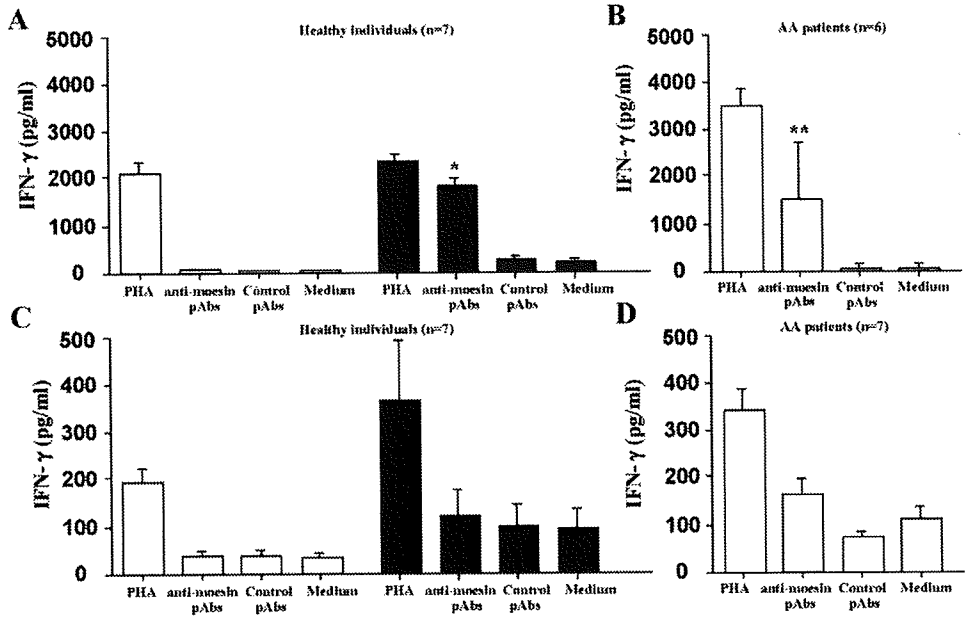

FIGURE 7. IFN-γ release from PBMCs or T cells stimulated by anti-moesin Abs. The PBMCs or isolated T cells were cultured for 48 h in the presence of 5 μg/ml of anti-moesin IgG pAbs or human control IgG pAbs isolated from the serum of AA patients or healthy individuals. Then, 10 μg/ml PHA was used as a positive control. Unprimed PBMCs (□) or CD3-primed PBMCs (■) were used for the culture. PBMCs were isolated from seven healthy individuals (*A*) and six AA patients (*B*). Uncostimulated T cells (□) or CD3-costimulated T cells (■) were used for the culture. T cells were isolated from the PBMCs of seven healthy individuals (*C*) and seven AA patients (*D*). The data represent the mean IFN-γ concentration ± SD. *, $p < 0.0001$ vs control Abs; **, $p = 0.04$ vs control Abs.

as well as ruffling of the cell membrane (17). This membrane-linking protein is expressed by various blood cells including megakaryocytes and granulocytes (23), but its expression was thought to be localized inside the cell membrane and not on the cell surface. Some studies revealed that anti-moesin Abs could bind to the surface of T cells (18) and macrophages (19) in keeping with our observation. However, none of the previous studies characterized the cell surface protein recognized by the anti-moesin Abs. Using biotin-labeled membrane proteins coupled with an avidin gel column and peptide massfinger printing, the present study identified the cell surface 80 kDa protein to be moesin. The decrease in the cell surface moesin induced by moesin shRNA has substantiated the presence of moesin on the cell surface of THP-1 cells.

Little is known about the function of anti-moesin Abs in vitro and in vivo. In contrast to our results, Amar et al. (24, 25) found that anti-moesin mAbs (clone 38) suppressed LPS-induced TNF-α secretion from monocytes through binding of moesin-like molecules on the cell surface. They used a different anti-moesin mAbs (clone 38) from the mAbs (clone 38/87) used in the present study. When we examined the effect of clone 38 mAbs on TNF-α secretion from THP-1 cells induced by LPS using the same condition as the one described by Amar et al. (24), a dose-dependent inhibition of TNF-α secretion was observed (data not shown). In contract to clone 38/87 mAb and pAbs from AA patients' sera, the clone 38 mAbs alone did not induce TNF-α secretion from THP-1 cells. Because the clone 38 preparation contains 1.5 mM sodium azide as a preservative, it is most likely that the dose-dependent inhibition of TNF-α secretion by clone 38 mAbs was due to toxic effect of sodium azide. Alternatively, clone 38 mAb which recognizes the C-terminal portion (554–564 amino acid residues) of moesin may exert a different effect on THP-1 cells from the effect of mAb clone 38/87 which recognizes the middle portion (317–398 amino acid residues) of moesin and from the effect of pAbs purified from AA patients' sera.

The present study revealed that both mAbs and pAbs specific to moesin stimulated the THP-1 cells to secrete TNF-α at an Ab concentration compatible to that in the serum of the AA patients. Moreover, anti-moesin pAbs were as potent as LPS in inducing TNF-α secretion from the PBMCs derived from healthy individuals and the AA patients. Our preliminary analyses on the activation of signaling pathways leading to TNF-α secretion from THP-1 cells showed the phosphorylation of ERK1/2 kinase induced by anti-moesin Abs (49th American Society of Hematology annual meeting abstract #1690, 2007 and submitted). In two patients from whom anti-moesin pAbs were purified, the Abs induced TNF-α release from autologous PBMCs. High concentrations of TNF-α were indeed present in the BM sera of two patients with high anti-moesin Ab titer. Although no difference in the serum TNF-α level was observed between anti-moesin Ab-positive and -negative patients, these findings suggest that anti-moesin Abs may induce a subtle amount of TNF-α from the monocytes or macrophages in the BM, thereby contributing to the pathogenesis of AA.

In contrast to TNF-α, IFN-γ was not induced by the anti-moesin pAbs alone from the PBMCs from healthy individuals, though anti-moesin pAbs augmented IFN-γ secretion from the PBMCs prestimulated with anti-CD3 mAbs. On the other hand, anti-moesin pAbs stimulated the PBMCs from the AA patients to secrete as much IFN-γ as did PHA. It has been shown that T cells from AA patients are in an activated state and are prone to produce IFN-γ in response to suboptimal stimuli (26). The amount of secreted TNF-α from isolated monocytes as well as the amount of secreted IFN-γ from isolated T cells was greatly reduced compared with those from unfractionated PBMCs. The inability to secrete a sufficient amount TNF-α and IFN-γ of isolated monocytes and T cells suggests that the interaction between monocytes and T cells may be required to efficiently respond to extrinsic stimuli as described by previous reports (27, 28). When the anti-moesin Abs titers in the serum were longitudinally measured in three patients, the Abs titer decreased in two patients in association with the response to immunosuppressive therapy, while the Abs titer increased in one patient who became dependent on transfusions due to the relapse of AA in comparison to the titer detected in remission (data not shown). The high titer TNF-α levels in BM plasma of patients showing high anti-moesin Abs titers and the decrease in the Ab titers in parallel with disease amelioration support the hypothesis that anti-moesin Abs are involved in the pathogenesis of AA by way of myelosuppressive cytokine induction from immunocompetent cells. One may wonder why high titer anti-moesin Abs in some AA patients do not induce hypercytokinemia. However, inability of T cell-stimulating Abs such as anti-CD3 Abs to induce IFN-γ secretion in vivo has been shown by previous reports (29, 30). There may be some regulatory mechanisms that mitigate T cell activation by stimulating Abs in vivo.

A previous study demonstrated the presence of anti-moesin Abs in 14–34% of patients with rheumatoid arthritis (11, 31), and a

case-control study on AA conducted by the International Agranulocytosis and Aplastic Anemia Study Group revealed that a past history of rheumatoid arthritis is significantly associated with the later development of AA (32). The anti-moesin pAbs derived from patients with rheumatoid arthritis also enhanced TNF-α secretion from THP-1 cells (data not shown). It is, therefore, possible that AA and rheumatoid arthritis may share pathogenetic mechanisms leading to a breakdown of immunologic tolerance to moesin. Anti-TNF-α therapy has been successfully used for patients with rheumatoid arthritis (33–35) as well as for some patients with myelodysplastic syndrome (36, 37). Recent reports have shown the efficacy of anti-CD20 Abs in restoring hematopoietic functions of AA (38, 39). Therefore, autoAbs capable of inducing cytokine secretion like anti-moesin Abs may be a new target of therapy for AA.

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Disclosures

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Brief report

Expansion of donor-derived hematopoietic stem cells with *PIGA* mutation associated with late graft failure after allogeneic stem cell transplantation

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A small population of CD55$^-$CD59$^-$ blood cells was detected in a patient who developed donor-type late graft failure after allogeneic stem cell transplantation (SCT) for treatment of aplastic anemia (AA). Chimerism and *PIGA* gene analyses showed the paroxysmal nocturnal hemoglobinuria (PNH)–type granulocytes to be of a donor-derived stem cell with a thymine insertion in *PIGA* exon 2. A sensitive mutation-specific polymerase chain reaction (PCR)–based analysis detected the mutation exclusively in DNA derived from the donor bone marrow (BM) cells. The patient responded to immunosuppressive therapy and achieved transfusion independence. The small population of PNH-type cells was undetectable in any of the 50 SCT recipients showing stable engraftment. The de novo development of donor cell–derived AA with a small population of PNH-type cells in this patient supports the concept that glycosyl phosphatidylinositol–anchored protein–deficient stem cells have a survival advantage in the setting of immune-mediated BM injury. (Blood. 2008;112:2160-2162)

Introduction

Although small populations of CD55$^-$CD59$^-$ blood cells are often detectable in patients with aplastic anemia (AA), it remains unclear how such paroxysmal nocturnal hemoglobinuria (PNH)-type cells arise.[1] We recently encountered a patient with immune-mediated late graft failure (LGF) following allogeneic stem cell transplantation (SCT) for treatment of AA. Analyses of the patient's peripheral blood (PB) and bone marrow (BM) showed hematopoietic stem cells (HSCs) of donor origin with mutant *PIGA*, supporting the concept that glycosyl phosphatidylinositol–anchored protein (GPI-AP)–deficient stem cells have a survival advantage in the setting of immune mediated BM injury.

Methods

Patients

A 59-year-old man underwent allogeneic PBSCT from a human leukocyte antigen (HLA)–matched sibling donor after conditioning with fludarabin (120 mg/m^2), cyclophosphamide (1200 mg/m^2), and antithymocyte globulin (60 mg/kg) for treatment of very severe AA in April 2002 (Table 1) and achieved complete donor chimerism with normal blood cell counts. In January 2006, he developed pancytopenia and was diagnosed as having LGF without residual recipient cells. The patient underwent a second PBSCT from the original donor without preconditioning on February 8, 2006. Pancytopenia resolved completely by day 16 after PBSCT. However, at approximately day 60, the blood counts decreased gradually, and the patient became transfusion-dependent. On day 196 after the second PBSCT, the white blood cell (WBC) count was 5.3×10^9/L with 17% neutrophils, the hemoglobin concentration was 75 g/L, and the platelet count was 22×10^9/L. Treatment with horse antithymocyte globulin (ATG) and cyclosporine was started on day 205 after the second PBSCT. Transfusions were terminated after 88 days of the immunosuppressive therapy. Although the patient presently receives low-dose tacrolimus for treatment of chronic graft-versus-host disease, which developed 1 year after the second PBSCT, his pancytopenia has markedly improved as shown in Table 1. PB and BM of the patient were subjected to analyses of chimerism and flow cytometry to detect CD55$^-$CD59$^-$ cells and *PIGA* gene analysis.

As controls, the PB from 51 SCT recipients (48 with hematologic malignancies and 3 with AA) who achieved a complete recovery of donor-derived hematopoiesis were subjected to flow cytometric analysis for the screening of CD55$^-$CD59$^-$ cells. Of the 51 patients, 4 and 23, respectively, had acute graft-versus-host disease (GVHD) of grade II or higher and chronic GVHD at sampling.

BM aspirates were obtained from the patient's donor and 10 healthy individuals for *PIGA* gene analysis. Informed consent was obtained from all patients and healthy individuals in accordance with the Declaration of Helsinki for blood examination, and the experimental protocol for *PIGA* gene analysis was approved by our participating institutional ethics committee (No.157).

Detection of PNH-type cells

To detect GPI-AP deficient (GPI-AP$^-$), PNH-type cells, we performed high-sensitivity 2-color flow cytometry of granulocytes and red blood cells (RBCs), as described previously.[1] The presence of 0.003% or more CD55$^-$CD59$^-$CD11b$^+$ granulocytes and 0.005% or more CD55$^-$CD59$^-$glycophorin-A$^+$ RBCs was defined as an abnormal increase based on the results in 183 healthy individuals.[2]

Cell sorting and chimerism analysis

CD3$^+$cells were isolated from the PB mononuclear cells of the patient using magnetic-activated cell sorting (MACS) CD3 Microbeads (Miltenyi Biotec, Auburn, CA). The CD55$^-$CD59$^-$CD11b$^+$ granulocytes were separated from the CD55$^+$CD59$^+$CD11b$^+$ granulocytes with a cell sorter (JSAN; Bay Bioscience, Yokohama, Japan). More than 95% of the sorted cells were

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*K.M. and C.S. contributed equally to this work.

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Table 1. Hematologic parameters of donor and recipient

	Donor		Recipient			
			Before 1st SCT	Before 2nd SCT	At ATG therapy	After 20 mo of ATG therapy
Date	Apr 2002	May 2008	Apr 2002	Jan 2006	Aug 2006	Apr 2008
WBC count, × 10^9/L	7.0	5.1	1.2	1.7	5.3	4.0
Neutrophil proportions, %	77	65	0	0	17	62
RBC count, × 10^{12}/L	4.21	4.43	2.20	2.75	2.07	3.04
Reticulocytes, × 10^9/L	not tested	35	2	3	26	61
Hemoglobin, g/L	146	150	72	89	75	120
Platelet count, × 10^9/L	261	230	19	52	22	54

$CD55^-CD59^-CD11b^+$. The *D1S80* locus was amplified from DNA of different cell populations with an AmpliFLP D1S80 PCR Amplification Kit (Perkin-Elmer Cetus, Norwalk, CT).

PIGA gene analysis

The coding regions of *PIGA* were amplified by seminested PCR or nested PCR from DNA extracted from the sorted PNH-type cells using 12 primer sets[3,4] (Table S1, available on the *Blood* website; see the Supplemental Materials link at the top of the online article), and 6 ligation reactions were used to transform competent *Escherichia coli* JM109 cells (Nippon Gene, Tokyo, Japan). Five clones were selected randomly from each group of transfectants and subjected to sequencing with a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) and an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems).

Amplification refractory mutation system PCR

On the basis of a mutant sequence detected in *PIGA* of the patient, a nested amplification refractory mutation system (ARMS) forward primer with a 3′-terminal nucleotide sequence complementary to the mutant sequence was prepared[5] (Table S1). To enhance the specificity, a mismatch at the penultimate nucleotide position of the mutation site was incorporated in the ARMS forward primer (P1).[6,7] P1 and a reverse primer (P3) were used to amplify a 127 bp fragment containing the mutant sequence from the exon 2 amplified product. PCR was conducted under the following conditions; denaturation for 30 seconds at 94°C, annealing for 60 seconds at 64°C and extension for 90 seconds at 72°C for 20 cycles. Another forward primer (P2), complementary to the wild-type *PIGA* sequence upstream of the mutation site, was used in combination with P3 to amplify an internal control according to the same condition of ARMS-PCR.

Results and discussion

PNH-type cells were not detected in the donor or the patient at the time of development of the first LGF, whereas 0.147% PNH-type granulocytes and 0.019% PNH-type RBCs were detected in the PB

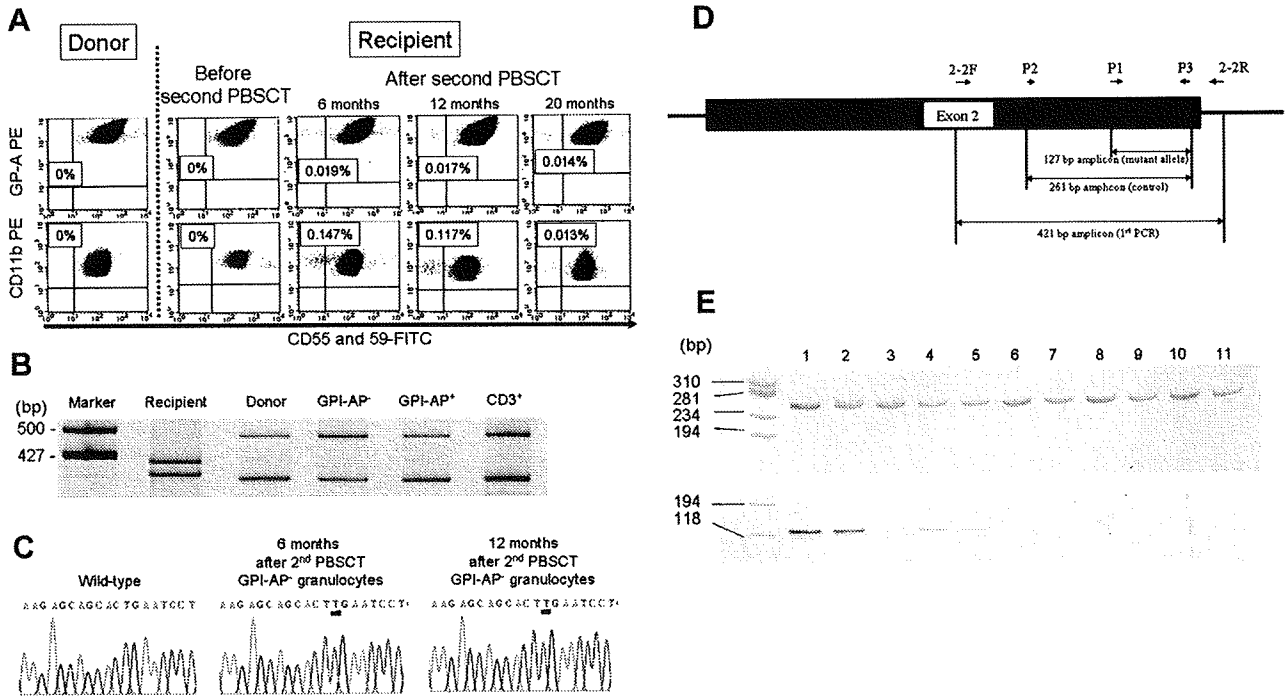

Figure 1. Analysis of PNH-type cells after the second PBSCT. (A) High-sensitivity flow cytometry detected small populations of $CD55^-CD59^-$ cells in both granulocytes and red blood cells at the development of the second LGF as well as in those obtained 6 and 12 months later, but did not detect PNH-type cells in the donor or in the recipient before the second PBSCT. The numbers denote the proportion of PNH-type cells in $CD11b^+$ granulocytes or glycophorin A^+ RBCs. (B) *D1S80* allelic patterns of sorted $GPI\text{-}AP^-$ granulocytes, $GPI\text{-}AP^+$ granulocytes, and $CD3^+$ lymphocytes. The polymerase chain reaction (PCR) products were subjected to 8% polyacrylamide gel electrophoresis and visualized by silver staining. (C) Nucleotide sequences of *PIGA* exon 2 in DNA from PNH-type granulocytes obtained 6 and 12 months after the second PBSCT. (D) A schematic illustration for ARMS-PCR is shown. Primer positions for the first, second are shown by short arrows. A black box and adjacent lines represent exon 2 and introns, respectively. (E) Amplified products of control PCR (the upper gel) and ARMS-PCR (the lower gel) were electrophoresed in 12.5% polyacrylamide gel and visualized by the silver staining. A pMD20-T vector containing the mutated exon 2 fragment was used as a positive control for ARMS-PCR. The template DNA derives from a plasmid containing the mutated exon 2 in lane 1, donor BM in lane 2, donor PB in lane 3, recipient BM in lane 4, recipient PB in lane 5, and BM from healthy individuals in lanes 6 to 11. PCR with a 5′ primer specific to the nucleotide sequence upstream of the mutated sequence amplified a 261 bp fragment from DNA of the donor and all healthy individuals.

obtained at the time of development of the second LGF (Figure 1A). Similar percentages of PNH-type blood cells were detectable in the PB of the patient 6 and 14 months later. When PB from 51 SCT recipients was examined, none of the patients were found to have detectable PNH-type cells (data not shown). PNH-type blood cells were also undetectable in a donor PB sample obtained 21 months later.

The *D1S80* locus allelic pattern of the PNH-type granulocytes in the patient was compatible to that of the donor (Figure 1B). The emergence of donor-derived PNH-type cells and hematologic improvement after immunosuppressive therapy suggest that LGF arises as a result of de novo development of AA which affects the donor-derived hematopoietic stem cells (HSCs).

PIGA gene analysis of the DNA prepared from the sorted PNH-type cells of the patient obtained at the development of LGF and 6 months later showed an insertion of thymine at position 593 (codon 198) in 3 of 5 clones and 5 of 5 clones examined, respectively (Figure 1C). Mutations in other exons were not identified. The presence of a single *PIGA* mutation in PNH-type granulocytes and its persistence over 6 months suggest that these PNH-type cells are derived from a mutant HSC rather than from a committed granulocyte progenitor cell. Moreover, an ARMS-PCR with a 5′ primer specific to the mutated sequence amplified a 127 bp fragment from DNA of the donor BM as well as of the recipient BM and PB while it failed to amplify the same fragment in donor PB and in BM of all 10 healthy individuals (Figure 1D).

These experiments demonstrate that *PIGA*-mutant HSCs were present in the BM of the donor in a dormant state and were transplanted into the recipient and provide, for the first time, in vivo evidence that *PIGA* mutant, GPI-AP–deficient HSCs have a survival advantage in the setting of immune mediated BM injury. Similarly, relative resistance to immune injury likely accounts for the high incidence of PNH observed in association with acquired AA.

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CASE REPORT

Graft rejection and hyperacute graft-versus-host disease in stem cell transplantation from non-inherited maternal antigen complementary HLA-mismatched siblings

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Abstract

Human leukocyte antigen (HLA)-mismatched stem cell transplantation from non-inherited maternal antigen (NIMA)-complementary donors is known to produce stable engraftment without inducing severe graft-versus-host disease (GVHD). We treated two patients with acute myeloid leukemia (AML) and one patient with severe aplastic anemia (SAA) with HLA-mismatched stem cell transplantation (SCT) from NIMA-complementary donors (NIMA-mismatched SCT). The presence of donor and recipient-derived blood cells in the peripheral blood of recipient (donor microchimerism) and donor was documented respectively by amplifying NIMA-derived DNA in two of the three patients. Graft rejection occurred in the SAA patient who was conditioned with a fludarabine-based regimen. Grade III and grade IV acute GVHD developed in patients with AML on day 8 and day 11 respectively, and became a direct cause of death in one patient. The findings suggest that intensive conditioning and immunosuppression after stem cell transplantation are needed in NIMA-mismatched SCT even if donor and recipient microchimerisms is detectable in the donor and recipient before SCT.

Key words graft-versus-host disease; rejection; graft failure; non-inherited maternal antigen; fetomaternal microchimerism

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In allogeneic stem cell transplantation (SCT) from human leukocyte antigen (HLA)-mismatched donors, severe acute graft-versus-host disease (GVHD) and graft rejections occur at a higher rate than SCT from HLA-matched donors (1, 2). Recently, allogeneic SCT from non-inherited maternal antigen (NIMA)-complementary donors has received attentions as one of the methods that potentially overcome the barrier of HLA incompatibility. It is well known that a small number of maternal blood cells exist in the newborn's blood, and in turn, blood cells derived from children can be detected in the mother's blood long after labor. This phenomenon is referred to as fetomaternal microchimerism. The existence of fetomaternal microchimerism suggests that immunological tolerance of hematopoietic cells takes place both in mother and child. Van Rood *et al.* (3) reported that the incidence of chronic GVHD was significantly lower in mother-to-child SCTs than in father-to-child SCTs. Their findings also suggest that HLA-haploidentical NIMA complementary siblings can be alternate donor candidates when there is no HLA-matched donor. Shimazaki *et al.* (4) reported five patients treated with allogeneic SCT from two or three loci-mismatched familiy donors who had a small number of recipient-derived cells in their blood before SCT. Engraftment occurred in all patients, and although acute GVHD developed in all five, their

severity was grade I or II except for one patient who developed grade III acute GVHD. Ichinohe *et al.* (5) reported that in HLA-haploidentical SCTs, NIMA mismatches in the graft-versus-host (GVH) direction, was associated with a lower risk of severe acute GVHD compared with IPA mismatches.

Based on these backgrounds, we treated two acute myeloid leukemia (AML) patients and one severe aplastic anemia (SAA) patient with SCT from NIMA-mismatched sibling donors. Graft rejection and severe acute GVHD occurred despite the fact that donor and recipient-derived microchimerisms were shown in donor and recipient.

Case report

Case 1

A 27-yr-old man was diagnosed as having chronic myloid leukemia (CML) in myeloid crisis. He underwent an HLA-matched unrelated bone marrow transplantation in September 1999. However, he relapsed with CML in blastic crisis in October 2000. He received allogeneic peripheral blood hematopoietic stem cell transplantation from a NIMA-complementary dizygotic sibling in November 2002. A fever of 38°C occurred on day 3 after transplantation and erythema developed in upper and lower extremities on day 8. A diagnosis of acute GVHD, which met the criteria of hyperacute GVHD (6, 7) was made through skin biopsy findings. The patient's GVHD responded to the treatment and both erythema and icterus disappeared on day 26. The complete donor chimerism was confirmed on day 17 by microsatellite marker analysis. Imatinib mesylate was administered on day 21 and he was in molecular remission on day 58. However, CML recurred as subcutaneous nodules on day 153 and the patient died of CML on day 203.

Case 2

A 15-yr-old woman was diagnosed as having SAA in 2000. She did not respond to all kinds of therapy including ATG and anabolic steroids, and required frequent transfusions. An HLA-matched donor was absent either in relatives or in the bone marrow banks. Allogeneic bone marrow transplantation from the NIMA-complementary sister was performed in September 2003. Microchimerism was revealed in both the patient and donor (8). Her neutrophil count rose to 750/μL on day 25, but it became 0/μL following high fever associated with hyperferritinemia (24 490 ng/dL). Virus-associated hemophagocytic syndrome was suspected and foscarnet was administered without any effect. A chimerism analysis performed on day 34 revealed the absence of donor-derived cells in both the peripheral blood and bone marrow, thus leading to the diagnosis of secondary graft failure. She received an infusion of 1.65×10^6/kg of peripheral blood $CD34^+$ cells collected from the marrow donor without conditioning due to the deteriorating clinical condition, but no hematological recovery occurred. She underwent a cord blood cell transplantation (CBT) following conditioning with fludarabine 125 mg/m^2; melphalan, 160 mg/m^2 and total body irradiation at 4 Gy on day 89 after the first transplantation. She achieved a complete reconstitution of hematopoiesis after CBT and remains well 33 months after CBT.

Case 3

In January 2002, a 32-yr-old man was diagnosed to have AML with a normal karyotype. He achieved a complete remission following standard chemotherapy. A year later, he relapsed with acute lymphocytic leukemia with the Philadelphia chromosome (Ph^+ALL). He was treated with chemotherapy consisting of daunorubicin, vincristine, L-asparaginase, and prednisolone, followed by the administration of imatinib mesylate, but did not achieve a complete remission. There was no HLA-matched family member. The microchimerism by NIMAs possessed by the patient was documented in the blood of one brother. He received allogeneic SCT from this NIMA-complementary brother. He became febrile from day 2 and erythema appeared diffusely on the generalized skin. He was diagnosed to have hyperacute GVHD. His skin GVHD deteriorated thus leading to a diagnosis of grade III acute GVHD. Bohrus methylprednisolone therapy could not improve the symptoms of acute GVHD. As a result, the patient died of thrombotic microangiopathy associated with acute GVHD on day 47.

Results and discussion

This study is observational. The incidence of grade II to IV acute GVHD and graft failure in patients who were transplanted from HLA-haploidentical NIMA-complementary siblings has been reported to be 40–50% and 0–18% respectively (3, 5). Based on the results of these studies, HLA-haploidentical siblings whose NIMA is complementary to that of a patient are thought to be a possible donor candidate when HLA-matched donors are unavailable. However, our experience of hyperacute GVHD and graft rejection in the present report raises a concern about the efficacy of HLA-mismatched SCT from NIMA-complementary siblings.

Tables 1 and 2 summarize the patient characteristics and outcome of SCT for the three patients. Although the HLA disparity was one locus in the GVH direction in case 1, acute GVHD appeared on day 8 before

Table 1 Patient Characteristics

Case	Age	Sex	Diagnosis	Status at SCT	Preconditioning regimen	GVHD prophylaxis	CD34+ cells ($\times 10^6$/kg)	HLA (A, B, DR)		Microchimerism	
								Recipient	Donor	patient	Donor
1	27	M	CML	BC, relapse after UR-BMT	Flu 150 mg/m^2 + BU 8 mg/kg + ATG 40 mg/kg	CSA	12.5	2/-, 51/38, 4/8	2/33, 51/44, 4/8	ND	ND
2	15	F	AA	Refractory to immunosuppressive therapy	Flu 150 mg/m^2 + CY 120 mg/kg + ATG 25 mg/kg + TBI 2Gy	CSA + sMTX	1.4	11/-, 55/67, 4/-	11/24, 52/67, 4/15	+	+
3	32	M	Ph + ALL	Resistant	TBI 12 Gy + CY 120 mg/kg	FK506 + sMTX	3.1	2/-, 52/51, 9/8	2/11, 51/60, 8/14	-	+

CML = chronic myeloid leukemia; AA = aplastic anemia; Ph + ALL = Philadelphia chromosome positive acute lymphoblastic leukemia; BC = blastic crisis; UR-BMT = unrelated bone marrow transplantation; Flu = fludarabine; BU = busulfan; ATG = antithymocyte globulin; TBI = total body irradiation; CY = cyclophosphamide; CSA = cyclosporine; FK506 = tacrolimus; sMTX = short term methotrexate; NIMA = non-inherited maternal antigens; ND = not done.

Table 2 Clinical outcome

Case	Engraftment		aGVHD		GVHD stage	Treatment of GVHD	Complications	Outcome	Survival after SCT (d)
	Neu (d)	Plt (d)	Onset (d)	Grade					
1	9	9	8	3	Skin 2, Liver 2	2 mg/kg of mPSL started on day 9, 15 mg/m^2 of MTX on day 11 and 1000 mg/d of MMF started on day15	-	Relapse on day 131	203
2	22	NR	-	-	-	-	HPS on day 23, graft rejection on day 27	CBT on day 89	993+
3	13	15	11	4	Skin 4, Liver 4, Gut 3	1 g/d of mPSL for 3 d	TMA, convulsion	Death by GVHD	47

Neu = neutrophil; Plt = platelet; G = granulocyte; T = T lymphocyte; aGVHD = acute graft-versus-host disease; HPS = homophagocytic syndrome; SCT = stem cell transplantation; mPSL = methylprednisolone; MTX = methotrexate; MMF = mycophenolate mofetil; TMA = thrombotic microangiopathy; CMV = cytomegalovirus.

neutrophil engraftment and rapidly progressed to grade III. Case 3 also developed hyperacute GVHD despite the fact that microchimerism was documented in the donor's blood. Acute GVHD is known to occur frequently before engraftment of neutrophil in recipient of HLA-mismatched SCT (6, 7). In the analysis of SCTs between NIMA-complementary family members described by Ichinohe *et al.* (5), the presence of acute GVHD was observed from day 10. The presence of the recipient-specific microchimerism did not necessary predict low incidence of acute GVHD in this study, in line with our experience. Our findings suggest the necessity of intensive immunosuppressive therapy to prevent acute GVHD such as ATG (9, 10) or alemtuzumab (11) even when a donor shows recipient-specific microchimerism. As case 1 had received HLA-matched unrelated bone marrow transplantation before undergoing the second SCT from a NIMA complementary sibling, recipient dendritic cells were probably replaced by the cells of the unrelated donor. The dendritic cells of a recipient play an important role in the development of acute GVHD (12, 13). The absence of the patient-derived dendritic cells, which were educated to tolerate donor T cells, may be responsible for hyperacute GVHD of the patients.

Case 2 was conditioned with fludarabine-based regimen which was known to ensure engraftment of bone marrow from HLA-matched unrelated donors in AA patients (14). Microchimerism by donor cells was documented in the patient. Nevertheless, SCT from the donor ended up with secondary graft rejection. Although the number of of $CD34^+$ cells infused (1.4×10^6/kg) was relatively low, the minimal number of $CD34^+$ cells in the successful SCT was 1.26×10^6/kg in the report by Shimazaki *et al.* (4) and 1.3×10^6/kg in the report by Ichinohe *et al.* (5). It is therefore necessary to intensify the conditioning regimen to prevent graft rejection when HLA-mismatched SCTs from NIMA-complementary siblings are administered to patients with AA even if microchimerism by donor cells is documented in the recipient.

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ELSEVIER

Safety and Efficacy of Foscarnet for Preemptive Therapy Against Cytomegalovirus Reactivation After Unrelated Cord Blood Transplantation

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ABSTRACT

In association with the increased use of unrelated cord blood transplantation (UCBT) in adults, numerous patients have developed cytomegalovirus (CMV) reactivation concomitant with cytopenia. Although foscarnet appears to offer similar efficacy and higher safety as a preemptive therapy against CMV infection than ganciclovir, little is known about the usefulness of foscarnet in such patients. Foscarnet was administered as preemptive therapy against CMV antigenemia in 10 UCBT recipients who were unable to receive ganciclovir due to cytopenia or poor response to ganciclovir. Fatal CMV disease developed in one patient, whereas CMV antigenemia resolved without progression to CMV disease in the remaining nine patients. Foscarnet was well tolerated without serious hematotoxicity and was not discontinued due to adverse events in any patient. Foscarnet represents a safe and effective agent for preemptive therapy against CMV infection and may offer a feasible alternative to ganciclovir in UCBT recipients.

COMPARED TO ALLOGENEIC bone marrow transplantation (BMT) and peripheral blood stem cell transplantation (PBSCT), the advantages of unrelated cord blood transplantation (UCBT) include ease and safety of cell collection, low risk of transmitting viral infections, prompt availability of stem cells, and reduced incidence and severity of graft-versus-host disease (GVHD). Conversely, UCBT is disadvantageous in that slow marrow recovery and immunological immaturity after transplantation lead to an increased risk of infectious complications, and these account for most transplant-related deaths in adult patients receiving UCBT. Patients undergoing UCBT not only develop cytomegalovirus (CMV) reactivation more frequently but also earlier posttransplant compared to patients undergoing BMT or PBSCT.[1,2] For patients who develop CMV reactivation in the early post-UCBT period, preemptive treatment with ganciclovir might promote neutropenia that could place the patient at risk of fatal infectious complications. In contrast to ganciclovir, the antiviral drug foscarnet (trisodium phosphonoformate) appears to lack significant hematotoxicity in allograft recipients.[3–5] Dose-limiting toxicities of foscarnet are nephrotoxicity and neurotoxicity. Foscarnet might thus represent a feasible alternative for preemptive therapy against CMV reactivation in patients after UCBT. To evaluate the safety and efficacy of foscarnet for preemptive therapy against CMV infection after UCBT, the present study used this drug as an alternative to ganciclovir for patients who were unable to receive ganciclovir due to cytopenia or poor response to ganciclovir.

MATERIALS AND METHODS

Patients

Patients with CMV-positive antigenemia detected at least once were considered eligible for the study if absolute neutrophil count (ANC) was $<1.0 \times 10^9$/L or platelet count was $<20 \times 10^9$/L or response of ganciclovir against CMV antigenemia was tardy as assessed by a physician. Exclusion criteria were serum creatinine clearance <40 mL/min or therapy with foscarnet before study inclusion. Among 28 consecutive adult patients receiving UCBT at Kanazawa University Hospital between 2001 and 2005, 10 patients entered this study. Written informed consent was obtained from all patients. Foscarnet was administered as initial preemptive therapy in 5 of the 10 patients due to cytopenia. Pharmacotherapy was switched from ganciclovir to foscarnet due to ganciclovir-induced

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cytopenia in three patients and tardy response to ganciclovir in two patients.

CMV Antigenemia Monitoring

Monitoring CMV-pp65 antigen levels assessed CMV antigenemia. CMV antigenemia assays were performed as previously described.[6] Degree of CMV antigenemia was expressed as the number of CMV antigen-positive cells per 5×10^4 leukocytes, and CMV antigenemia was defined as ≥1 antigen-positive cell.

Preemptive Therapy With Foscarnet for Prevention of CMV Disease

Foscarnet was intravenously administered over 2 hours at 60 mg/kg every 12 hours for 14 days as induction treatment. Treatment was stopped if two consecutive CMV antigenemia assays yielded negative results. When CMV antigenemia remained detectable in peripheral blood assay after induction treatment, patients then received intravenous foscarnet at a maintenance dosage of 90 mg/kg/d over 3 hours while antigenemia persisted. Dosage of foscarnet was adjusted according to decreased renal function based on predetermined guidelines. Renal impairment was considered present for an increase in serum creatinine ≥100% or a decrease in creatinine clearance ≥50% from baseline values.

Statistical Analysis

Parameters in the two groups were compared by Fisher exact test.

RESULTS

Efficacy of Foscarnet

The 10 patients had a total of 28 episodes of CMV antigenemia one to five episodes a patient). Median duration from transplantation to initiation of foscarnet treatment was 43 days (range, 14 to 52 days; Table 1). Median duration of exposure to foscarnet in one episode was 10 days (range, 7 to 20 days). In 9 of the 10 patients CMV antigenemia ended, which was defined as attainment of no CMV antigenemia for ≥3 months without antiviral treatment, at a median of 60 days (range, 7 to 104 days) after starting foscarnet treatment. The remaining one patient received foscarnet starting on day 14 due to ganciclovir-associated neutropenia, but it resulted in no response and the patient died of interstitial pneumonia (IP) on day 83.

Safety of Foscarnet

Although one patient developed impaired renal function during treatment with foscarnet, only dose adjustment was required. Although hypocalcemia, hypomagnesemia, hypokalemia, and hypophosphatemia occurred in seven, five, seven, and one patients, respectively, electrolyte disturbances were improved by supplementation through intravenous infusions, and no clinical symptoms or signs attributable to changes in serum electrolyte levels were identified. No seizures or parasthesias occurred. Nausea and vomiting occurred in two patients and were resolved using antiemetic treatments. Median ANC on onset of foscarnet was 1.3×10^9 (range, 0.4×10^9/L to 4.3×10^9/L), and median maximal drop in ANC was 0.2×10^9/L (range, -1.6×10^9/L to 12.9×10^9/L). No patient developed foscarnet-induced severe neutropenia ($<0.5 \times 10^9$/L), although one patient whose ANC was 0.4×10^9/L at the start of foscarnet therapy received transient treatment with granulocyte colony-stimulating factor in association with foscarnet. No adverse events required discontinuation of foscarnet treatment.

Comparison Between UCBT Patients and Unrelated BMT Patients

When the UCBT patients were compared to seven unrelated BMT patients who received foscarnet as preemptive therapy against CMV infection, there was no statistical difference between the two groups in parameters on the efficacy and toxicity of foscarnet (Table 1).

DISCUSSION

CMV infection is still a major concern following allogeneic hematopoietic transplantation because CMV pneumonia is fatal in 70% of patients, even when treated with a combination of antiviral therapies and CMV hyperimmune immunoglobulin.[7] The determinants for development of CMV infection and disease are seropositivity for CMV prior to transplant, GVHD, HLA-mismatched donor, unrelated bone marrow or cord blood donor, treatment with steroids, and modifications of the graft such as in vitro and in vivo T-cell depletion. In contrast to patients treated with high-

Table 1. Efficacy and Toxicity of Foscarnet

	UCBT (n = 10)	Unrelated BMT (n = 7)
Median time from SCT to treatment start with foscarnet, d (range)	43 (14–52)	34 (25–70)
Median time of foscarnet treatment, d (range)	10 (7–20)	8 (6–60)
Patients with clearance of CMV antigenemia (%)	90	100
Patients with CMV disease (type)	1 (IP)	1 (enteritis, before the initiation of foscarnet)
Transplant-related deaths		
Infections	2	1
CMV-induced IP	1	0
Median ANC on onset of foscarnet (range), $\times 10^9$/L	3 (0.4–15.4)	3.1 (1.7–10.5)
Median platelet counts on onset of foscarnet (range), $\times 10^9$/L	26 (13–113)	75 (40–223)
Median maximal drop in ANC from the start of foscarnet during foscarnet treatment (range), $\times 10^9$/L	0.2 (−1.6–12.9)	0 (−2.4–6.9)
Patients with discontinuation of foscarnet	0	0

dose chemotherapy and autologous stem cell transplantation, patients after allogeneic stem cell transplantation are at a much higher risk of development of CMV infection because of the delayed recovery of T- and B-cell functions. Thus the rate by which immune function recovers after hematopoietic reconstitution significantly influences the incidence of CMV infection and disease after stem cell transplantation.

The current study has shown that foscarnet can be effective as a preemptive therapy against CMV reactivation after UCBT. Only 1 of 10 patients developed CMV disease and CMV antigenemia resolved in the remaining nine patients during the study period, appearing comparable to treatment results with ganciclovir and foscarnet for recipients of allogeneic BMT and PBSCT.[3,5,8–10]

Despite effectiveness in preventing CMV disease after BMT and PBSCT, use of ganciclovir is associated with marked toxicity to the bone marrow, and severe neutropenia is reported in up to 35% of patients who receive ganciclovir treatment after allogeneic BMT or PBSCT.[11–14] This long duration of neutropenia places patients receiving ganciclovir at significant risk of bacterial and fungal infection,[14] and 8% also develop CMV disease.[13] Furthermore, neutropenia in ganciclovir recipients has been shown to represent an independent risk factor for mortality.[11]

Foscarnet was well tolerated and was not associated with any serious hematotoxicity, even in patients with cytopenia at the start of treatment. In addition, foscarnet did not interfere with hematopoietic engraftment despite being administered in the early posttransplant period after UCBT. These results suggest that foscarnet not only can be used from the very early phases of engraftment but also might be started before transplantation for prophylaxis against CMV reactivation. Of note is the fact that foscarnet was not discontinued due to adverse events in any patient. All adverse events, including nephrotoxicity, were resolved with supportive medication or a reduction in foscarnet dose.

In conclusion, the present results may suggest that foscarnet offers an effective and safe alternative to ganciclovir for CMV prophylaxis in recipients of allogeneic UCBT who are unable to receive treatment with ganciclovir due to cytopenia or poor response to ganciclovir. Randomized, comparative studies between ganciclovir and foscarnet are warranted for better evaluation of the preemptive and treatment roles of these agents in patients after UCBT.

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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.

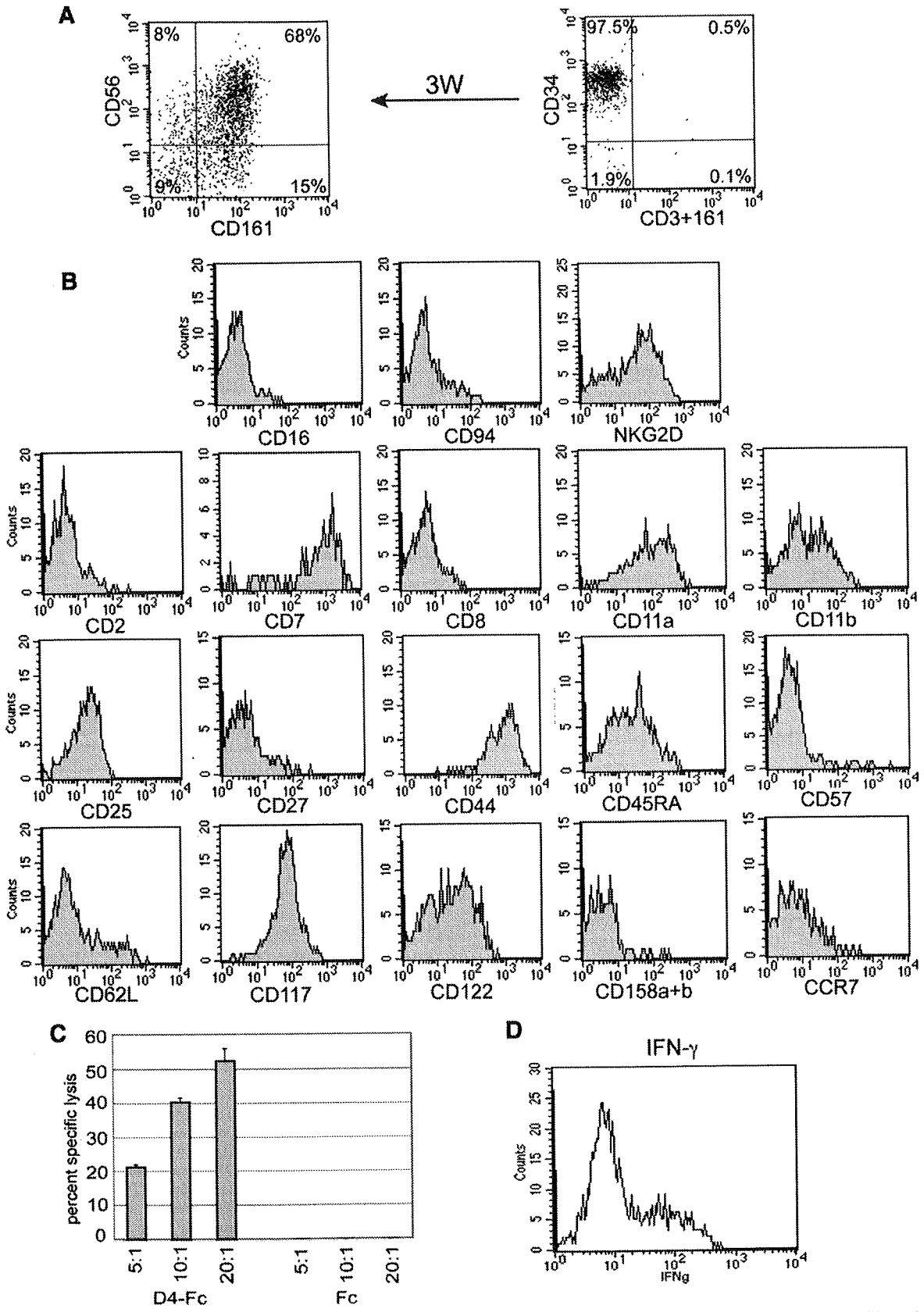

FIGURE 1. Phenotypic and functional analysis of cells derived from $CD34^+$ cells on Delta4-Fc-coated plates. *A*, Representative dot plot illustrating CD161 vs CD56 expression in the cells generated on Delta4-Fc-coated plates from $CD34^+$ CB cells after culture for 3 wk, and dot plot illustrating CD161/CD3 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.

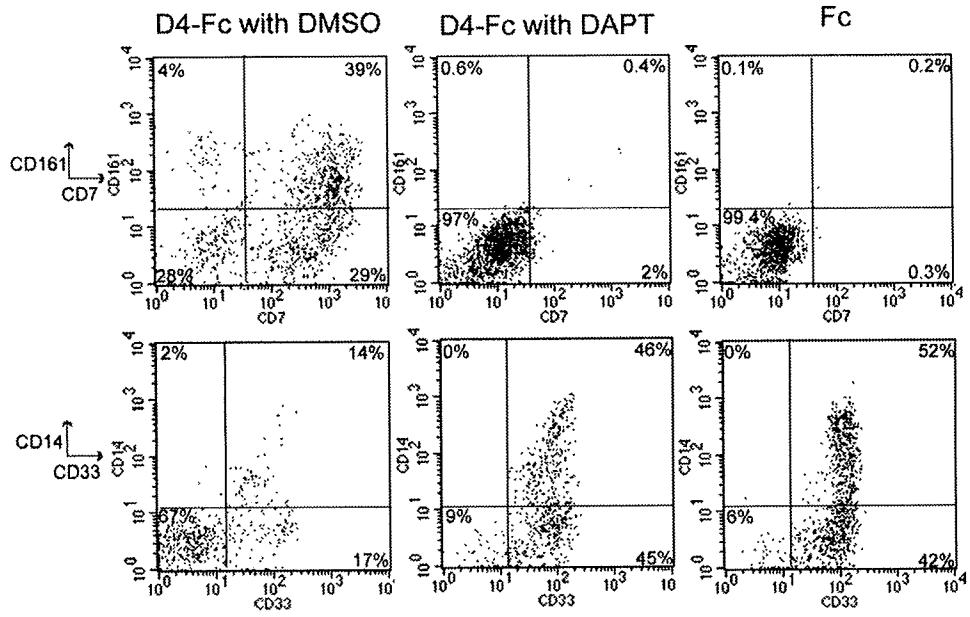

FIGURE 2. Phenotypic analysis of cells cultured in the presence of γ-secretase inhibitors. Representative dot plots of CB CD34$^+$ cells that were cultured for 2.5 wk on Delta4-Fc-coated plates with DMSO (the solvent for the γ-secretase inhibitors: D4-Fc with DMSO), Delta4-Fc-coated plates with DAPT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

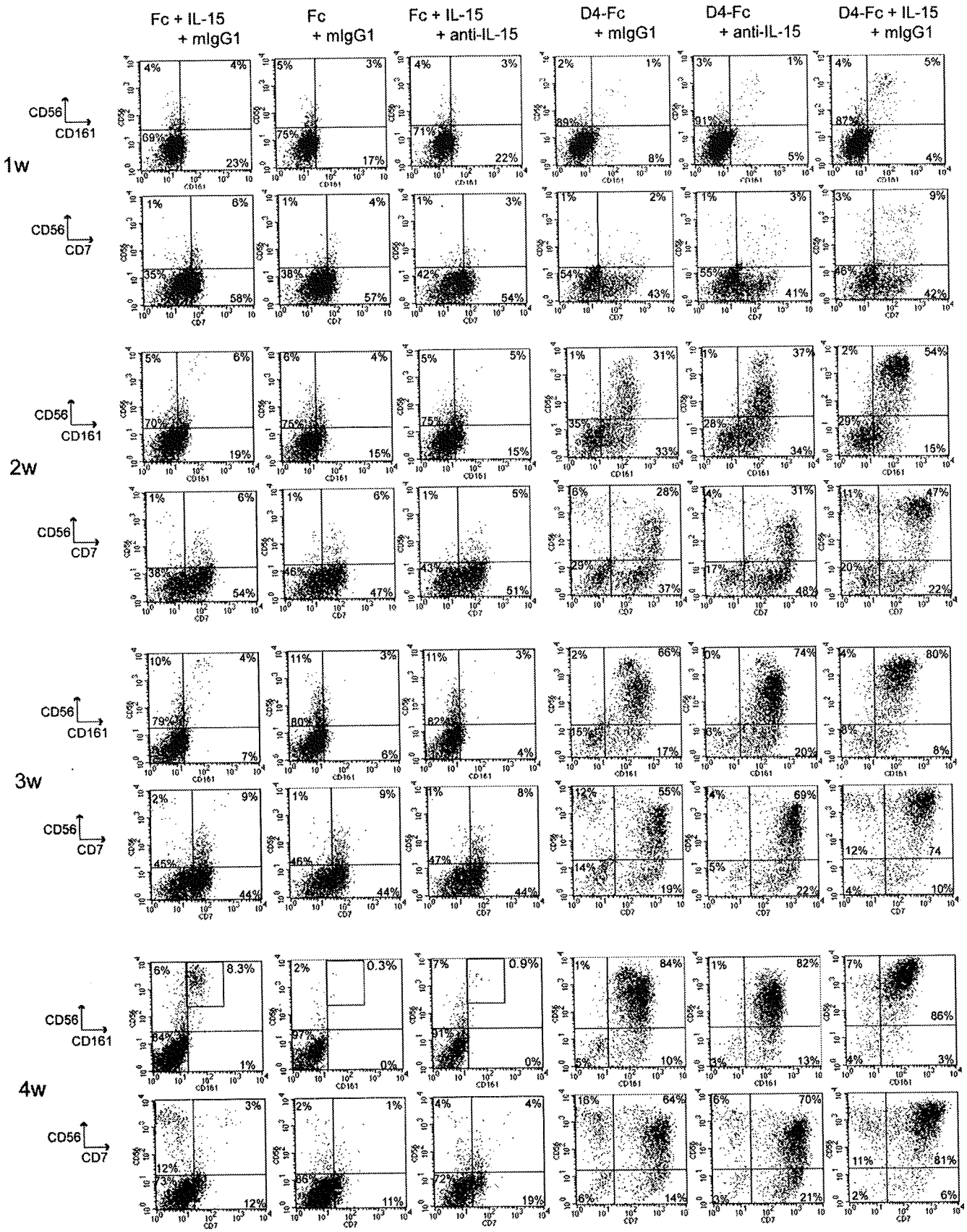

FIGURE 3. Phenotypic analysis during culture under several culture conditions. Representative dot plots illustrating CD161 vs CD56 and CD7 vs CD56 of cells that were cultured from CB CD34$^+$ cells for the indicated number of weeks on Fc-coated plates with IL-15 and mouse (m) IgG1-containing medium (Fc + 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.

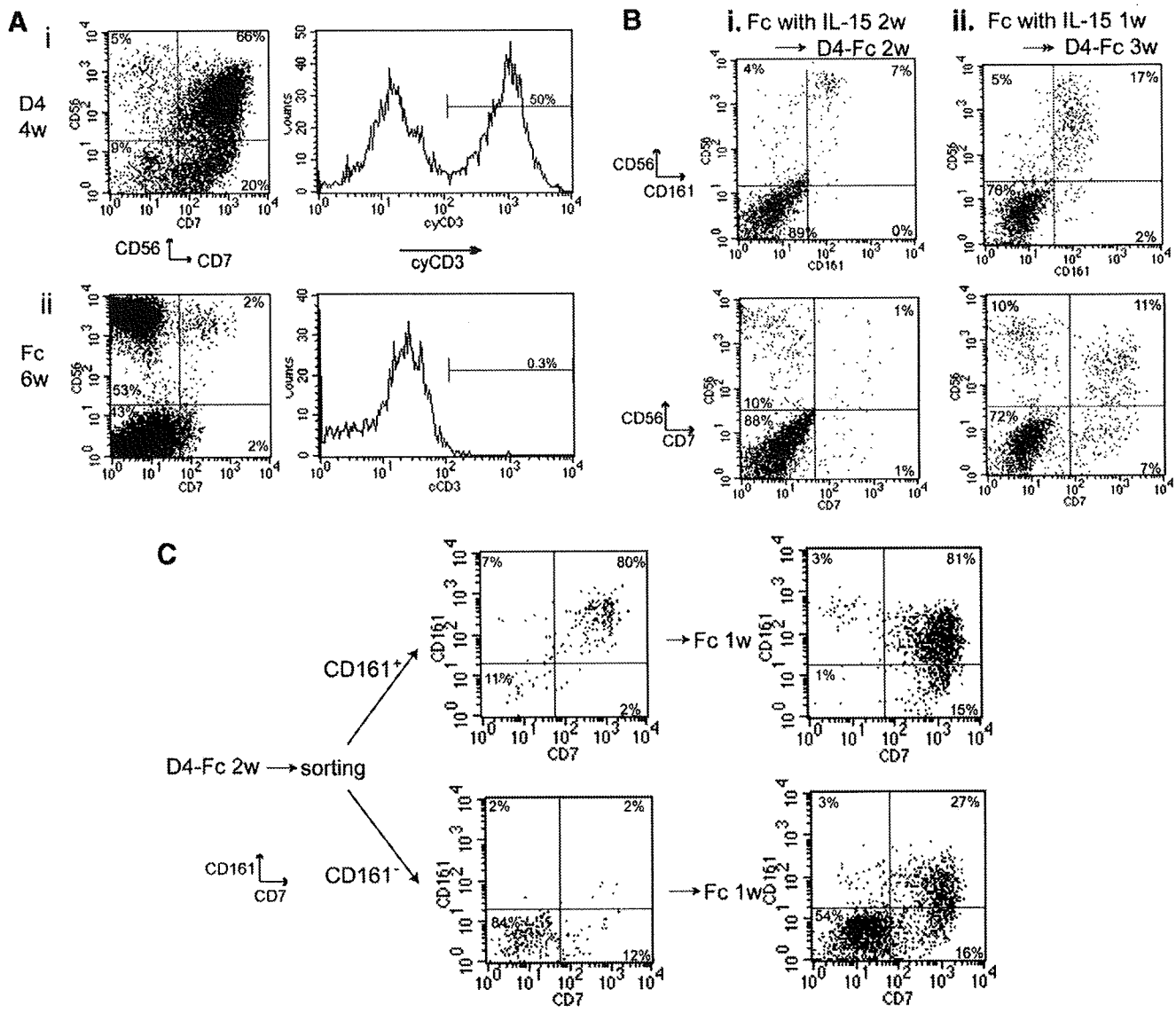

FIGURE 4. Phenotypic analysis of cells after various culture conditions. *A*, Representative dot plots illustrating CD7 vs CD56 cells that were cultured from CB CD34$^+$ cells for 4 wk on Delta4-Fc-coated plates (D4, *Ai*) and for 6 wk on Fc-coated plates in the presence of IL-15 (Fc, *Aii*). Histogram 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

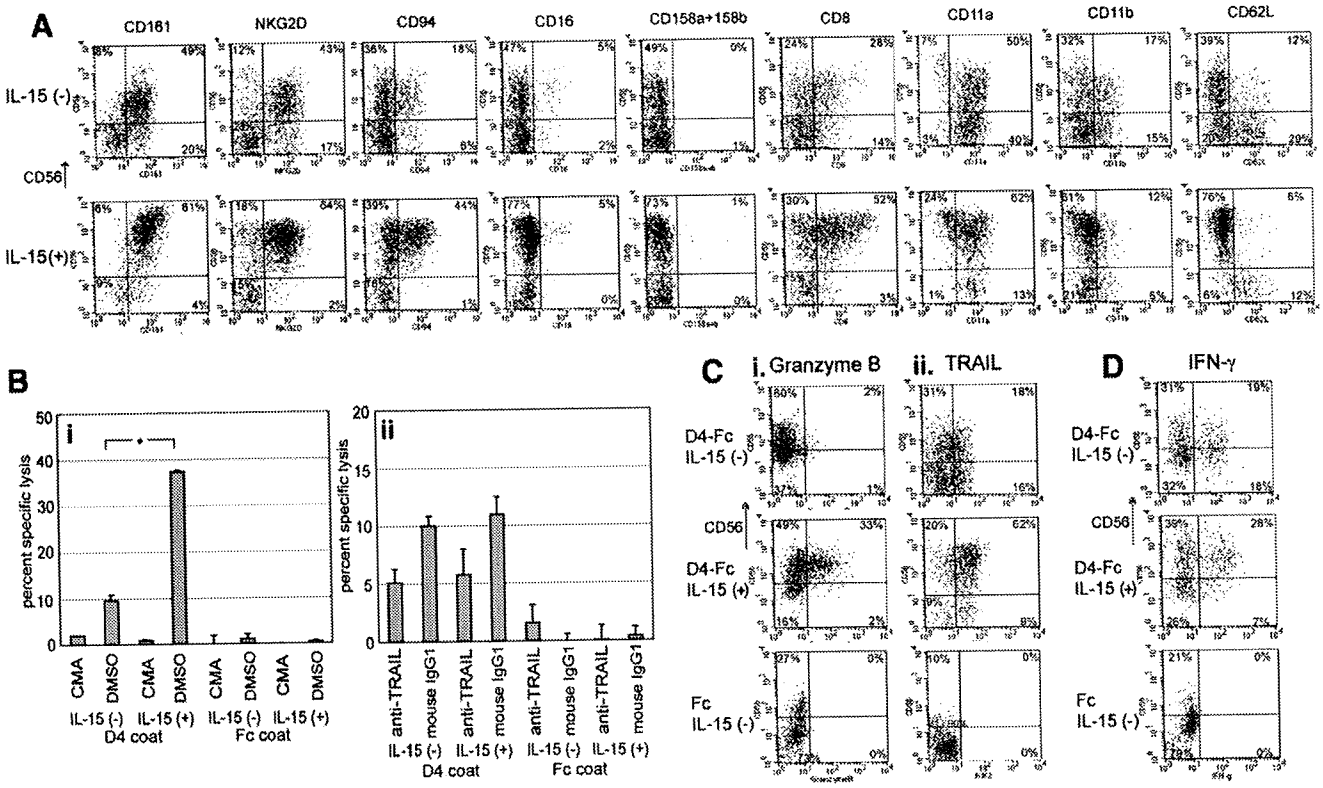

FIGURE 5. Phenotypic and functional differences between cells cultured in IL-15-containing and IL-15-free medium on Delta4-Fc-coated plates. *A*, Representative dot plots illustrating CD56 vs indicated Ags of cells cultured for 3 wk from CB CD34$^+$ cells in IL-15-containing or IL-15-free medium on Delta4-Fc-coated 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

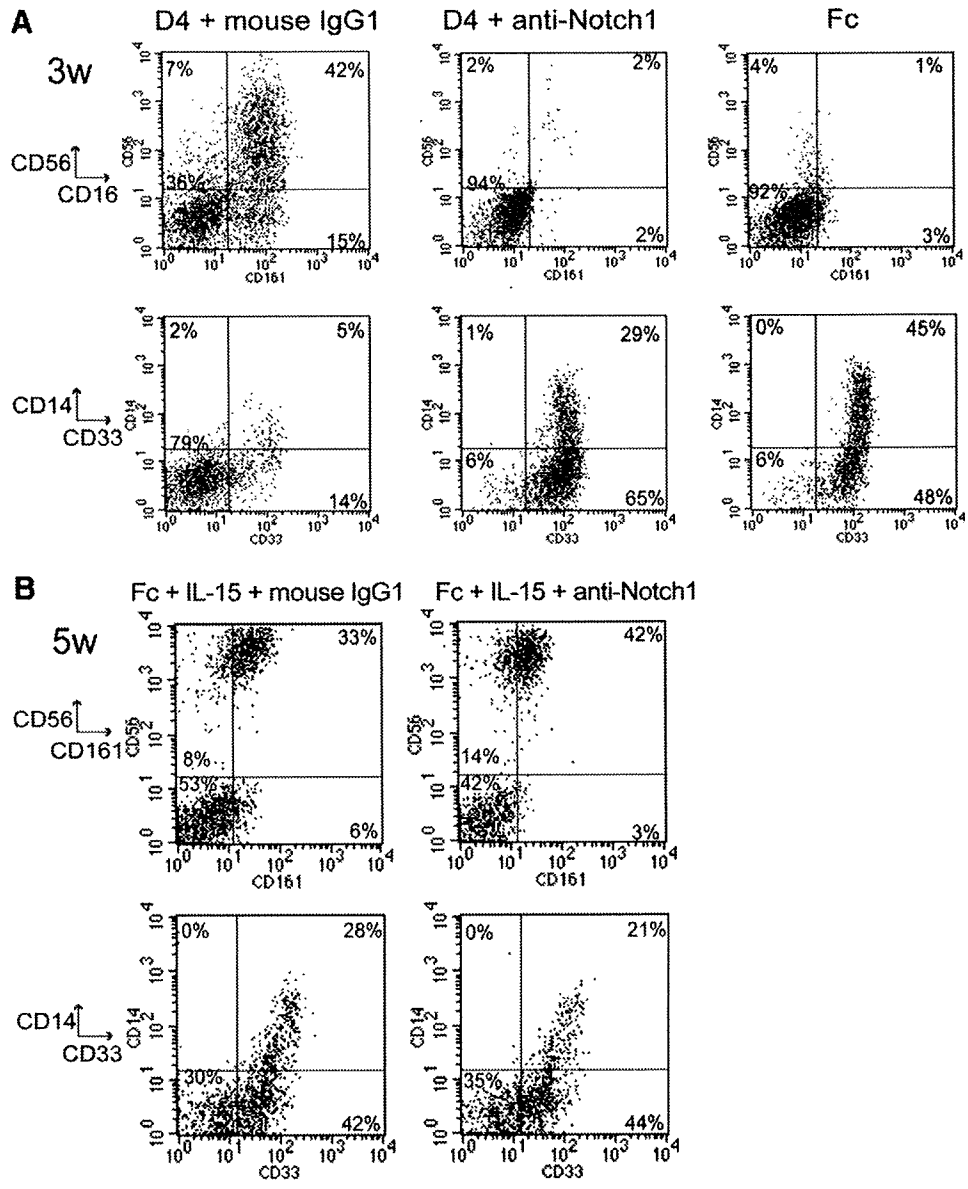

FIGURE 6. Phenotypic analysis of cells cultured in the presence of human Notch1-blocking Ab. *A*, Representative dot plots of cells that were cultured for 3 wk from CB CD34$^+$ cells on Delta4-Fc-coated plates with mouse IgG1-containing medium, Delta4-Fc-coated plates with anti-human Notch1-containing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