16. Tachikawa Y, Abe Y, Choi I, Ohtsuka R, Nagasawa E, Shibata K, et al. Second nonmyeloablative allogeneic peripheral blood stem cell transplantation with more immunosuppressive conditioning regimen for the late graft failure of the patient with acute myeloid leukemia. Fukuoka Igaku Zasshi. 2005;96:378–82.
17. Tanaka H, Ohwada C, Sakaida E, Takeda Y, Abe D, Oda K, et al. Successful engraftment by second cord blood transplantation with reduced-intensity conditioning after graft rejection due to hemophagocytic syndrome following initial CBT. Bone Marrow Transplant. 2007;40:995–6. doi:10.1038/sj.bmt.1705842.
18. Nakamura Y, Tanaka Y, Ando T, Sato Y, Yujiri T, Tanizawa Y. Successful engraftment of the second reduced-intensity conditioning cord blood transplantation (CBT) for a patient who developed graft rejection and infectious complications after the first CBT for AML. Bone Marrow Transplant. 2007;40:395–6. doi:10.1038/sj.bmt.1705732.
19. Mizutani E, Narimatsu H, Murata M, Tomita A, Kiyoi H, Naoe T. Successful second cord blood transplantation using fludarabine and cyclophosphamide as a preparative regimen for graft rejection following reduced-intensity cord blood transplantation. Bone Marrow Transplant. 2007;40:85–7. doi:10.1038/sj.bmt.1705684.
20. Ohwada C, Nakaseko C, Ozawa S, Takeuchi M, Shono K, Koizumi M, et al. Second cord blood transplantation (CBT) with reduced-intensity conditioning for graft failure after the first CBT for AML. Bone Marrow Transplant. 2004;34:999–1000. doi:10.1038/sj.bmt.1704696.
21. Nishihira H, Kato K, Isoyama K, Takahashi TA, Kai S, Kato S, et al. The Japanese cord blood bank network experience with cord blood transplantation from unrelated donors for haematological malignancies: an evaluation of graft-versus-host disease prophylaxis. Br J Haematol. 2003;120:516–22. doi:10.1046/j.1365-2141.2003.04115.x.

The Journal of Immunology

Anti-Moesin Antibodies in the Serum of Patients with Aplastic Anemia Stimulate Peripheral Blood Mononuclear Cells to Secrete TNF-{alpha} and IFN-{gamma}

Hiroyuki Takamatsu, J. Luis Espinoza, Xuzhang Lu, Zhirong Qi, Katsuya Okawa and Shinji Nakao

J. Immunol. 2009;182;703-710
http://www.jimmunol.org/cgi/content/full/182/1/703

The Journal of Immunology is published twice each month by The American Association of Immunologists, Inc., 9650 Rockville Pike, Bethesda, MD 20814-3994.

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

Anti-Moesin Antibodies in the Serum of Patients with Aplastic Anemia Stimulate Peripheral Blood Mononuclear Cells to Secrete TNF-α and IFN-γ[1]

Hiroyuki Takamatsu,[2*†] J. Luis Espinoza,[2*] Xuzhang Lu,[*] Zhirong Qi,[*] Katsuya Okawa,[‡] and Shinji Nakao[3*]

Moesin is an intracellular protein that links the cell membrane and cytoskeleton, while also mediating the formation of microtubules and cell adhesion sites as well as ruffling of the cell membrane. To determine the roles of anti-moesin Abs derived from the serum of patients with aplastic anemia (AA) in the pathophysiology of bone marrow failure, we studied the expression of moesin on various blood cells and the effects of anti-moesin Abs on the moesin-expressing cells. The proteins recognized by anti-moesin mAbs were detectable on the surface of T cells, NK cells, and monocytes from healthy individuals as well as on THP-1 cells. The peptide mass fingerprinting of the THP-1 cell surface protein and the knock-down experiments using short hairpin RNA proved that the protein is moesin itself. Both the anti-moesin mAbs and the anti-moesin polyclonal Abs purified from the AA patients' sera stimulated THP-1 cells and the PBMCs of healthy individuals and AA patients to secrete 60–80% as much TNF-α as did LPS 100 ng/ml. Although the polyclonal Abs induced IFN-γ secretion from the PBMCs of healthy individuals only when the PBMCs were prestimulated by anti-CD3 mAbs, the anti-moesin Abs were capable of inducing IFN-γ secretion from the PBMCs of AA patients by themselves. Anti-moesin Abs may therefore indirectly contribute to the suppression of hematopoiesis in AA patients by inducing myelosuppressive cytokines from immunocompetent cells. ***The Journal of Immunology,*** **2009, 182: 703–710.**

Acquired aplastic anemia (AA)[4] is a syndrome characterized by pancytopenia and bone marrow (BM) hypoplasia (1). The T cell-mediated suppression of hematopoiesis is considered to be the most important mechanism responsible for the development of this syndrome because approximately 70% of AA patients respond to immunosuppressive therapy, such as antithymocyte globulin and cyclosporine (2, 3). In addition to a large body of evidence for T cell involvement in the pathogenesis of AA (4–7), recent studies have revealed the presence of Abs specific to self-Ags in the serum of AA patients (8–11). Although some of these Abs are directed toward Ags that are abundant in hematopoietic cells (e.g., kinectin (Ref. 8) and DRS-1 (Ref. 9)), their roles in the pathophysiology of AA are unclear.

Of the various autoAbs detected in the autoimmune diseases, some are known to exhibit stimulatory effects on the target cells rather than inhibitory effects, such as anti-thyroglobulin Abs in Basedow's disease (12) and anti-desmoglein Abs in pemphigus vulgaris (12, 13). The autoAbs specific to platelet-derived growth factor receptors in patients with scleroderma and those with extensive chronic graft-vs-host diseases trigger an intracellular loop, involving Ha-Ras-ERK 1 and 2 (ERK 1/2)-reactive oxygen species (Ha-Ras-ERK 1/2-ROS), and augment collagen gene expression as well as myofibroblast phenotype conversion of normal human primary fibroblasts (14, 15). The anti-proteinase 3 Abs detected in Wegener's granuloma stimulate monocytes through the binding of cell surface proteinase 3 to secrete IL-8 (16). The autoAbs detected in AA patients may also be involved in the pathophysiology of BM failure by way of other mechanisms than the direct toxicity against the hematopoietic cells, though there has been no evidence for such functional autoAbs in AA patients.

We previously demonstrated that Abs specific to moesin, a membrane-cytoskeleton linker protein in the cytoplasm, were detectable in approximately 40% of AA patients (11). Moesin is an intracellular protein that links the cell membrane and cytoskeleton, and mediates the formation of microtubules and cell adhesion sites as well as ruffling of the cell membrane (17). On the other hand, some reports have identified molecules that were recognized by anti-moesin mAbs on the surface of blood cells such as T cells and macrophages (18, 19). Because these immune cells are an important source of myelosuppressive cytokines such as TNF-α and IFN-γ, it is conceivable that anti-moesin Abs in AA patients may bind such moesin-like molecules on these immune cells and affect the cytokine secretion from these cells.

To test these hypotheses, we studied the expression of moesin on blood cells and the effects of anti-moesin Abs on the moesin-expressing

*Cellular Transplantation Biology, Kanazawa University Graduate School of Medical Science, and †Internal Medicine, NTT WEST Kanazawa Hospital, Kanazawa, Ishikawa, and ‡Biomolecular Characterization Unit, Frontier Technology Center, Kyoto University Graduate School of Medicine, Kyoto, Japan

Received for publication August 28, 2008. Accepted for publication October 22, 2008.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, Technology, Sports and Culture of Japan (KAKENHI No. 19659243) and grants from the Research Committee for Idiopathic Hematopoietic Disorders, the Ministry of Health, Labor, and Welfare, Japan.

[2] H.T. and J.L.E. contributed equally to this work.

[3] Address correspondence and reprint requests to Dr. Shinji Nakao, Cellular Transplantation Biology, Kanazawa University Graduate School of Medical Science, Kanazawa, Ishikawa 920-8641, Japan. E-mail address: snakao@med3.m.kanazawa-u.ac.jp

[4] Abbreviations used in this paper: AA, aplastic anemia; BM, bone marrow; pAb, polyclonal Ab; PB, peripheral blood.

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 co-stimulation 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

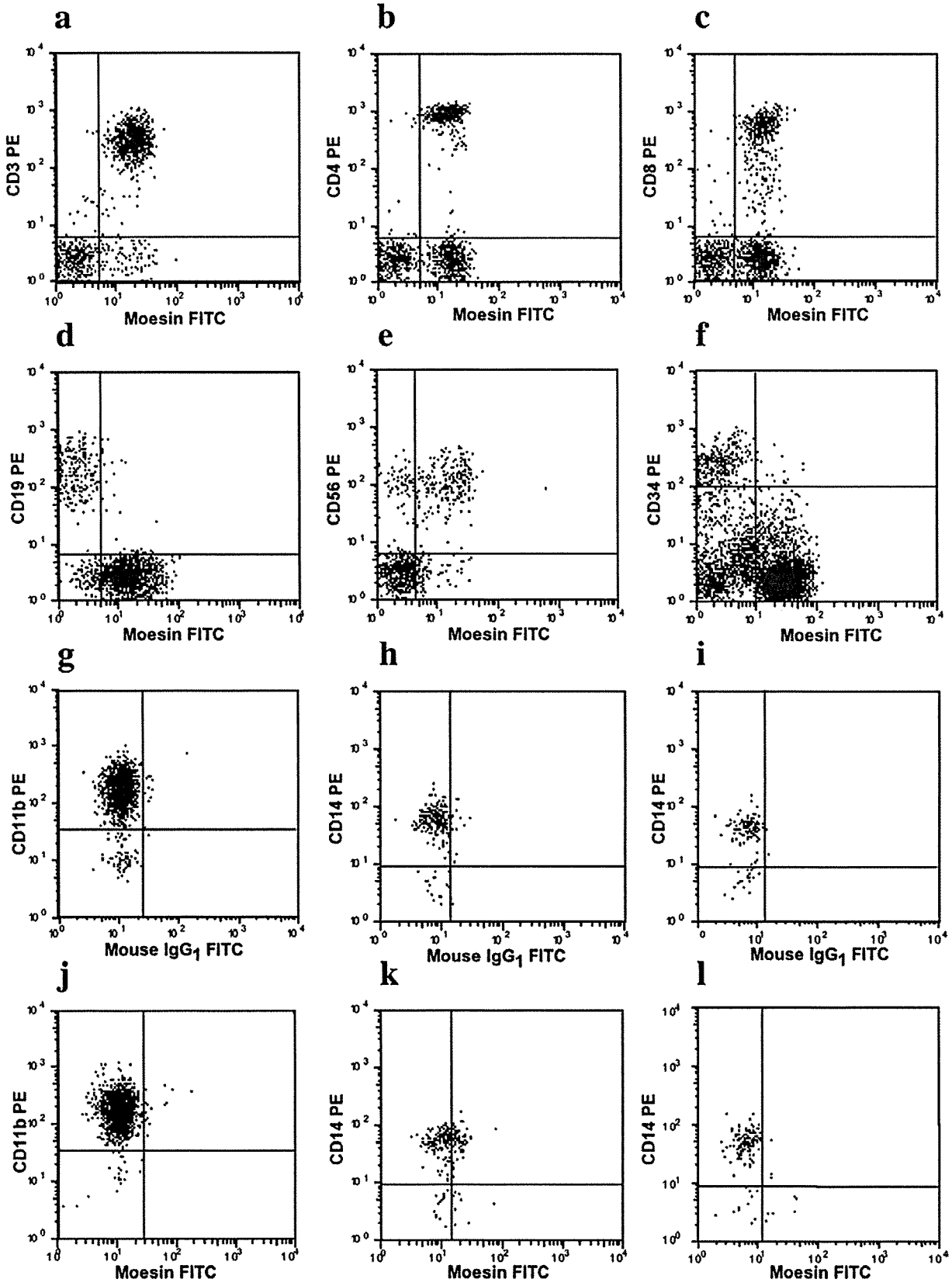

FIGURE 1. Expression of moesin-like molecules on the surface of various blood cells. PB lymphocytes, granulocytes, and monocytes, as well as BM mononuclear cells, of a healthy individual and a patient with AA were analyzed by flow cytometry. The gate was set up for lymphocytes (*a–d*), $CD3^-$ lymphocytes (*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.

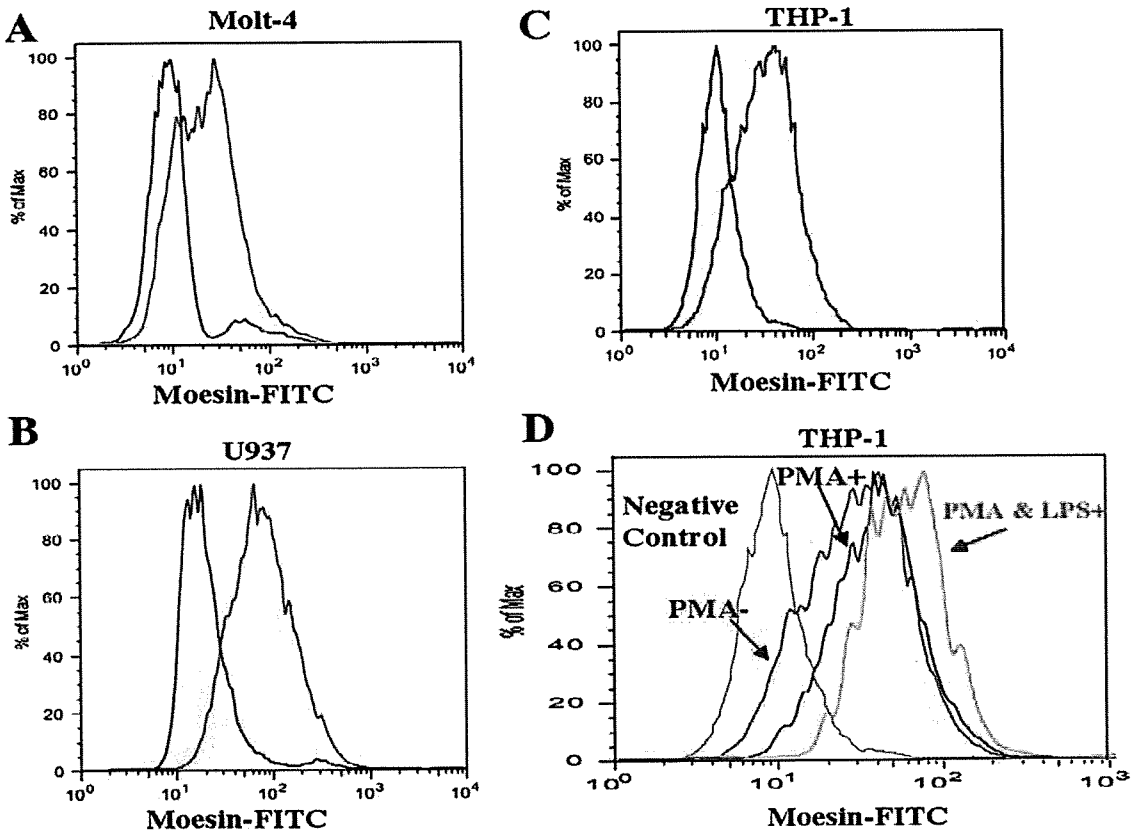

FIGURE 2. Expression of moesin-like molecules on the surface of T cell and monocytic leukemia cell lines. *A–C*, Three leukemia cell lines were examined for the cell surface expression of moesin-like molecules. Left lines, mouse IgG used as negative control; right lines, FITC-labeled anti-moesin mAbs. *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;

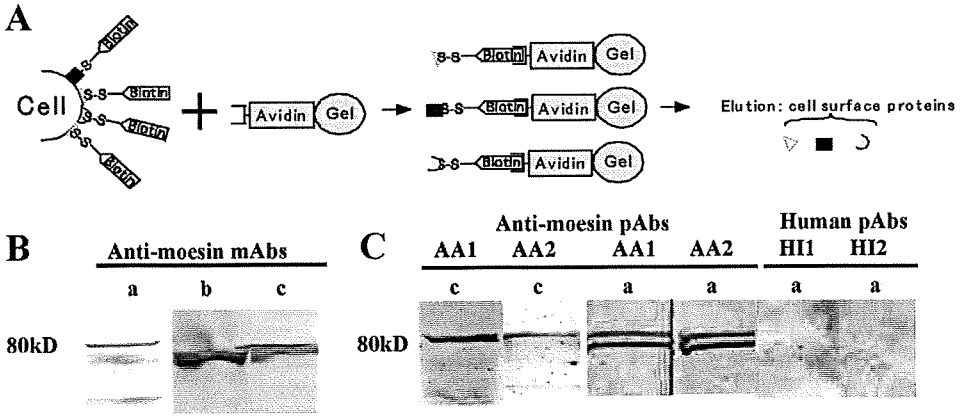

FIGURE 3. Isolation and identification of proteins on THP-1 cells recognized by anti-moesin Abs. *A*, THP-1 cells were treated with sulfo-NHS-SS-biotin and the cell surface proteins were isolated with avidin-fixed columns. *B*, Three different protein lysates (*a*, whole cells; *b*, cytoplasmic proteins; 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 ± 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 ± 2.2 and 6.6 ± 2.1, respectively, and the difference was significant (mean fluorescence intensity ± 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

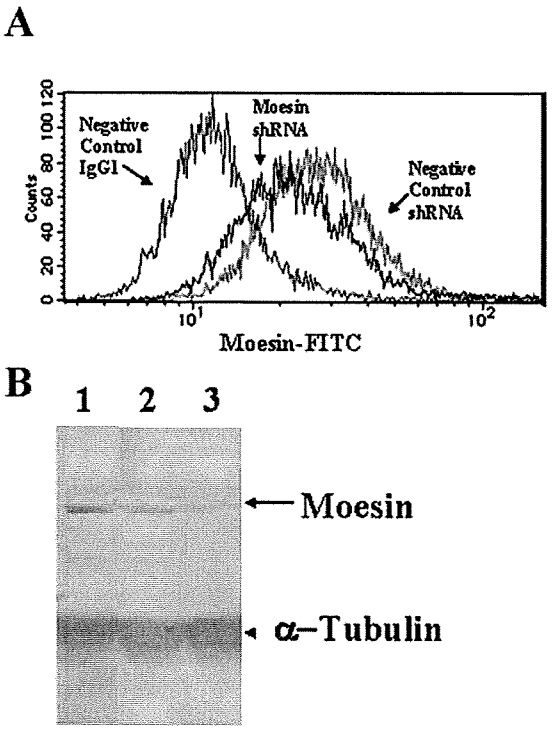

FIGURE 4. Effect of moesin shRNA transfection on the expression of moesin by THP-1 cells. *A*, THP-1 cells transfected with 5 μg of moesin shRNA or control shRNA were examined for the expression of moesin with flow cytometry. The blue line, non-transfected THP-1 cells stained with control mouse IgG1 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.

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.

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

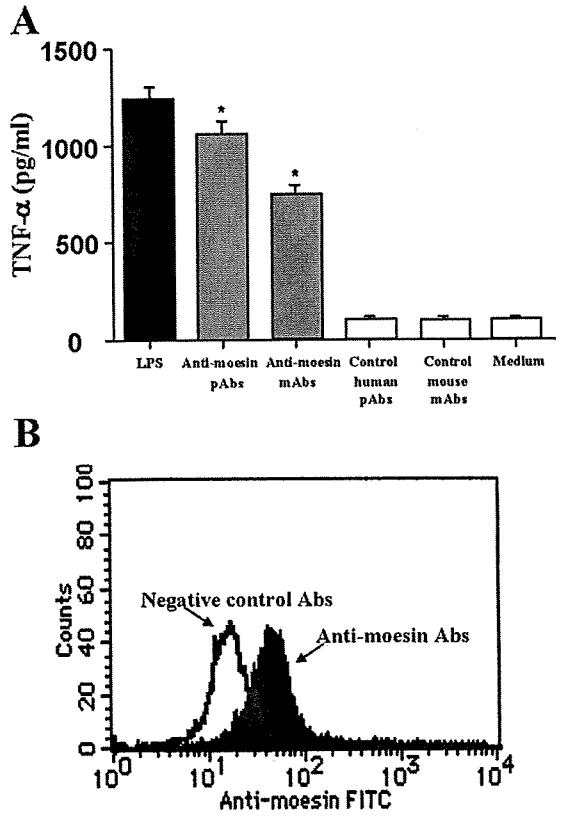

FIGURE 5. TNF-α release from THP-1 cells stimulated by anti-moesin Abs. *A*, THP-1 cells were cultured for 48 h with 5 μg/ml of anti-moesin Abs or control Abs. Anti-moesin pAbs, anti-moesin polyclonal IgG isolated from the serum of AA patients; control human pAbs, control human IgG pAbs isolated from 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.

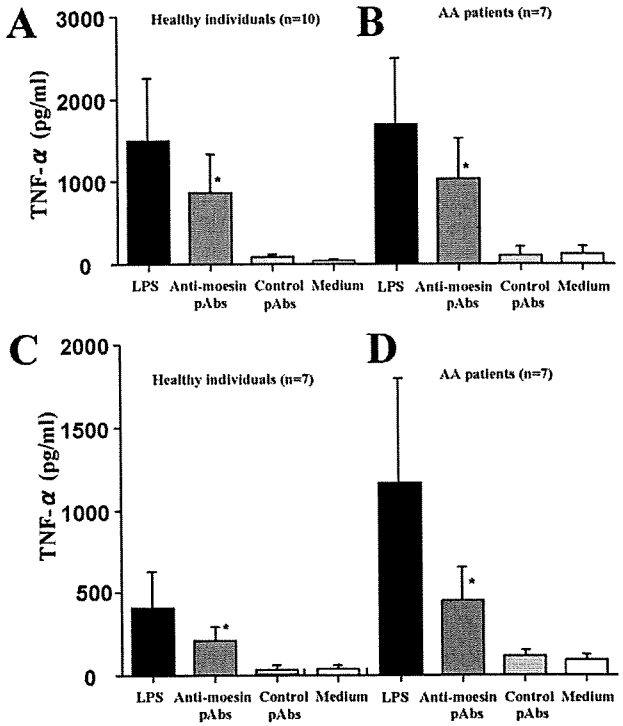

FIGURE 6. TNF-α release from PBMCs or monocytes stimulated by anti-moesin pAbs. The PBMCs or isolated monocytes were cultured for 48 h in the presence of 5 μg/ml of anti-moesin IgG pAbs or human control IgG pAbs isolated from the serum of AA patients or healthy individuals. Then, 100 ng/ml of LPS was 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

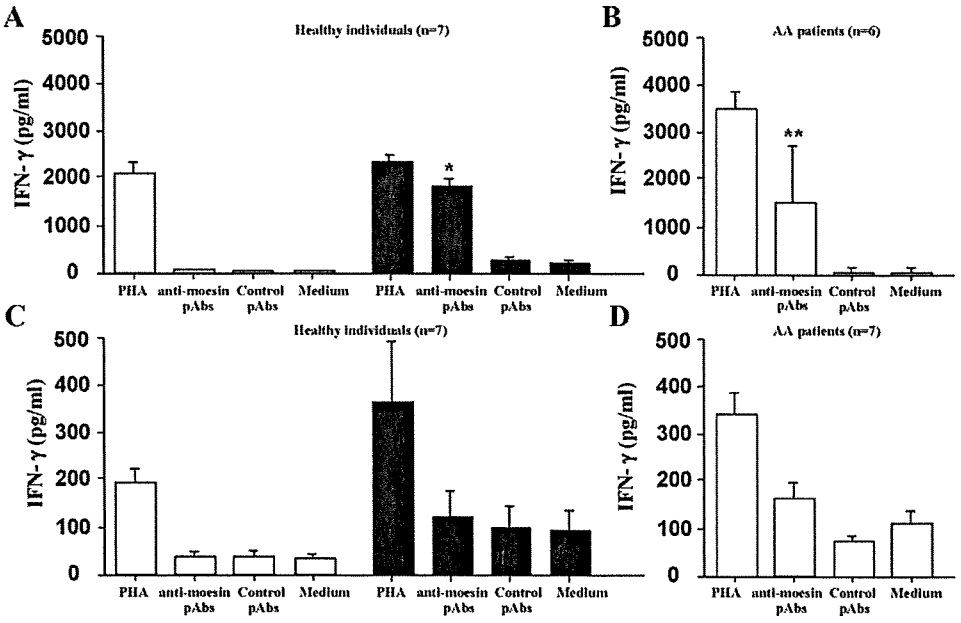

FIGURE 7. IFN-γ release from PBMCs or T cells stimulated by anti-moesin Abs. The PBMCs or isolated T cells were cultured for 48 h in the presence of 5 μg/ml of anti-moesin IgG pAbs or human control IgG pAbs isolated from the serum of AA patients or healthy individuals. Then, 10 μg/ml PHA was used as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

Acknowledgments

We gratefully acknowledge Prof. A. Yachie of Kanazawa University for suggestions and helpful discussion. We also thank M. Yoshii, A. Hamano, R. Oumi, and T. Tanaka of Cellular Transplantation Biology of Kanazawa University for technical assistance.

Disclosures

The authors have no financial conflict of interest.

References

1. Young, N. S. 2002. Acquired aplastic anemia. *Ann. Intern. Med.* 136: 534–546.
2. Bacigalupo, A., G. Broccia, G. Corda, W. Arcese, M. Carotenuto, A. Gallamini, F. Locatelli, P. G. Mori, P. Saracco, G. Todeschini, et al. 1995. Antilymphocyte globulin, cyclosporin, and granulocyte colony-stimulating factor in patients with acquired severe aplastic anemia (SAA): a pilot study of the EBMT SAA Working Party. *Blood* 85: 1348–1353.
3. Rosenfeld, S. J., J. Kimball, D. Vining, and N. S. Young. 1995. Intensive immunosuppression with antithymocyte globulin and cyclosporine as treatment for severe acquired aplastic anemia. *Blood* 85: 3058–3065.
4. Hoffman, R., E. D. Zanjani, J. D. Lutton, R. Zalusky, and L. R. Wasserman. 1977. Suppression of erythroid-colony formation by lymphocytes from patients with aplastic anemia. *N. Engl. J. Med.* 296: 10–13.
5. Nissen, C., P. Cornu, A. Gratwohl, and B. Speck. 1980. Peripheral blood cells from patients with aplastic anemia in partial remission suppress growth of their own bone marrow precursors in culture. *Br. J. Haematol.* 45: 233–243.
6. Nakao, S., A. Takami, H. Takamatsu, W. Zeng, N. Sugimori, H. Yamazaki, Y. Miura, M. Ueda, S. Shiobara, T. Yoshioka, et al. 1997. Isolation of a T-cell clone showing HLA-DRB1*0405-restricted cytotoxicity for hematopoietic cells in a patient with aplastic anemia. *Blood* 89: 3691–3699.
7. Zeng, W., J. P. Maciejewski, G. Chen, and N. S. Young. 2001. Limited heterogeneity of T cell receptor BV usage in aplastic anemia. *J. Clin. Invest.* 108: 765–773.
8. Hirano, N., M. O. Butler, M. S. Von Bergwelt-Baildon, B. Maecker, J. L. Schultze, K. C. O'Connor, P. H. Schur, S. Kojima, E. C. Guinan, and L. M. Nadler. 2003. Autoantibodies frequently detected in patients with aplastic anemia. *Blood* 102: 4567–4575.
9. Feng, X., T. Chuhjo, C. Sugimori, T. Kotani, X. Lu, A. Takami, H. Takamatsu, H. Yamazaki, and S. Nakao. 2004. Diazepam-binding inhibitor-related protein 1: a candidate autoantigen in acquired aplastic anemia patients harboring a minor population of paroxysmal nocturnal hemoglobinuria-type cells. *Blood* 104: 2425–2431.
10. Hirano, N., M. O. Butler, E. C. Guinan, L. M. Nadler, and S. Kojima. 2005. Presence of anti-kinectin and anti-PMS1 antibodies in Japanese aplastic anaemia patients. *Br. J. Haematol.* 128: 221–223.
11. Takamatsu, H., X. Feng, T. Chuhjo, X. Lu, C. Sugimori, K. Okawa, M. Yamamoto, S. Iseki, and S. Nakao. 2007. Specific antibodies to moesin, a membrane-cytoskeleton linker protein, are frequently detected in patients with acquired aplastic anemia. *Blood* 109: 2514–2520.
12. Weetman, A. P. 2003. Grave's disease 1835–2002. *Horm. Res.* 59 (*Suppl.* 1): 114–118.
13. Kitajima, Y., and Y. Aoyama. 2007. A perspective of pemphigus from bedside and laboratory-bench. *Clin. Rev. Allergy Immunol.* 33: 57–66.
14. Baroni, S. S., M. Santillo, F. Bevilacqua, M. Luchetti, T. Spadoni, M. Mancini, P. Fraticelli, P. Sambo, A. Funaro, A. Kazlauskas, et al. 2006. Stimulatory autoantibodies to the PDGF receptor in systemic sclerosis. *N. Engl. J. Med.* 354: 2667–2676.
15. Svegliati, S., A. Olivieri, N. Campelli, M. Luchetti, A. Poloni, S. Trappolini, G. Moroncini, A. Bacigalupo, P. Leoni, E. V. Avvedimento, and A. Gabrielli. 2007. Stimulatory autoantibodies to PDGF receptor in patients with extensive chronic graft-versus-host disease. *Blood* 110: 237–241.
16. Ralston, D. R., C. B. Marsh, M. P. Lowe, and M. D. Wewers. 1997. Antineutrophil cytoplasmic antibodies induce monocyte IL-8 release: role of surface proteinase-3, α1-antitrypsin, and Fcγ receptors. *J. Clin. Invest.* 100: 1416–1424.
17. Tsukita, S., and S. Yonemura. 1999. Cortical actin organization: lessons from ERM (ezrin/radixin/moesin) proteins. *J. Biol. Chem.* 274: 34507–34510.
18. Ariel, A., R. Hershkoviz, I. Altbaum-Weiss, S. Ganor, and O. Lider. 2001. Cell surface-expressed moesin-like receptor regulates T cell interactions with tissue components and binds an adhesion-modulating IL-2 peptide generated by elastase. *J. Immunol.* 166: 3052–3060.
19. Matsuyama, A., N. Sakai, H. Hiraoka, K. Hirano, and S. Yamashita. 2006. Cell surface-expressed moesin-like HDL/apoA-I binding protein promotes cholesterol efflux from human macrophages. *J. Lipid Res.* 47: 78–86.
20. Elkord, E., P. E. Williams, H. Kynaston, and A. W. Rowbottom. 2005. Human monocyte isolation methods influence cytokine production from in vitro generated dendritic cells. *Immunology* 114: 204–212.
21. Jensen, O. N., A. Podtelejnikov, and M. Mann. 1996. Delayed extraction improves specificity in database searches by matrix-assisted laser desorption/ionization peptide maps. *Rapid Commun. Mass Spectrom.* 10: 1371–1378.
22. Krawetz, R., M. J. MacKenzie, Q. Sun, P. A. Walton, and G. M. Kelly. 2006. Gα13 activation rescues moesin-depletion induced apoptosis in F9 teratocarcinoma cells. *Exp. Cell. Res.* 312: 3224–3240.
23. Masumoto, J., J. Sagara, M. Hayama, E. Hidaka, T. Katsuyama, and S. Taniguchi. 1998. Differential expression of moesin in cells of hematopoietic lineage and lymphatic systems. *Histochem. Cell Biol.* 110: 33–41.
24. Amar, S., K. Oyaisu, L. Li, and T. Van Dyke. 2001. Moesin: a potential LPS receptor on human monocytes. *J. Endotoxin Res.* 7: 281–286.
25. Tohme, Z. N., S. Amar, and T. E. Van Dyke. 1999. Moesin functions as a lipopolysaccharide receptor on human monocytes. *Infect. Immun.* 67: 3215–3220.
26. Solomou, E. E., K. Keyvanfar, and N. S. Young. 2006. T-bet, a Th1 transcription factor, is up-regulated in T cells from patients with aplastic anemia. *Blood* 107: 3983–3991.
27. Debets, J. M., C. J. van der Linden, I. E. Spronken, and W. A. Buurman. 1988. T cell-mediated production of tumour necrosis factor-α by monocytes. *Scand. J. Immunol.* 27: 601–608.
28. Tsukaguchi, K., B. de Lange, and W. H. Boom. 1999. Differential regulation of IFN-γ, TNF-α, and IL-10 production by CD4$^+$ $\alpha\beta$TCR$^+$ T cells and vδ2$^+$ $\gamma\delta$ T cells in response to monocytes infected with *Mycobacterium tuberculosis*-H37Ra. *Cell Immunol.* 194: 12–20.
29. Herold, K. C., J. B. Burton, F. Francois, E. Poumian-Ruiz, M. Glandt, and J. A. Bluestone. 2003. Activation of human T cells by FcR nonbinding anti-CD3 mAb, hOKT3γ1(Ala-Ala). *J. Clin. Invest.* 111: 409–418.
30. Gaston, R. S., M. H. Deierhoi, T. Patterson, E. Prasthofer, B. A. Julian, W. H. Barber, D. A. Laskow, A. G. Diethelm, and J. J. Curtis. 1991. OKT3 first-dose reaction: association with T cell subsets and cytokine release. *Kidney Int.* 39: 141–148.
31. Wagatsuma, M., M. Kimura, R. Suzuki, F. Takeuchi, K. Matsuta, and H. Watanabe. 1996. Ezrin, radixin and moesin are possible auto-immune antigens in rheumatoid arthritis. *Mol. Immunol.* 33: 1171–1176.
32. Kaufman, D. W., J. P. Kelly, M. Levy, and S. Shapiro. 1991. *The Drug Etiology of Agranulocytosis and Aplastic Anemia.* Oxford University Press, New York.
33. Lipsky, P. E., D. M. van der Heijde, E. W. St. Clair, D. E. Furst, F. C. Breedveld, J. R. Kalden, J. S. Smolen, M. Weisman, P. Emery, M. Feldmann, et al. 2000. Infliximab and methotrexate in the treatment of rheumatoid arthritis: anti-tumor necrosis factor trial in rheumatoid arthritis with Concomitant Therapy Study Group. *N. Engl. J. Med.* 343: 1594–1602.
34. Klareskog, L., D. van der Heijde, J. P. de Jager, A. Gough, J. Kalden, M. Malaise, E. Martin Mola, K. Pavelka, J. Sany, L. Settas, et al. 2004. Therapeutic effect of the combination of etanercept and methotrexate compared with each treatment alone in patients with rheumatoid arthritis: double-blind randomized controlled trial. *Lancet* 363: 675–681.
35. Maini, R., E. W. St. Clair, F. Breedveld, D. Furst, J. Kalden, M. Weisman, J. Smolen, P. Emery, G. Harriman, M. Feldmann, and P. Lipsky. 1999. Infliximab (chimeric anti-tumour necrosis factor α monoclonal antibody) versus placebo in rheumatoid arthritis patients receiving concomitant methotrexate: a randomized phase III trial. ATTRACT Study Group. *Lancet* 354: 1932–1939.
36. Deeg, H. J., J. Gotlib, C. Beckham, K. Dugan, L. Holmberg, M. Schubert, F. Appelbaum, and P. Greenberg. 2002. Soluble TNF receptor fusion protein (etanercept) for the treatment of myelodysplastic syndrome: a pilot study. *Leukemia* 16: 162–164.
37. Raza, A., A. Candoni, U. Khan, L. Lisak, S. Tahir, F. Silvestri, J. Billmeier, M. I. Alvi, M. Mumtaz, S. Gezer, P. Venugopal, P. Reddy, and N. Galili. 2004. Remicade as TNF suppressor in patients with myelodysplastic syndromes. *Leuk. Lymphoma* 45: 2099–2104.
38. Hansen, P. B., and A. M. Lauritzen. 2005. Aplastic anemia successfully treated with rituximab. *Am. J. Hematol.* 80: 292–294.
39. Castiglioni, M. G., P. Scatena, C. Pandolfo, S. Mechelli, and M. Bianchi. 2006. Rituximab therapy of severe aplastic anemia induced by fludarabine and cyclophosphamide in a patient affected by B-cell chronic lymphocytic leukemia. *Leuk. Lymphoma* 47: 1985–1986.

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

J. Luis Espinoza,[1] Akiyoshi Takami,[1] Makoto Onizuka,[2] Hiroshi Sao,[3] Hideki Akiyama,[4] Koichi Miyamura,[5] Shinichiro Okamoto,[6] Masami Inoue,[7] Yoshinobu Kanda,[8] Shigeki Ohtake,[1] Takahiro Fukuda,[9] Yasuo Morishima,[10] Yoshihisa Kodera,[11] and Shinji Nakao,[1] for the Japan Marrow Donor Program

[1]Department of Hematology and Oncology, Kanazawa University Hospital, Kanazawa; [2]Department of Hematology and Oncology, Tokai University School of Medicine, Isehara; [3]Department of Hematology, Meitetsu Hospital, Nagoya; [4]Hematology Division, Tokyo Metropolitan Cancer and Infectious Diseases Center, Komagome Hospital, Tokyo; [5]Department of Hematology, Japanese Red Cross Nagoya First Hospital, Nagoya; [6]Division of Hematology, Department of Medicine, Keio University School of Medicine, Tokyo; [7]Department of Hematology and Oncology, Osaka Medical Center and Research Institute for Maternal and Child Health, Osaka; [8]Division of Hematology, Saitama Medical Center, Jichi Medical University, Saitama; [9]Hematopoietic Stem Cell Transplantation Unit, National Cancer Center Hospital, Tokyo; [10]Department of Hematology and Cell Therapy, Aichi Cancer Center Hospital, Nagoya; [11]Department of Promotion for Blood and Marrow Transplantation, Aichi Medical University, Nagoya, Japan

Acknowledgments: we are indebted to Drs. Hiroko Oshima, Masanobu Oshima and Atsushi Hirao, Mrs. Shinichi Ohmae and Katsuya Nakata, and Ms. Kaori Matsuura at Kanazawa University, and Drs. Keitaro Matsuo and Takakazu Kawase at the Aichi Cancer Center Research Institute for their technical assistance. We thank all of the JMDP transplant teams who contributed patients and donors to this study.

Funding: this study was supported by grants from the Ministry of Health, Labor and Welfare, and the Ministry of Education, Culture, Sports and Technology, and funds from the Mitani Research and Development Assistance Organization (Kanazawa, Japan) and by the Japan Leukemia Research Fund (Tokyo, Japan).

Manuscript received on March 5, 2009. Revised version arrived on April 13, 2009. Manuscript accepted on April 29, 2009.

Correspondence: Akiyoshi Takami, M.D., Ph.D., Department of Hematology & Oncology, Kanazawa University Hospital, 13-1 Takaramachi, Kanazawa, 920-8641, Japan. E-mail: takami@med3.m.kanazawa-u.ac.jp

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.

Citation: Espinoza JL, Takami A, Onizuka M, Sao H, Akiyama H, Miyamura K, Okamoto S, Inoue M, Kanda Y, Ohtake S, Fukuda T, Morishima Y, Kodera Y, and Nakao S, for the Japan Marrow Donor Program. NKG2D *gene polymorphism has a significant impact on transplant outcomes after HLA-fully-matched unrelated bone marrow transplantation for standard risk hematologic malignancies. Haematologica 2009;94:1427-1434. doi:10.3324/haematol.2009.008318*

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%)					High-risk disease (n=52, 36%)				
	Donor NKG2D haplotype					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, $\times 10^8$/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.

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).

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.**

Variable	Overall survival			Transplant-related mortality			Relapse			Grades II-IV acute GVHD			Chronic GVHD		
	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-

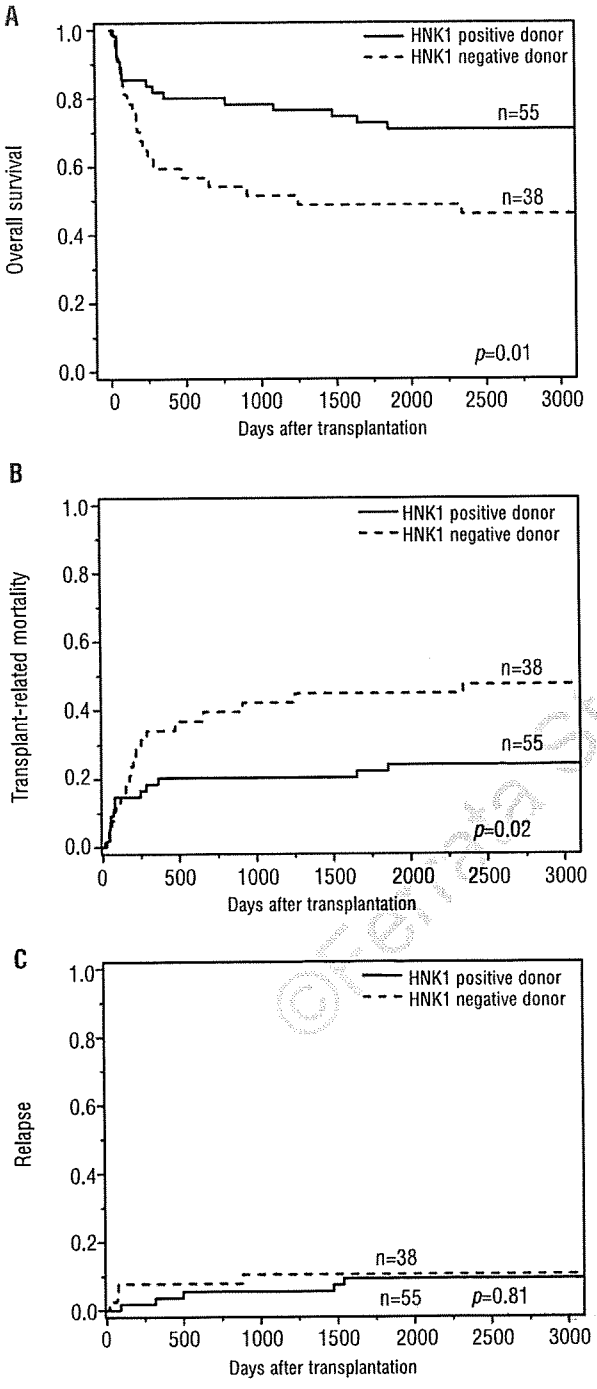

Figure 1. **Kaplan-Meier analysis of (A) overall survival, (B) cumulative incidence of transplant-related mortality and (C) disease relapse after transplantation according to the donor *NKG2D* polymorphism in patients with standard-risk disease. Patients with donors with the *HNK1* haplotype had better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

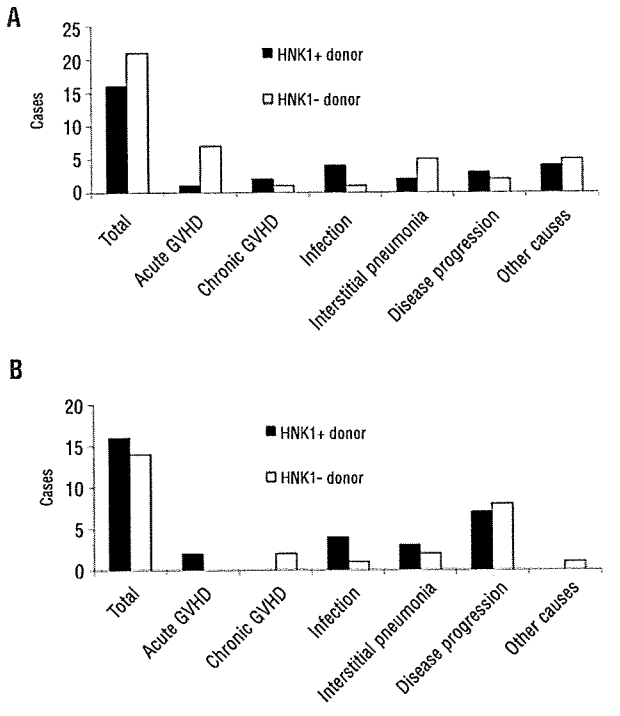

Figure 2. **Main causes of death after transplantation according to the *NKG2D* polymorphism in patients with (A) standard-risk disease (B) high-risk disease.**

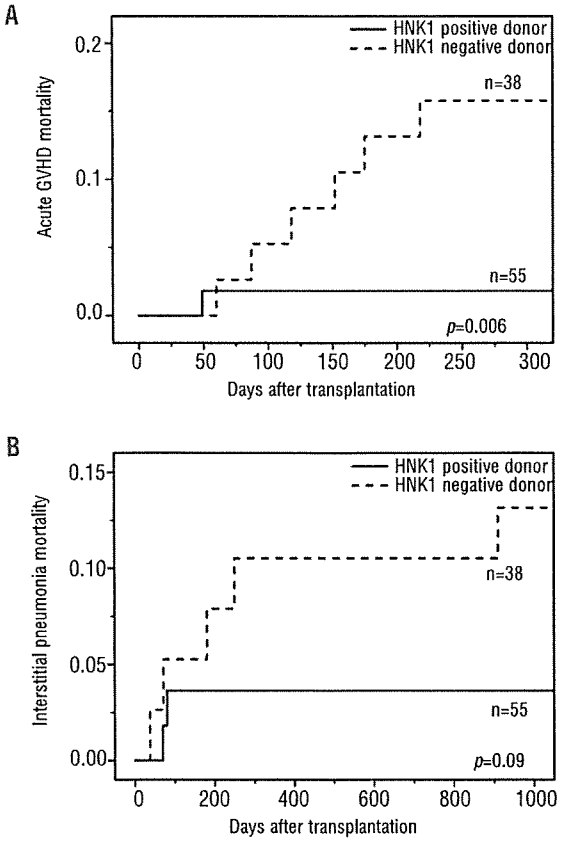

Figure 3. **Cumulative incidence of deaths due to (A) acute GVHD and (B) interstitial pneumonia after transplantation in patients with standard-risk disease. The *HNK1* haplotype in donors was associated with a significantly lower incidence of deaths due to acute GVHD (p=0.006) as well as a trend toward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

References

1. Weisdorf DJ, Anasetti C, Antin JH, Kernan NA, Kollman C, Snyder D, et al. Allogeneic bone marrow transplantation for chronic myelogenous leukemia: comparative analysis of unrelated versus matched sibling donor transplantation. Blood 2002; 99:1971-7.
2. Gratwohl A, Brand R, Frassoni F, Rocha V, Niederwieser D, Reusser P, et al. Cause of death after allogeneic haematopoietic stem cell transplantation (HSCT) in early leukaemias: an EBMT analysis of lethal infectious complications and changes over calendar time. Bone Marrow Transplant 2005;36:757-69.
3. Dickinson AM, Middleton PG, Rocha V, Gluckman E, Holler E. Genetic polymorphisms predicting the outcome of bone marrow transplants. Br J Haematol 2004; 127:479-90.
4. Bochud PY, Chien JW, Marr KA, Leisenring WM, Upton A, Janer M, et al. Toll-like receptor 4 polymorphisms and aspergillosis in stem-cell transplantation. N Engl J Med 2008; 359:1766-77.
5. Elmaagacli AH, Koldehoff M, Landt O, Beelen DW. Relation of an interleukin-23 receptor gene polymorphism to graft-versus-host disease after hematopoietic-cell transplantation. Bone Marrow Transplant 2008; 41:821-6.
6. Gerbitz A, Hillemanns P, Schmid C, Wilke A, Jayaraman R, Kolb HJ, et al. Influence of polymorphism within the heme oxygenase-1 promoter on overall survival and transplantation-related mortality after allogeneic stem cell transplantation. Biol Blood Marrow Transplant 2008;14:1180-9.
7. Holler E, Rogler G, Brenmoehl J, Hahn J, Herfarth H, Greinix H, et al. Prognostic significance of NOD2/CARD15 variants in HLA-identical sibling hematopoietic stem cell transplantation: effect on long-term outcome is confirmed in 2 independent cohorts and may be modulated by the type of gastrointestinal decontamination. Blood 2006;107: 4189-93.
8. Holler E, Rogler G, Herfarth H, Brenmoehl J, Wild PJ, Hahn J, et al. Both donor and recipient

NOD2/CARD15 mutations associate with transplant-related mortality and GvHD following allogeneic stem cell transplantation. Blood 2004;104:889-94.
9. Mayor NP, Shaw BE, Hughes DA, Maldonado-Torres H, Madrigal JA, Keshav S, et al. Single nucleotide polymorphisms in the NOD2/CARD15 gene are associated with an increased risk of relapse and death for patients with acute leukemia after hematopoietic stem-cell transplantation with unrelated donors. J Clin Oncol 2007;25:4262-9.
10. Mullighan CG, Heatley S, Doherty K, Szabo F, Grigg A, Hughes TP, et al. Mannose-binding lectin gene polymorphisms are associated with major infection following allogeneic hemopoietic stem cell transplantation. Blood 2002;99:3524-9.
11. Noori-Daloii MR, Rashidi-Nezhad A, Izadi P, Hossein-Nezhad A, Sobhani M, Derakhshandeh-Peykar P, et al. Transforming growth factor-β1 codon 10 polymorphism is associated with acute GVHD after allogenic BMT in Iranian population. Ann Transplant 2007;12:5-10.
12. Rocha V, Franco RF, Porcher R, Bittencourt H, Silva WA Jr, Latouche A, et al. Host defense and inflammatory gene polymorphisms are associated with outcomes after HLA-identical sibling bone marrow transplantation. Blood 2002;100:3908-18.
13. Shimada M, Onizuka M, Machida S, Suzuki R, Kojima M, Miyamura K, et al. Association of autoimmune disease-related gene polymorphisms with chronic graft-versus-host disease. Br J Haematol 2007;139:458-63.
14. Soydan E, Topcuoglu P, Dalva K, Arat M. The impact of methylenetetrahydrofolate reductase (MTHFR) C677T gene polymorphism on transplant-related variables after allogeneic hematopoietic cell transplantation in patients receiving MTX as GVHD prophylaxis. Bone Marrow Transplant 2008;42:429-30.
15. Viel DO, Tsuneto LT, Sossai CR, Lieber SR, Marques SB, Vigorito AC, et al. IL2 and TNFA gene polymorphisms and the risk of graft-versus-host disease after allogeneic haematopoietic stem cell transplantation. Scand J Immunol 2007;66: 703-10.
16. Burgess SJ, Maasho K, Masilamani M, Narayanan S, Borrego F, Coligan JE. The NKG2D receptor: immunobiology and clinical implications. Immunol Res 2008;40:18-34.
17. Hyka-Nouspikel N, Phillips JH. Physiological roles of murine DAP10 adapter protein in tumor immunity and autoimmunity. Immunol Rev 2006;214:106-17.
18. Jamieson AM, Diefenbach A, McMahon CW, Xiong N, Carlyle JR, Raulet DH. The role of the NKG2D immunoreceptor in immune cell activation and natural killing. Immunity 2002;17:19-29.
19. Raulet DH. Roles of the NKG2D immunoreceptor and its ligands. Nat Rev Immunol 2003;3:781-90.
20. Groh V, Wu J, Yee C, Spies T. Tumour-derived soluble MIC ligands impair expression of NKG2D and T-cell activation. Nature 2002;419: 734-8.
21. Hayashi T, Imai K, Morishita Y, Hayashi I, Kusunoki Y, Nakachi K. Identification of the NKG2D haplotypes associated with natural cytotoxic activity of peripheral blood lymphocytes and cancer immunosurveillance. Cancer Res 2006;66: 563-70.
22. Imai K, Matsuyama S, Miyake S, Suga K, Nakachi K. Natural cytotoxic activity of peripheral-blood lymphocytes and cancer incidence: an 11-year follow-up study of a general population. Lancet 2000;356:1795-9.
23. Furue H, Kumimoto H, Matsuo K, Suzuki T, Hasegawa Y, Shinoda M, et al. Opposite impact of NKG2D genotype by lifestyle exposure to risk of aerodigestive tract cancer among Japanese. Int J Cancer 2008; 123:181-6.
24. Furue H, Matsuo K, Kumimoto H, Hiraki A, Suzuki T, Yatabe Y, et al. Decreased risk of colorectal cancer with the high natural killer cell activity NKG2D genotype in Japanese. Carcinogenesis 2008; 29:316-20.
25. Kawase T, Morishima Y, Matsuo K, Kashiwase K, Inoko H, Saji H, et al. High-risk HLA allele mismatch combinations responsible for severe acute graft-versus-host disease and implication for its molecular mechanism. Blood 2007;110:2235-41.
26. Sasazuki T, Juji T, Morishima Y, Kinukawa N, Kashiwabara H, Inoko H, et al. Effect of matching of class I HLA alleles on clinical outcome after transplantation of hematopoietic stem cells from an unrelated donor. Japan Marrow Donor Program. N Engl J Med 1998;339:1177-85.
27. Livak KJ. Allelic discrimination using fluorogenic probes and the 5' nuclease assay. Genet Anal 1999;14:143-9.
28. Przepiorka D, Weisdorf D, Martin P, Klingemann HG, Beatty P, Hows J, et al. 1994 Consensus Conference on Acute GVHD Grading. Bone Marrow Transplant 1995;15:825-8.
29. Shulman HM, Sullivan KM, Weiden PL, McDonald GB, Striker GE, Sale GE, et al. Chronic graft-versus-host syndrome in man. A long-term clinicopathologic study of 20 Seattle patients. Am J Med 1980;69:204-17.
30. Scrucca L, Santucci A, Aversa F. Competing risk analysis using R: an easy guide for clinicians. Bone Marrow Transplant 2007;40:381-7.
31. Gooley TA, Leisenring W, Crowley J, Storer BE. Estimation of failure probabilities in the presence of competing risks: new representations of old estimators. Stat Med 1999;18: 695-706.
32. Kim DH, Jung HD, Lee NY, Sohn SK. Single nucleotide polymorphism of CC chemokine ligand 5 promoter gene in recipients may predict the risk of chronic graft-versus-host disease and its severity after allogeneic transplantation. Transplantation 2007;84:917-25.
33. Dulphy N, Haas P, Busson M, Belhadj S, Peffault de Latour R, Robin M, et al. An unusual CD56(bright) CD16(low) NK cell subset dominates the early posttransplant period following HLA-matched hematopoietic stem cell transplantation. J Immunol 2008; 181:2227-37.
34. Hamby K, Trexler A, Pearson T, Larsen C, Rigby M, Kean L. NK cells rapidly reject allogeneic bone marrow in the spleen through a perforin- and Ly49D-dependent, but NKG2D-independent mechanism. Am J Transplant 2007;7:1884-96.
35. Ruggeri L, Capanni M, Urbani E, Perruccio K, Shlomchik W, Tosti A, et al. Effectiveness of donor natural killer cell alloreactivity in mismatched hematopoietic transplants. Science 2002;295:2097-100.
36. Ruggeri L, Mancusi A, Burchielli E, Capanni M, Carotti A, Aloisi T, et al. NK cell alloreactivity and allogeneic hematopoietic stem cell transplantation. Blood Cells Mol Dis 2008;40: 84-90.
37. Hsu K, Keever-Taylor C, Wilton A, Pinto C, Heller G, Arkun K, et al. Improved outcome in HLA-identical sibling hematopoietic stem-cell transplantation for acute myelogenous leukemia predicted by KIR and HLA genotypes. Blood 2005;105: 4878-84.
38. Morishima Y, Yabe T, Matsuo K, Kashiwase K, Inoko H, Saji H, et al. Effects of HLA allele and killer immunoglobulin-like receptor ligand matching on clinical outcome in leukemia patients undergoing transplantation with T-cell-replete marrow from an unrelated donor. Biol Blood Marrow Transplant 2007;13: 315-28.
39. Kimura F, Sato K, Kobayashi S, Ikeda T, Sao H, Okamoto S, et al. Impact of ABO-blood group incompatibility on the outcome of recipients of bone marrow transplants from unrelated donors in the Japan Marrow Donor Program. Haematologica 2008;93: 1686-93.
40. Goldstone AH, Richards SM, Lazarus HM, Tallman MS, Buck G, Fielding AK, et al. In adults with standard-risk acute lymphoblastic leukemia, the greatest benefit is achieved from a matched sibling allogeneic transplantation in first complete remission, and an autologous transplantation is less effective than conventional consolidation/maintenance chemotherapy in all patients: final results of the International ALL Trial (MRC UKALL XII/ECOG E2993). Blood 2008;111: 1827-33.
41. Collins RW. Human MHC class I chain related (MIC) genes: their biological function and relevance to disease and transplantation. Eur J Immunogenet 2004;31:105-14.

included refractory anemia (RA) (n=4), refractory anemia with excess blasts (RAEB) (n=7), RAEB-t (n=2), and MDS-related secondary AML (n=21). All patients received four fractionated 12 Gy total body irradiation and chemotherapy as myeloablative conditioning. All patients received standard cyclosporine and methotrexate as a GVHD prophylaxis. The median age was 40 years (range, 19-52 years), the median weight was 56 kg (range, 43-75 kg), and the median number of cryopreserved nucleated cells was 2.44 x 10^7/kg (range, 1.71-4.10 x 10^7/kg) and the median number of cryopreserved CD34 positive cells was 0.85 x 10^6/kg (range, 0.40-2.14 x 10^6/kg). 31 patients had myeloid reconstitution and the median time to more than 0.5 x 109/L absolute neutrophil count was 22 days. A self-sustained platelet count more than 50 x 10^9/L was achieved in 30 patients at a median time of 50 days. Grades III-IV acute GVHD occurred in 5 of 31 evaluable patients and chronic GVHD in 26 of 29 evaluable patients. Among 26 chronic GVHD patients, in 13 patients the disease was extensive. No factor was associated with hematopoietic recovery and the incidence of acute and chronic GVHD. The 5-year cumulative incidence of transplant related-mortality (TRM) and relapse was 14.5%, 16.6%, respectively. No factor was associated with TRM and relapse. 25 patients are alive and free of disease at between 3.4 and 125 months after transplantation. Median follow-up was 69 months. The probability of event-free survival (EFS) at 5 years was 71.3%. Poor-risk karyotype at diagnosis was significantly associated with worse EFS (p=0.037). Cell dose and HLA matching had no impact on any transplant outcomes. These results suggest that adult MDS patients without suitable related or unrelated bone marrow donors should be considered as candidates for CBT.

P485

Efficacy of plerixafor plus G-CSF compared to G-CSF plus placebo for mobilisation of CD34+ haematopoietic progenitor cells in patients older than 60 years with non-Hodgkin's lymphoma or multiple myeloma

I.N. Micallef (1), J.F. DiPersio (2), A.P. Nademanee (3), P.J. Stiff (4), E.A. Stadtmauer (5), R.T. Maziarz (6), B.J. Bolwell (7), J. Angell (8), G. Bridger (8), G. Calandra (8)

(1)Mayo Clinic (Rochester, US); (2)Washington University (Saint Louis, US); (3)City of Hope (Duarte, US); (4)Loyola University Medical Center (Maywood, US); (5)University of Pennsylvania (Philadelphia, US); (6)Oregon Health & Science University (Portland, US); (7)Cleveland Clinic Foundation (Cleveland, US); (8)Genzyme Corporation (Cambridge, US)

Advanced age and intensive previous chemotherapy are two independent risk factors that are associated with poor CD34+ hematopoietic stem cell (HSC) mobilization in patients undergoing autologous stem cell transplantation (ASCT). The purpose of this report is to evaluate the efficacy of plerixafor plus G-CSF (G) to G alone in non-Hodgkin's lymphoma (NHL) and multiple myeloma (MM) patients >60 years of age undergoing CD34+ HSC mobilization for ASCT.

Adult NHL and MM patients requiring ASCT were eligible to participate in a phase III multi-center, randomized, placebo controlled trial. Non-Hodgkin's lymphoma patients participated in study 3101 and MM patients in study 3102. In each of the trials, patients were randomized to receive plerixafor plus G or G plus placebo. All patients received G (10mcg/kg/day) for 4 days; on the evening of day 4 they received either plerixafor (240mcg/kg) or placebo. Patients underwent apheresis on day 5 after a morning dose of G. Patients continued to receive evening study treatment followed by morning G and apheresis for up to a pre-specified number of apheresis days or until a target number of CD34+ cells/kg were collected. In 3101, the primary endpoint was the percentage of patients who achieved 5x10^6 CD34+ cells/kg in 4 or less apheresis days. In 3102, the primary endpoint was the percentage of patients who achieved 6x 10^6 CD34+ cells/kg in 2 or less apheresis days.

Of the 298 patients enrolled in study 3101, 124 NHL patients were older than 60 years of age. The primary endpoint in this subgroup of patients was met in 29/57 (50.9%) and 17/67 (25.4%) of the patients in the plerixafor plus G and G plus placebo groups, respectively (p<0.001). Median time to neutrophil engraftment in the plerixafor plus G group was 10 days and 11 days in the G plus placebo group. Median time to platelet engraftment was 20 days and 21 days in the G plus plerixafor and G plus placebo groups, respectively.

Of the 302 patients enrolled in study 3102, 145 MM patients were older than 60 years of age. The primary endpoint in this subgroup of MM patients was met in 48/69 (69.6%) and 18/76 (23.7%) of the patients in the plerixafor plus G and G plus placebo groups, respectively (p<0.001). Median time to neutrophil and platelet engraftment in both groups was 11 and 18 days, respectively.

In these studies, the addition of plerixafor to G in NHL or MM patients greater than 60 years old undergoing ASCT is superior to G alone.

P486

Prospective analysis for antigen-specific cellular immune reconstitution after cord blood transplantation: immuno response to CMV is not affected by HLA disparity

S. Takahashi, M. Ishige, N. Watanabe, T. Yamaguchi, J. Ooi, N. Tsukada, S. Kato, A. Sato, F. Monma, R. Yamazaki, A. Fujita, N. Toki, H. Tanaka, T. Uohara, K. Fujimaki, K. Oshima, Y. Aisa, K. Motohashi, R. Hyo, Y. Najima, H. Kanamori, Y. Nannya, R. Sakai, S. Takada, T. Kobayashi, T. Hoshino, K. Matsumoto, K. Hidoyuki, J. Kato, A. Yokota, N. Nakamura, M. Tanaka, T. Mori, H. Sakamaki, S. Okamoto on behalf of Kanto Study Group for Cell Therapy (KSGCT)

Study purpose: Cytomegalovirus (CMV) infection is still serious problem in cord blood transplant (CBT) and T cell immunity has an important role in control of virus replication and prevention. The one of crucial questions in CBT is whether naivity of lymphocytes could gain antigen-specific cellular immunity during early phase of human leukocyte antigen (HLA)-mismatched transplant. To answer this, we have analyzed the CMV-specific immune reconstitution process for first 6 months.

Patients and Methods: During 2006 and 2008, 40 adults has received myeloablative regimens including 12 Gy of total body irradiation followed by CBT and a standard cyclosporine and methotrexate combination as GVHD prophylaxis in the Institute of Medical Science, University of Tokyo (IMSUT), for 19 patients and in 9 different facilities which participated for the prospective study using IMSUT regimen for 21 patients. CMV-specific CD4+ and CD8+ T cell recoveries were assessed by detection of interferon-gamma (IFN-g) producing cells with CMV antigen stimulation using intracellular cytokine staining or tetramers for CMV pp65 in whom HLA-A0201, -A0206 or -A2402 positive patients. The positive was defined as >0.03% IFN-g positive cells among CD4+ or CD8+ T cell population and >0.01% positive in tetramer assay.

Results: CMV-reactive (IFN-g positive) CD4+ T cells were detected in 65% at 1 month, 88% at 2 months, 92% at 3 months, 92% at 4 months and 95% at 6 months after CBT which were comparable to CMV-positive age-adjusted healthy control (100%). CMV-reactive (IFN-g positive) and CMV-specific (tetramer-positive) CD8+ T cells were detected in 53/5% at 1 month, 71/44% at 2 months, 68/36% at 3 months 75/50% at 4 months, 65/50% at 6 months (39/67% in the control). Next, we looked the effect of HLA disparity (HLA-DR for CD4+ and HLA-A/ -B for CD8+ T cell) in graft-versus-host direction with low resolution typing (LRT) and in high resolution typing (HRT). CMV-reactive CD4+ T cells were detected in 94% with matched (0MM), 81% with one mismatched (1MM) in LRT and 100% with 0MM, 89% with 1MM, 80% with 2MM in HRT at 2 months. CMV-specific CD8+ T cells were detected in 33% with 0MM, 38% with 1MM, 56% with 2MM in LRT and 38% with 1MM, 50% with 2MM, 67% with 3MM in HRT at 2 months, respectively.

Conclusion: Post-thymic naive T cells in cord blood might obtain memory and effector function in vivo with antigen-specific

manner during early phase of post-transplant without effect of HLA disparity.

P487

HHV6 infection is a hallmark of cord blood transplant in adults and may participate to delayed engraftment: a comparison with matched unrelated donors as stem cell source

P. Chevallier, T. Guillaume, L. Plancho, I. Hoblo-Follah, C. Bressolette-Bodin, F. Rialland, M. Costo-Burel, S. Ayari, J. Delaunay, V. Horvais, M. Mohty, P. Moreau, J.-L. Harousseau, B. Imbert-Marcille
CHU Hotel-Dieu (Nantes, FR)

Herpesvirus infections after cord blood transplant (CBT) in adults have been little studied thus far. To address this issue, we performed a comparison between 15 patients who received a CBT (CBT group) with 40 patients who received an allogeneic transplant from a matched unrelated donor (MUD group) in our centre.
The two groups were comparable except for the use of ATG and the median number of CD34+ cells infused and were stringently monitored through CMV, EBV, and HHV6 DNA quantifications before and up to 9 months after transplant. An active infection was defined by a viral load >3 log/mL of blood or two consecutive PCR between 2 and 3 log/mL.
Considering the all cohort, 22, 29 and 29 patients experienced at least one positive PCR for CMV, EBV and HHV6. Incidence of HHV6 infection was significantly higher in the CBT group (80% vs 42.5%, P<0.0001),Incidence of EBV infection was significantly higher in the MUD group (62% vs 27%, P<0.0001). Incidences of CMV were similar between the 2 groups.
In multivariate analysis, the use of a CBT and a myeloablative conditioning regimen were found to increase the risk of HHV6 infection (OR=5.4, 95% CI:1.2-23.0; P=0.02; OR=3.5, 95%CI: 1.03-12.05, P= 0.04) while the use of a MUD transplant was found to increase the risk of EBV infection (OR=0.31, 95%CI, 0.07-1.37; P=0.04). HHV6 infection started at a median of 36 days post-transplant in the CBT group vs 58 days in the MUD group (P=NS). In the CBT group, all patients with HHV6 infection (n=12/12, 100%) were still positive at the end of their follow-up (7 patients alive at 9 months post transplant and 5 patients dead before month +9) compared to only 7 out of 17 (41%) in the MUD group (P<0.009). Overall, four patients were still EBV positive at the end of the follow-up (1 patient in the CBT group, and 3 patients in the MUD group, P=NS).
Interestingly, the occurrence of HHV6 reactivation translated towards delayed engraftment (median time to neutrophils and platelets recovery: 37 vs 16 days; P=0.03; 98 vs. 12 days; P=0.0001).
Overall, these results show that after allogeneic transplant, the pattern of HHV6 and EBV infections is dependant on the source of stem cells. A specific relationship is suggested between HHV6 infection and the use of cord blood cells. In addition to a potential delayed engraftment, the clinical implications of the latter finding need to be refined and prospective screening and antiviral prophylaxis approaches are warranted in the context of CBT.

P488

A novel P-glycoprotein (Pgp) -dependent ex-vivo approach for expansion of human umbilical cord blood CD133+ cells significantly enriches the CD34+/CD38- fraction

H. Galski, I. Bar, A. Naglar
Chaim Sheba Medical Center (Tel Hashomer, IL)

Introduction: While umbilical cord blood (UCB) is an attractive source for transplantation, major disadvantage is its relative low number of hematopoietic stem cells (HSCs). One approach thus involves ex-vivo expansion of UCB-derived HSCs. However, the current expansion processes are laborious, expensive and providing unwanted differentiated sub-populations. AIMES. As the ABC transporter Pgp (gene product of ABCB1) is over-expressed in various stem cells relatively to their differentiated progeny, we reasoned that higher Pgp activity in CD133+ HSCs would protect them from the anti-proliferative effect of the Pgp substrate colchicine (COL) and thus could be applicable to their selection and enrichment.
Methods: To this end, we isolated CD133+ HSCs from UCB by CD133-immunomagnetic separation (MACS). Pgp-expression level was measured by flow cytometry using the Pgp-antibodies MRK-16, and its activity was measured by efflux assay of the Pgp-substrate Rh123. We further analyzed the relative distribution of various CD133+ subsets during 8 weeks of standard procedure of their expansion, in the absence or the presence of COL.
Results: Analyses of freshly isolated CD133+ HSCs indicated that 92% of CD133+ cells express functional Pgp on the cell surface. At 8 weeks of expansion, the CD133+ cell number increased from 105 cells to 0,56±0,19 x10^9 and 1.60±0.41 x10^9 in the absence and the presence of COL, respectively (2.9 ± 0.5 fold increase, n=6). The long exposure of CD133+ HSCs to COL at the expansion process did not affect their ability to form various hematopoietic colonies in semisolid culture after COL removal (Table 1). Furthermore, FACS analyses indicated that ex-vivo expansion in the presence of COL preferentially enrich the CD133+/Pgp+ subset and specifically the CD34+/CD38- HSCs (Figure1).
Conclusion: We established a novel expansion approach that specifically enriches the CD133+/CD34+/CD38- fraction of UCB-derived HSCs.

CFU-	Total	BFU-E	CFU-GM	CFU-GEMM
Control	125 ± 3	16 ± 2	16 ± 1	93 ± 3
Treated	640 ± 12	9 ± 3	21 ± 5	130 ± 35

Table 1: CFU measurements (mean ± SD, /4E3) of control and COL exposed CD133 cells. In each case, 10^3 CD133 cells/ well were seeded

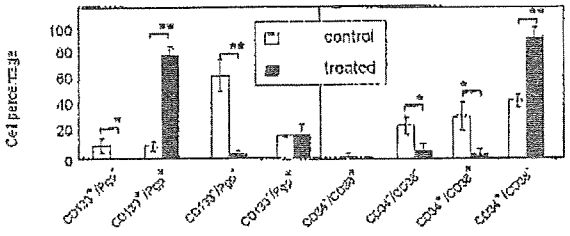

Figure 1 Cell subsets distribution of UCB-derived CD133+ cells after expansion with or without colchicine treatment.Means (n=6) ± SD; * p<0.05; ** p<0.01.

P489

Clinical expansion of cord blood derived T-cells for use as donor lymphocyte infusion after cord blood transplantation

M. Okas, J. Gertow, O. Ringdén, J. Mattsson, M. Uhlin
Karolinska Institutet (Stockholm, SE)

Background: When no HLA-identical donor is available, cord blood transplantation (CBT) is an attractive option due to the rapid availability of the graft and its biological properties. One of the disadvantages associated with CBT is lack of possibility for donor lymphocyte infusion (DLI) after CBT. Here we report expansion and characterization of CD3 positive lymphocytes from CB grafts in connection to CBT.
Material and Methods: Lymphocytes from 13 CB grafts were expanded with CD3/CD28 beads and 200 IU/ml rIL2. Expanded