cells. The present study revealed a novel function of autoAbs, which may contribute to the pathophysiology of BM failure.

Materials and Methods

Study subjects (patients)

Sera were obtained from 19 patients with AA and 4 healthy individuals. BM plasma was obtained from five patients with AA and three healthy individuals. All AA patients had severe AA and were positive for anti-moesin Abs. The samples were cryopreserved at −80°C until use. Peripheral blood (PB) was obtained from 7 patients with AA and 10 healthy individuals and BM was aspirated from 3 healthy individuals. The PBMCs were isolated using lymphoprep (Nycomed). All patients and healthy volunteers provided an informed consent before sampling according to the Declaration of Helsinki. This study was approved by the human research committee of Kanazawa University Graduate School of Medical Science.

Cell lines

Molt-4, THP-1, U937, K562, Daudi, and Jurkat cell lines were purchased from the Health Science Research Resources Bank. A megakaryoblastic leukemia cell line UT-7, a myeloid leukemia cell lines OUN-1, and a myelodysplastic syndrome cell line TF-1 were provided by Dr. N. Komatsu of Jichi Medical School, Dr. M.Yasukawa of Ehime University, and Dr. S. Ogawa of the University of Tokyo, respectively.

Purification of anti-moesin Abs in the sera of patients with AA

The anti-moesin polyclonal Abs (pAbs) were purified from the patients' serum with affinity chromatography using a protein G column (mAb Trap kit, no. 17-1128-0; GE Healthcare) and recombinant moesin protein (11) fixed on an agarose-gel column (1 ml, HiTrap NHS-activated HP, no. 17-0716-01; GE Healthcare) according to the manufacturer's instruction. In brief, 20 ml of serum from AA patients was applied to the Protein G column. After washing with the binding buffer, the whole IgG was eluted with the elution buffer and neutralized with the neutralizing buffer. The purified IgG was then applied to the recombinant moesin-fixed affinity chromatography column. After washing with the binding buffer (75 mM sodium phosphate (pH 8.0)), anti-moesin pAbs were eluted with the elution buffer (100 mM glycine-HCl and 500 mM NaCl (pH 2.7)). The purified anti-moesin pAbs were dialyzed in PBS at 4°C overnight using a spectra/Por Float-A-Lyzer column (Spectrum Laboratories; no. 235118). The purity of the isolated anti-moesin pAbs was confirmed by PAGE followed by Coomassie Brilliant Blue staining.

Flow cytometry

Mouse anti-moesin mAb (clone 38/87; Neomarkers) which was labeled with FITC (Immuno-Biological Laboratories) was used in combination with mAbs specific to CD3-PE (BD Pharmingen; no. 555333), CD19-PE (BD Pharmingen; No. 555413), CD4-PE (BD Pharmingen; No. 347327), CD-8-PE (BD Pharmingen; no. 555367), CD14-PE (BD Pharmingen; No. 555398), CD11b-PE (BD Pharmingen; No. 555388), CD34-PE (BD Pharmingen; no. 348057), and CD3-Cy-Chrome (BD Pharmingen; no. 555334). Isotype-matched control mAbs (BD Pharmingen) were used as negative controls. For the detection of moesin-like molecules on leukocytes and leukemia cell lines, 1 μl of anti-moesin mAbs and 2 μl of PE-labeled mAbs were added to 50 μl of cell suspension containing 1×10^6 cells and the suspension was incubated on ice for 30 min. For the detection of moesin-like molecules by pAbs, 1×10^6 THP-1 cells were washed twice with PBS containing 1% BSA (Sigma-Aldrich; no. A8022) and resuspended in 200 μl of a PBS containing 2% FCS, 2% goat serum, and 2% BSA. The cell suspension was incubated for 30 min at 4°C. Then, 5 μg/ml anti-moesin pAbs or isotype control human IgG pAbs isolated from healthy individuals were added to the cell suspension and incubated for 1 h at 4°C. The cells were washed twice with PBS containing 1% BSA followed by incubation with a secondary Ab (goat anti-human IgG FITC-labeled Ab; Sigma-Aldrich; no. F5512) diluted 1/100 in PBS containing 2% goat serum and were incubated at 4°C for 30 min. Finally, the cells were washed twice with PBS containing 1% BSA and subjected to flow cytometry.

Stimulation of THP-1 cells with PMA/LPS

THP-1 cells (10^6) were suspended in 2 ml of RPMI 1640 containing 10% FCS and 20 ng/ml PMA (Wako Chemicals; no. 545-00261) and incubated for 24 h at 37°C in a CO_2 incubator. A total of 10 ng/ml LPS (Sigma-Aldrich; no. L2880) was added to the cell suspension and further incubated for 20 h. The cultured cells were analyzed for the expression of moesin-like molecules by flow cytometry using anti-moesin mAbs (clone 38/87; Neomarkers).

Isolation of monocytes and T cells

Monocytes were isolated by plastic adherence as previously described (20). In brief, 5×10^6 PBMCs/well were distributed into 12-well plates (Corning) and allowed to adhere in a 5% CO_2 incubator at 37° for 2 h in RPMI 1640 supplemented with 10% (v/v) heat-inactivated FCS, 100 U/ml penicillin - 0.1 mg/ml streptomycin (Invitrogen; No.15140-148) and 10 μg/ml polymyxin B (Aerosporin) (Sigma-Aldrich; no. 194538). Nonadherent cells were removed and the remaining adherent cells on the plates were used as monocytes. T cells were purified by negative selection using the Human T Cell Enrichment Columns (R&D Systems; no. HTCC-500) following the manufacturer instructions. The purity of enriched T cells and monocytes was approximately 90% as determined by flow cytometry using anti-CD3-PE and anti-CD-14-PE mAbs, respectively.

Stimulation of THP-1 cells, PBMCs, monocytes, and T cells with anti-moesin Abs

THP-1 cells, PBMCs, monocytes, or T cells were suspended in RPMI 1640 supplemented with 10% (v/v) heat-inactivated FCS. Polymyxin B (Aerosporin) (Sigma-Aldrich; no. 194538) was added at 10 μg/ml to eliminate any contaminating endotoxin. The endotoxin concentration in the pAbs and the reagents used for culture was <10 pg/ml as demonstrated by chromogenic *Limulus* amebocyte lysate assay (Seikagaku). The cells (5×10^5) were incubated for 48 h in the presence of 5 μg/ml of anti-moesin mouse mAbs (clone 38/87, IgG1; Neomarkers) or 5–10 μg/ml of pAbs isolated from the serum of AA patients as described above. Mouse mAbs (Coulter Clone; IgG1, no. 6602872) and control human IgG pAbs isolated from healthy individuals were used as negative controls. This Ab concentration was selected based on an estimated concentration of anti-moesin pAbs in the serum of an AA patient. For prestimulation of PBMCs to induce IFN-γ secretion, the cells were incubated for 1 h in the presence of 100 ng/ml of anti-CD3 mAbs (Clone OKT3) (eBioscience; functional grade no. 16-0037). For costimulation of isolated T cells to induce IFN-γ secretion, the cells were cultured for 48 h on a 48-well tissue culture plate that was coated with 100 ng/ml of anti-CD3 mAbs (clone OKT3) (eBioscience; functional grade no. 16-0037) overnight at 4°C and washed. Then, 100 ng/ml LPS or 10 μg/ml PHA (Sigma-Aldrich; no. L4144) was included instead of anti-moesin Abs as the positive controls for the induction of TNF-α or IFN-γ secretion, respectively, and control human IgG pAbs isolated from healthy individuals were added as a negative control.

Western blotting

Western blotting was performed using THP-1 cell lysates. The specific bands were visualized by anti-moesin mAbs, pAbs from AA patients, or control human IgG pAbs from healthy individuals as described in a previous report (11). The expression levels of α-tubulin were determined as an internal control using Western blotting with anti-α-tubulin mAbs (Sigma-Aldrich; no. T 5168).

Isolation of proteins on the surface of THP-1 cells

The THP-1 cells were treated with sulfo-NHS-SS-biotin, and the cell surface proteins were isolated with avidin-fixed columns according to the manufacturer's instructions (Pierce). Thereafter, 1×10^7 cells were washed twice with 8 ml of ice-cold PBS. The cells were suspended in 10 ml PBS containing 2.5 mg sulfo-NHS-SS-Biotin and incubated for 30 min at 4°C. Then, 500 μl of quenching solution was added to the cell suspension and the cells were washed with 10 ml TBS twice. The cell pellet was lysed in 500 μl of lysis buffer containing 60 μl of preotease inhibitor cocktail (Sigma-Aldrich; no. P-8340) and then disrupted by sonication. The biotin-labeled membrane proteins were isolated by an immobilized NeutrAvidin Gel column. The isolated membrane proteins were subjected to Western blotting and peptide mass fingerprinting.

Peptide mass fingerprinting

Mass spectrometric identification of 80- and 75-kDa proteins on the surface of the THP-1 cells was performed as previously described (21). In brief, the proteins fractionated by SDS-PAGE were visualized by Coomassie Brilliant Blue staining and the 80- and 75-kDa bands were excised from gels, followed by in-gel digestions with trypsin (Promega) in a buffer containing 50 mM ammonium bicarbonate (pH 8.0) and 2% acetonitrile overnight at 37°C. Molecular mass analyses of the tryptic

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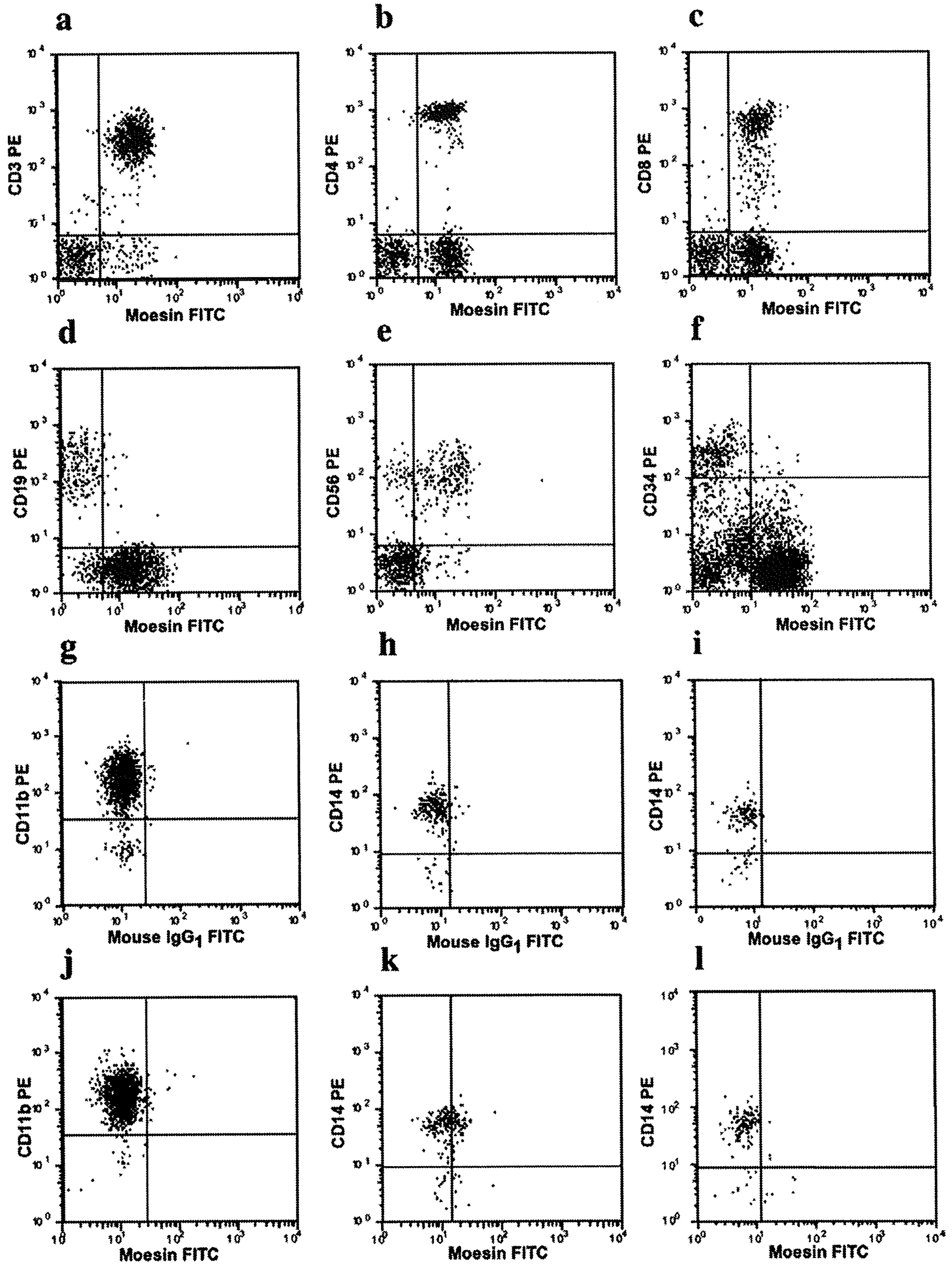

FIGURE 1. Expression of moesin-like molecules on the surface of various blood cells. PB lymphocytes, granulocytes, and monocytes, as well as BM mononuclear cells, of a healthy individual and a patient with AA were analyzed by flow cytometry. The gate was set up for lymphocytes (*a–d*), $CD3^-$ lymphocytes (*e*), granulocytes (*g* and *j*), and monocytes (*h* and *k*) derived from a healthy individual and monocytes (*i* and *l*) derived from an AA patient. BM mononuclear cells (*f*) of a healthy individual were included in the analysis. One representative result of three experiments is shown.

peptides were performed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry using an ultraflex TOF/TOF (Bruker Daltonics). The proteins were identified by comparisons between the molecular weights determined by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry and the theoretical peptide masses of proteins registered in NCBInr.

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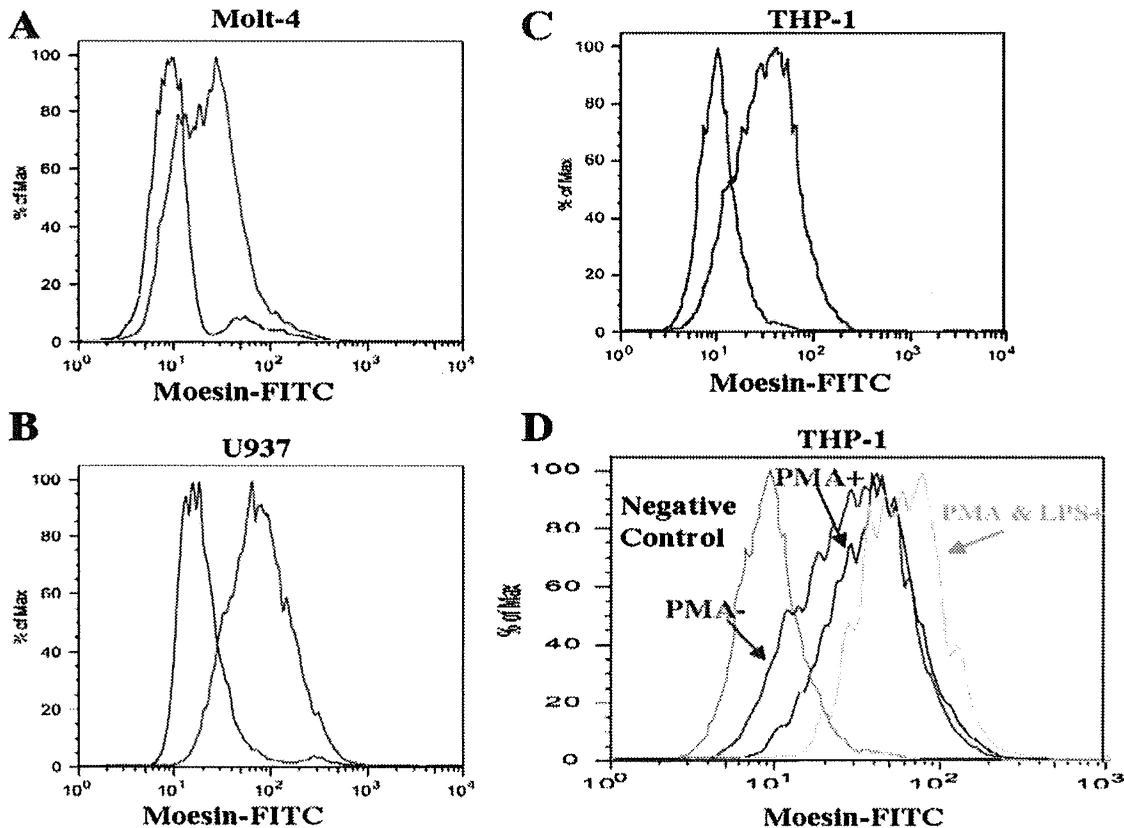

FIGURE 2. Expression of moesin-like molecules on the surface of T cell and monocytic leukemia cell lines. *A–C*, Three leukemia cell lines were examined for the cell surface expression of moesin-like molecules. Left lines, mouse IgG used as negative control; right lines, FITC-labeled anti-moesin mAbs. *D*, THP-1 cells were cultured in the presence or absence of 20 ng/ml PMA for 24 h and then the PMA-stimulated cells were further cultured in the presence of 10 ng/ml LPS for 20 h. The cultured cells were analyzed for the expression of moesin-like molecules by flow cytometry. One representative result is shown.

Transfection of moesin short hairpin (shRNA)

Moesin shRNA plasmid (pENTR/moesin-shRNA-264) (22) was kindly provided by Dr. G. M. Kelly of the University of Western Ontario (Ontario, Canada). THP-1 cells were transfected by electroporation using a Gene Pulser II Electroporation System (Bio-Rad). In brief, 3–5 μg of moesin shRNA plasmid or control shRNA (pENTR/U6-GW/lacZ^{shRNA}) was mixed with 800 μl of Opti-Mem I medium (Invitrogen) containing 1×10^6 THP-1 cells and incubated on ice for 10 min. The cells were electroporated in a 4-mm cuvette (Bio-Rad) at the setting of 300 V of voltage pulse and 960 μF of capacitance. Immediately after electroporation, the transfected THP-1 cells were left on ice for 10 min and then 3 ml of RPMI 1640 containing 10% FCS was added to the cell suspension followed by overnight incubation at 37°C. The cells were rinsed and cultured in 3 ml of fresh RPMI 1640 containing 10% FCS for 72 h at 37°C in a CO_2 incubator and were analyzed for the expression of moesin-like molecules by flow cytometry using FITC-labeled anti-moesin mAb (clone 38/87; Neomarkers).

ELISA

The TNF-α and IFN-γ concentration in the culture supernatant, as well as in PB serum and BM plasma was measured using ELISA kits (Mabtech;

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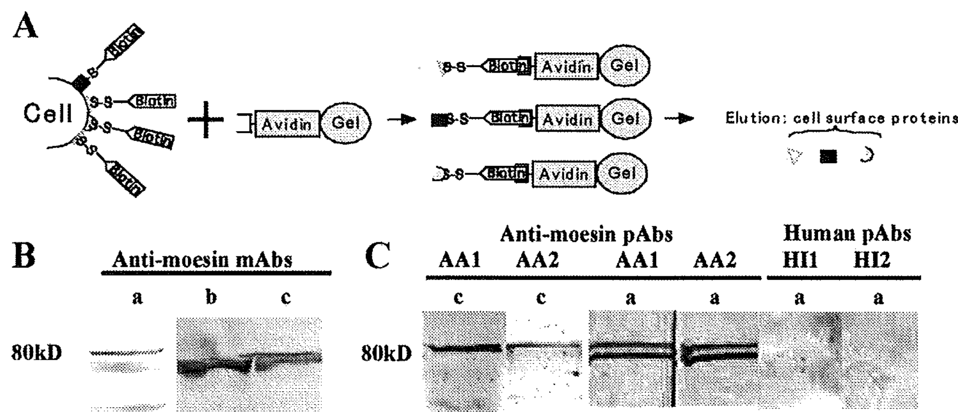

FIGURE 3. Isolation and identification of proteins on THP-1 cells recognized by anti-moesin Abs. *A*, THP-1 cells were treated with sulfo-NHS-SS-biotin and the cell surface proteins were isolated with avidin-fixed columns. *B*, Three different protein lysates (*a*, whole cells; *b*, cytoplasmic proteins; and *c*, surface proteins) were subjected to Western blotting with anti-moesin mAbs. *C*, THP-1 cell lysates (*a*) and surface proteins (*c*) isolated from THP-1 cells were subjected to Western blotting using anti-moesin pAbs purified from two AA patients' sera (AA1 and AA2) or non-specific control human IgG pAbs purified from two healthy individuals' sera (HI1 and HI2).

AB, No. 3510-1H-20, and Mabtech; AB, No. 3420-1H-6) according to the manufacturer's instructions. The OD absorbance at 450 nm was determined using a SLTEAR 340 ATELISA reader (SLT-Labinstruments). For determination of cytokine levels in the PB serum and BM plasma, the following additional procedures were performed. Samples were centrifuged at 10,000 rpm for 10 min. ELISA plates were covered with 200 μl/well of TNF-α assay diluent (eBioscience; No. 00-4202-AD) or IFN-γ assay diluent (Mabtech; No. 3652-D) for 1 h at room temperature before adding samples to block nonspecific reactions. TNF-α assay diluent (eBioscience; No. 00-4202-AD) and IFN-γ assay diluent (Mabtech; No. 3652-D) were used to dilute biotinylated mAb TNF-α-II solution and biotinylated mAb 7-B6-1, respectively.

Statistics

The results are given as the mean $\pm$ SD. Comparisons were made using the paired t test.

Results

Expression of moesin-like molecules on the surface of various blood cells

To confirm the expression of moesin-like molecules on the PB and BM cells, various leukocyte subsets were examined using flow cytometry with anti-moesin mAbs. Fig. 1 shows the representative results of flow cytometry on one healthy individual. Moesin-like molecules were detectable on T cells, NK cells, and monocytes on their surface but not on B cells, neutrophils, and BM CD34$^+$ cells as shown in Fig. 1. All three healthy individuals and the three AA patients showed similar results except that moesin-like molecules were not detectable on monocytes derived from the three AA patients. The mean fluorescence intensity values of the monocytes from healthy individuals and AA patients were 11.5 $\pm$ 2.2 and 6.6 $\pm$ 2.1, respectively, and the difference was significant (mean fluorescence intensity $\pm$ SD, $p < 0.05$, unpaired t test). In addition to the leukocyte subsets from the healthy individuals, moesin-like molecules were detectable on a T cell leukemia cell line Molt-4 as well as on monocytic leukemia cell lines U937 and THP-1 (Fig. 2), while they were undetectable on myeloid leukemia cell lines such as K562, UT-7, OUN-1, and TF-1. They were either undetectable on the Burkitt lymphoma cell line, Daudi, or T cell lymphoma cell line, Jurkat (data not shown). The treatment of THP-1 with 20 ng/ml PMA for 24 h and/or 10 ng/ml LPS for 20 h augmented the expression of moesin-like molecules (Fig. 2*D*), thus, indicating an up-regulation of the moesin-like molecules associated with the differentiation of THP-1 cells into macrophages.

Identification of moesin on the surface of THP-1 cells

To identify the proteins on THP-1 cells recognized by anti-moesin Abs, the THP-1 cells were treated with sulfo-NHS-SS-biotin and the cell surface proteins were isolated with avidin-fixed columns (Fig. 3*A*). Western blotting of the isolated proteins with anti-moesin mAbs showed two clear bands of which the sizes were 75 and 80 kDa (Fig. 3*B*). Mass fingerprinting of the eluted protein revealed the 80 kDa protein to be moesin. The 75 kD band proved to be nucleolin and eukaryotic translation elongation factor 2. To confirm that anti-moesin pAbs in the serum of AA patients can bind to this cell surface moesin, anti-moesin pAbs were purified from the AA patients' sera (AA1 and AA2) with recombinant moesin proteins using affinity chromatography and then were used for Western blotting. As shown in Fig. 3*C*, the serum-derived anti-moesin pAbs bound to moesin derived from the surface proteins of THP-1.

Effect of moesin-specific shRNA on the expression of moesin on THP-1 cells

To further confirm the expression of moesin on the surface of THP-1 cells, the cells were transfected with moesin shRNA using electroporation. Flow cytometry showed a decrease in the moesin expression level on the surface of the THP-1 cells transfected with moesin shRNA in comparison to the THP-1 cells transfected with negative control shRNA (Fig. 4*A*). When the THP-1 cells transfected with different dosages of moesin-specific shRNA were examined by Western blotting, the moesin expression by the THP-1 cells was decreased in a dose-dependent manner. The control shRNA specific to LacZ had no effect on moesin expression.

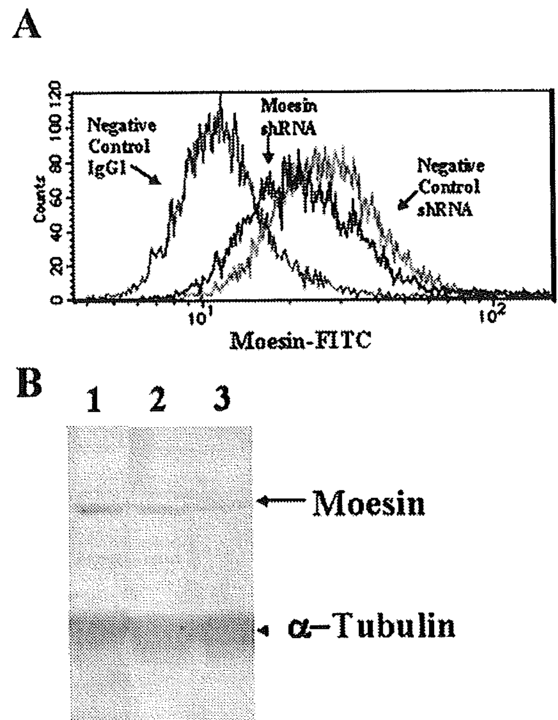

FIGURE 4. Effect of moesin shRNA transfection on the expression of moesin by THP-1 cells. *A*, THP-1 cells transfected with 5 μg of moesin shRNA or control shRNA were examined for the expression of moesin with flow cytometry. The blue line, non-transfected THP-1 cells stained with control mouse IgG1 mAbs; the green line, moesin shRNA transfected cells stained with anti-moesin IgG1 mAbs; the red line, negative control shRNA transfected cells stained with anti-moesin IgG1 mAbs. *B*, Negative control shRNA or moesin-specific shRNA transfected THP-1 cell lysates were examined by Western blotting. 1, 5 μg control shRNA; 2, 3 μg moesin shRNA; 3, 5 μg moesin shRNA.

Effect of anti-moesin Abs on THP-1 cells

To determine whether anti-moesin Abs have some effects on THP-1 cells, the THP-1 cells were cultured in the presence of anti-moesin Abs or control IgG for 48 h and the TNF-α concentration of the culture supernatant was measured using ELISA. Both the anti-moesin mAbs and pAbs induced a significantly greater amount of TNF-α from the THP-1 cells than did the control IgG (Fig. 5*A*). The amount of TNF-α induced by anti-moesin pAbs (5 μg/ml) was almost comparable to that induced by LPS (100 ng/ml) (Fig. 5*A*). The anti-moesin pAbs' binding to moesin on the THP-1 cells was ascertained by flow cytometry (Fig. 5*B*).

Effect of anti-moesin pAbs on PBMCs, monocytes, and T cells from healthy individuals and AA patients

The expression of moesin on the T cells and monocytes as well as the TNF-α secretion from the THP-1 cells induced by anti-moesin pAbs suggested that anti-moesin pAbs in the AA patients' sera might also stimulate these immune cells from healthy individuals and AA patients to secrete cytokines. When the PBMCs from healthy individuals were incubated for 48 h in the presence of 5

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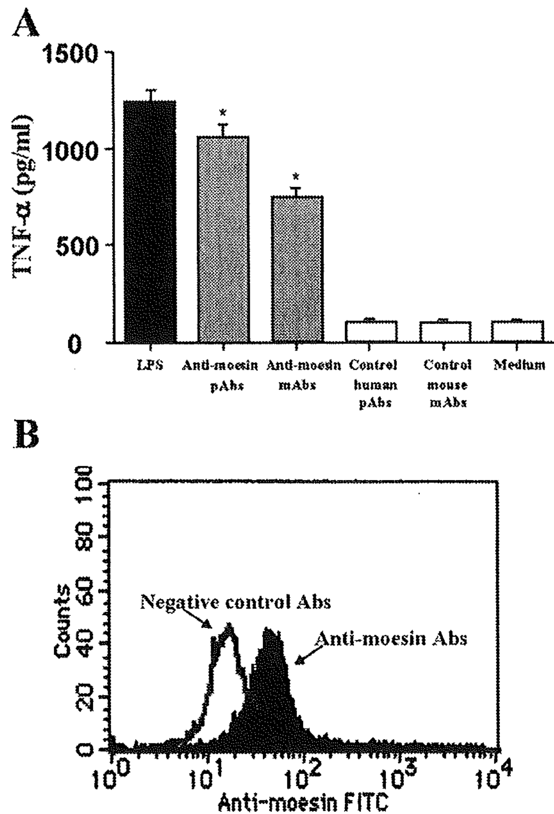

FIGURE 5. TNF-α release from THP-1 cells stimulated by anti-moesin Abs. *A*, THP-1 cells were cultured for 48 h with 5 μg/ml of anti-moesin Abs or control Abs. Anti-moesin pAbs, anti-moesin polyclonal IgG isolated from the serum of AA patients; control human pAbs, control human IgG pAbs isolated from healthy individuals; anti-moesin mAbs, anti-moesin mouse IgG1 mAbs (clone 38/87); control mouse mAbs, control mouse IgG1 mAbs. Then, 100 ng/ml LPS was used as a positive control. The data represent the mean TNF-α concentration ± SD of three experiments. *, $p < 0.01$ vs control Abs. *B*, The detection of moesin on THP-1 cells by anti-moesin pAbs purified from the serum of an AA patient.

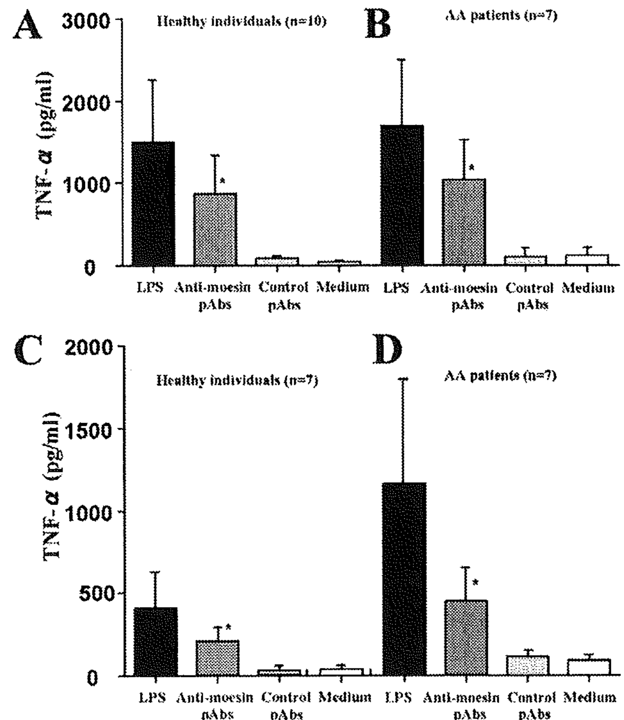

FIGURE 6. TNF-α release from PBMCs or monocytes stimulated by anti-moesin pAbs. The PBMCs or isolated monocytes were cultured for 48 h in the presence of 5 μg/ml of anti-moesin IgG pAbs or human control IgG pAbs isolated from the serum of AA patients or healthy individuals. Then, 100 ng/ml of LPS was used as a positive control. PBMCs isolated from 10 healthy individuals (*A*) and 7 AA patients (*B*), and monocytes separated from the PBMCs of 7 healthy individuals (*C*) and 7 AA patients (*D*), were used as targets. The data represent the mean TNF-α concentration ± SD. *, $p < 0.005$ vs control Abs.

μg/ml of anti-moesin pAbs, the amount of TNF-α in the culture medium was approximately 10 times more than those of control cultures and was more than half of that of the culture stimulated by 100 ng/ml of LPS (Fig. 6*A*). The same concentration of anti-moesin pAbs induced a similar amount of TNF-α from the PBMCs from AA patients (Fig. 6*B*). On the other hand, when monocytes isolated from the PBMC of healthy individuals or AA patients were used as a target, anti-moesin pAbs induced less than half the amount of TNF-α of that induced from whole PBMCs (Fig. 6, *C* and *D*).

The unexpectedly high inducibility of TNF-α secretion from the PBMCs by the anti-moesin pAbs prompted studies on the inducibility of IFN-γ secretion from the PBMCs by the Abs. Fig. 7, *A* and *B*, shows the effect of anti-moesin Abs on the IFN-γ secretion from PBMCs. Although anti-moesin pAbs alone could not induce IFN-γ secretion from the PBMCs derived from healthy individuals, the Abs stimulated PBMCs that were prestimulated with anti-CD3 mAbs to secrete nearly as much IFN-γ as that PHA did. In contrast, the PBMCs from the AA patients could secrete IFN-γ in response to anti-moesin pAbs without the prestimulation by anti-CD3 mAbs, and the amount of IFN-γ was approximately 40% as much as that of the culture stimulated by 10 μg/ml of PHA. In contrast, T cells isolated from the PBMC of healthy individuals or AA patients could not secrete a significantly larger amount of IFN-γ in response to anti-moesin pAbs compared with that in response to control IgG pAbs (Fig. 7, *C* and *D*), and the amount of IFN-γ secreted by T cells was one-tenth as much as that by PBMCs.

When the sera of the 16 AA patients comprising 7 anti-moesin Ab-positive and 9 anti-moesin Ab-negative patients were examined using ELISA, no significant differences in TNF-α and IFN-γ concentrations were observed between the 2 groups (TNF-α: 88.0 ± 106.3 pg/ml in anti-moesin Abs-positive patients, 90.1 ± 161.3 in anti-moesin Abs-negative patients; IFN-γ: 44.6 ± 33.8 pg/ml in anti-moesin Abs-positive patients, 47.5 ± 44.9 pg/ml in anti-moesin Abs-negative patients). None of the sera derived from four healthy donors showed detectable levels of TNF-α (>5 pg/ml) and IFN-γ (>5 pg/ml). On the other hand, when the BM plasma from five patients with AA was examined using ELISA, three anti-moesin Abs-positive patients showed higher levels of TNF-α (129, 338, and 349 pg/ml) compared with those of TNF-α (13 and 128 pg/ml) in two anti-moesin Abs-negative patients. IFN-γ concentrations of three anti-moesin Abs-positive patients were 29, 123, and 133 pg/ml, while those of two anti-moesin Abs-negative patients were 13 and 80 pg/ml. None of the BM plasma derived from three healthy donors showed detectable levels of TNF-α (>5 pg/ml) and IFN-γ (>5 pg/ml).

Discussion

The present study revealed that the proteins recognized by the anti-moesin Abs are detectable on the surface of various leukocytes subsets including T cells, NK cells, and monocytes as well as on T lymphocytic and monocytic leukemia cell lines. Moesin is an intracellular protein that links the cell membrane and cytoskeleton, and mediates the formation of microtubules and cell adhesion sites

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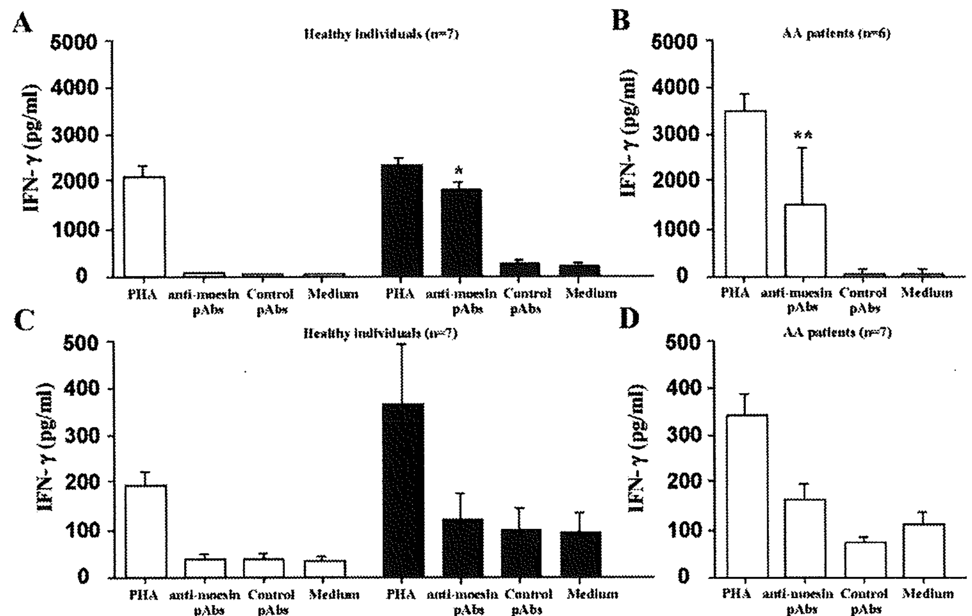

FIGURE 7. IFN-γ release from PBMCs or T cells stimulated by anti-moesin Abs. The PBMCs or isolated T cells were cultured for 48 h in the presence of 5 μg/ml of anti-moesin IgG pAbs or human control IgG pAbs isolated from the serum of AA patients or healthy individuals. Then, 10 μg/ml PHA was used as 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 coupied 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

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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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NKG2D gene polymorphism has a significant impact on transplant outcomes after HLA-fully-matched unrelated bone marrow transplantation for standard risk hematologic malignancies

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ABSTRACT

Background
NKG2D, an activating and co-stimulatory receptor expressed on natural killer cells and T cells, plays pivotal roles in immunity to microbial infections as well as in cancer immunosurveillance. This study examined the impact of donor and recipient polymorphisms in the *NKG2D* gene on the clinical outcomes of patients undergoing allogeneic T-cell-replete myeloablative bone marrow transplantation using an HLA-matched unrelated donor.

Design and Methods
The *NKG2D* polymorphism was retrospectively analyzed in a total 145 recipients with hematologic malignancies and their unrelated donors. The patients underwent transplantation following myeloablative conditioning; the recipients and donors were matched through the Japan Marrow Donor Program.

Results
In patients with standard-risk disease, the donor *NKG2D-HNK1* haplotype, a haplotype expected to induce greater natural killer cell activity, was associated with significantly improved overall survival (adjusted hazard ratio, 0.44; 95% confidence interval, 0.23 to 0.85; p=0.01) as well as transplant related mortality (adjusted hazard ratio, 0.42; 95% confidence interval, 0.21 to 0.86; p=0.02), but had no impact on disease relapse or the development of grade II-IV acute graft-versus-host disease or chronic graft-versus-host disease. The *NKG2D* polymorphism did not significantly influence the transplant outcomes in patients with high-risk disease.

Conclusions
These data suggest an association between the donor *HNK1* haplotype and better clinical outcome among recipients, with standard-risk disease, of bone marrow transplants from HLA-matched unrelated donors.

Key words: *NKG2D*, *HNK1*, *LNK1*, unrelated donor; bone marrow transplantation, single nucleotide polymorphism.

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Introduction

Hematopoietic stem cell transplantation (SCT) is a potentially curative treatment for a range of hematologic malignancies. Although the use of an HLA-matched unrelated donor is well accepted when an HLA-identical sibling donor is unavailable, the risk of transplantation- related complications may be increased.[1] Despite improvements in clinical and supportive care, transplant- related life-threatening complications, including graft-versus-host disease (GVHD), infections and disease relapse, remain an enormous obstacle to overcome.[2] Although HLA matching is the major genetic determinant of clinical outcome after allogeneic SCT, recent evidence suggests that non-HLA immune-associated genes are also implicated.[3] Previous investigations have revealed that several single nucleotide polymorphisms (SNP) which affect individual immune response to infections and inflammatory reactions are associated with the risk of GVHD and transplant outcomes.[4-15]

NKG2D is an activating and co-stimulatory receptor belonging to the C-type lectin-like family of transmembrane proteins and is expressed as a homodimer on natural killer (NK) cells, $CD8^+$ $\alpha\beta^+$ T cells, $\gamma\delta^+$ T cells and activated macrophages.[16-18] The ligands for NKG2D, such as MHC class I-chain related proteins (MICA and MICB), UL16 binding proteins are usually absent or expressed at very low levels in normal cells but are up-regulated by cellular stress including heat shock and microbial infections and are frequently expressed in epithelial tumor cells.[19] Ligand engagement of NKG2D triggers cell-mediated cytotoxicity and co-stimulates cytokine production through a DAP10-phosphoinositol 3-kinase dependent pathway and plays an important role in the elimination of tumors and infected cells.[16-18,20]

Recently, SNP were identified between *LNK1* and *HNK1* haplotypes of the *NKG2D* gene.[21] In Japanese individuals, the *HNK1* haplotype is associated with greater activity of NK cells in the peripheral blood[21,22] and a lower prevalence of cancers originating from epithelial cells.[21,23,24] The present study investigates the impact of donor and recipient polymorphisms in the *NKG2D* gene on the clinical outcomes of patients undergoing allogeneic myeloablative bone marrow transplantation using an HLA allele-matched unrelated donor.

Design and Methods

Patients

NKG2D genotyping was performed on a total 145 recipients with hematologic malignancies and their unrelated donors who were part of the Japan Marrow Donor Program (JMDP). The recipients underwent transplantation, following myeloablative conditioning, with T-cell-replete marrow from an HLA-A, -B, -C, -DRB1 allele-matched donor between November 1995 and March 2000. HLA genotypes of the HLA-A, -B, -C, and -DRB1 alleles of the patients and donors were determined by the Luminex microbead method described previously. (Luminex 100 System; Luminex, Austin, TX, USA).[25,26] No patient had a history of prior transplantation. The final clinical survey of these patients was completed by November 1, 2007. Diagnoses were acute myeloid leukemia (n=49; 34%), acute lymphoblastic leukemia (n=37; 26%), chronic myeloid leukemia (n=41; 28%), myelodysplastic syndrome (n=11; 8%) and malignant lymphoma (n=7; 5%), (Table 1). The recipients were defined as having standard risk disease if they had acute myeloid or lymphoblastic leukemia in first complete remission, malignant lymphoma in complete remission, chronic myeloid leukemia in any chronic phase or myelodysplastic syndrome. All other patients were designated as having high-risk disease. Myeloid malignancies included acute myeloid leukemia, chronic myeloid leukemia and myelodysplastic syndrome, whereas lymphoid malignancies included acute lymphoblastic leukemia and malignant lymphomas. Cyclosporine or tacrolimus- based regimens were used in all patients for GVHD prophylaxis whereas anti-T-cell therapy, such as anti-thymocyte globulin and *ex vivo* T-cell depletion, was not. All patients and donors gave their written informed consent to molecular studies, according to the declaration of Helsinki, at the time of transplantation. The project was approved by the Institutional Review Board of Kanazawa University Graduate School of Medicine and the JMDP.

NKG2D *genotyping*

NKG2D was genotyped using the TaqMan-Allelic discrimination method[27] with a 9700-HT real time polymerase chain reaction (PCR) system (Applied Biosystems, Foster City, CA, USA) and results were analyzed using allelic discrimination software (Applied Biosystems). The genotyping assay was conducted in 96-well PCR plates. The amplification reaction contained template DNA, TaqMan universal master mix and a specific probe (product No. C_9345347_10; Applied Biosystems) for rs1049174, a single locus featuring a G-C substitution to distinguish between the *HNK1* (G) and *LNK1* (C) haplotypes of the *NKG2D* gene.[21,23,24]

Data management and statistical analysis

Data were collected by the JMDP using a standardized report form. Follow-up reports were submitted at 100 days, 1 year and annually after transplantation. Pre-transplant cytomegalovirus serostatus was routinely tested only in patients but not in their donors. Engraftment was confirmed by an absolute neutrophil count of more than 0.5×10^9/L for at least 3 consecutive days. Acute and chronic GVHD were diagnosed and graded using established criteria.[28,29] Overall survival was defined as the number of days from transplantation to death from any cause. Disease relapse was defined as the number of days from transplantation to disease relapse. Transplant-related mortality was defined as death without relapse. Any patients who were alive at the last-follow-up date were censored. When collecting data, only the main cause of death was recorded if two or more causes were combined. Data on etiological agents of infections, post-mortem changes and supportive care (including prophylaxis of infections and therapy of GVHD, which were given on an institutional basis), were not available for this

cohort of patients. The analysis was performed using Excel 2007 (Microsoft Corp, Redmond, WA, USA), OriginPro version 8.0J (Lightstone Inc, Tokyo, Japan), and R (The R Foundation for Statistical Computing, Perugia, Italy).[30] The probability of overall survival was calculated using the Kaplan-Meier method and compared using the log-rank test. The probabilities of transplant-related mortality, disease relapse, acute GVHD, chronic GVHD, and each cause of death were compared using the Grey test[31] and analyzed using cumulative incidence analysis,[30] considering relapse, death without disease relapse, death without acute GVHD, death without chronic GVHD, and death without each cause as respective competing risks. The analysis was stratified for patients with standard-risk disease and high-risk disease to take into account the already recognized prognostic differences. The variables considered were recipient age at time of transplantation, sex, recipient cytomegalovirus serosta-

Table 1. Characteristics of the donors and recipients.

	Standard-risk disease (n=93, 64%) Donor NKG2D haplotype					High-risk disease (n=52, 36%) Donor NKG2D haplotype				
	HNK1 negative n=55, 59%		*HNK1* negative n=38, 41%			*HNK1* positive n=28, 54%		*HNK1* negative n=24, 46%		
Variable	N.	Ratio	N.	Ratio	*p*	N.	Ratio	N.	Ratio	*p*
Age, years										
Recipient					0.08					0.39
Median	31		23			23		22		
Range	1-50		1-50			7-46		2-48		
Donor					0.54					
Median	33		28			34		29		0.02
Range	22-49		21-50			21-47		21-50		
Recipient *NKG2D* haplotype					0.17				0.48	
HNK1 positive	33	60%	28	74%		19	68%	14	58%	
HNK1 negative	22	40%	10	26%		9	32%	10	42%	
Sex, male					0.37					0.77
Recipient	30	55%	23	61%		19	68%	15	63%	
Donor	42	76%	23	61%		19	68%	13	54%	
Recipient/donor sex					0.23					0.86
Sex matched	31	56%	20	53%		18	64%	16	67%	
Male/female	6	11%	9	24%		5	18%	5	21%	
Female/male	18	33%	9	24%		5	18%	3	13%	
Disease					0.86					0.99
Acute myeloid leukemia	14	25%	9	24%		14	50%	12	50%	
Acute lymphoblastic leukemia	10	18%	8	21%		10	36%	9	38%	
Myelodysplastic syndrome	6	11%	5	13%		0	0%	0	0%	
Malignant lymphoma	2	4%	3	8%		1	4%	1	4%	
Chronic myeloid leukemia	23	42%	13	34%		3	11%	2	8%	
ABO matching					0.37					0.18
Matched	35	64%	19	50%		14	50%	17	71%	
Major mismatch	11	20%	10	26%		6	21%	5	21%	
Minor mismatch	9	16%	9	24%		8	29%	2	8%	
Bi-directional	0	0%	1	3%		0	0%	1	4%	
Conditioning regimen					0.93.					0.51
With total body irradiation	43	78%	30	79%		26	93%	21	88%	
Without total body irradiation	12	22%	8	21%		2	7%	3	13%	
Pretransplant CMV serostatus				0.30					0.99	
CMV-negative recipient	14	25%	5	13%		6	27%	5	21%	
Missing data	4	7%	2	5%		5	18%	4	17%	
GVHD prophylaxis					0.58					0.11
With cyclosporine	51	93%	34	89%		27	96%	20	83%	
With tacrolimus	4	7%	4	11%		1	4%	4	17%	
TNC, ×10⁸/kg					0.40					0.04
Median	5.4		5.8			5.8		8.2		
Range	2.3-14.6		2.3-57.6			2.9-20.0		2.4-42.8		
Engraftment	53	96%	38	100%	0.23	28	100%	23	96%	0.28

CMV: cytomegalovirus; TNC: total nucleated cell count harvested.

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tus before transplantation, disease characteristics (disease type and disease lineage), donor characteristics (age, sex, sex compatibility, and ABO compatibility), transplant characteristics (total body irradiation-containing regimen, tacrolimus versus cyclosporine, and total nucleated cell count harvested per recipient weight). The median was used as the cut-off point for continuous variables. The χ^2 test and Mann-Whitney test were used to compare results of two groups. The Hardy-Weinberg equilibrium for the *NKG2D* gene polymorphism was tested using the Haploview program.[32] Multivariate Cox models were used to evaluate the hazard ratio associated with the NKG2D polymorphism. Co-variates found to be statistically significant in univariate analyses ($p \leq 0.10$) were included in the models. For both the univariate and multivariate analyses, p values were two-sided and outcomes were considered to be statistically significant with $p \leq 0.05$.

Results

Frequencies of NKG2D haplotype

The *NKG2D* gene polymorphism was analyzed in 145 pairs of unrelated donors-recipients of bone marrow following myeloablative conditioning (Table 1). The haplotype frequencies of *LNK1/LNK1*, *HNK1/LNK1* and *HNK1/HNK1* were 43%, 42% and 15%, respectively in donors and 35%, 45% and 20%, respectively in recipients. These frequencies were similar to those reported in previous studies in Japanese populations[21,24] and were in accordance with the Hardy-Weinberg equilibrium (p=0.80).

Transplant outcomes according to NKG2D haplotype

With a median follow-up of 115 months among survivors (range, 74 to 140 months), 30 recipients (21%) had relapsed or progressed and 62 (47%) had died. Three patients (2%) died before engraftment. The analysis of the influence of the *NKG2D* genotype on clinical outcomes after transplantation was stratified according to whether the recipients had standard-risk disease or high-risk disease to account for the already recognized prognostic difference. The overall survival at 5 years in patients with standard-risk disease was 63% while that of patients with high-risk disease was 44% (p=0.06). The 5-year cumulative incidences of trasplant-related mortality were 32% and 27%, respectively (p=0.33) and those of disease relapse were 10% and 31%, respectively (p=0.0006).

The transplant outcomes according to *NKG2D* genotype are summarized in Table 2. Patients with standard-risk disease receiving transplants from donors with the *HNK1* haplotype had a significantly better 5-year overall survival (73% *vs.* 49%, p=0.01; Figure 1A) and lower transplant-related mortality rate (22% *vs.* 45%, p=0.02; Figure 1B) than those receiving transplants from donors without the *HNK1* haplotype. No difference was noted in disease relapse in relation to the donors' polymorphism (9% *vs.* 11%, p=0.81; Figure 1C) or in the development of grades II to IV acute GVHD (28% *vs.* 41%, p=0.25) or chronic GVHD (37% *vs.* 41%, p=0.83). When patients with acute myeloid leukemia or myelodysplastic syndrome were separately analyzed, there was still no difference in disease relapse in relation to NKG2D polymorphisms (*data not shown*). In patients with high-risk disease, the donor *HNK1* haplotype had no significant effects on transplant outcomes (Table 2).

Multivariate analysis

Any factors found to be significant in univariate analyses were included in the multivariate analysis. When patients with standard-risk disease were analyzed, the *HNK1* haplotype in donors remained statistically significant in multivariate analyses for both overall survival and transplant-related mortality (Table 3). The presence of the *HNK1* haplotype in the donor resulted in better overall survival (hazard ratio, 0.44; 95% confidence interval, 0.23 to 0.85; p=0.01) and transplant-related mortality (hazard ratio, 0.42; 95% confidence interval, 0.21 to 0.86; p=0.02).

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Table 2. Univariate analysis of the association of *NKG2D* polymorphisms with clinical outcomes after transplantation.

	N.	5-year OS	p	5-year TRM	p	5-year relapse	p	Grade II-IV acute GVHD	p	Chronic GVHD	p
Standard-risk disease											
Donor *NKG2D* haplotype			0.01		0.02		0.81		0.25		0.83
HNK1-positive	55	73%		22%		9%		28%		37%	
HNK1-negative	38	49%		45%		11%		41%		41%	
Recipient *NKG2D* haplotype			0.39		0.31		0.93		0.48		0.98
HNK1-positive	61	62%		33%		10%		37%		39%	
HNK1-negative	32	66%		28%		9%		25%		38%	
High-risk disease											
Donor *NKG2D* haplotype			0.91		0.77		0.93		0.08		0.47
HNK1-positive	28	43%		26%		33%		54%		44%	
HNK1-negative	24	46%		29%		29%		30%		35%	
Recipient *NKG2D* haplotype			0.41		0.43		0.10		0.40		0.68
HNK1-positive	33	42%		23%		39%		39%		37%	
HNK1-negative	19	47%		35%		18%		50%		47%	

OS: overall survival; TRM: transplant-related mortality.

The donor and recipient *HNK1* haplotype did not significantly influence the transplant outcomes in patients with high-risk disease.

Main causes of death

The main causes of death according to the *HNK1* haplotype of the donors and recipients are illustrated in Figure 2A for patients with standard-risk disease, and in Figure 2B for those with high-risk disease. In patients with standard-risk disease receiving transplants from *HNK1*-negative donors, the most frequent cause of death was acute GVHD, followed by interstitial pneumonia. Transplants from *HNK1*-positive donors resulted in a statistically significantly reduced incidence of death attributed to acute GVHD (Figure 3A; p=0.006) as well as a trend toward a lower incidence of death attributed to interstitial pneumonia (Figure 3B; p=0.09). Other causes of death did not differ according to the *HNK1* haplotype.

Discussion

The current study showed an association between the *NKG2D-HNK1* haplotype in unrelated donors of HLA-matched myeloablative bone marrow transplants (haplotype frequency, 61%) and a significantly reduced transplant-related mortality and better overall survival for their recipients with standard-risk disease. The polymorphism of the donor *NKG2D* gene did not influence disease relapse or the development of grades II to IV acute GVHD or chronic GVHD in the patients. One possible explanation for the absence of the beneficial effects of the *HNK1* haplotype in patients with high-risk disease may be that the number of cases in the study was insufficient for a meaningful assessment of the effect. Alternatively, disease progression may precede the emergence of the potential advantageous effects of the *HNK1* donor haplotype that could protect the recipient from severe transplant-related complications. There was a larger difference in disease relapse between patients with standard-risk disease and those with high-risk disease: 10% and 31% at 3 years after transplantation, respectively.

NKG2D plays important roles in immunity to microbial infections and is especially prominent in controlling viral and bacterial infections.[16] Therefore, the reduced transplant-related mortality in patients with standard-risk disease receiving grafts from donors with the *HNK1* haplotype in this study might be a consequence of increased resistance to infections in the recipients. However, the hypothesis is too speculative because of the unavailability of data on causes of infections in this cohort. Further studies will be needed to clarify whether the *HNK1* haplotype in donors can effectively protect patients against infections.

Several studies have shown that NK cell activity has an important role in the outcomes of patients undergoing allogeneic transplantation.[33,34] Alloreactive NK cells reduced the risk of relapse of acute myeloid leukemia without increasing the incidence of GVHD, resulting in a marked improvement of event-free survival in a series of haploidentical transplant recipients.[35,36] In HLA-identical sibling transplants, the absence of HLA-C and HLA-B ligand for donor-inhibitory killer immunoglobulin-like receptors (KIR) provided benefits in terms of survival and relapse of patients with acute myeloid leukemia and myelodysplastic syndrome in recipients of T-cell-depleted SCT.[37] On the other hand, the JMDP found that KIR ligand mismatch was unfavorably correlated with relapse of leukemia and survival in patients undergoing T-cell-replete unrelated bone marrow transplants.[38] All patients in the present study received grafts from an HLA-A, -B, and -C allele-matched donor, implying KIR ligand match between each patient and donor. It is an open question whether the *NKG2D* polymorphism could affect the outcomes of patients undergoing transplantation with KIR-mismatched grafts.

In this study, major and minor ABO incompatibilities between the donor and recipient tended to be associated with poorer transplant outcomes, regardless of the risk

Table 3. Multivariate analysis of the association of *NKG2D* polymorphisms with clinical outcomes after transplantation.

	Overall survival			Transplant-related mortality			Relapse			Grades II-IV acute GVHD			Chronic GVHD		
Variable	HR	95% CI	p	HR	95% CI	p	HR	95% CI	p	HR	95% CI	p	HR	95% CI	p
Standard-risk disease															
HNK1-positive donor	0.44	0.23-0.85	0.01	0.42	0.21-0.86	0.02	0.71	0.19-2.67	0.61	0.83	0.39-1.75	0.63	0.83	0.39-1.75	0.62
HNK1-positive recipient	1.22	0.60-2.50	0.58	1.32	0.61-2.87	0.48	1.11	0.28-4.48	0.88	1.54	0.66-3.57	0.32	1.06	0.49-2.31	0.88
Donor age, >31 years	-	-	-	-	-	-	-	-	-	2.17	0.95-4.96	0.07	-	-	-
Major ABO incompatibility	-	-	-	-	-	-	-	-	-	3.12	1.49-6.56	0.003	0.50	0.17-1.45	0.20
Minor ABO incompatibility	2.42	1.17-5.03	0.02	-	-	-	-	-	-	-	-	-	0.29	0.07-1.24	0.10
High-risk disease															
HNK1-positive donor	0.68	0.30-1.51	0.34	0.62	0.20-1.91	0.40	1.25	0.41-3.80	0.69	1.87	0.69-5.07	0.22	1.55	0.60-4.01	0.37
HNK1-positive recipient	1.41	0.65-3.07	0.39	0.76	0.25-2.29	0.63	2.35	0.66-8.44	0.19	0.47	0.18-1.22	0.12	0.92	0.35-2.38	0.86
Age, >26 years	1.95	0.93-4.09	0.08	6.30	1.86-21.32	0.003	-	-	-	-	-	-	-	-	-
Donor age, >31 years	-	-	-	-	-	-	0.53	0.17-1.65	0.27	-	-	-	-	-	-
Minor ABO incompatibility	2.94	1.19-7.25	0.02	-	-	-	-	-	-	5.10	2.08-12.52	0.004	-	-	-

category of the disease. These findings are compatible with those of a previous study by the JMDP,[39] although the impact of ABO incompatibilities on SCT outcomes is controversial.

This study also identified age as a significant predictive factor for transplant-related mortality in the patients with standard-risk disease. This is consistent with the results of a previous study[40] showing that age over 35 years increased the risk of transplant-related mortality after allogeneic myeloablative SCT in high-risk patients.

A possible limitation of this study is the fact that no direct evidence is yet available regarding the ability of *NKG2D* polymorphisms to protect against microbial infections. The association observed between the *NKG2D* haplotype and transplant outcome might be due to another genetic polymorphism in linkage disequilibrium responsible for a better transplant outcome. One candidate gene is *NKG2F* (*KLRC4*), which is located in the NK complex region adjacent to the *NKG2D* gene, because an intrinsic SNP (rs2617171) in the gene has been reported to be in complete linkage with the *NKG2D* genotype.[24] Alternatively, polymorphisms may not be directly associated with controlling infection, but rather may be associated with other factors, such as sensitivity to treatment against GVHD or protection against organ toxicities related to transplants, which also influence the transplant outcome. These hypotheses have yet to be verified give the insufficient evidence.

Polymorphisms in genes encoding for nucleotide-binding oligomerization domain 2 (NOD2)/caspase recruitment domain 15 (CARD15),[9] heme oxygenase-1 (HO-1) promoter,[6] the Toll-like receptor 4,[4] CC chemokine ligand (CCL) 5 promoter,[32] transforming growth factor (TGF) β1,[11] interleukin (IL) 12, tumor necrosis factor (TNF) α,[15] IL-23,[5] mannose-binding lectin (MBL),[10] Fcγ receptor IIa (FcγRIIa), myeloperoxidase (MPO), FcγRIIIb, IL-1Ra, IL-10,[12] Fc receptor-like 3 (FCRL3), peptidylarginine deimi-

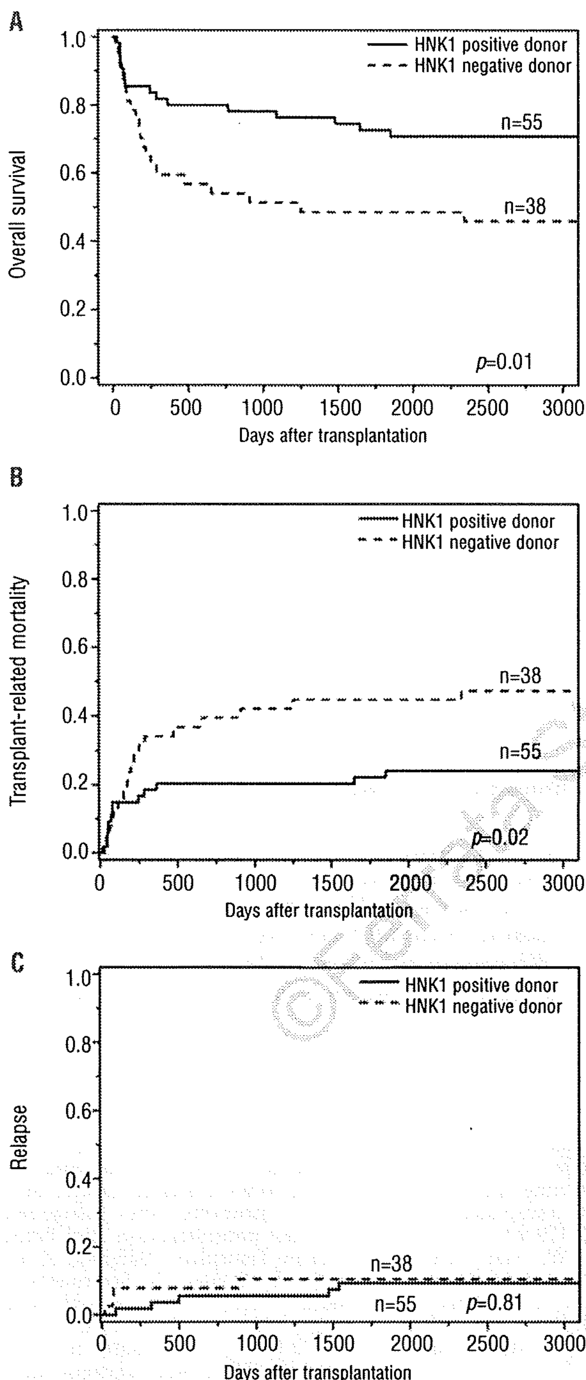

Figure 1. Kaplan-Meier analysis of (A) overall survival, (B) cumulative incidence of transplant-related mortality and (C) disease relapse after transplantation according to the donor *NKG2D* polymorphism in patients with standard-risk disease. Patients with donors with the *HNK1* haplotype had better overall survival and lower transplant-related mortality. Donor haplotype had no significant impact on disease relapse.

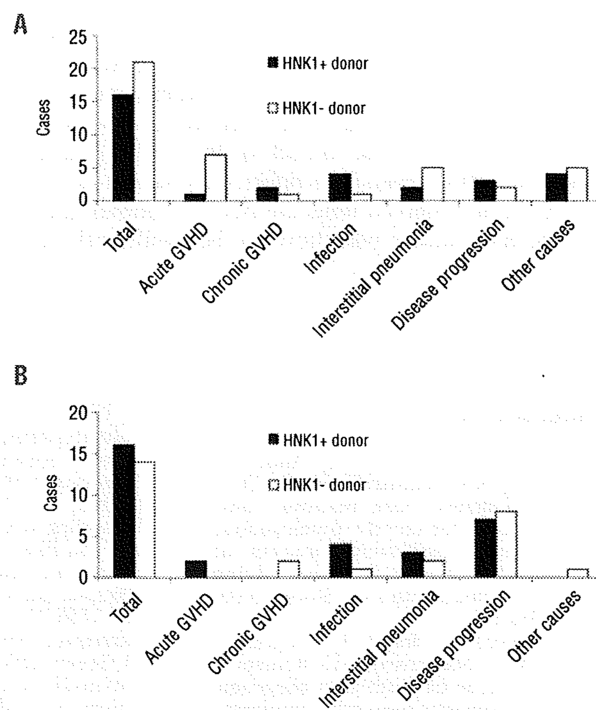

Figure 2. Main causes of death after transplantation according to the *NKG2D* polymorphism in patients with (A) standard-risk disease (B) high-risk disease.

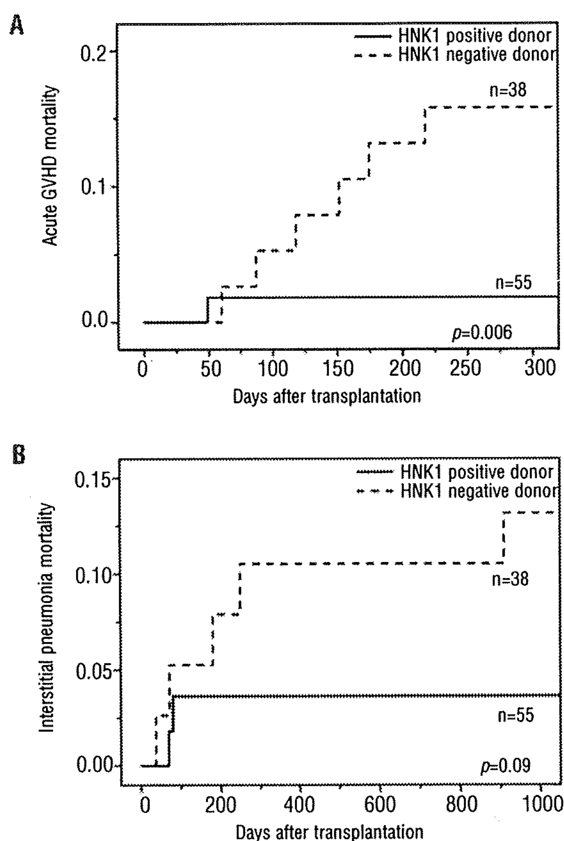

Figure 3. **Cumulative incidence of deaths due to (A) acute GVHD and (B) interstitial pneumonia after transplantation in patients with standard-risk disease. The *HNK1* haplotype in donors was associated with a significantly lower incidence of deaths due to acute GVHD (p=0.006) as well as a trend toward a lower incidence of deaths due to interstitial pneumonia (p=0.09).**

nase citullinating enzymes 4 (PADI4)[13] and methylenetetrahydrofolate reductase (MTHFR)[14] have been shown to influence the outcome after allogeneic SCT. Most of them are associated with the development of GVHD. Only the *NOD2/CARD15* and *HO-1* promoter polymorphisms have a significant impact on overall survival after SCT. Furthermore, the impact of the *HO-1* promoter polymorphisms depends on donor cells but not on recipient cells, as observed with the *NKG2D* polymorphism which, in the donor, was shown to be significantly associated with overall survival in the present study. This may prompt the determination of the donor *NKG2D* polymorphism prior to SCT in order to choose the best donor, expected to minimize transplant-related mortality after SCT, when multiple donors for a patient are available. Otherwise, prior information on the donor *NKG2D* polymorphism may be helpful in selecting risk-specific appropriate precautions following transplantation.

In conclusion, the present data suggest that the *NKG2D* polymorphism, in addition to HLA disparity between recipients and donors, affects prognosis after a bone marrow transplant from an unrelated donor. However, care should be made in drawing conclusions because the number of patients in the present study was small. The finding of a gene polymorphism may not be equivalent to differences in gene expression, which may be influenced by multiple factors because the NKG2D receptor is found on many tissues and cells.[41] Experimental evidence is required to substantiate the effect of the *NKG2D* polymorphism on immune function. We next plan to conduct a prospective study to confirm these results and to extend this investigation to other transplantation settings, such as related donor SCT, reduced-intensity SCT, HLA-mismatched SCT and SCT for patients with non-hematologic malignancies.

Authorship and Disclosures

JLE and AT designed and performed the research, and contributed to the same aspects of the work; AT, JLE and SN wrote the paper; AT, YKa, and SOh performed the statistical analyses; MO, HS, HA, KM, SOk, MI, TF, YM, and YKo contributed to data collection.

The authors reported no potential conflicts of interest.

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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.**

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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.

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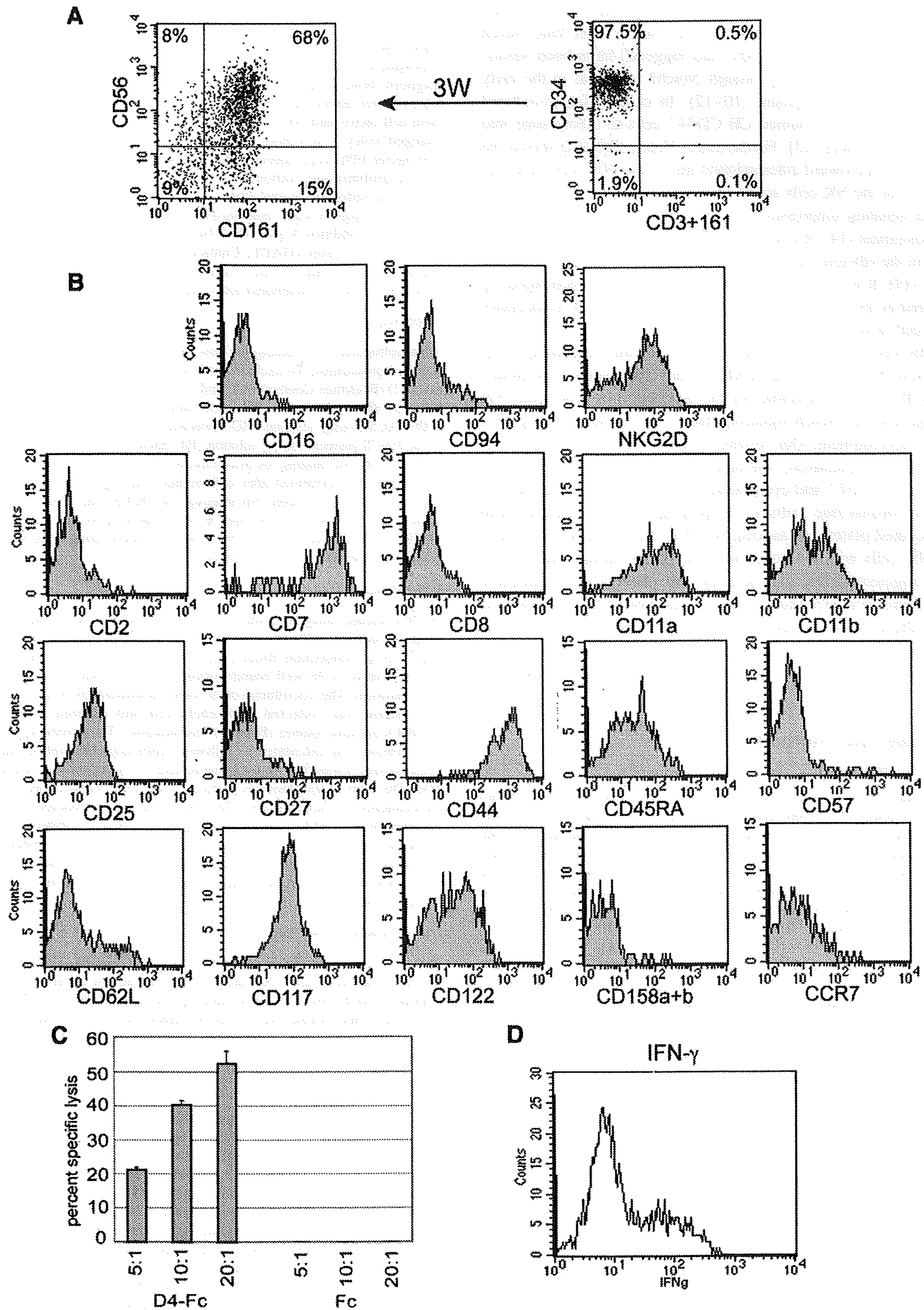

FIGURE 1. Phenotypic and functional analysis of cells derived from CD34$^+$ cells on Delta4-Fc-coated plates. *A*, Representative dot plot illustrating CD161 vs CD56 expression in the cells generated on Delta4-Fc-coated plates from CD34$^+$ CB cells after culture for 3 wk, and dot plot illustrating CD161/CD3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

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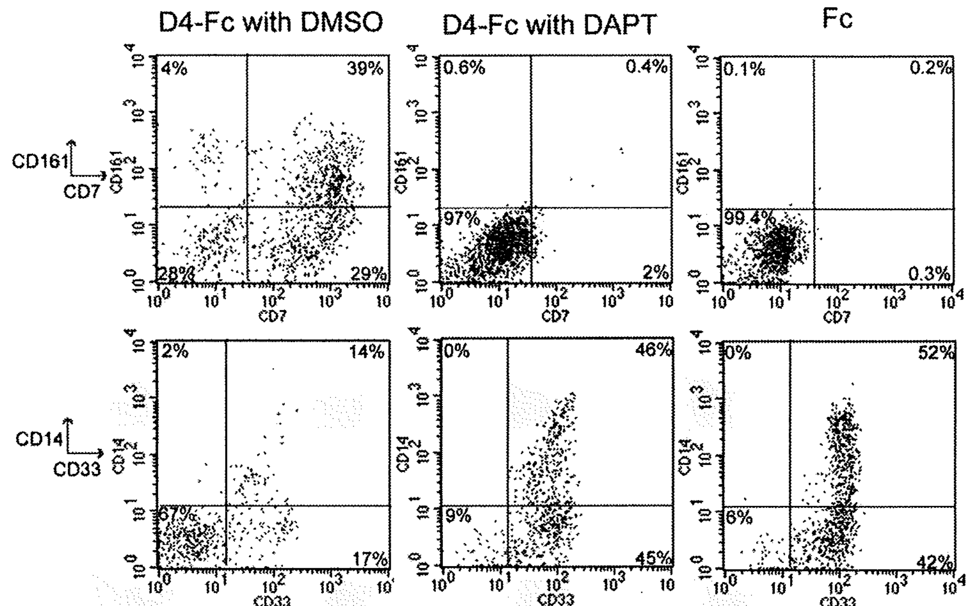

FIGURE 2. Phenotypic analysis of cells cultured in the presence of γ-secretase inhibitors. Representative dot plots of CB CD34$^+$ cells that were cultured for 2.5 wk on Delta4-Fc-coated plates with DMSO (the solvent for the γ-secretase inhibitors: D4-Fc with DMSO), Delta4-Fc-coated plates with DAPT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

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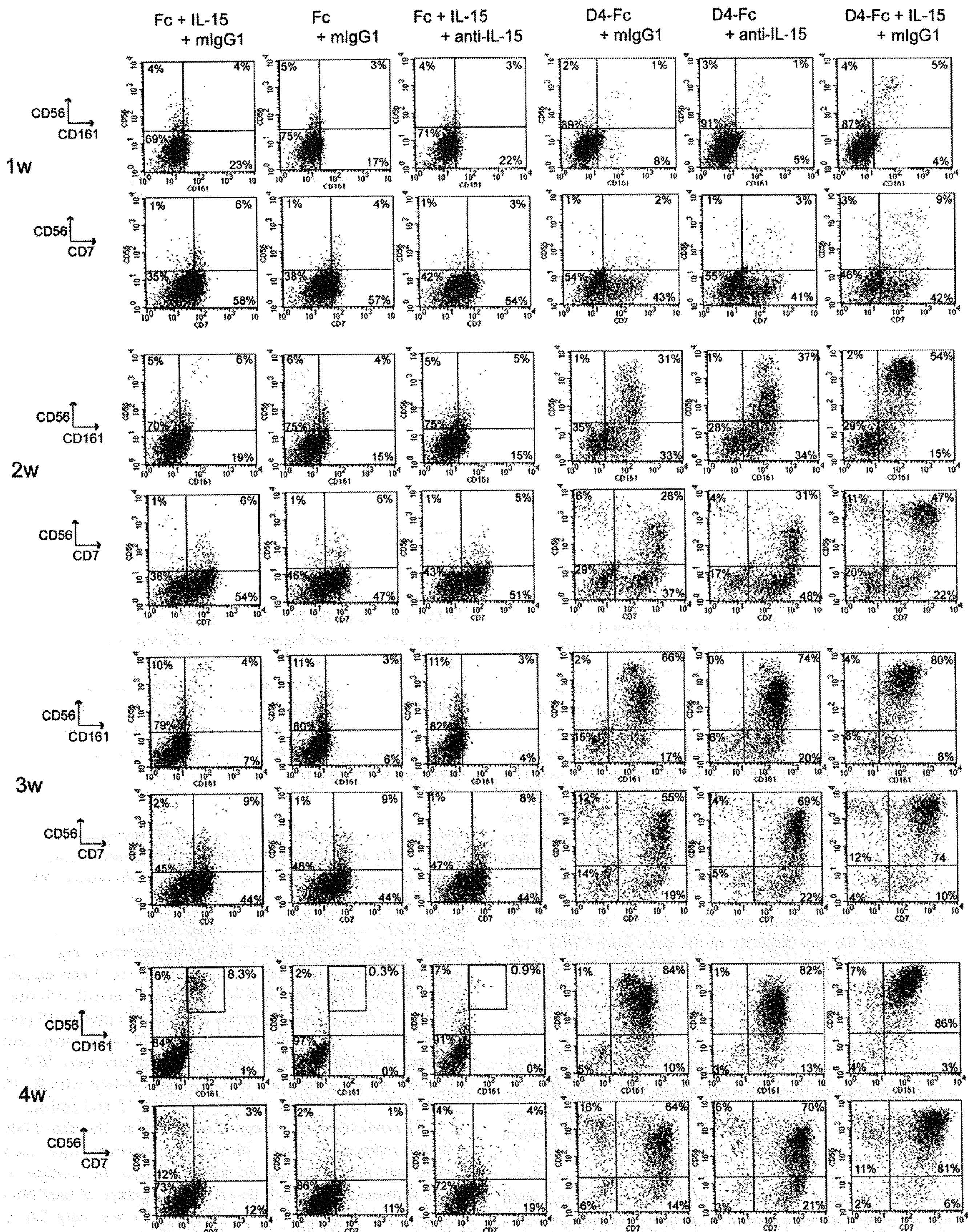

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FIGURE 3. Phenotypic analysis during culture under several culture conditions. Representative dot plots illustrating CD161 vs CD56 and CD7 vs CD56 of cells that were cultured from CB CD34^{+} cells for the indicated number of weeks on Fc-coated plates 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.