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Short-Term Culture of Umbilical Cord Blood-Derived CD34 Cells Enhances Engraftment into NOD/SCID Mice Through Increased CXCR4 Expression

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Human umbilical cord blood (CB) has been used successfully in stem cell transplantation. A subpopulation of $CD34^+$ cells expresses chemokine receptor CXCR4 on the cell surface that is critical for bone marrow engraftment in human hematopoietic stem cells. Here, we demonstrate the effect of short-term culture on CXCR4 expression on umbilical CB-derived $CD34^+$ cells and subsequent engraftment capability in nonobese diabetic/severe combined immunodeficient (NOD/SCID) mice. Surface CXCR4 expression on $CD34^+$ cells increased after incubating the cells in medium alone for 2 h; this effect was blocked by the addition of AMD3100. No difference in CXCR4 mRNA expression was noted after incubating $CD34^+$ cells in culture for 2 h, although these cells showed significantly increased transmigrational activity toward SDF-1 and homing activity in NOD/SCID mice. Furthermore, cultured human $CD34^+$ cells showed improved engraftment into the bone marrow of NOD/SCID mice compared to noncultured or AMD3100-treated $CD34^+$ cells. These observations suggest that increased cell surface expression of CXCR4 on $CD34^+$ cells improved the engraftment of human umbilical CB cells into bone marrow through enhanced homing activity.

Introduction

HUMAN CORD BLOOD (CB), collected from the postpartum placenta and umbilical cord, is a rich source of hematopoietic stem cells (HSCs) and provides an attractive alternative to bone marrow or mobilized peripheral blood transplantation. However, a major disadvantage of CB transplantation is the relatively low number of HSCs in each CB unit that severely limits its usefulness in clinical transplantation [1]. Therefore, the development of ex vivo culture systems to expand CB HSC numbers is important to stem cell research and clinical application. Previous studies showed that the transplantation of HSCs into nonobese diabetic/severe combined immunodeficient (NOD/SCID) mice was shown to be a reliable model for the detection of regenerative human HSCs [2,3].

CXCR4 is the seven-transmembrane receptor of SDF-1 and is widely expressed in a variety of hematopoietic cell types, neuronal cells, and immature $CD34^+$ progenitor cells. The chemokine receptor, CXCR4, and its ligand, stromal cell-derived factor-1 (SDF-1, also known as CXCL12), play a central role in the migration, proliferation, differentiation, and survival of both murine and human hematopoietic stem/progenitor cells [4–7]. The SDF-1–CXCR4 axis has been proposed to be essential for the homing and repopulation of HSCs transplanted into immunodeficient NOD/SCID mice [8,9]. Recently, Kollet et al. [10] demonstrated that $CD34^+/CXCR4^-$ cells expressed intracellular CXCR4, the cell surface expression of which was stimulated by cytokines. The overexpression of CXCR4 on $CD34^+$ cells via gene transfer improved human stem cell motility, retention, and multilineage repopulation [11].

In the present study, we examined the effect of short-term culture on CXCR4 expression and engraftment in CB-derived $CD34^+$ cells transplanted into NOD/SCID mice. Our results demonstrated that short-term culture increased cell surface CXCR4 expression in $CD34^+$ cells and enhanced the retention of these human cells in mouse bone marrow through enhanced homing activity.

Materials and Methods

Isolation of $CD34^+$ cells

Umbilical CB was obtained from normal full-term deliveries, after first obtaining informed consent from all participants and the approval of the Chugoku-Shikoku Regional Cord Blood Bank (Hiroshima, Japan). Mononuclear cells

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(MNCs) from CB samples were isolated using Lymphoprep (Axis-Shield PoC AS, Oslo, Norway) density gradient centrifugation and washed three times in phosphate-buffered saline (PBS). MNCs were then enriched for $CD34^+$ cells using the CD34 Progenitor Cell Selection System (Dynal, Oslo, Norway) according to the manufacturer's instructions. The purity of $CD34^+$ cells (>90%) was determined by flow cytometry.

Cell cultures

Enriched $CD34^+$ cell populations were divided into three aliquots. They were cultured with or without AMD3100 (10 mM; Sigma Chemicals) for 2 h in RPMI-1640 (Sigma Chemicals, St. Louis, MO) without serum at 1×10^6 cells/ml (10^5 to 2×10^5 cells per well) in 96-well round bottom microtiter plates (Costar; Corning, Inc., Corning, NY). All cultures were performed at 37°C under a humidified atmosphere of 5% CO_2. A third aliquot was neither treated nor cultured and used immediately for all experiments without preservation.

Flow cytometric analysis

The presence of cell surface antigens was determined with a FACSCalibur (BD Biosciences, San Jose, CA) using the following fluorescein-conjugated monoclonal antibodies (mAbs): FITC-labeled anti-CD45 (2D1; BD Biosciences), PE-labeled anti-CD34 (AC136; Miltenyi Biotec, Bergisch-Gladbach, Germany), APC-labeled anti-CXCR4 (12G5; BD Biosciences), PE-labeled anti-CD26 (M-A261; BD Biosciences), APC-labeled anti-CD38 (HIT2; BD Biosciences), PE-labeled anti-CD33 (WM53; BD Biosciences), PE-labeled anti-CD19 (SJ25C1; BD Biosciences), and PE-labeled anti-CD3 (UCHT1; BD Biosciences). To identify and disregard dead cells from our analyses, cell cultures were stained with propidium iodide (PI; Sigma Chemicals, St. Louis, MO) at a concentration of 1 μg/mL.

Reverse transcription-polymerase chain reaction (RT-PCR) analysis

RT-PCR was used to determine *CXCR4* mRNA expression in cultured versus noncultured cells. Total RNA from cultured $CD34^+$ cells was extracted using the acid–phenol technique. RT-PCR was performed according to the manufacturer's protocol (Takara Bio, Inc., Shiga, Japan). The following specific primers were used to amplify *CXCR4*: sense, 5′-ggc cct caa gac cac agt ca-3′; antisense, 5′-tta gct gga gtg aaa act tga ag-3′ [12]. As an internal control, we also examined the expression of glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) in all samples. Amplified DNA fragments were then electrophoresed on an agarose gel and visualized by ethidium bromide staining.

Migration assay

Cells were incubated for 30 min at 37°C in 5% CO_2 in the upper chambers of a 96-well transwell apparatus (1×10^5 cells per well; QCM™ Chemotaxis 5 μm 96-Well Cell Migration Assay; Chemicon, Temecula, CA) containing RPMI-1640 medium supplemented with 10% fetal calf serum (FCS; StemCell Technologies, Vancouver, BC, Canada) and then allowed to migrate to the lower chamber containing 125 ng/ml SDF-1α (Sigma Chemicals). The number of cells that migrated to the lower chamber was scored visually with a light microscope.

Transplantation into mice

NOD/Shi-scid jic mice obtained from CLEA (Kawasaki, Japan) were bred and maintained under controlled conditions in individually ventilated (high-efficiency particle-arresting filtered air), sterile microisolator cages at the Hiroshima University Animal Institute. Before transplantation, 6- to 8-week-old mice were subjected to a sublethal dose of 300 cGy total-body irradiation. Then, 1×10^5 cultured or noncultured $CD34^+$ cells were injected into the tail veins of irradiated mice.

Homing assay

Sixteen hours after tail vein injection, the mice were killed and PBS was used to flush bone marrow cells from femurs and tibias. The cells obtained in this manner were analyzed by flow cytometry for the presence of human cells using human-specific anti-CD45-FITC and anti-CD34-PE. At least 1×10^6 cells were analyzed.

Engraftment analysis

To assess the engraftment of human cells into murine bone marrow, cell populations obtained from the murine marrows were incubated with antihuman, fluorescein-conjugated mAbs against CD45, CD34, CD33, CD19, and CD3 and then analyzed via flow cytometry. Engraftment analysis was performed 8 weeks after transplantation.

Secondary transplantation

Bone marrow cells were harvested from NOD/SCID mice in which CB $CD34^+$ cells had previously engrafted. Three mice were injected with 1×10^7 MNCs after sublethal irradiation at 300 cGy. Eight weeks after secondary transplantation, human cells in murine bone marrow were labeled with the same antihuman mAbs used in the primary engraftment analysis and subjected to flow cytometry.

Statistics

All data are expressed as the mean ±standard deviation (SD). Statistically significant differences within the data set were detected using the Student's *t*-test and Wilcoxon's signed-ranks test. All analyses were performed using the StatView software (version 5.0; SAS Institute, Cary, NC).

Results

Flow cytometric analysis of CXCR4 expression on $CD34^+$ cells from umbilical cord blood

Consistent with previous studies, a subpopulation of $CD34^+$ cells from umbilical CB constantly expressed CXCR4

TABLE 1. PERCENTAGE OF CB CD34$^+$ CELLS THAT ARE CXCR4$^+$, CD26$^+$, AND CD38$^-$

	Incubation		
	(−)	(+)	
		AMD3100	
Surface markers		(−)	(+)
CXCR4$^+$	8.0 ± 5.0	19.8 ± 8.7*	5.2 ± 3.4
CD26$^+$	7.7 ± 3.5	7.7 ± 4.7	7.6 ± 3.6
CD38$^+$	4.3 ± 2.0	5.5 ± 3.2	5.3 ± 2.8

Data represent the mean ±SD of six independent experiments. CD34$^+$ cells from CB were incubated with or without 10 mM AMD3100 for 2 h. *$p < 0.05$, compared to noncultured and AMD3100-treated cells.

on the cell surface (7.7 ± 3.6%). The cell surface expression of CXCR4 increased after culturing CD34$^+$ cells for 2 h (19.8 – 8.6%, Fig. 1, $p < 0.05$). It increased to >30% after 6 h in culture, but PI staining showed that the nonviable cells at 6 h were >30% relative to <10% at 2 h. Viability of the cultured cells at for 2 h was not different from that of the noncultured cells. Culturing for 6 h, therefore, was not done after this first experiment. The increase in the CXCR4 level was not exhibited in the presence of AMD3100, a CXCR4 antagonist. Next, we cultured CD34+ cells in the presence or absence of AMD3100 and examined the expression of CD26, CD38, and CXCR4. As shown in Table 1, a 2-h incubation with AMD3100 did not affect the expression of CD26 or CD38.

RT-PCR analysis of CXCR4 expression

The expression of *CXCR4* mRNA during short-term incubation was examined via RT-PCR. As shown in Figure 2, the addition of AMD3100 had no affect on *CXCR4* mRNA expression after a 2-h incubation. A quantitative real-time RT-PCR analysis using the same samples showed no difference in *CXCR4* expression after a 2-h incubation (data not shown). Thus, the observed increase in cell surface expression was not the result of increased mRNA expression.

Migration and homing activity of cultured cells

Because 2-h incubation increased CXCR4 expression on the surface of CD34$^+$ cells, we examined the in vitro migration of these cells toward the CXCR4 ligand, SDF-1. As shown in Figure 3, over 25% of CD34$^+$ cells cultured for 2 h showed transmigration in response to SDF-1. However, transmigrational activity was significantly lower in noncultured cells and in cells which had been cultured in the presence of AMD3100 ($p < 0.05$). Furthermore, the effect of increased CXCR4 expression on homing activity was determined by evaluating the presence of human CD45+/CD34+ cells in mouse bone marrow 16 h after tail vein injection. As shown in Figure 4A, a cluster of CD45+/CD34+ cells (R1) was detected in the bone marrow of mice injected with cultured CD34+ cells. Homing activity was compared among cells cultured in the presence and absence of AMD3100 and

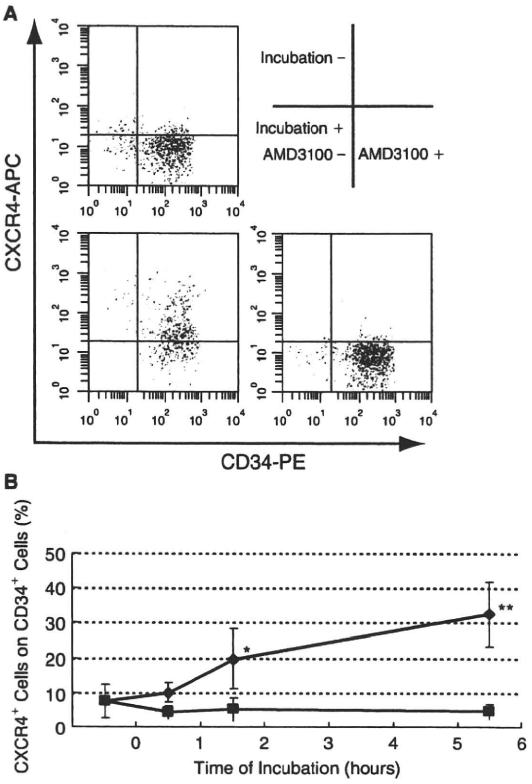

FIG. 1. Effect of short-term culture with or without AMD3100 treatment on CXCR4 expression on CD34$^+$ cells. Representative flow cytometry profiles for CXCR4 expression in cultured cells with or without AMD3100 treatment (**A**). CXCR4 expression on CD34$^+$ cells during short-term incubation with AMD3100 (◆) or without AMD3100 (◆) (**B**). Data represent the mean ±SD of six independent experiments. And, CXCR4 expression increased significantly on the surface of CD34$^+$ cells (*$p < 0.05$, **$p < 0.01$).

noncultured cells. Cultured CD34+ cells demonstrated significantly greater homing activity compared to noncultured cells and to cells cultured in the presence of AMD3100 ($p < 0.05$ in each case).

Engraftment of cultured CD34$^+$ cells into NOD/SCID mice

Figure 5 shows a representative FACS analysis of human marrow cells in bone marrow from identical NOD/SCID mice after CD34$^+$ cell transplantation. The presence of human cells was detected by the cell surface expression of CD45, CD34, CD33, CD19, and CD3. The recipients exhibited CD45$^+$/CD19$^+$ B-lymphoid cells, CD45$^+$/CD33$^+$ granulomonopoietic cells, and CD45$^+$/CD34$^+$ immature cells, but not CD3$^+$ T cells. The percentage of CD34$^+$/CD19$^+$ positive cells

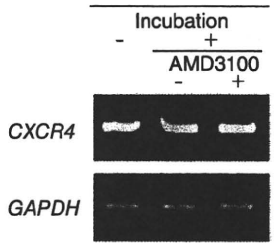

FIG. 2. Effect of short-term incubation on *CXCR4* mRNA expression in CD34⁺ cells. Total RNA was extracted from cells after the cells were incubated under the indicated culture conditions, RT-PCR was performed. *GAPDH* was used as an internal control. Short-term culture had no effect on *CXCR4* mRNA expression in CD34⁺ cells.

in the CD45⁺ cells was 6–12%, consistent with the report of Kobari et al. [13]. Next, at 8 weeks, we performed a secondary transplant of engrafted cells from previously transplanted mice to determine if long-term engraftment of self-renewing cultured CB cells had occurred. Successful secondary engraftment occurred as shown by the presence of CD45⁺/CD19⁺ cells and possibly CD45⁺/CD34⁺ cells (Fig. 5B).

To assess the effect of short-term culture and AMD3100 interference on engraftment, CD34⁺ cells were cultured with or without AMD3100 as indicated and injected into NOD/SCID mice via the tail vein. Eight weeks after transplantation, engraftment was assessed based on the expression of the human CD45 antigen. As shown in Figure 6, cultured CD34⁺ cells showed increased engraftment into murine bone marrow compared to noncultured CD34⁺ cells (32.0 ± 21.4% vs. 17.1 ± 15.7%), $p < 0.01$, or to CD34⁺ cells from AMD3100-treated cultures (12.9 ± 11.1%), $p < 0.01$, as determined by Wilcoxon's signed-ranks test.

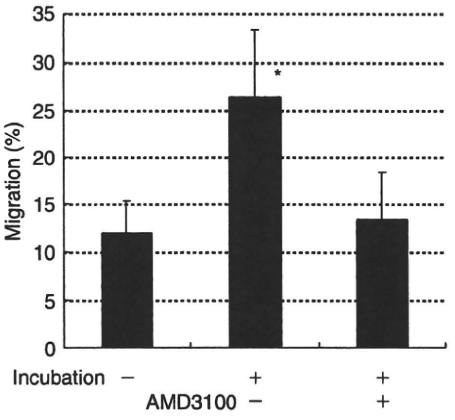

FIG. 3. Effect of short-term incubation and AMD3100 treatment on transmigrational activity in CD34⁺ cells. CD34⁺ cells were loaded into the upper wells of a transwell apparatus and incubated for 2 h. Migration was assessed according to the percentage of cells migrated to lower wells containing SDF-1. The data represent the mean ± SD of six independent experiments. * $p < 0.05$, significant increase compared to noncultured and AMD3100-treated cells.

Discussion

Umbilical CB contains hematopoietic cells capable of engrafting into NOD/SCID mice in vivo. Our results demonstrate that short-term culture in the absence of serum and/or cytokines induced the expression of CXCR4 on umbilical CB-derived CD34⁺ cells, resulting in enhanced homing activity and subsequently improved engraftment into NOD/SCID mice. The enhanced homing and engraftment of short-term cultured CD34+ cells was abolished by the addition of AMD3100 to the cultures, suggesting that improved engraftment was dependent upon increased CXCR4 expression.

Lataillade et al. [14] reported that cell surface expression of CXCR4 increased significantly when purified progenitor cells were incubated overnight in serum. Peled et al. [9] demonstrated that human CD34⁺ cells incubated with stem cell factor and IL-6 for 48 h showed increased CXCR4 expression, resulting in enhanced in vitro migration toward an

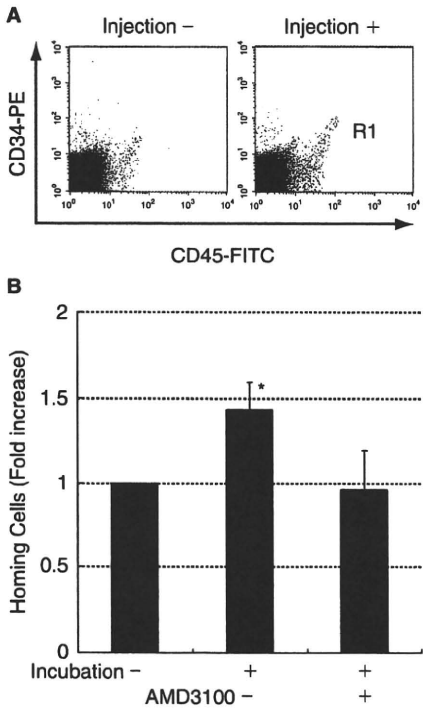

FIG. 4. Homing activity of CD34⁺ cells in NOD/SCID mice. CD34⁺ cells were cultured as indicated and then injected into the tail veins of NOD/SCID mice. After 16 h, the mice were killed and human-derived cells in the bone marrow were quantified based on the presence of a CD45⁺/CD34⁺ cluster (R1) in flow cytometry (**A**). The fold-increase compared to noninjected mice was calculated (**B**). The data represent the mean ±SD of six independent experiments. *$p < 0.05$, significant increase compared to noncultured and AMD3100-treated cells.

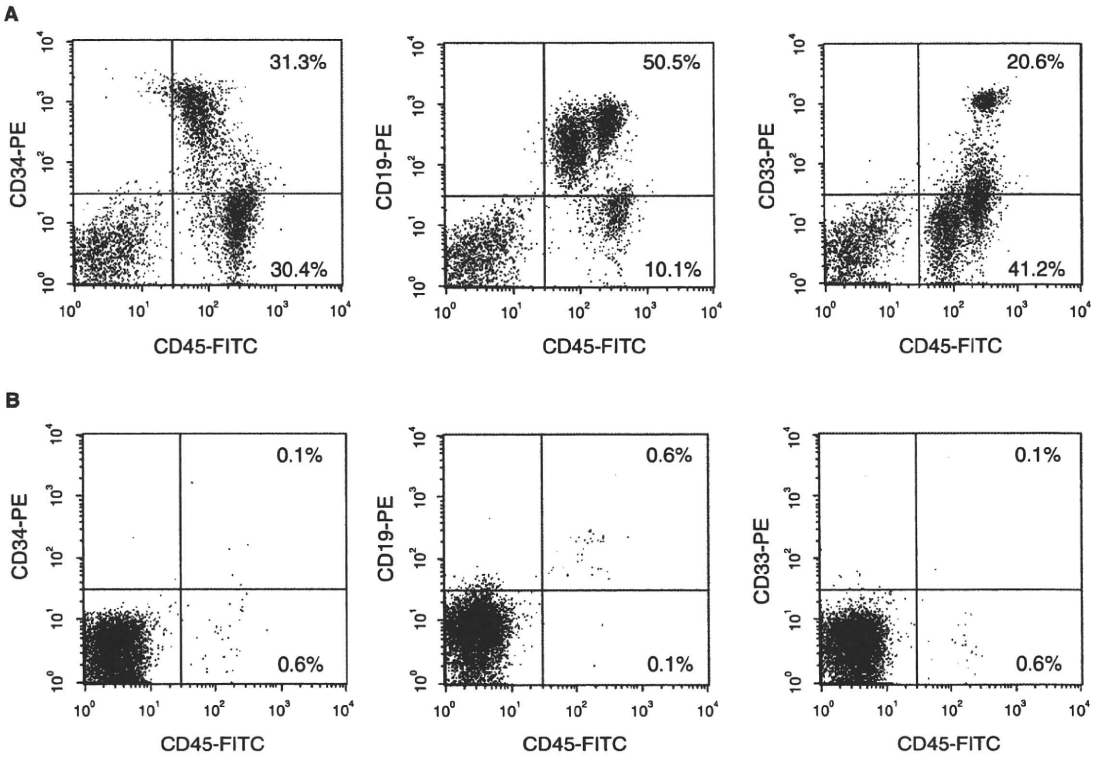

FIG. 5. Representative flow cytometry profiles of human CD45⁺, CD34⁺, CD33⁺, and CD19⁺ cells obtained at 8 weeks after transplantation. The following fluors were coupled to each antibody: CD45-FITC, CD34-PE, CD33-PE, and CD19-PE. Similar results were obtained in 12 additional experiments (**A**). Secondary transplantation of bone marrow cells obtained from previously transplanted mice (**B**). Marrow cells obtained at 8 weeks.

SDF-1 gradient and increased homing and repopulating potential in NOD/SCID and NOD/SCID/beta2-microglobulin null mice. In contrast, the long-term culture of CD34⁺ cells in a cytokine cocktail decreased CXCR4 expression and led to reduced repopulating potential in immunodeficient mice [15]. The mechanism underlying CXCR4 up-regulation during incubation remains unclear, although various factors, including cell surface adhesion molecules, cell–cell contact between CD34⁺ and low-density MNCs, growth factor production, and adhesion molecule interactions, may be involved [4,5,16]. Our RT-PCR results showed that cell surface CXCR4 expression increased in cultured CD34⁺ cells without concomitant increases in mRNA expression.

CXCR4 is thought to play a crucial role in the homing and retention of HSCs in murine bone marrow [9] and is required for the retention of B-lineage and myeloid precursors in the bone marrow [16]. Recently, Kahn et al. [11] reported that the overexpression of CXCR4 in human CD34⁺ cells via gene transfer increased proliferation, migration, and NOD/SCID repopulation. Similarly, we confirmed the importance of CXCR4 expression in CD34⁺ cell in NOD/SCID marrow homing through the inhibitory effect of AMD3100. AMD3100, a small bicyclam molecule, reversibly inhibits SDF-1–CXCR4 binding [17].

In conclusion, we demonstrated that short-term culture of CB-derived CD34⁺ cells increased CXCR4 expression on these cells, resulting in increases in in vivo homing and engraftment into NOD/SCID mice. Thus, short-term culture of hematopoietic stem/progenitor cells in the absence of sera and cytokines may serve as a simple but effective way to enhance their repopulating activity.

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There is no possible conflict of interest (including financial and other relationship) for each author.

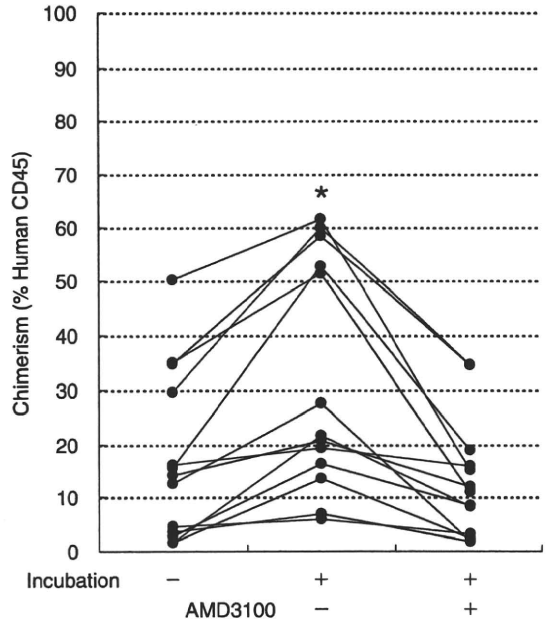

FIG. 6. Engraftment of CD34⁺ cells with or without the presence of AMD3100 into NOD/SCID mice. CD34⁺ cells were cultured as indicated and then injected into the tail veins of NOD/SCID mice. Eight weeks after transplantation, the mice were killed and percentage of human CD45⁺ cells was determined. The data represent the percentage of human CD45⁺ cells in 13 independent experiments. Statistically significant differences among cultured, noncultured, and AMD3100-treated cells were analyzed using Wilcoxon's signed-ranks test. $^*p < 0.01$, significant increase compared to noncultured and AMD3100-treated cells.

References

1. American Academy of Pediatrics Section on Hematology/Oncology; American Academy of Pediatrics Section on Allergy/Immunology, Lubin BH and WT Shearer. (2007). Cord blood banking for potential future transplantation. Pediatrics 119:165–170.
2. McCune JM, R Namikawa, H Kaneshima, LD Shultz, M Lieberman and IL Weissman. (1988). The SCID-hu mouse: murine model for the analysis of human hematolymphoid differentiation and function. Science 241:1632–1639.
3. Mosier DE, RJ Gulizia, SM Baird and DB Wilson. (1988). Transfer of a functional human immune system to mice with severe combined immunodeficiency. Nature 335:256–259.
4. Roland J, BJ Murphy, B Ahr, V Robert-Hebmann, V Delauzun, KE Nye, C Devaux and M Biard-Piechaczyk. (2003). Role of the intracellular domains of CXCR4 in SDF-1-mediated signaling. Blood 101:399–406.
5. Martin C, PC Burdon, G Bridger, JC Gutierrez-Ramos, TJ Williams and SM Rankin. (2003). Chemokines acting via CXCR2 and CXCR4 control the release of neutrophils from the bone marrow and their return following senescence. Immunity 19:583–593.
6. Suratt BT, JM Petty, SK Young, KC Malcolm, JG Lieber, JA Nick, JA Gonzalo, PM Henson and GS Worthen. (2004). Role of the CXCR4/SDF-1 chemokine axis in circulating neutrophil homeostasis. Blood 104:565–571.
7. Semerad CL, F Liu, AD Gregory, K Stumpf and DC Link. (2002). G-CSF is an essential regulator of neutrophil trafficking from the bone marrow to the blood. Immunity 17, 413–423.
8. Lapidot T and O Kollet. (2002). The essential roles of the chemokine SDF-1 and its receptor CXCR4 in human stem cell homing and repopulation of transplanted immune-deficient NOD/SCID and NOD/SCID/B2m (null) mice. Leukemia 16:1992–2003.
9. Peled A, I Petit, O Kollet, M Magid, T Ponomaryov, T Byk, A Nagler, H Ben-Hur, A Many, L Shultz, O Lider, R Alon, D Zipori and T Lapidot. (1999). Dependence of human stem cell engraftment and repopulation of NOD/SCID mice on CXCR4. Science 283:845–848.
10. Kollet O, I Petit, J Kahn, S Samira, A Dar, A Peled, V Deutsch, M Gunetti, W Piacibello, A Nagler and T Lapidot. (2002). Human CD34(+) CXCR4(-) sorted cells harbor intracellular CXCR4, which can be functionally expressed and provide NOD/SCID repopulation. Blood 100:2778–2786.
11. Kahn J, T Byk, L Jansson-Sjostrand, I Petit, S Shivtiel, A Nagler, I Hardan, V Deutsch, Z Gazit, D Gazit, S Karlsson and T Lapidot. (2004). Overexpression of CXCR4 on human CD34+ progenitors increases their proliferation, migration, and NOD/SCID repopulation. Blood 103:2942–2949.
12. Bajetto A, R Bonavia, S Barbero, P Piccioli, A Costa, T Florio and G Schettini. (1999). Glial and neuronal cells express functional chemokine receptor CXCR4 and its natural ligand stromal cell-derived factor 1. J Neurochem 73:2348–2357.
13. Kobari L, F Pflumio, M Giarratana, X Li, M Titeux, B Izac, F Leteurtre, L Coulombel and L Douay. (2000). In vitro and in vivo evidence for the long-term multilineage (myeloid, B, NK, and T) reconstitution capacity of ex vivo expanded human CD34(+) cord blood cells. Exp Hematol 28:1470–1480.
14. Lataillade JJ, D Clay, C Dupuy, S Rigal, C Jasmin, P Bourin and MC Le Bousse-Kerdilès. (2000). Chemokine SDF-1 enhances circulating CD34(+) cell proliferation in synergy with cytokines: possible role in progenitor survival. Blood 95:56–768.
15. Denning-Kendall P, S Singha, B Bradley and J Hows. (2003). Cytokine expansion culture of cord blood CD34⁺ cells induces marked and sustained changes in adhesion receptor and CXCR4 expressions. Stem Cells 21:61–70.
16. Herrera C, J Sanchez, A Torres, A Pascual, A Rueda and MA Alvarez. (2004). Pattern of expression of CXCR4 and adhesion molecules by human CD34⁺ cells from different sources: role in homing efficiency in NOD/SCID mice. Haematologica 89:1037–1045.
17. Ma Q, D Jones and TA Springer. (1999). The chemokine receptor CXCR4 is required for the retention of B lineage and granulocytic precursors within the bone marrow microenvironment. Immunity 10:463–471.

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Notch Activation Induces the Generation of Functional NK Cells from Human Cord Blood CD34-Positive Cells Devoid of IL-15[1]

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The development of NK cells from hematopoietic stem cells is thought to be dependent on IL-15. In this study, we demonstrate that stimulation of human cord blood CD34$^+$ cells by a Notch ligand, Delta4, along with IL-7, stem cell factor, and Fms-like tyrosine kinase 3 ligand, but no IL-15, in a stroma-free culture induced the generation of cells with characteristics of functional NK cells, including CD56 and CD161 Ag expression, IFN-γ secretion, and cytotoxic activity against K562 and Jurkat cells. Addition of γ-secretase inhibitor and anti-human Notch1 Ab to the culture medium almost completely blocked NK cell emergence. Addition of anti-human IL-15-neutralizing Ab did not affect NK cell development in these culture conditions. The presence of IL-15, however, augmented cytotoxicity and was required for a more mature NK cell phenotype. CD56$^+$ cells generated by culture with IL-15, but without Notch stimulation, were negative for CD7 and cytoplasmic CD3, whereas CD56$^+$ cells generated by culture with both Delta4 and IL-15 were CD7$^+$ and cytoplasmic CD3$^+$ from the beginning and therefore more similar to in vivo human NK cell progenitors. Together, these results suggest that Notch signaling is important for the physiologic development of NK cells at differentiation stages beyond those previously postulated. *The Journal of Immunology,* **2009, 182: 6168–6178.**

Natural killer cells are critical for host immunity because they rapidly mediate cellular cytotoxicity against pathogen-infected or malignantly transformed cells and produce a wide variety of cytokines and chemokines that influence other components of the immune system. Unlike other lymphocytic lineages, however, the continuous staging scheme of human NK cell development in vivo has yet to be elucidated (1). One reason for this may be the difficulty in closely correlating our knowledge of mouse NK cell biology with human NK cell biology (2), because mouse NK cells do not express a homolog of CD56, which is the marker most representative of human NK cells; instead, the most widely used markers of NK cells in various mouse strains are NK1.1 and DX5, mouse-specific Ags. Among the molecules involved in NK cell development, IL-15 has a particularly important role. For example, IL-15-deficient mice lack NK1.1$^+$ cells (3), indicating that IL-15 is essential for NK cell development in mice. The requirement of IL-15 for mouse NK cell development has also been demonstrated by other studies (4, 5). In humans, IL-15 is considered to be required for in vitro NK cell development and virtually most current protocols for human NK cell differentiation culture depend on IL-15. IL-15-independent NK cell differentiation has been reported in which human cord blood (CB)[9] cells are cocultured with murine stromal cell lines (6). Signaling, however, substituting IL-15 signaling that is responsible for the NK cell differentiation in this culture system was not described.

NK cells are thought to be derived from hematopoietic stem cells through a T/NK precursor stage. The Notch signaling pathway influences cell fate decisions in numerous cellular systems,

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[9] Abbreviations used in this paper: CB, cord blood; cy, cytoplasmic; FL, Fms-like kinase 3 ligand; DAPT, *N*-[*N*-(3,5-difluorophenacetyl-L-alanyl)]-*S*-phenylglycine *tert*-butyl ester; CMA, concanamycin A.

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including various hematopoietic and immune cells (7–9). To date, four Notch receptors (Notch1– Notch4) and at least four Notch ligands (Delta1, Delta4, Jagged1, and Jagged2) have been identified in mammals. Signaling through Notch1 is crucial in the early stages of T cell development (10–12). In culture, ligand-induced Notch signaling drives human CB $CD34^+$ cells to differentiate into T/NK cell precursors (13). Furthermore, Notch signaling drives the T/NK precursors toward differentiation into T and NK cells, although the results for the NK cells are controversial. For example, inhibition of Notch signaling suppresses T cell development and stimulates NK cell development (14–16), whereas activation of Notch signaling contributes to the efficient development of NK cells in mice (17, 18) and humans (19). It is not concluded, however, whether Notch signaling is involved in the function of NK cells or whether IL-15 is necessary for NK cell development in culture.

In this report, to gain further insight into the physiologic significance of Notch signaling in NK cell development, we examined whether IL-15 is dispensable for the generation of functional NK cells and whether Notch signaling has a role in the later stages of NK cell development. Our results indicated that Notch signaling, but not IL-15 stimulation, was essential for inducing $CD34^+$ cells to give rise to $CD7^+$ and cytoplasmic (cy) $CD3^+$ cells that express CD56 in stroma-free culture. Surprisingly, cells cultured with Delta4-coated plates, but lacking IL-15 in the medium, were functional NK cells with cytotoxic activity. IL-15, along with Delta4, further augmented NK cell activity and phenotypic maturation. The addition of IL-15 without exogenous Notch ligand, however, did not allow $CD34^+$ cells to take a NK cell developmental pathway resembling physiologic NK cell precursors. Notch signaling might have a significant role in the development of NK cells in vivo.

Materials and Methods

Reagents and Abs

Recombinant human Delta4-Fc chimeric protein was generated as described previously (20). Recombinant human IL-7 and IL-15 were purchased from R&D Systems. Human stem cell factor and human Fms-like kinase 3 ligand (FL) were a gift from Amgen. Human IL-6/IL-6 receptor fusion protein (FP6) and human thrombopoietin were provided by Kirin Pharma. Anti-IL-15 Ab (MAB2471) and isotype control mouse IgG1 were purchased from R&D Systems. Anti-CD3 (UCHT1), CD8 (SK1), CD14 (M5E2), CD44 (G44-26), CD45 (HI30), CD45RA (HI100), CD56 (B159), CD94 (HP-3D9), CD161 (DX12), NKG2D (1D11), CCR7 (3D12), granzyme B (GB11), and IFN-γ (25723.1) Abs were purchased from BD Biosciences. Anti-CD2 (T11), CD4 (13B8.2), CD7 (8H8.1), CD11a (25.3), CD11b (Bear1), CD25 (B1.49.9), CD27 (1A4CD27), CD33 (D3HL60.251), CD57 (NC1), CD62L (DREG56), CD117 (YB5.B8), CD122 (CF1), CD158a (EB6), and CD158b (GL183) Abs were purchased from Beckman Coulter. Anti-CD34 and CD133 Abs were purchased form Miltenyi Biotec. RIK-2, anti-TRAIL mAb, was prepared as described previously (21).

Isolation of $CD34^+$ and $CD133^+$ cells

Human CB samples were collected from normal full-term deliveries. The parents of all donors provided written informed consent to participate in the study. The procedures were approved by the institutional review board. Mononuclear cells were separated from blood samples by density gradient centrifugation (Lymphoprep; AXIS-SHIELD PoC). CD34- and CD133-enriched cells were separated from mononuclear cells using a MACS Direct CD34 Progenitor Cell Isolation Kit and MACS CD133 MicroBead Kit (Miltenyi Biotec), respectively, according to the manufacturer's protocol. The purity of the $CD34^+$ and $CD133^+$ cells was 97.3 ± 2.3% (n = 15) and 95.4 ± 3.2% (n = 4), respectively. Residual $CD3^+$ and $CD56^+$ cells were 0.73 ± 0.42% and 0.41 ± 0.32%, respectively, in either purification strategy.

Cell culture

Nontissue culture-type 24-well plates were precoated by applying 10 μg/ml Delta4-Fc or control Fc fragments of human Ig G (Fc) (Athens Research & Technology) to the plates at 37°C for 1 h. Cells were cultured in MEM Eagle, α modification (Sigma-Aldrich) supplemented with 20% FBS (Thermo Trace) and penicillin-streptomycin at 37°C in a humidified atmosphere flushed with 5% CO_2 in air. The number of CD34 or CD133 magnetic bead-sorted cells seeded in each well was 0.25–1.2 × 10^5. Cytokines were added at concentrations of 10 ng/ml for IL-7, 100 ng/ml for stem cell factor and 100 ng/ml for FL. one-half of the culture medium was changed every 3 or 4 days. Ten nanograms of thrombopoietin per ml and 100 ng/ml FP6 were added only into the starting culture medium for effective proliferation, although they were not essential (data not shown). IL-15 was added at 5 ng/ml when indicated. Anti-IL-15 or isotype IgG was added at 10 μg/ml when indicated. To inhibit Notch signaling, 10 μmol/L γ-secretase inhibitor *N*-[*N*-(3,5-difluorophenacetyl-L-alanyl)]-*S*-phenylglycine *tert*-butyl ester (DAPT; Calbiochem) was added to the culture medium. $CD161^+$ and $CD161^-$ cells from the culture were isolated using FACSAria (BD Biosciences) after staining with anti-CD161-PE Ab.

Phenotyping assay

Immunofluorescence staining for flow cytometry was performed according to standard procedures. To exclude dead cells from the analysis, 7-aminoactinomycin D (Beckman Coulter) was used. Cytoplasmic staining was performed as follows: after staining the cells with anti-CD56-allophycocyanin and fixing with FACS lysing solution (BD Biosciences), the cells were permeabilized using FACS permeabilizing solution (BD Biosciences) and stained with anti-CD3-PE Ab. For staining for granzyme B, the same fixing and permeabilizing procedure was performed after cell surface staining with anti-CD56-PE and anti-CD3-allophycocyanin. For staining for TRAIL, the cells were incubated with 1 μg of RIK-2 for 30 min at 4°C followed by anti-mouse IgG1-PE (A85-1). Cells were analyzed by flow cytometry using FACSCalibur and CellQuest software (BD Biosciences).

Cytotoxicity assays

A ^{51}Cr release assay to determine cytotoxicity was performed using standard procedures. In brief, 5 × 10^3 K562 or Jurkat cells were labeled with $Na_2{}^{51}CrO_3$ (Amersham Biosciences) and cocultured with effector cells at various ratios in 96-well round-bottom microtiter plates in 200 μl of culture medium. The cocultured cells were incubated for 4 h, and 100 μl of supernatant was collected from each well and counted with a Packard COBRA gamma counter (Packard Instruments). The percentage of specific ^{51}Cr release was calculated as follows: [cpm experimental release − cpm spontaneous release]/(cpm maximal release − cpm spontaneous release) × 100. The ratio of spontaneous release to maximal release was <20% in all experiments. In experiments to test the mode of cytotoxicity, we used concanamycin A (CMA; Sigma-Aldrich) as a selective inhibitor of the perforin-mediated cytotoxicity, and anti-TRAIL Ab RIK-2. Effectors were pretreated with 100 nmol/L CMA for 2 h before the cytotoxicity assays (22). RIK-2 was added at a final concentration of 10 μg/ml at the start of the cytotoxicity assay.

Intracellular cytokines

The cells were stimulated by PMA (25 ng/ml; Sigma-Aldrich) and ionomycin (1 μg/ml; Sigma-Aldrich) in the presence of monensin (2 μmol/L; Sigma-Aldrich) for 4 h. After staining the cells with anti-CD56-PE, they were fixed and permeabilized as described above and stained with anti-IFN-γ-FITC Ab. The cells were analyzed on a FACSCalibur using CellQuest software.

Anti-Notch Abs

For cell surface staining, we used biotinylated Abs and streptavidin-PE (BD Biosciences). To block Notch1, we added 10 (μg/ml) MHN1-519 to the medium. Mouse IgG1 (R&D Systems) was used as the control. The anti-human Notch1 (MHN1-519, mouse IgG1), Notch2 (MHN2-25, mouse IgG2a), and Notch3 (MHN3–21, mouse IgG1) mAbs were generated by immunizing BALB/c mice with human Notch1-Fc (R&D Systems), Notch2-Fc (the Fc portion of human IgG1 was fused to the 22nd epidermal growth factor repeat of the extracellular region of human Notch2), or Notch3-Fc (R&D Systems) and screening hybridomas producing mAbs specific for Notch1-Fc, Notch2-Fc, or Notch3-Fc by ELISA. MHN1-519, MHN2-25, and MHN3-21 reacted with CHO(r) cells (23) expressing human Notch1, Notch2, and Notch3, respectively, as demonstrated by flow cytometry (supplemental Fig. S4*A*[10]). MHN1-519 and MHN3h21 blocked Notch1-Fc and Notch3-Fc binding to CHO(r) cells expressing human Delta4, respectively, but MHN2-25 did not block Notch2-Fc binding (supplemental Fig. S4*B*).

[10] The online version of this article contains supplemental material.

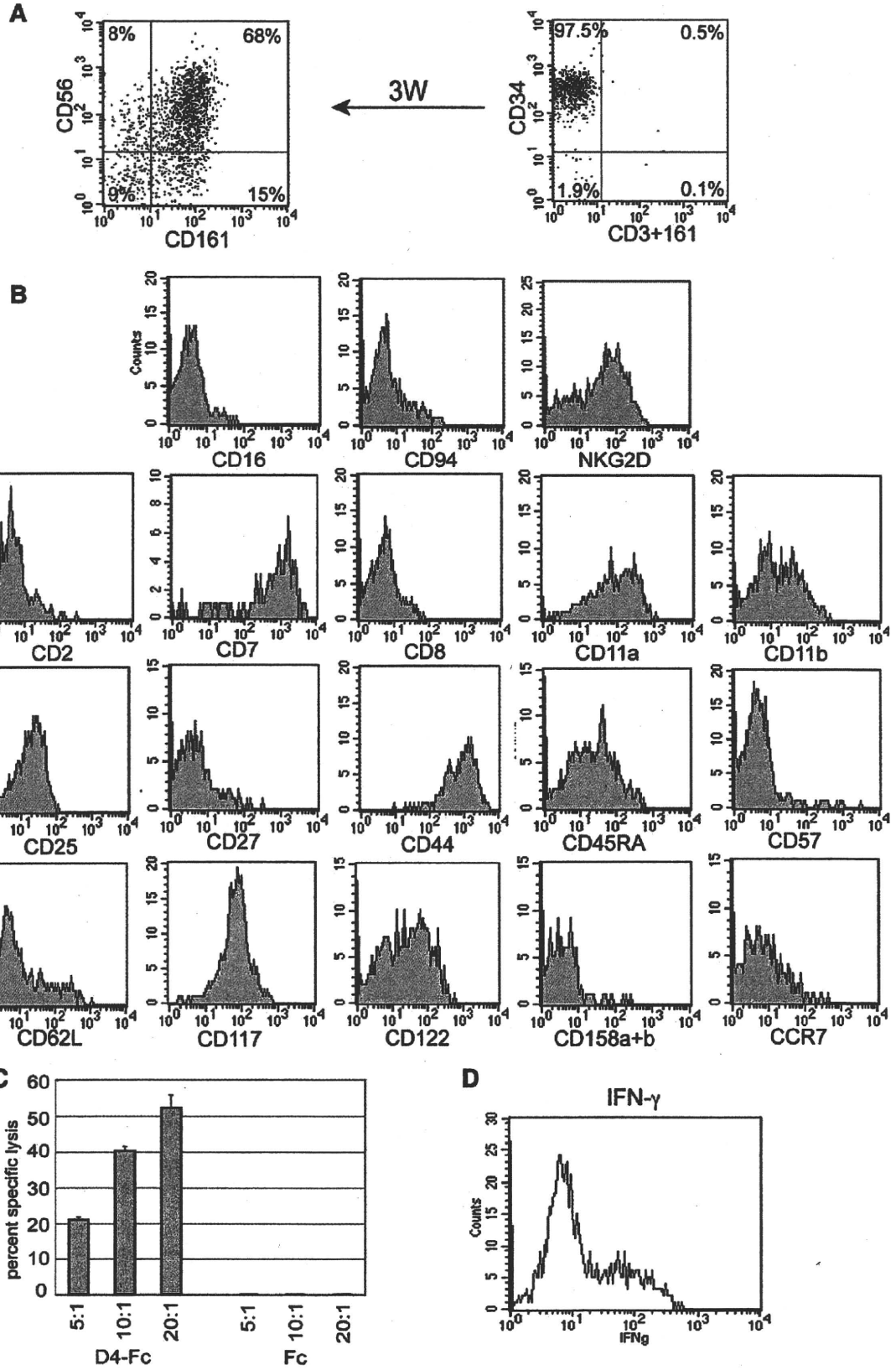

FIGURE 1. Phenotypic and functional analysis of cells derived from CD34$^+$ cells on Delta4-Fc-coated plates. *A*, Representative dot plot illustrating CD161 vs CD56 expression in the cells generated on Delta4-Fc-coated plates from CD34$^+$ CB cells after culture for 3 wk, and dot plot illustrating CD161/CD3 vs CD34 of the sorted CB population before culture. *B*, Various phenotypic analyses of the 3-wk cultured cells that were gated on CD161$^+$ events. Results are representative of at least four experiments. *C*, The 2.5-wk cultured cells were cytotoxic against K562 target cells at the indicated E:T ratios. The ratio of CD161$^+$ cells cultured on Delta4-Fc-coated plates and those Fc-coated plates in this experiment was 40 and 0%, respectively. Results are representative of four experiments. *D*, IFN-γ production by the 3-wk culture cells, as analyzed by intracellular expression. The histogram plots were gated on CD56$^+$events. Results are representative of five experiments.

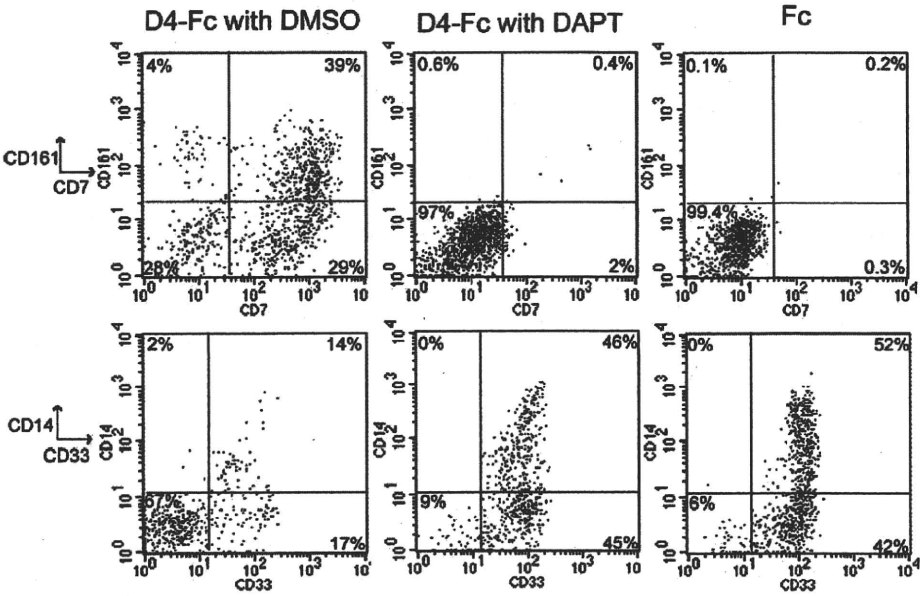

FIGURE 2. Phenotypic analysis of cells cultured in the presence of γ-secretase inhibitors. Representative dot plots of CB $CD34^+$ cells that were cultured for 2.5 wk on Delta4-Fc-coated plates with DMSO (the solvent for the γ-secretase inhibitors: D4-Fc with DMSO), Delta4-Fc-coated plates with DAPT (D4-Fc with DAPT), and Fc-coated plates (Fc). Results are representative of three experiments.

Results

Human CB $CD34^+$ and $CD133^+$ cells gave rise to functional NK cells by Notch signaling in a stroma-free culture without exogenous IL-15

$CD34^+$ or $CD133^+$ cells were cultured on Delta4-Fc-coated plates. The cells became almost immunophenotypically homogeneous after culture for ~3 wk (Fig. 1*A*). The proliferation efficiency depended on CB batches; fold increases in the cell number after the 3-wk culture were 10.3 ± 7.74-fold (n = 11). These cells expressed CD56 and CD161, but did not express surface CD3 or TCRα/β (data not shown). CD56/CD161 double-positive cells also expressed NKG2D and CD117, but were essentially negative for CD16 and killer Ig-like receptors (CD158a and CD158b). The cells had cytotoxic activity against K562 (Fig. 1*C*) and Jurkat cells (see Fig. 5*Bii*), and secreted IFN-γ (Fig. 1*D*). These results indicate that the culture products meet the general criteria for functional NK cells. The products generated from CB $CD34^+$ and $CD133^+$ had the same characteristics (data not shown).

Virtually no NK cells developed in culture on control Fc-coated plates; the vast majority of the cells were $CD33^+$ myeloid cells, a significant part of which expressed CD14 (Fig. 2). The absolute cell numbers with control Fc are ~5-fold higher than that with Delta4-Fc, and the fold increases in the cell number after the 3-wk culture were 45.7 ± 31.6-fold (n = 11). To confirm that the NK cell differentiation was Notch dependent, we added a γ-secretase inhibitor, DAPT, which strongly inhibits ligand-dependent Notch activation (24, 25). The cells cultured on Delta4-Fc-coated plates in the presence of DAPT had the same immunophenotype as those cultured on the control Fc-coated plates and did not give rise to NK cells (Fig. 2), indicating that the observed NK cell development was Notch activation dependent. The number of cells generated increased to the level of that in the control Fc protein-coated plates (data not shown).

We cultured $CD34^+$ cells and $CD133^+$ cells purified from G-CSF-mobilized peripheral blood cells. Both cell types gave rise to $CD56^+CD161^+$ NK cells that were similar to those derived from CB $CD34^+$ or $CD133^+$ cells. The amount of time required for mobilized peripheral blood $CD34^+$ or $CD133^+$ cells (~5 wk) to develop to a major population of $CD56^+CD161^+$ NK cells was greater than that required for CB $CD34^+$ or $CD133^+$ cells (supplemental Figs. S1*A* and S2 and Fig. 3), although the time courses varied to some degree from batch to batch (supplemental Fig. S2 and data not shown).

We next examined the effects of other soluble Notch ligands, human Delta1-Fc and Jagged1-Fc, on NK cell development from CB $CD34^+$ cells. Delta1-Fc had an effect similar to that of Delta4-Fc, although with lower efficiency (supplemental Fig. S1*B*), and Jagged1-Fc showed no potential to induce NK cell development (data not shown). Therefore, we used Delta4-Fc as the soluble Notch ligand and CB $CD34^+$ cells as the starting material for the remaining experiments.

IL-15 is dispensable for in vitro NK cell development from CB $CD34^+$ cells in the presence of Delta4 stimulation, whereas Notch stimulation appears to be essential for physiologic NK cell development

When IL-15 was added to the culture medium on control Fc-coated plates, $CD56^+CD161^+$ NK cells emerged (Fig. 3 and supplemental Fig.S2, Fc plus IL-15; cf with Fig. 3 and supplemental Fig.S2, Fc); this effect was blocked by anti-IL-15- neutralizing Ab (Fig. 3 and supplemental Fig.S2, Fc plus IL-15 plus anti-IL-15). IL-15 does not affect the absolute cell number; fold increases in the cell number after the 3-wk culture were 46.8 ± 36.3-fold, 43.1 ± 35.7-fold, and 48.4 ± 9.48-fold with IL-15 (n = 7), without IL-15 (n = 7), and with IL-15 and anti-IL-15 (n = 3) in the control Fc-coated plate condition. The rate of NK cell development by IL-15 stimulation, however, was much slower than that by Delta4-Fc stimulation. In the absence of Notch stimulation, but with IL-15, the percentage of total NK-lineage cells represented by positive CD161 was only 2.6 ± 2.9%, 6.3 ± 4.6%, and 9.0 ± 4.5% at 2, 3, and 4 wk, respectively (Fig. 3 and supplemental Fig.S2, Fc plus IL-15); whereas in Delta4-Fc with IL-15 (Fig. 3 and supplemental Fig.S2, D4-Fc plus IL-15) or without IL-15 (Fig. 3 and supplemental Fig.S2, D4-Fc), the percentage of total NK-lineage cells was 56 ± 17%, 77 ± 11%, and 81 ± 5.8% (with IL-15) or 52 ± 18%, 74 ±

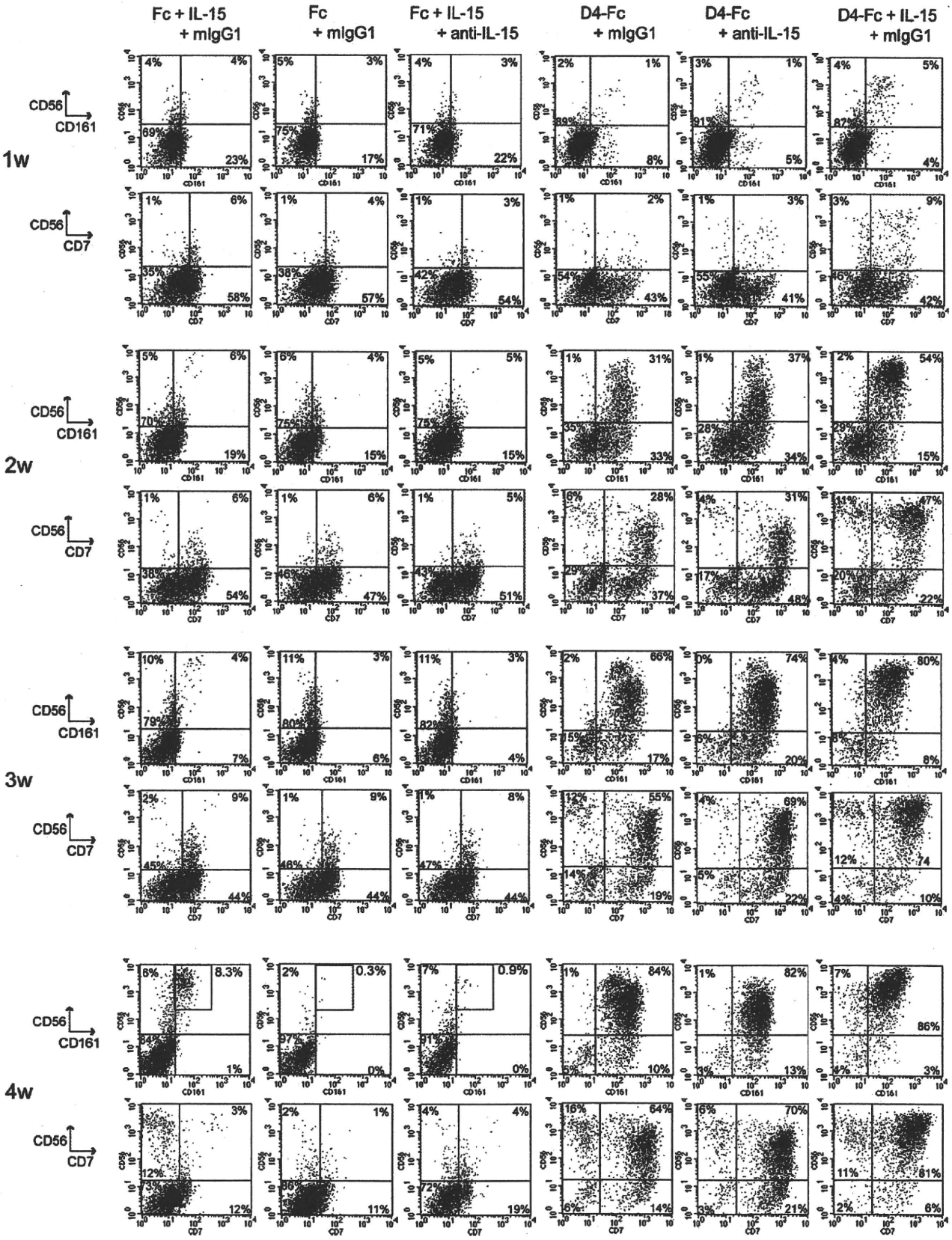

FIGURE 3. Phenotypic analysis during culture under several culture conditions. Representative dot plots illustrating CD161 vs CD56 and CD7 vs CD56 of cells that were cultured from CB CD34$^+$ cells for the indicated number of weeks on Fc-coated plates with IL-15 and mouse (m) IgG1-containing medium (Fc + IL-15 + mIgG1), Fc-coated plates with mouse IgG1-containing medium (Fc + mIgG1), Fc-coated plates with anti-IL-15 Ab-containing medium (Fc + anti-IL-15), Delta4-Fc-coated plates with mouse IgG1-containing medium (D4-Fc + mIgG1), Delta4-Fc-coated plates with anti-IL-15 Ab-containing medium (D4-Fc + anti-IL-15), and Delta4-Fc-coated plates with IL-15 and mouse IgG1-containing medium (D4-Fc + IL-15 + mIgG1). Results are representative of at least three experiments. The means and SD of each CD161 vs CD56 quadrant in replicate experiments are shown in supplemental Fig. S2.

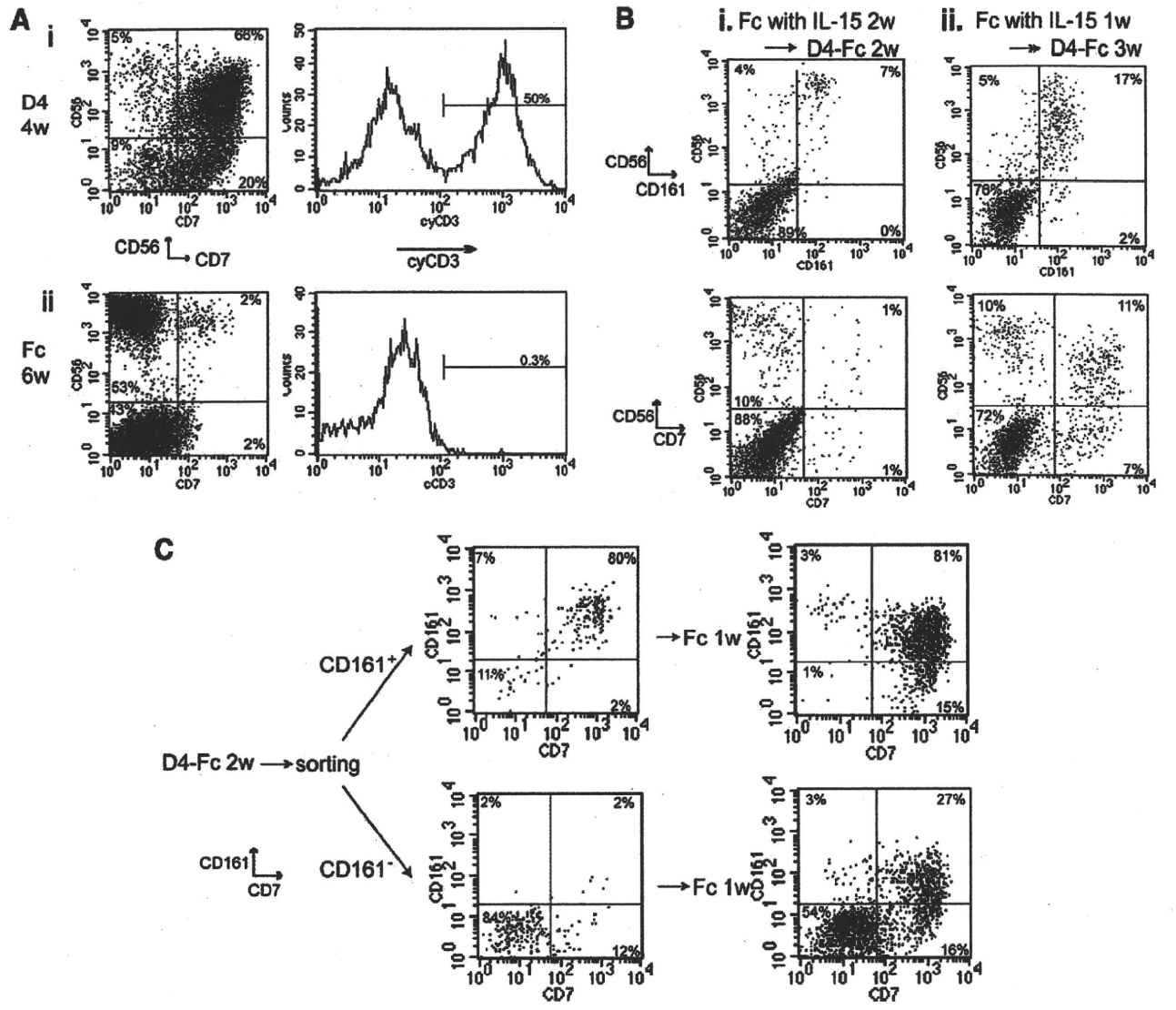

FIGURE 4. Phenotypic analysis of cells after various culture conditions. *A*, Representative dot plots illustrating CD7 vs CD56 cells that were cultured from CB CD34$^+$ cells for 4 wk on Delta4-Fc-coated plates (D4, *Ai*) and for 6 wk on Fc-coated plates in the presence of IL-15 (Fc, *Aii*). Histogram plots illustrating cyCD3 of the same cells that were gated on CD56$^+$ events. Results are representative of six and five experiments, respectively. *B*, Representative dot plots of cells that were cultured from CB CD34$^+$ cells for 2 or 1 wk on Fc-coated plates with IL-15-containing medium and were then transferred to Delta4-Fc-coated plates and cultured for 2 or 3 wk, respectively, with IL-15-free medium (*Bi* and *Bii*). Results are representative of three experiments. *C*, Representative dot plots illustrating CD7 vs CD161 expression in the cells that were sorted into CD161$^+$ or CD161$^-$ after 2-wk culture from CB CD34$^+$ cells on Delta4-Fc-coated plates, and dot plots of cells that were cultured another week on Fc-coated plates with IL-15-free medium. Results are representative of three experiments.

11%, and 88 ± 6.7% (without IL-15) at 2, 3, and 4 wk, respectively. (supplemental Fig. S2*Bi*) The differences were statistically significant between the D4-Fc group and the Fc group ($p < 0.001$). The adjusted absolute numbers of NK-lineage cells cultured on Delta4-Fc tended to be greater than those cultured on Fc with IL-15, although the differences were not always statistically significant (supplemental Fig. S3*C*). CD56$^+$ CD161$^+$ NK cells eventually comprised a major population after 6 wk of culture with IL-15 but without Notch stimulation (Fig. 4*Ai*). No CD56$^+$CD7$^+$ (Fig. 3, Fc plus IL-15) or CD56$^+$ cyCD3$^+$ (Fig. 4*Aii*) cells were detected during culture with IL-15 but without Delta4-Fc, whereas Delta4-Fc stimulation induced the generation of CD7$^+$cyCD3$^+$ cells, which could represent naturally arising T/NK cell progenitors (26, 27), at the early phase of the culture. Although CD7^{low} cells appeared in culture with IL-15 alone, they might represent monocytes, because a substantial amount of CD14$^+$ cells emerged regardless of the presence of IL-15 when Delta4-Fc was absent and peripheral blood monocytes express CD7 at low levels.

Delta4-Fc stimulation without IL-15 efficiently induced NK cell development (Figs. 1 and 3 and supplemental Fig. S2, D4-Fc). Most of the cells became CD7^{high} in the first 2 wk. A few CD161$^+$ cells were detected at the first week, the number of which increased at the next week. Only a part of the CD161$^+$ cells was positive for CD56 during the early phase of the culture, but at the later time points, most CD161$^+$ cells were CD56$^+$. This observation may indicate that CD161$^+$CD56$^-$ cells emerge at first and they gradually become CD161$^+$ CD56$^+$, although there is another interpretation such as simultaneous generation of double-positive and CD161 single-positive cells, expansion of double-positive cells, and apoptotic disappearance of the single-positive cells. Given the previous

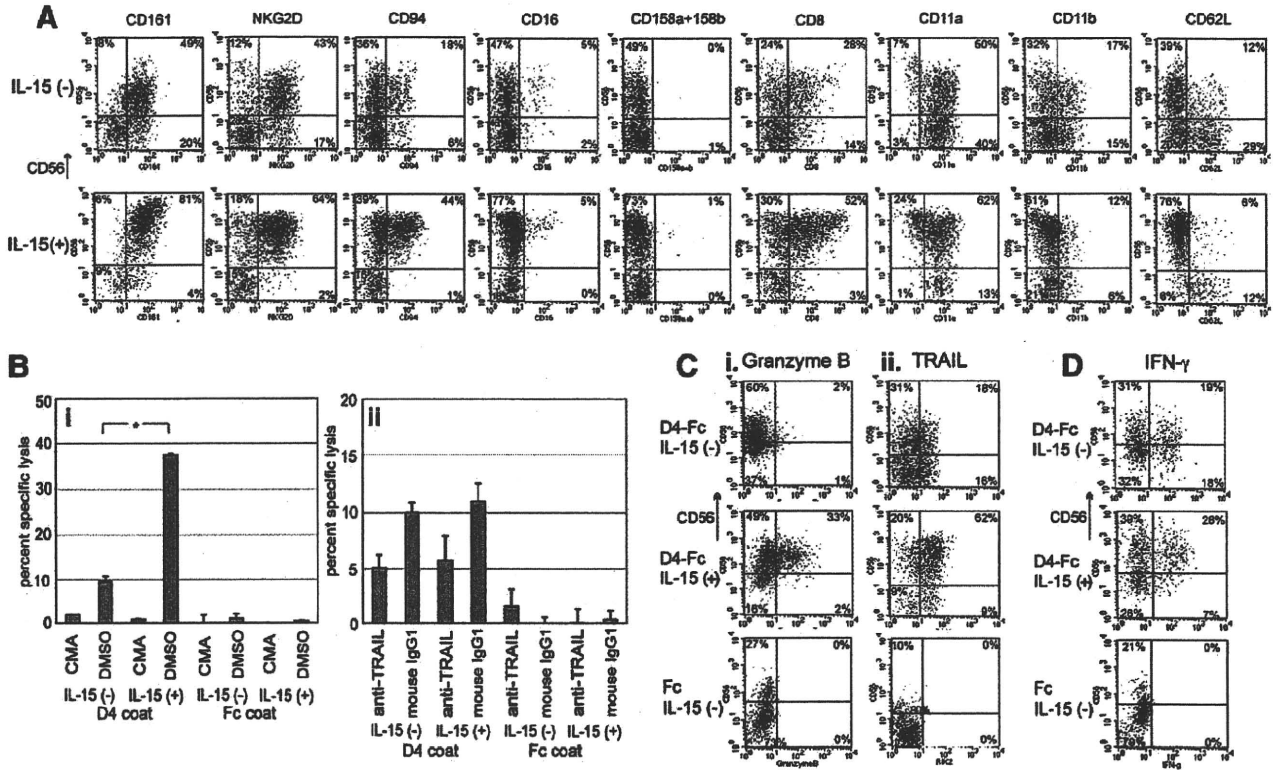

FIGURE 5. Phenotypic and functional differences between cells cultured in IL-15-containing and IL-15-free medium on Delta4-Fc-coated plates. *A*, Representative dot plots illustrating CD56 vs indicated Ags of cells cultured for 3 wk from CB CD34$^+$ cells in IL-15-containing or IL-15-free medium on Delta4-Fc-coated plates. Results are representative of six experiments. *B*, Cytotoxicity against K562 (*Bi*) or Jurkat (*Bii*) target cells at an E:T ratio of 5:1. Effectors were developed in the indicated conditions for 2.5 wk. In this experiment, the ratio of CD161$^+$ cells cultured on Delta4-Fc-coated plates with or without IL-15 condition and those cultured on Fc-coated plates with or without IL-15 condition were 53, 46, 0.6, and 0%, respectively. Effectors were pretreated with CMA or DMSO (the solvent for CMA) (*Bi*). Anti-TRAIL RIK-2 or its isotype control mouse IgG1 was added at the start of the cytotoxicity assay (*Bii*). Results are representative of three (*Bi*) and six (*Bii*) experiments. Batch to batch variation can be seen by comparing this figure with Fig. 1. *C*, Representative dot plots illustrating intracellular granzyme B (*Ci*) or TRAIL (*Cii*) vs CD56 of the cells cultured for 3 wk in medium with or without IL-15 on Delta4-Fc-coated plates and without IL-15 on Fc-coated plates. Results are representative of four experiments. *D*, Representative dot plots illustrating intracellular IFN-γ vs CD56 of cells cultured for 3 wk in medium with or without IL-15 on Delta4-Fc-coated plates and without IL-15 on Fc-coated plates. Results are representative of four experiments.

demonstration that CD161 is expressed on the cell surface earlier than CD56 (28), the former possibility appears more likely. To explore the possibility that IL-15 is secreted by a certain population of cells during culture and contributes to NK cell development, we added anti-IL-15-neutralizing Ab to the culture. The addition of anti-IL-15-neutralizing Ab to the culture medium blocked NK cell development in the presence of IL-15 (Fig. 3, IL-15 plus anti-IL-15), but did not affect either the rate or efficiency of Delta4-Fc-dependent NK cell emergence (Fig. 3, D4-Fc plus anti-IL-15, fold increase in the cell number after 3-wk culture on Delta4-coated plate with anti-IL-15 was 8.75 ± 4.18-fold (n = 5), which was not statistically different from those cultured on Delta4-coated plates without anti-IL-15 or with IL-15), further supporting the possibility that IL-15 is dispensable for NK cell development from human CB CD34$^+$ cells.

IL-2 is also suggested to be involved in the NK cell development. To examine whether IL-2, which might be secreted by a certain population of the cells, was present in the culture, the IL-2 concentration in the supernatant was measured by ELISA. No IL-2 was detected (cutoff level, 7 pg/ml; data not shown), indicating that IL-2 was not involved in the NK cell development induced by Delta4-Fc.

To examine the NK cell developmental stages that are critically dependent on Notch signaling, we cultured CB CD34$^+$ cells on control Fc-coated plates with IL-15 for 1 or 2 wk and then transferred them onto Delta4-Fc-coated plates and cultured them further for 3 or 2 wk without IL-15, respectively (culturing for a total of 4 wk). Approximately 50% of the CD56$^+$ CD161$^+$ population expressed CD7$^+$ at 4 wk in the 1-wk IL-15 condition (Fig. 4*Bii*). In contrast, very few CD56$^+$ cells that emerged in the 2-wk IL-15 condition expressed CD7 (Fig. 4*Bi*). These observations indicated that CB CD34$^+$ cells cultured with IL-15, but without Notch stimulation, for 1 wk retained the capacity to generate CD56$^+$CD7$^+$ cells, but that they lost this capacity when cultured without Notch stimulation for 2 wk. We also examined whether the Notch stimulation at early phases of the culture irreversibly determines NK cell developmental fate. To examine the early phase of NK cell development, we cultured CB CD34$^+$ cells for 2 wk on Delta4-Fc-coated plates and sorted the product into CD161$^+$ and CD161$^-$ cells, because CD161 is known to be expressed earlier than CD56 on the cell surface (28). We then transferred each population onto control Fc-coated plates and cultured them for another week without IL-15. More than 80% of the population derived from the CD161$^+$ cells expressed CD7$^+$. Interestingly, the CD161$^-$ cells also gave rise to CD161$^+$CD7$^+$ cells among one of the two major populations (Fig. 4*C*). These observations indicate that Notch activation irreversibly drives a subset of CD34$^+$ cell

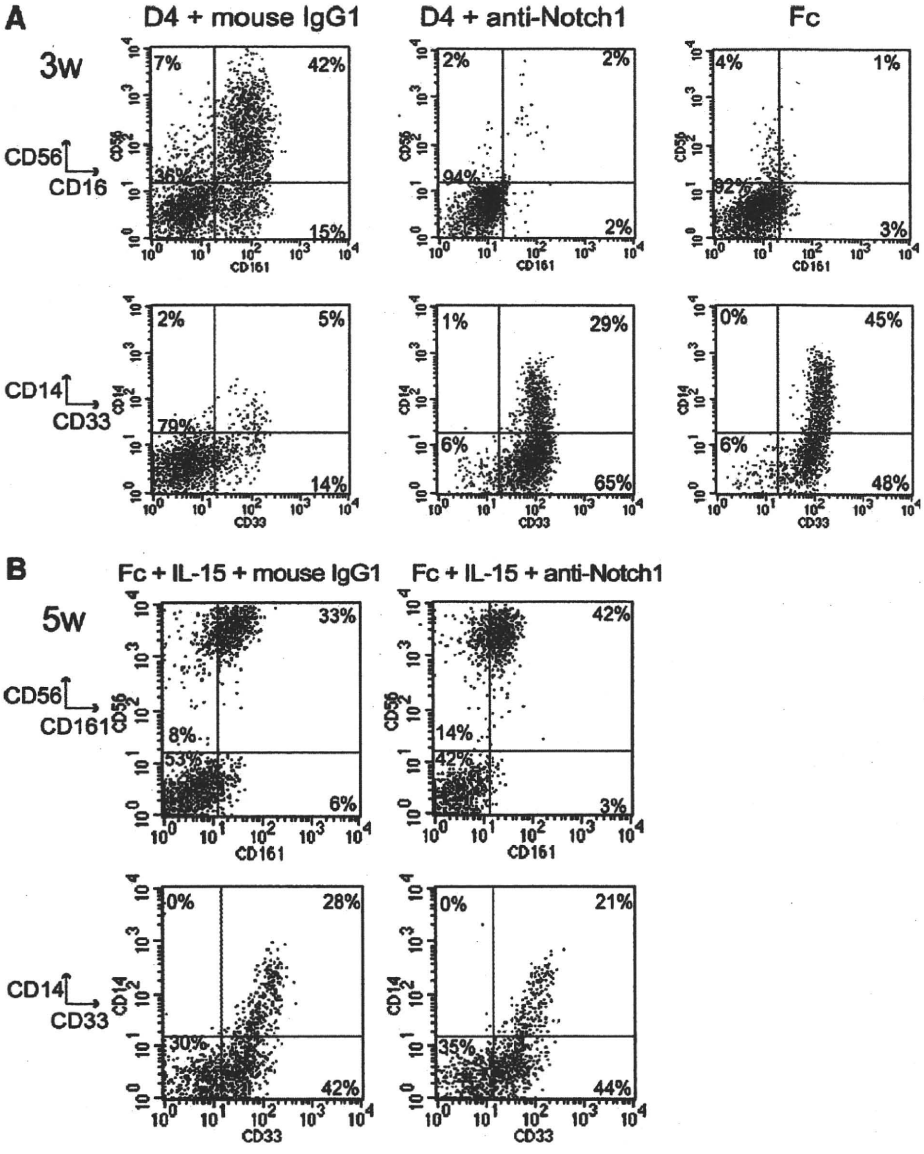

FIGURE 6. Phenotypic analysis of cells cultured in the presence of human Notch1-blocking Ab. *A*, Representative dot plots of cells that were cultured for 3 wk from CB $CD34^+$ cells on Delta4-Fc-coated plates with mouse IgG1-containing medium, Delta4-Fc-coated plates with anti-human Notch1-containing medium, and Fc-coated plates. Results are representative of six experiments. *B*, Representative dot plots of cells that were cultured for 5 wk from CB $CD34^+$ cells on Fc-coated plates with IL-15 and mouse IgG1-containing medium and Fc-coated plates with IL-15 and anti-human Notch1-containing medium. Results are representative of three experiments.

progenies to the $CD161^+CD7^+$ NK cell fate within 2 wk, presumably before $CD161^+$ is expressed.

IL-15, along with Delta4 stimulation, induces phenotypic maturation and functional augmentation of CB $CD34^+$ cell-derived NK cells

We compared the immunophenotype of the CB $CD34^+$ cell-derived NK cells generated in the culture with Delta4-Fc but lacking IL-15 (D4-Fc) and in culture with Delta4-Fc and IL-15 (D4-Fc plus IL-15). IL-15 does not affect the absolute cell number; fold increases in the cell number after the 3-wk culture were 10.6 ± 6.16-fold and 10.2 ± 6.71-fold with and without IL-15 in the D4-Fc-coated plate condition (n = 8). The cells grew slightly faster with D4-Fc plus IL-15 than with D4-Fc alone, but there were no significant differences in the frequency of $CD56^+CD161^+$ population in both conditions after 3 wk (cf Fig. 3 and supplemental Fig. S2*A*, D4-Fc and D4-Fc plus IL-15; supplemental Fig. S2*Bii*; and Fig. 5). The expression levels of CD7 and NKG2D were similar. CD94 was expressed at a higher level in the D4-Fc plus IL-15 condition. CD16 and CD158 were not expressed in the D4-Fc condition, but were expressed at low levels in the D4-Fc plus IL-15 condition. The expression levels of adhesion molecules, i.e., CD11a, CD11b, and CD62L, were higher in the D4-Fc condition (Fig. 5*A*). The other markers shown in Fig. 1 (CD2, CD7, CD25, CD27, CD44, CD45RA, CD57, CD117, CD122, and CCR7; data not shown), as well as IFN-γ (Fig. 5*D*), were expressed at similar levels under both conditions. There was a remarkable difference in the expression level of CD56, which was markedly higher in the D4-Fc plus IL-15 condition.

Cytotoxic activity against K562 cells was significantly higher in NK cells generated in the D4-Fc plus IL-15 condition than that in the D4-Fc condition. CMA, an inhibitor of perforin-mediated cytotoxicity, had a stronger suppressive effect on the cytotoxic activities of NK cells generated in the D4-Fc plus IL-15 condition (Fig. 5*Bi*). Interestingly, granzyme B, which enhances the perforin-mediated cytotoxicity and whose expression was not detected in the D4-Fc condition, was up-regulated in the D4-Fc plus IL-15 condition (Fig. 5*Ci*). This might explain the stronger suppression of NK cell cytotoxic activity by CMA when generated in the D4-Fc plus IL-15 condition compared with the D4-Fc condition. In contrast, there was no significant difference in the killing activities against Jurkat cells of the NK cells generated under

either condition (Fig. 5*Bii*), and CMA did not affect the cytotoxic activities against Jurkat cells, irrespective of the culture conditions (data not shown). This finding suggests that perforin or granzyme B does not have a major role in killing Jurkat cells. We evaluated whether TRAIL had a role by adding anti-TRAIL-blocking Ab RIK2 to the medium. RIK2 partially but clearly suppressed the cytotoxic activities against Jurkat cells generated in both conditions without significant differences (Fig. 5*Bii*), although TRAIL expression was slightly higher in the NK cells generated in the D4-Fc plus IL-15 condition (Fig. 5*Cii*). From these observations, we concluded that IL-15 does not influence the killing activity through TRAIL but does enhance the killing activity through perforin/granzyme B. The cytotoxic activity of immature NK cells is TRAIL dependent, while that of mature NK cells is mainly dependent on perforin (29). Therefore, IL-15 might contribute to the maturation of NK cells and confer on them the capacity to exact perforin/granzyme B-medicated cytotoxicity.

Inhibitory effect of anti-Notch1 Ab on Delta4-dependent NK cell development

We prepared mAbs specific for the extracellular domain of Notch1, Notch2, and Notch3 (supplemental Fig. S4*A*). The expression patterns of Notch1, Notch2, and Notch3 in fresh CB mononuclear cells, $CD34^+$ cells, and products during the culture of $CD34^+$ cells are shown in supplemental Fig. S3, *A* and *B*. Notch1 was expressed at higher levels on NK and T cells than on B cells and monocytes. Notch2 was expressed at higher levels on monocytes than on lymphocytes. Notch3 expression was virtually negative on all types of lymphocytes and positive on monocytes. Notch1 and Notch2, but not Notch3, were expressed on $CD34^+$ cells. The $CD34^+$ cell-derived $CD56^+$ NK cells also expressed Notch1 and Notch2, but not Notch3. All three Notch receptors were expressed on cells grown on the control Fc-coated plates (supplemental Fig. S3*B*).

Because $CD34^+$ cells expressed Notch1 and Notch2, but not Notch3 (supplemental Fig. S3*B*), and the established anti-Notch1 Ab, but not anti-Notch2 Ab, blocked binding of the cognate soluble Notch receptor to the ligands (supplemental Fig. S4*B*), we cultured CB $CD34^+$ cells on Delta4-Fc-coated plates in anti-Notch1 Ab-containing medium. Remarkably, the immunophenotype of the cells grown under the presence of anti-Notch1 Ab was almost the same as that of cells grown on control Fc-coated plates, indicating that the effect of Delta4 was completely blocked and NK cell development was shut down by the anti-Notch1 Ab (Fig. 6*A*). Anti-Notch2 Ab did not have such an effect, consistent with the fact that it did not block ligand binding to the cognate receptors (data not shown). CB $CD34^+$ cells cultured with IL-15 on Fc-coated plates in the presence of the anti-Notch1 Ab gave rise to NK cells in a manner indistinguishable from that of cells grown without the Ab (Fig. 6*B*). These results suggest that Notch1 might be a physiologic Notch receptor that mediates Delta4 signaling for NK cell development from CB $CD34^+$ cells and further support the notion that Notch signaling has a role distinct from that of IL-15.

Discussion

In the present study, we demonstrated that functional NK cells developed from CB $CD34^+$ cells when stimulated with the Notch ligand Delta4. Previous reports indicated that NK cells can be derived from in vitro culture of human $CD34^+$ cells prepared from fetal liver, bone marrow, or CB with either IL-2 or IL-15 (30–33), which signal through the shared IL-2/IL-15 receptor β-chain and the common γ-chain. IL-15 has been considered to have a more physiologic role than IL-2 in NK development (30). Notably, IL-15-independent NK cell differentiation has recently been published (6). This culture system, however, has been reported to be stromal cell dependent while the potential molecules and signaling pathways are unknown and, thus, the conclusion whether IL-15 is indispensable is yet to be determined. Notch signaling has been examined in the context of NK cell development as well and appears to affect the very early phase of progenitor development (17–19). In studies of human NK cell development, however, culture systems containing IL-15 and/or a coculture system with the fetal thymus organ or stromal cells are used exclusively. A novel and unexpected finding in the present study was the fact that stimulation of CB $CD34^+$ cells with a soluble Notch ligand, Delta4-Fc, coated onto the plate in the presence of stem cell factor, FL, and IL-7 was sufficient to induce the development of functional NK cells.

Our data do not officially exclude the possibility that endogenous IL-15 is involved in NK cell development in a manner, e.g., that cell-autonomously produced IL-15 activated the signaling by binding to the receptor intracellularly. Given the fact, however, that the exogenous addition of IL-15 resulted in the qualitative rather than quantitative difference in the NK cells developed in the presence of Delta4-Fc, in addition to inefficient blockade by anti-IL-15-neutralizing Ab, IL-15 is likely to be dispensable for human NK cell development in the presence of Delta4-Fc.

The finding that IL-15 is not necessary for human NK cell development in culture contrasts with the absolute necessity of IL-15 signaling for NK development in some mouse phenotypes; mice lacking a gene for IL-15 (3) (34, 35), IL-15 receptor α-chain (36), common β-chain (37), or common γ-chain (38, 39) lack NK cells. This might be due to differences between the in vitro culture conditions and the in vivo environment in which NK cells develop. Another explanation might be a difference between mice and humans, as in the case of IL-7 requirement for T cell development; IL-7 is required for the V-D-J rearrangement of the TCR β-chain gene in humans, whereas it is dispensable in mouse T cell development (40).

Previous studies reported that the effect of Notch signaling in the presence of IL-15 on NK cell development is confined to the very early stages of development. In the present study, we demonstrated that Notch signaling confers CD7 expression competence on cells cultured with or without IL-15 for 1 wk or less, but not for 2 wk, unless also stimulated by Notch. This finding is similar to that in a previous report demonstrating that Notch signaling confers cyCD3 expression competence only on prethymic but not thymic NK cell progenitors or peripheral blood $cyCD3^-$ NK cells (19). We confirmed the Notch signal dependency of cyCD3 expression during NK cell development. Coexpression of CD7 and CD45RA on $CD34^+$ cells might be associated with a restriction toward NK cell development (26, 33). Our data strongly suggest that the vast majority, if not all, of the NK cells derived from $CD34^+$ cells without Notch signaling were generated through $CD7^-$ cells. Therefore, although it is yet to be elucidated whether all of the NK cell progenitors are $CD7^+$ (41), NK cells established in vitro without Notch stimulation might not develop from a physiologic NK progenitor or might skip the physiologic NK/T progenitor stage. Furthermore, our data suggest that the effect of Notch stimulation on CD7 expression is imprinted on cells only if it is administered at the initial stage of the $CD34^+$ cell culture. We, however, failed to prospectively identify the subpopulations in the $CD34^+$ cells that are targets of Delta4 to develop NK-lineage cells. Delta4 stimulation induced NK cell development from both the most immature $CD34^+CD38^-$ and more mature $CD34^+CD38^+$ progenitor populations and both $CD34^+$

$CD45RA^+$ lymphoid progenitors and $CD34^+CD45RA^-$ populations (data not shown).

The findings of the present study extend our understanding to more mature stages of NK cell differentiation: the presence of Notch signaling induces generation of functional NK cells in culture conditions that do not generate $CD56^+$ cells without Notch stimulation per se. The precise stages of NK cell development during which Notch signaling determines the progression toward functional NK cells is not known.

In our experiments, even cells cultured with a Notch ligand alone had cytotoxic activity. The level of this activity, however, was weaker than that in NK cells generated by Notch stimulation with IL-15. Indeed, the perforin-mediated cytotoxicity of NK cells generated in the absence of IL-15 was significantly weaker, despite the fact that this is the major pathway of mature NK cells to kill target cells (42). In contrast, the TRAIL-mediated cytotoxicity was almost the same regardless of presence or absence of IL-15. This finding, along with the change in the expression level of CD56, might indicate that IL-15 induces the maturation of $CD56^{low}$ $CD161^+$ immature NK cells generated by Notch stimulation without IL-15. Another difference between the cells cultured with or without IL-15 was the down-regulation of adhesion molecules (CD11a, CD11b, CD62L) on the cell surface. These molecules might be important for homing of the NK cells to the sites at which they function.

To our surprise, cytotoxic activities were not detected in the cell populations generated in the control Fc plus IL-15 condition at either 3 or 6 wk (Fig. 5*B* and data not shown), although these results might be affected by the facts that the frequency of $CD56^+CD161^+$ cells was very low at 3 wk and that culture for 6 wk might be too long to evaluate cytotoxic activities while the frequency of $CD56^+CD161^+$ cells was much greater. In any case, when clinical application of progenitor-derived NK cells is considered, a Delta4-Fc-coating system would give a significant advantage.

In conclusion, Notch stimulation by Delta4 (or Delta1) was required for initial NK cell differentiation and the development of $CD161^+CD56^{low}$ immature NK cells. Among Notch receptors, Notch1 might be essential for physiologic NK cell development, although the involvement of other Notch receptors is yet to be elucidated. IL-15 was not essential for differentiation, but was necessary for maturation. IL-15 might have an indispensable role only in the later part of the NK development. This knowledge might be useful for future approaches toward the ex vivo generation and manipulation of NK cells and their therapeutic application.

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Disclosures

The authors have no financial conflict of interest.

References

1. Freud, A. G., and M. A. Caligiuri. 2006. Human natural killer cell development. *Immunol. Rev.* 214: 56–72.
2. Hayakawa, Y., N. D. Huntington, S. L. Nutt, and M. J. Smyth. 2006. Functional subsets of mouse natural killer cells. *Immunol. Rev.* 214: 47–55.
3. Kennedy, M. K., M. Glaccum, S. N. Brown, E. A. Butz, J. L. Viney, M. Embers, N. Matsuki, K. Charrier, L. Sedger, C. R. Willis, et al. 2000. Reversible defects in natural killer and memory CD8 T cell lineages in interleukin 15-deficient mice. *J. Exp. Med.* 191: 771–780.
4. Williams, N. S., J. Klem, I. J. Puzanov, P. V. Sivakumar, J. D. Schatzle, M. Bennett, and V. Kumar. 1998. Natural killer cell differentiation: insights from knockout and transgenic mouse models and in vitro systems. *Immunol. Rev.* 165: 47–61.
5. Yokoyama, W. M., S. Kim, and A. R. French. 2004. The dynamic life of natural killer cells. *Annu. Rev. Immunol.* 22: 405–429.
6. McCullar, V., R. Oostendorp, A. Panoskaltsis-Mortari, G. Yun, C. T. Lutz, J. E. Wagner, and J. S. Miller. 2008. Mouse fetal and embryonic liver cells differentiate human umbilical cord blood progenitors into CD56-negative natural killer cell precursors in the absence of interleukin-15. *Exp. Hematol.* 36: 598–608.
7. Chiba, S. 2006. Concise review: Notch signaling in stem cell systems. *Stem Cells* 24: 2437–2447.
8. Artavanis-Tsakonas, S., M. D. Rand, and R. J. Lake. 1999. Notch signaling: cell fate control and signal integration in development. *Science* 284: 770–776.
9. Suzuki, T., and S. Chiba. 2005. Notch signaling in hematopoietic stem cells. *Int. J. Hematol.* 82: 285–294.
10. Radtke, F., A. Wilson, G. Stark, M. Bauer, J. v. Meerwijk, H. R. MacDonald, and M. Aguet. 1999. Deficient T cell fate specification in mice with an induced inactivation of Notch1. *Immunity* 10: 547–558.
11. Wilson, A., H. R. MacDonald, and F. Radtke. 2001. Notch 1-deficient common lymphoid precursors adopt a B cell fate in the thymus. *J. Exp. Med.* 194: 1003–1012.
12. Pui, J. C., D. Allman, L. Xu, S. DeRocco, F. G. Karnell, S. Bakkour, J. Y. Lee, T. Kadesch, R. R. Hardy, J. C. Aster, and W. S. Pear. 1999. Notch1 expression in early lymphopoiesis influences B versus T lineage determination. *Immunity* 11: 299–308.
13. Jaleco, A. C., H. Neves, E. Hooijberg, P. Gameiro, N. Clode, M. Haury, D. Henrique, and L. Parreira. 2001. Differential effects of Notch ligands Delta-1 and Jagged-1 in human lymphoid differentiation. *J. Exp. Med.* 194: 991–1002.
14. De Smedt, M., I. Hoebeke, K. Reynvoet, G. Leclercq, and J. Plum. 2005. Different thresholds of Notch signaling bias human precursor cells toward B-, NK-, monocytic/dendritic-, or T-cell lineage in thymus microenvironment. *Blood* 106: 3498–3506.
15. van den Brandt, J., K. Voss, M. Schott, T. Hunig, M. S. Wolfe, and H. M. Reichardt. 2004. Inhibition of Notch signaling biases rat thymocyte development towards the NK cell lineage. *Eur. J. Immunol.* 34: 1405–1413.
16. Garcia-Peydro, M., V. G. de Yebenes, and M. L. Toribio. 2006. Notch1 and IL-7 receptor interplay maintains proliferation of human thymic progenitors while suppressing non-T cell fates. *J. Immunol.* 177: 3711–3720.
17. Rolink, A. G., G. Balciunaite, C. Demoliere, and R. Ceredig. 2006. The potential involvement of Notch signaling in NK cell development. *Immunol. Lett.* 107: 50–57.
18. Carotta, S., J. Brady, L. Wu, and S. L. Nutt. 2006. Transient Notch signaling induces NK cell potential in Pax5-deficient pro-B cells. *Eur. J. Immunol.* 36: 3294–3304.
19. De Smedt, M., T. Taghon, I. Van de Walle, G. De Smet, G. Leclercq, and J. Plum. 2007. Notch signaling induces cytoplasmic CD3e expression in human differentiating NK cells. *Blood* 110: 2696–2703.
20. Karanu, F. N., B. Murdoch, T. Miyabayashi, M. Ohno, M. Koremoto, L. Gallacher, D. Wu, A. Itoh, S. Sakano, and M. Bhatia. 2001. Human homologues of Delta-1 and Delta-4 function as mitogenic regulators of primitive human hematopoietic cells. *Blood* 97: 1960–1967.
21. Kayagaki, N., N. Yamaguchi, M. Nakayama, A. Kawasaki, H. Akiba, K. Okumura, and H. Yagita. 1999. Involvement of TNF-related apoptosis-inducing ligand in human $CD4^+$ T cell-mediated cytotoxicity. *J. Immunol.* 162: 2639–2647.
22. Kataoka, T., N. Shinohara, H. Takayama, K. Takaku, S. Kondo, S. Yonehara, and K. Nagai. 1996. Concanamycin A, a powerful tool for characterization and estimation of contribution of perforin- and Fas-based lytic pathways in cell-mediated cytotoxicity. *J. Immunol.* 156: 3678–3686.
23. Shimizu, K., S. Chiba, K. Kumano, N. Hosoya, T. Takahashi, Y. Kanda, Y. Hamada, Y. Yazaki, and H. Hirai. 1999. Mouse Jagged1 physically interacts with Notch2 and other Notch receptors. *J. Biol. Chem.* 274: 32961–32969.
24. Geling, A., H. Steiner, M. Willem, L. Bally-Cuif, and C. Haass. 2002. A γ-secretase inhibitor blocks Notch signaling in vivo and causes a severe neurogenic phenotype in zebra fish. *EMBO Rep.* 3: 688–694.
25. Cheng, H.-T., J. H. Miner, M. Lin, M. G. Tansey, K. Roth, and R. Kopan. 2003. γ-Secretase activity is dispensable for mesenchyme-to-epithelium transition but required for podocyte and proximal tubule formation in developing mouse kidney. *Development* 130: 5031–5042.
26. Haddad, R., P. Guardiola, B. Izac, C. Thibault, J. Radich, A.-L. Delezoide, C. Baillou, F. M. Lemoine, J. C. Gluckman, F. Pflumio, and B. Canque. 2004. Molecular characterization of early human T/NK and B-lymphoid progenitor cells in umbilical cord blood. *Blood* 104: 3918–3926.
27. Sanchez, M., M. Muench, M. Roncarolo, L. Lanier, and J. Phillips. 1994. Identification of a common T/natural killer cell progenitor in human fetal thymus. *J. Exp. Med.* 180: 569–576.
28. Huntington, N. D., C. A. J. Vosshenrich, and J. P. Di Santo. 2007. Developmental pathways that generate natural-killer-cell diversity in mice and humans. *Nat. Rev. Immunol.* 7: 703–714.
29. Zamai, L., M. Ahmad, I. M. Bennett, L. Azzoni, E. S. Alnemri, and B. Perussia. 1998. Natural killer (NK) cell-mediated cytotoxicity: differential use of TRAIL and Fas ligand by immature and mature primary human NK cells. *J. Exp. Med.* 188: 2375–2380.
30. Mrozek, E., P. Anderson, and M. Caligiuri. 1996. Role of interleukin-15 in the development of human $CD56^+$ natural killer cells from $CD34^+$ hematopoietic progenitor cells. *Blood* 87: 2632–2640.
31. Jaleco, A., B. Blom, P. Res, K. Weijer, L. Lanier, J. Phillips, and H. Spits. 1997. Fetal liver contains committed NK progenitors, but is not a site for development of $CD34^+$ cells into T cells. *J. Immunol.* 159: 694–702.

32. Lotzova, E., C. Savary, and R. Champlin. 1993. Genesis of human oncolytic natural killer cells from primitive $CD34^+CD33^-$ bone marrow progenitors. *J. Immunol.* 150: 5263–5269.
33. Miller, J., K. Alley, and P. McGlave. 1994. Differentiation of natural killer (NK) cells from human primitive marrow progenitors in a stroma-based long-term culture system: identification of a $CD34^+7^+$ NK progenitor. *Blood* 83: 2594–2601.
34. Ohteki, T., H. Yoshida, T. Matsuyama, G. S. Duncan, T. W. Mak, and P. S. Ohashi. 1998. The transcription factor interferon regulatory factor 1(IRF-1) is important during the maturation of natural killer 1.1^+ T cell receptor-α/β^+ ($NK1^+$ T) cells, natural killer cells, and intestinal intraepithelial T cells. *J. Exp. Med.* 187: 967–972.
35. Ogasawara, K., S. Hida, N. Azimi, Y. Tagaya, T. Sato, T. Yokochi-Fukuda, T. A. Waldmann, T. Taniguchi, and S. Taki. 1998. Requirement for IRF-1 in the microenvironment supporting development of natural killer cells. *Nature* 391: 700–703.
36. Lodolce, J. P., D. L. Boone, S. Chai, R. E. Swain, T. Dassopoulos, S. Trettin, and A. Ma. 1998. IL-15 receptor maintains lymphoid homeostasis by supporting lymphocyte homing and proliferation. *Immunity* 9: 669–676.
37. Suzuki, H., G. S. Duncan, H. Takimoto, and T. W. Mak. 1997. Abnormal development of intestinal intraepithelial lymphocytes and peripheral natural killer cells in mice lacking the IL-2 receptor β chain. *J. Exp. Med.* 185: 499–506.
38. Cao, X., E. W. Shores, J. Hu-Li, M. R. Anver, B. L. Kelsall, S. M. Russell, J. Drago, M. Noguchi, A. Grinberg, E. T. Bloom, et al. 1995. Defective lymphoid development in mice lacking expression of the common cytokine receptor γ chain. *Immunity* 2: 223–238.
39. DiSanto, J., W. Muller, D. Guy-Grand, A. Fischer, and K. Rajewsky. 1995. Lymphoid development in mice with a targeted deletion of the interleukin 2 receptor γ chain. *Proc. Natl. Acad. Sci. USA* 92: 377–381.
40. Spits, H. 2002. Development of $\alpha\beta$ T cells in the human thymus. *Nat. Rev. Immunol.* 2: 760–772.
41. Freud, A. G., A. Yokohama, B. Becknell, M. T. Lee, H. C. Mao, A. K. Ferketich, and M. A. Caligiuri. 2006. Evidence for discrete stages of human natural killer cell differentiation in vivo. *J. Exp. Med.* 203: 1033–1043.
42. Smyth, M. J., E. Cretney, J. M. Kelly, J. A. Westwood, S. E. A. Street, H. Yagita, K. Takeda, S. L. H. van Dommelen, M. A. Degli-Esposti, and Y. Hayakawa. 2005. Activation of NK cell cytotoxicity. *Mol. Immunol.* 42: 501–510.

Lipopolysaccharide-Induced Monocytic Cell Death for the Diagnosis of Mild Neonatal-Onset Multisystem Inflammatory Disease

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In this report, we describe a boy who showed mild symptoms of neonatal-onset multisystem inflammatory disease. Although his symptoms and laboratory findings were similar to those of systemic juvenile idiopathic arthritis, further examinations revealed papilledema, meningitis, and a *NLRP3* mutation. His peripheral blood monocytes died within 24 hours after lipopolysaccharide stimulation, a test that may be useful for diagnosis even in mild cases. *(J Pediatr 2008;152:885-7)*

Cryopyrin-associated periodic syndromes (CAPS), which include neonatal-onset multisystem inflammatory disease (NOMID), Muckle-Wells syndrome, and familial cold autoinflammatory syndrome, are autosomal-dominant autoinflammatory disorders caused by *NLR family pyrin domain containing 3* (*NLRP3*) mutations.[1] NOMID, the most severe form among them, is characterized by early onset of rash, fever, uveitis, chronic meningitis, and joint manifestations.[1-5] Head enlargement, wide fontanel, hydrocephalus, hearing loss, blindness, poor growth, and mental retardation are also observed.[1-5] Because the diagnosis of CAPS is primarily clinical, it may be difficult when the patients show atypical manifestations.

Interleukin (IL)-1ß oversecretion is the principal mechanism of CAPS.[1-5] Disease-associated *NLRP3* mutations, most of which were found in exon 3, have a profound intrinsic proinflammatory effect through oligomerization of NLRP3. This leads to its binding to ASC (apoptosis-associated speck-like protein containing a card), the formation of a molecular platform for caspase-1 activation called an inflammasome, and oversecretion of IL-1β that is cleaved from pro–IL-1β by activated caspase-1.[6-8] It was recently reported that overexpression of mutated *NLRP3* or LPS stimulation lead to a rapid death of human THP-1 monocytic cells by necrosis.[9]

We report a boy with spiking fever and recurrent erythematous rashes that started when he was 10 months old. We found a novel mutation of *NLRP3* and a significant lipopolysaccharide (LPS)-induced death of monocytes in the patient.

CASE REPORT

An 11-month-old boy was referred to our hospital because he had prolonged spiking fever and recurrent erythematous rashes for 1 month.

On physical examination, numerous areas of erythema were disseminated on the trunk and extremities. Wide fontanel (3.5 × 4.7 cm) was observed. His joints were normal. Leukocyte count was 23.74 × 10^9/L, with 67.0% neutrophils. The erythrocyte sedimentation rate was 73 mm/h. Serum C-reactive protein concentration was 10.5 mg/dL. There was no evidence of infection or malignant diseases. These clinical findings strongly suggested systemic juvenile idiopathic arthritis (sJIA).

The ophthalmologic examination revealed bilateral papilledema, which is not usually observed in sJIA. Therefore, we decided to analyze the possibility of CAPS in this patient. Histologic examination of the skin showed mild perivascular and interstitial infiltrations of lymphocytes and polymorphonuclear cells with mild edema (data not shown), which did not seem to be the typical findings of CAPS. On the other hand, the cerebrospinal fluid (CSF) examination showed pleocytosis (198 cells/μL, with 80.8% polymorphonuclear cells) with normal protein and glucose concentrations. Concentrations

CAPS	Cryopyrin-associated periodic syndromes	*NLRP3*	NLR family, pyrin-domain containing 3
CSF	Cerebrospinal fluid	NOMID	Neonatal-onset multisystem inflammatory disease
IL	Interleukin		
LPS	Lipopolysaccharide	sJIA	Systemic juvenile idiopathic arthritis

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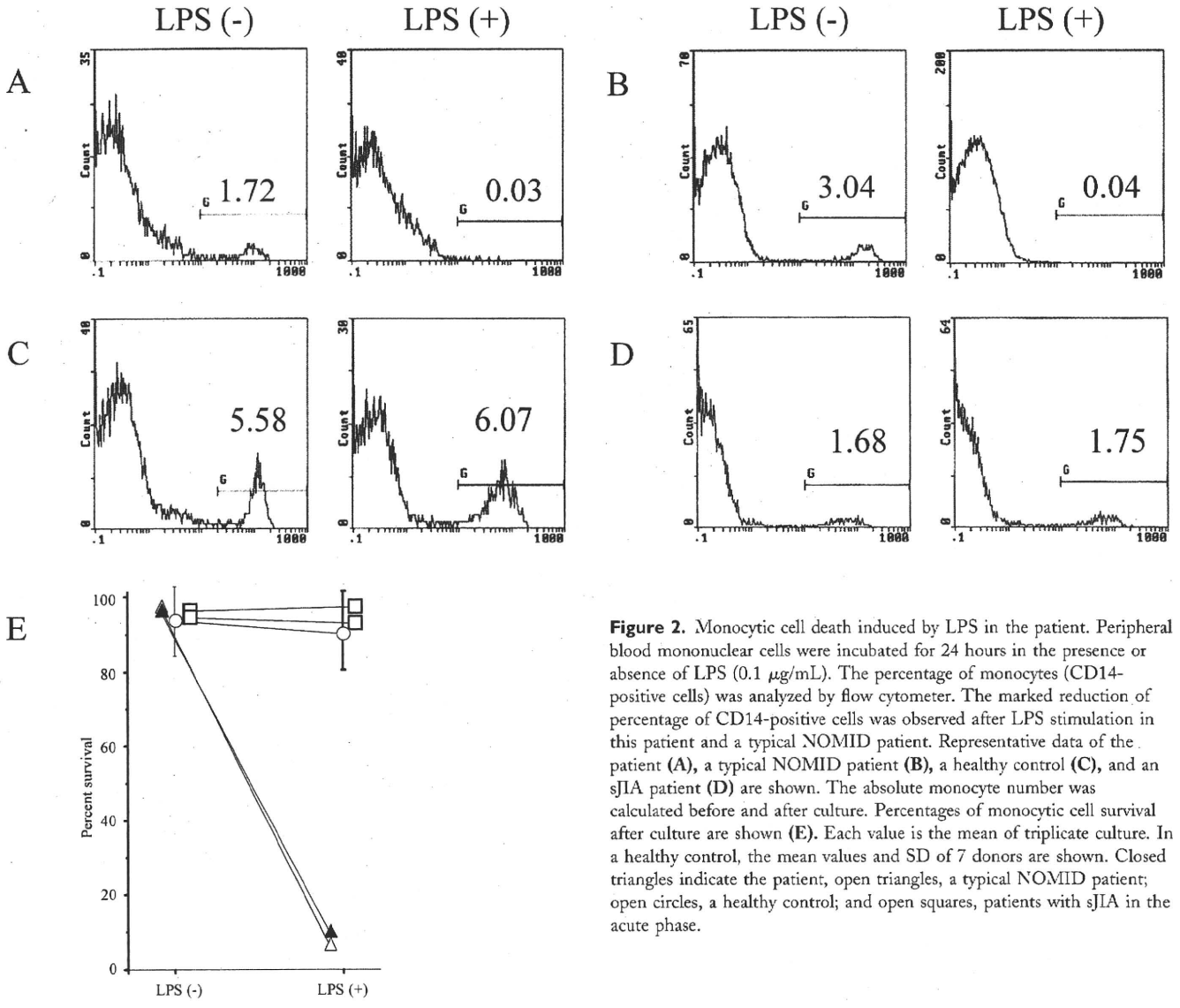

Figure 2. Monocytic cell death induced by LPS in the patient. Peripheral blood mononuclear cells were incubated for 24 hours in the presence or absence of LPS (0.1 μg/mL). The percentage of monocytes (CD14-positive cells) was analyzed by flow cytometer. The marked reduction of percentage of CD14-positive cells was observed after LPS stimulation in this patient and a typical NOMID patient. Representative data of the patient **(A)**, a typical NOMID patient **(B)**, a healthy control **(C)**, and an sJIA patient **(D)** are shown. The absolute monocyte number was calculated before and after culture. Percentages of monocytic cell survival after culture are shown **(E)**. Each value is the mean of triplicate culture. In a healthy control, the mean values and SD of 7 donors are shown. Closed triangles indicate the patient, open triangles, a typical NOMID patient; open circles, a healthy control; and open squares, patients with sJIA in the acute phase.

of serum and CSF IL-1β were 538.6 pg/mL and 245.6 pg/mL, respectively, and those of IL-6 were 27.3 pg/mL and 126.5 pg/mL, respectively (normal range: <20 pg/mL in serum or CSF for both cytokines).

METHODS

All coding regions and intronic flanking sequences of the *NLRP3* gene were amplified by PCR[2] and sequenced using ABI PRISM 3100 Genetic Analyzer (Perkin-Elmer; Foster City, CA).

Peripheral blood mononuclear cells were incubated 24 hours in the presence or absence of LPS (0.1 μg/mL) (Sigma-Aldrich; St Louis, MO). The cells were stained with PE-cyanin 5·1 (PC5)-conjugated anti-CD14 monoclonal antibody (Beckman Coulter; Miami, FL), and the percentage of monocytes was analyzed by flow cytometer (EPICS XL, Beckman Coulter).

This study was approved by the Regional Committee of Ethics for Human Research at the Faculty of Medicine of Kyushu University.

RESULTS

NLRP3 Mutation in the Patient

We considered the possibility that the patient had NOMID because he had prolonged fever, rash, wide fontanel, papilledema, and meningitis. We found a heterozygous mutation (1075 T > G, L359V) in his *NLRP3* gene within the NACHT (NAIP, CIITA, HET-E, and TP-1) domain in exon 3, which was not reported previously (Figure 1; available at www.jpeds.com). The mutation was not observed in parents or in 100 healthy control subjects by allele-specific PCR (data not shown).

Increased Monocytic Cell Death with LPS Stimulation in the Patient

It was recently reported that monocytes of CAPS patients underwent rapid cell death after LPS stimulation.[9] We analyzed the survival of monocytes with LPS stimulation to examine whether this feature is observed in the patient.

Monocytes of the patient underwent cell death similar to those from a typical NOMID patient.[10] The cell death was not observed in patients with sJIA (Figure 2).

DISCUSSION

The patient had fever, rash, wide fontanel, papilledema, and chronic meningitis as manifestations of NOMID. All his symptoms did not appear until he was 10 months of age. He has had no other symptoms including joint manifestation at 2 years of age, and typical histological findings were not observed in the skin. We diagnosed our patient as having variant or mild NOMID by his symptoms and physical, laboratory, and genetic findings. It is possible that the novel *NLRP3* mutation is directly related to the mild manifestations. Frenkel et al[11] reported variant NOMID caused by an *NLRP3* mutation within the leucin-rich repeat domain in a patient who was well until 2 years of age. Other genetic or environmental factors may have had a role in the mild presentation because it is reported that the clinical manifestation can differ even with the same *NLRP3* mutation.[2]

It may be difficult to diagnose atypical or mild NOMID cases, and some of these patients might be misdiagnosed as having sJIA, especially in early infancy. Our case suggests that NOMID should be considered even if a child has only a limited set of symptoms such as prolonged fever, rash, and wide fontanel. Ophthalmologic examination and CSF analysis would guide diagnosis. Early diagnosis and treatment are necessary because NOMID patients can have serious complications such as stroke and seizures, poor growth, mental retardation, blindness, hearing loss, and amiloidosis.[8] Fujisawa et al[9] reported that monocytes from CAPS patients rapidly die by lysosomal cathepsin B–dependent necrosis after LPS stimulation. *CIAS1* overexpression caused by LPS stimulation may lead to cell death.[9] Although the significance of this feature is to be clarified in vivo, we have found that the test was abnormal even in variant or mild NOMID and may be useful for diagnosis.

REFERENCES

1. Hull KM, Shoham N, Chae JJ, Aksentijevich I, Kastner DL. The expanding spectrum of systemic autoinflammatory disorders and their rheumatic manifestations. Curr Opin Rheumatol 2003;15:61-9.
2. Hoffman HM, Mueller JL, Broide DH, Wanderer AA, Kolodner RD. Mutation of a new gene encoding a putative pyrin-like protein causes familial cold autoinflammatory syndrome and Muckle-Wells syndrome. Nat Genet 2001;29:301-5.
3. Aganna E, Martinon F, Hawkins PN, Ross JB, Swan DC, Booth DR, et al. Association of mutations in the *NALP3/CIAS1/PYPAF1* gene with a broad phenotype including recurrent fever, cold sensitivity, sensorineural deafness and AA amyloidosis. Arthritis Rheum 2002;46:2445-52.
4. Aksentijevich I, Nowak M, Mallah M, Chae JJ, Watford WT, Hofmann SR, et al. De novo *CIAS1* mutations, cytokine activation, and evidence for genetic heterogeneity in patients with neonatal-onset multisystem inflammatory disease (NOMID). Arthritis Rheum 2002;46:3340-8.
5. Neven B, Callebaut I, Prieur AM, Feldmann J, Bodemer C, Lepore L, et al. Molecular basis of the spectral expression of *CIAS1* mutations associated with phagocytic cell-mediated autoinflammatory disorders CINCA/NOMID, MWS, and FCU. Blood 2004;103:2809-15.
6. Agostini L, Martinon F, Burns K, McDermott MF, Hawkins PN, Tschopp J. NALP3 forms an IL-1 beta-processing inflammasome with increased activity in Muckle-Wells autoinflammatory disorder. Immunity 2004;20:319-25.
7. Dowds TA, Masumoto J, Zhu Li, Inohara N, Nunez G. Cryopyrin-induced interleukin 1β secretion in monocytes. J Biol Chem 2004;279:21924-8.
8. Hoffman HM, Rosengren S, Boyle DL, Cho JY, Nayar J, Mueller JL, et al. Prevention of cold-associated acute inflammation in familial cold autoinflammatory syndrome by interleukin-1 receptor antagonist. Lancet 2004;364:1779-85.
9. Fujisawa A, Kambe N, Saito M, Nishikomori R, Tanizaki H, Kanazawa N, et al. Disease-associated mutations in *CIAS1* induce cathepsin B-dependent rapid cell death of human THP-1 monocytic cells. Blood 2007;109:2903-11.
10. Takada H, Kusuhara K, Nomura A, Ohga S, Hayashi M, Furue M, Hara T. A novel CIAS1 mutation in a Japanese patient with chronic infantile neurological cutaneous and articular syndrome. Eur J Pediatr 2005;164:785-6.
11. Frenkel J, van Kempen MJ, Kuis W, van Amstel HK. Variant chronic infantile neurologic, cutaneous, articular syndrome due to a mutation within the leucine-rich repeat domain of *CIAS1*. Arthritis Rheum 2004;50:2719-20.