greater than that of NK cell single cultures. In addition, exogenous IL-12 (10 ng/ml) and IL-18 (10 ng/ml) enhanced the cytotoxicity of NK cells against K562 and Jurkat cells (Fig. 10, C and D). These data suggest that DC-derived endogenous IL-12 and IL-18 both play important roles in DC-stimulated NK cell cytotoxicity.

Effects of anti-IFN-γ mAb on DC-stimulated NK cell cytotoxicity
To examine whether production of endogenous IFN-γ by DC-stimulated NK cells is directly responsible for the enhancement of NK cell cytotoxicity, we incubated DC-stimulated NK cell cultures with various concentrations of anti-IFN-γ Ab and then measured NK cell cytotoxicity against K562 and Jurkat cells. Addition of anti-IFN-γ Ab inhibited DC-stimulated NK cell cytotoxicity against Jurkat cells in a dose-dependent manner (Fig. 11B); however, only minor inhibition of K562 cell cytotoxicity was observed (Fig. 11A). These data indicate that IFN-γ plays an important role in NK cell-mediated apoptosis of Jurkat cells.

Involvement of CD40 in DC-stimulated NK cell cytotoxicity
Recently, some studies have suggested that costimulatory molecules such as CD40 in humans can trigger NK cell-mediated cytotoxicity in vitro (30). We finally examined whether the high levels of CD40 on DCs are involved in the DC-stimulated NK cell cytotoxicity. As represented in Fig. 12, DC-stimulated NK cell cytotoxicity against K562 cells was inhibited in a dose-dependent manner by anti-CD40 mAb (Fig. 12A). In contrast, no significant inhibition was observed against Jurkat cells (Fig. 12B). These results indicate that CD40 expressed on DCs are responsible for mediating DC-stimulated NK cell cytotoxicity against K562 cells.

Discussion
The findings presented in this manuscript have shown that 1) DCs up-regulated HUCB CD34+ cell-derived NK cell cytotoxicity against K562 and Jurkat cells but not against Daudi cells (Fig. 3); 2) IFN-γ expression in DC-stimulated NK cells and IFN-γ production in NK-DC direct contact coculture supernatants were not increased when NK cells and DCs were grown together separated by a transwell insert (Figs. 3 and 4); 4) CMA completely abolished DC-stimulated NK cell cytotoxicity against K562 cells (Fig. 5); 5) neutralizing anti-FasL mAb markedly reduced DC-stimulated NK cell cytotoxicity against Jurkat cells (Fig. 6); 6) DCs markedly increased mRNA expression and protein production of FasL (Fig. 8) but did not affect mRNA expression of perforin and granzyme B and production of perforin (Fig. 7); 7) preadministration of neutralizing anti-IL-12 and anti-IL-18 mAb markedly reduced DC-mediated enhancement of NK cell cytotoxicity (Fig. 10); 8) endogenous IFN-γ production played an important role in NK cell-mediated apoptosis of Jurkat cells (Fig. 11); and 9) CD40 expressed on DCs mediated the DC-stimulated NK cell cytotoxicity against K562 cells (Fig. 12).
shown that IL-15 combined with the early acting cytokine SCF produced a large number of higher purity NK cells from HUCB CD34+ cells, and IL-15 (minimum 10 ng/ml) is essential to maintain their survival (data not shown).

It is well known that NK cells use multiple mechanisms to lyse different target cells. The perforin/granzyme B-based pathway appears to be the predominant cytolytic pathway (2, 39). Active perforin is secreted from NK cells in the presence of calcium. It inserts into the membrane of the target cells, forms a transmembrane pore, and causes osmotic lysis. Furthermore, the perforin-formed pore could also allow granule components, such as granzyme B, to enter target cells and trigger apoptosis (40). Killing of K562 cells by NK cells is mainly mediated by the perforin/granzyme B-based pathway (41); therefore, we used K562 cells as the target cells to examine the effect of DCs on perforin/granzyme B-based cytolytic activity of NK cells. In agreement with previous reports (17, 42), our results showed that IL-15 combined with the early acting cytokine SCF produced a large number of higher purity NK cells from HUCB CD34+ cells, and IL-15 (minimum 10 ng/ml) is essential to maintain their survival (data not shown).

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The FasL/Fas–based cytolytic pathway is also involved in activated NK cell cytotoxicity (45). PMA–ionomycin–activated peripheral NK cells (46) and IL-2 stimulated NK cells (45) to express FasL protein, and killed Fas-positive tumor cells via FasL/Fas–based pathway (3). An absence of expression of FasL protein in the HUCB CD34+–derived resting NK cells has been reported (17). In agreement with this report, our experiments demonstrated no FasL protein expression in single cultured NK cells (Fig. 8) and only a weak cytolytic activity of these cells against Fas–positive Jurkat cells (Fig. 3B). However, cytolytic activity of NK cells against Jurkat cells was significantly increased after coculture with DCs (Fig. 3B), and anti-Fasl mAb significantly reduced this effect (Fig. 6B). FasL mRNA expression and Fasl protein expression were up-regulated in DC-stimulated NK cells (Fig. 8). These findings suggest that DCs enhance FasL/Fas–based NK cell cytotoxicity.

DCs are considered to be the most potent APCs and to play a critical role in the initiation of a primary immune response (47). Some cytokines can be secreted from DCs and play pivotal roles in adaptive immune responses. Among them, IL-12 and IL-18 have
been reported to promote NK cell cytotoxicity through up-regulation of NK cell-derived IFN-γ production (4, 6, 48). Moreover, IL-12 enhanced NK cytotoxicity primarily by inducing expression of perforin, and IL-18 was reported to induce Fas-mediated apoptosis of tumor cells, both by up-regulating FasL expression on NK cells and augmenting IFN-γ-enhanced Fas susceptibility of target cells (13, 49). In these studies, we observed that CD34⁺ cell-derived DCs produced intracytoplasmic IL-18 and IL-12 (Fig. 9), and these DCs significantly enhanced endogenous IFN-γ production by NK cells (Fig. 4). Thus, it was of interest to determine whether DC-derived endogenous IL-12 and IL-18 play important roles in DC-stimulated NK cell cytotoxicity.

It is well known that IFN-γ is an important factor for enhancing NK cell activities (50). In these studies, we evaluated the role of endogenous IFN-γ production in DC-stimulated NK cell cytotoxicity. Anti-IFN-γ had only minor inhibitory effects on DC-stimulated anti-K562 cytotoxicity; however, NK cell cytotoxicity against Jurkat cells was significantly blocked by anti-IFN-γ in a dose-dependent manner (Fig. 10, A and B). These results indicate that DC-derived endogenous IL-12 and IL-18 play important roles in DC-stimulated NK cell cytotoxicity.

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**FIGURE 8.** Effect of DCs on FasL expression of CD34⁺ cell-derived NK cells. A, Expression of FasL mRNA was detected by RT-PCR analysis using total RNAs isolated from NK cells, DCs, and DC-stimulated NK cells. Representative results from three independent experiments for 35 cycles amplification with different set of primers for FasL and GAPDH genes are shown. Marker represents the m.w. standard. “No RT” indicates the negative control for RT-PCR performed in absence of reverse transcriptase. B, Flow cytometric analysis for the expression of FasL on DCs, NK cells, and DC-stimulated NK cells. The cells were stained with FITC-conjugated anti-CD56 mAb, and then stained with biotinylated anti-FasL mAb followed by streptavidin-PE. The numbers in each quadrant represent the percentage of double positive (FasL and CD56) cells from one experiment, which was representative of three independent experiments. C, Western blot analysis of FasL protein in cell lysates from NK cells and DC-stimulated NK cells. Molecular mass markers are indicated right in kilodaltons.

**FIGURE 9.** Expression of IL-12 and IL-18 on CD34⁺ cell-derived DCs. Intracellular IL-12 and IL-18 expressions on CD34⁺ cell-derived DCs were detected by flow cytometry as described in Materials and Methods. Representative results from three independent experiments are shown. The open histograms represent the levels of fluorescence obtained with an isotype control. The shaded histograms represent the levels of fluorescence of IL-12 mAb (A) and IL-18 mAb (B).

**FIGURE 10.** Effects of anti-IL-12 mAb and anti-IL-18 mAb on DC-stimulated NK cell cytotoxicity. CD34⁺ cell-derived NK cells were cocultured with DCs for 48 h in the presence of neutralizing anti-IL-12 mAb alone, anti-IL-18 mAb alone, as well as combinations of both anti-IL-12 and IL-18 mAbs. NK cell cytotoxicity against K562 (A and C) and Jurkat (B and D) target cells were analyzed using the ⁵¹Cr release assay at the indicated E:T ratios. Normal murine IgG from the pooled sera of nonimmunized mice was used as isotype control. Results are presented as the mean ± SEM of specific lysis from three donors. CD34⁺ cell-derived NK cells exposed to exogenous IL-12 (10 ng/ml) and IL-18 (10 ng/ml) for 48 h were used as positive control.
dose-dependent manner (Fig. 11). As endogenous IFN-γ enhances NK cell cytotoxicity (51, 52) as well as inducing target cell susceptibility to apoptosis (53, 54), it is likely that IFN-γ produced by activated NK cells, in response to DC stimulation, is a final mediator in the enhancement of NK cell Fas-mediated cytotoxicity.

Consistent with previous observations (14), our results have shown that NK cell cytotoxicity and IFN-γ production were not increased when NK cells and DCs were grown together separated by a transwell insert (Figs. 3 and 4). Therefore, direct cell-to-cell contact between DCs and NK cells is necessary for the enhancement of NK cell cytotoxicity. No soluble IL-12 or IL-18 in the supernatants of NK-DC cocultures was found by ELISA (data not shown). Combinations of anti-IL-18 and anti-IL-12 mAbs did not completely abolish the effect of DCs on NK cell cytotoxicity. These results lead us to speculate that IL-12 and IL-18 are packaged in vesicles that attach to the inner cell membrane of DCs and then come into contact with NK cells that are bound directly to the outer membrane (Fig. 2, A and B, c and d) and thus activate NK cells.

The molecular mechanisms underlying DC/NK cell interaction are unclear. Several recent studies suggest that interaction of NK cells with DC through costimulatory molecules activates NK cells, in response to DC stimulation, as a final mediator in the enhancement of NK cell Fas-mediated cytotoxicity.

Cord blood is now being used as an alternative source to bone marrow of stem cells for hemologic reconstitution (59). Because of the reduced incidence and/or severity of graft-vs-host disease following cord blood transplants, as well as the altered biology of human cord blood T cells, activated NK cells play a number of recognized roles in transplantation. They are especially beneficial in the graft-vs-leukemia effect following HUCB transplantation (59, 60). Enhancement of the antineoplastic cytotoxicity of NK cells and infusion of selected NK cells as alternatives to CTL seem to be very promising in the treatment of hemologic patients with low tumor burden (e.g., after stem cell transplantation) (61). The findings presented in this study are expected to be useful for designing this new immunotherapy. Moreover, the culture systems established in this study appear to be a valuable hemopoiesis.
model in analyzing the interaction between human NK cells and DCs.

In conclusion, DCs enhance HUCB CD34+ cell-derived NK cell cytotoxicity via both the perforin/granzyme B- and FasL/Fas-based pathways. IFN-γ, produced by activated NK cells in response to DC stimulation, is a final mediator in the enhancement of NK cell Fas-mediated cytotoxicity. Direct interaction between DCs and NK cells, via CD40 binding, and DC-derived IL-18 and IL-12 is necessary for all of these effects to occur.

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