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ORIGINAL ARTICLE

CD34+CD38+CD19+ as well as CD34+CD38−CD19+ cells are leukemia-initiating cells with self-renewal capacity in human B-precursor ALL

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The presence of rare malignant stem cells supplying a hierarchy of malignant cells has recently been reported. In human acute myelogenous leukemia (AML), the leukemia stem cells (LSCs) have been phenotypically restricted within the CD34+CD38− fraction. To understand the origin of malignant cells in primary human B-precursor acute lymphocytic leukemia (B-ALL), we established a novel *in vivo* xenotransplantation model. Purified CD34+CD38+CD19+, CD34+CD38−CD19+ and CD34+CD38−CD19− bone marrow (BM) or peripheral blood (PB) cells from three pediatric B-ALL patients were intravenously injected into sublethally irradiated newborn NOD/SCID/IL2rγ^{null} mice. We found that both CD34+CD38+CD19+ and CD34+CD38−CD19+ cells initiate B-ALL in primary recipients, whereas the recipients of CD34+CD38−CD10−CD19− cells showed normal human hematopoietic repopulation. The extent of leukemic infiltration into the spleen, liver and kidney was similar between the recipients transplanted with CD34+CD38+CD19+ cells and those transplanted with CD34+CD38−CD19+ cells. In each of the three cases studied, transplantation of CD34+CD38+CD19+ cells resulted in the development of B-ALL in secondary recipients, demonstrating self-renewal capacity. The identification of CD34+CD38+CD19+ self-renewing B-ALL cells proposes a hierarchy of leukemia-initiating cells (LICs) distinct from that of AML. Recapitulation of patient B-ALL in NOD/SCID/IL2rγ^{null} recipients provides a powerful tool for directly studying leukemogenesis and for developing therapeutic strategies.
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Introduction

Acute lymphocytic leukemia (ALL) is the most common hematological malignancy in childhood. On the basis of ontogenic classification, pediatric ALL is divided into T-ALL, B-precursor ALL and mature B-ALL. B-precursor ALL accounts for 80–85% of total pediatric ALL cases.[1,2] Recent reports suggest that at least some cases of human leukemia and cancer, including acute myelogenous leukemia (AML), selectively develop from a rare fraction of malignant stem cells.[3–5] Unlike AML, however, whether the malignant clone arises from such a leukemic stem cell fraction has not been clarified in B-precursor ALL.

To identify human leukemia stem cells (LSCs), the *in vivo* leukemia-initiating capacity of purified cells has been evaluated in various xenotransplantation systems using immunocompromized mice. These leukemia-initiating cells (LICs) have been considered equivalent to LSCs, although not all studies have demonstrated other properties of stem cells, that is, differentiation and self-renewal capacities. In AML, the cell surface phenotype defined by the markers CD34 and CD38, that is, CD34+CD38− analogous to normal hematopoietic stem cells (HSCs), have been used to identify LIC-enriched cell population.[6,7] Although the engraftment of B-ALL CD34+ cells in NOD/SCID mice has been reported,[8–10] markers for further enrichment of B-precursor ALL-initiating cells have not been identified. CD38 is expressed by a variety of normal and malignant leukocytes and functions in cell adhesion and signaling. In normal hematopoiesis and in AML, its absence on CD34+ cells highly enriches a primitive self-renewing stem cell population.[6,7] Similarly, Cobaleda *et al.*[10] have reported that in Ph+ ALL, CD34+CD38− cells exclusively initiate leukemia in NOD/SCID recipients. In this study, we aimed to clarify the significance of CD38 expression in B-precursor ALL-initiating cells.

For this purpose, we used the newborn NOD/SCID/IL2rγ^{null} xenotransplantation model. This model takes advantage of the absence of acquired immunity accompanied by multiple defects in innate immunity in a novel NOD/SCID strain carrying a complete null mutation in the cytokine receptor common γ chain.[11] The use of this severely immunocompromised strain overcomes the limitations of existing SCID-repopulating assays using CB17-*scid*, NOD/SCID and NOD/SCID/β2m^{null} mice in engraftment levels of human normal and primary leukemic cells, and differentiation from normal or leukemic stem cells into progeny.[12,13] Especially, when human cells are intravenously injected into newborn recipients, differentiation and self-renewal capacities are efficiently detected both in normal and malignant hematopoiesis, making this model an ideal system for creating mouse models of primary human hematological malignancies.[12,13]

Using the newborn NOD/SCID/IL2rγ^{null} xenotransplantation model, we demonstrate that CD34+CD38+CD19+ cells as well as CD34+CD38−CD19+ cells have the capacities to initiate B-ALL, to infiltrate into non-hematopoietic organs *in vivo* and to self-renew. Leukemia initiation by self-renewing CD34+CD38+CD19+ primary human ALL cells demonstrates a distinct pathogenesis of B-precursor ALL from that of AML, which may provide new insight into the development of

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novel strategies for the treatment of pediatric B-precursor ALL. Furthermore, the use of anti-human CD19 antibody may discriminate normal HSCs and LSCs within CD34+CD38− stem fraction, enabling autologous BM transplantation without LIC contamination in patients with B-precursor ALL.

Materials and methods

Mice

NOD.Cg-*Prkdc*^{scid}*IL2rg*^{tm1Wjl}/Sz (NOD/SCID/IL2rγ^{null}) mice[11] were developed at The Jackson Laboratory (Bar Harbor, ME, USA). The NOD/SCID/IL2rγ^{null} strain was established by backcrossing a complete null mutation of the γ chain locus onto the NOD.Cg-*Prkdc*^{scid} strain. These mice have been bred and maintained under defined flora with irradiated food at the animal facility at RIKEN Research Center for Allergy and Immunology (RCAI). All experimental procedures were performed according to the guidelines established by the Institutional Animal Committee at RCAI.

Cell purification and xenogeneic transplantation

Bone marrow (BM; Cases 1 and 3) or peripheral blood (PB; Case 2) samples were obtained from three pediatric patients with newly diagnosed B-precursor ALL after written informed consent. The patient ages at the time of diagnosis were 6 years, 3 months and 10 months old, respectively, for Cases 1, 2 and 3. The white blood cell count at the time of diagnosis were 260.0, 134.0 and 196.0 ($\times 10^9$ ml^{-1}), respectively, for Cases 1, 2 and 3. MLL rearrangement was identified in Cases 2 and 3. BM and PB mononuclear cells (MNCs) were isolated by density centrifugation using lymphocyte separation medium (ICN Biomedicals, Oh, USA). BMMNCs or PBMNCs were stained with mouse anti-human CD10, CD19, CD3, CD4, CD8, CD34 and CD38 monoclonal antibodies (BD Immunocytometry, San Jose, CA, USA). Samples were analyzed and sorted using FACSAria (Becton Dickinson, San Jose, CA, USA). Nonviable cells were excluded by 7-aminoactinomycin D (BD Immunocytometry) staining. Within the viable CD3−CD4−CD8− BMMNCs or PBMNCs, CD34+CD38−CD19+, CD34+CD38+CD19+ and CD34+CD38−CD10−CD19− populations were sorted and injected into sublethally irradiated (1.5 Gy) NOD/SCID/IL2rγ^{null} mice through the facial vein within 48 h of birth. The purity of each cell population was higher than 97%. As control, normal human HSCs (Lin−CD34+CD38− cells) were purifed from cord blood MNCs and intravenously transplanted into NOD/SCID/IL2rγ^{null} newborns. Cord blood was obtained from Tokyo Cord Blood Bank after written consent was obtained from donors, and experimental plans were evaluated at IRB.

Evaluation of hematopoietic chimerism by flow cytometry

Starting 4 weeks after transplantation, PB was harvested from retro-orbital plexus of the recipients every 2–4 weeks. Recipients were killed when they became moribund and their BM, spleen and PB were evaluated for the repopulation of human normal or leukemic cells with mouse anti-human CD3, CD4, CD8, CD10, CD19, CD20, CD33, CD34, CD38, CD41a, CD45, HLA-DR and surface IgM monoclonal antibodies (BD Immunocytometry). Multicolor flow cytometric analyses were performed using FACSAria or FACSCanto II (Becton Dickinson). Engraftment of human B-ALL was defined by the frequency of the hCD45+hCD19+ cells.

Histological analysis

The liver, kidney and spleen tissues of the recipients were fixed with 4% paraformaldehyde for 1 h, dehydrated with 70% ethanol, embedded in paraffin and 5 μm sections were prepared. Hematoxylin-eosin staining was performed on each tissue section derived from the recipient mice. Immunostaining with mouse anti-human CD19 primary antibody (AbD SeroTec, Oxford, UK) and Cy3-conjugated donkey anti-mouse secondary antibody (Jackson Immunoresearch, West Grove, PA, USA) was performed after dehydration with graded alcohol and antigen retrieval with heated citrate buffer. Each section was examined using light microscopy (Zeiss Axiovert 200, Carl Zeiss, Germany) and laser scanning confocal microscopy (Leica TCS, Leica, Germany) to identify the infiltrating B-ALL cells.

Serial transplantation

For serial transplantation, either sorted human CD45+CD34+CD38+CD19+ cells or magnetic bead-enriched human CD34+ cells (Miltenyi Biotec, Germany) were obtained from the recipient BM and spleen, and 10^4–10^6 purified cells were intravenously transplanted into newborn NOD/SCID/IL2rγ^{null} mice. Human engraftment was evaluated in the serially transplanted recipients at 4–12 weeks post-transplantation.

Statistical analysis

Continuous variables were expressed as mean ± s.d. The data were analyzed by SPSS software version 13.0 (SPSS Inc., Chicago, IL, USA).

Results

Phenotypic characterization of primary human B-ALL cells

It is essential to understand leukemogenesis of B-precursor ALL, the most common hematological malignancy in children. While AML-initiating cells and Ph+ ALL-initiating cells are highly enriched within the CD34+CD38− population, the significance of CD38 expression in B-precursor ALL-initiating cells has not been clarified. To address this question, we first examined the phenotypic characteristics of B-precursor ALL cells in three patient samples. We analyzed the frequency of each fraction expressing CD34, CD38, CD10 and CD19 antigens by multicolor flow cytometry (Figure 1). As expected, considerable heterogeneity in the expression of cell surface antigens was observed in the three patient samples examined. Within the CD3−CD4−CD8− BM or PB MNC populations, consistent with the diagnosis of B-precursor ALL, CD34+ cells accounted for 90.5 ± 3.6%. CD34+CD38− and CD34+CD38+ cells accounted for 5.2 ± 6.0 and 85.3 ± 7.7%, respectively. In contrast with normal BM and cord blood samples, CD19+ cells accounted for 66.9 ± 44.4 and 93.1 ± 11.1% of CD34+CD38− and CD34+CD38+ populations, respectively. The CD34+CD38−CD10−CD19− subfraction was present in low frequency (0.18 ± 0.17%) in all three samples tested. The complete phenotype is shown in Figure 1.

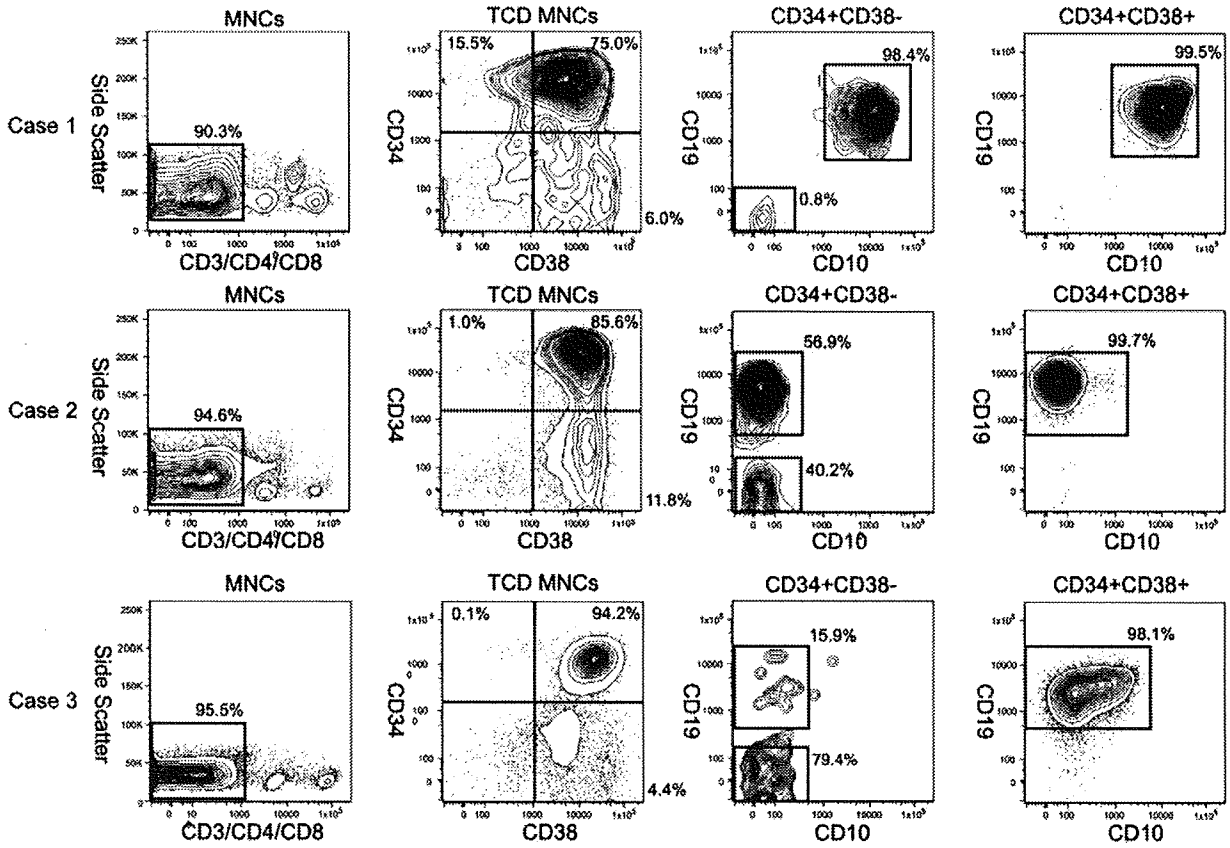

Figure 1 Phenotypic analysis of primary B-precursor ALL cells. BM (Cases 1 and 3) and PB (Case 2) from three cases of B-precursor ALL were analyzed for the expressions of CD34 and CD38 within T-cell-depleted MNCs. Within CD34+CD38− and CD34+CD38+ populations, the expression patterns of CD10 and CD19 were analyzed. Three subfractions (CD34+CD38−CD19+, CD34+CD38+CD19+ and CD34+CD38−CD10−CD19− cells) from each sample were transplanted into newborn NOD/SCID/IL2rγ^{null} mice. ALL, acute lymphocytic leukemia; BM, bone marrow; MNCs, mononuclear cells; PB, peripheral blood; TCD, T-cell-depleted.

Both CD34+CD38+CD19+ and CD34+CD38−CD19+ primary human B-ALL cells have long-term engraftment and leukemia-initiating capacity

On the basis of phenotypic characterization above, we simultaneously purified CD34+CD38+CD19+, CD34+CD38−CD19+, CD34+CD38−CD10−CD19− cells from B-precursor ALL patient BM (Cases 1 and 3) and PB (Case 2), and intravenously transplanted these purified populations into sublethally irradiated NOD/SCID/IL2rγ^{null} newborns. The information on each recipient is summarized in Table 1. The engraftment of human B-ALL cells was monitored by flow cytometric analysis of hCD45+CD19+ cells in the recipient PB. In contrast with previous reports on AML[7] and Ph+ ALL,[10,14] the injection of either CD34+CD38−CD19+ or CD34+CD38+CD19+ cells resulted in efficient engraftment of human B-ALL (Table 1). The engraftment of B-ALL in the PB of recipient mice following injection of CD34+CD38+CD19+ cells was seen for long term, with human B-ALL cells being observed to increase over time for up to 15 weeks post-transplantation. Purified CD34+CD38−CD10−CD19− cells did not initiate B-ALL in the recipients, but showed engraftment of normal human hematopoietic cells.

When the recipient mice exhibited ruffled fur and lethargy, we killed them to analyze the engraftment levels of human B-ALL in the BM and the spleen. As observed in the PB, BM and spleen showed efficient engraftment of ALL both in the recipients transplanted with CD34+CD38+CD19+ cells and those transplanted with CD34+CD38−CD19+ cells (Table 1 and Figure 2). The significance of CD38 expression on leukemia-initiating capacity is totally different between adult AML and pediatric B-precursor ALL.

Transplanted primary human B-ALL cells infiltrate into recipient organs

As we demonstrated leukemia-initiating capacity both in CD34+CD38−CD19+ and CD34+CD38+CD19+ cells, we next performed histological analyses of the recipient organs to examine the infiltration of B-ALL cells in the liver, kidney and spleen. In the liver and the kidney, infiltration of monomorphic MNCs was detected with hematoxylin-eosin staining in the recipients transplanted either with CD34+CD38+CD19+ or CD34+CD38−CD19+ cells derived from all three cases (Figure 3). When compared with the organs from normal human HSC recipient, there are sheets and clusters of monomorphic MNCs in the organs of the B-ALL recipients. The infiltrating cells exhibited cellular morphology similar to that of cells engrafted in the spleen. These cells also expressed CD19 on their surface, suggesting that they are human B-ALL cells. No infiltrating CD19+ cells were detected in the liver and kidney of the normal human HSC recipient. The degree of infiltration in the recipients transplanted with CD34+CD38−CD19+ cells and those transplanted with CD34+CD38+CD19+ cells was not significantly different.

Table 1 Serial transplantation of primary human B-ALL CD34+CD38+CD19+ and CD34+CD38-CD19+ cells

Patient ID	Transplant round	Graft cell type	Graft cell dose	Survival post-transplant (weeks)	% BM hCD45+CD19+	% spleen hCD45+CD19+	% PB hCD45+CD19+
(a)							
1	Primary	34+38+19+	6.5E+04	13	63.7	27.8	ND
		34+38+19+	6.5E+04	13	74.8	33.4	ND
		34+38+19+	1.0E+05	15	93.5	46.9	78.7
	Secondary	34+38+19+	8.0E+04	12	97.8	65.3	92.0
		34+38+19+	3.0E+04	11	ND	ND	95.0
		34+	2.0E+05	8	ND	ND	89.9
		34+	2.0E+05	9	95.7	90.2	42.8
2	Primary	34+38+19+	5.0E+03	6	99.9	ND	69.4
		34+38+19+	5.0E+04	5	ND	ND	73.8
		34+38+19+	5.0E+04	5	99.9	99.4	82.3
		34+38+19+	8.0E+04	5	54.9	10.8	10.7
	Secondary	34+38+19+	3.0E+04	7	86.3	87.4	60.3
		34+	1.0E+04	5	99.6	99.5	92.2
		34+	1.0E+05	4	98.4	89.1	ND
		34+	4.0E+06	4	90.3	83.3	ND
	Tertiary	34+	1.0E+05	5	ND	ND	73.4
		34+	1.0E+05	4	94.9	73.2	75.3
		34+	1.0E+06	4	93.9	72.2	94.8
		34+	1.0E+06	4	96.1	87.4	94.0
3	Primary	34+38+19+	2.5E+04	10	98.1	87.9	72.3
		34+38+19+	2.5E+05	7	98.8	52.7	61.2
		34+38+19+	5.0E+05	11	99.1	77.1	87.7
		34+38+19+	1.0E+04	13	97.9	44.4	23.3
		34+38−19+	6.5E+04	10	90.0	ND	70.0
	Secondary	34+38+19+	1.0E+05	6	91.2	ND	ND
		34+38+19+	1.0E+06	4	ND	94.7	ND
		34+	1.0E+05	9	72.8	ND	71.5
	Tertiary	34+	1.0E+05	9	ND	ND	98.2
		34+	1.0E+05	9	ND	ND	90.3
		34+	1.0E+06	7	99.7	96.1	91.5
		34+	1.0E+06	8	98.7	99.9	56.9
(b)							
1	Primary	34+38−19+	6.5E+04	12	52.4	37.2	ND
		34+38−19+	1.0E+05	13	90.3	42.2	76.0
	Secondary	34+	2.8E+05	4	97.6	99.3	ND
		34+	2.8E+05	4	95.2	99.9	ND
		34+	2.8E+05	4	98.8	ND	ND
		34+	2.8E+06	4	98.2	99.3	ND
		34+	2.8E+06	4	98.5	99.6	ND
		34+	2.8E+06	4	99.5	99.1	ND
2	Primary	34+38−19+	4.0E+04	5	6.0	1.8	1.1
		34+38−19+	4.0E+04	5	7.9	4.8	1.1
		34+38−19+	5.0E+03	7	98.7	77.6	85.6
		34+38−19+	5.0E+03	7	ND	ND	85.7
	Secondary	34+	2.0E+05	4	13.8	ND	ND
		34+	2.0E+05	4	7.6	36.0	ND
		34+	2.0E+05	4	11.0	41.0	ND
		34+	2.0E+05	8	87.8	49.2	47.0
3	Primary	34+38−19+	6.5E+04	10	90.0	ND	70.0

Abbreviations: ALL, acute lymphocytic leukemia; BM, bone marrow; ND, not determined; PB, peripheral blood.
PB, BM and spleen engraftment levels in primary, secondary and tertiary recipients from: (a) primary recipients of CD34+CD38+CD19+ B-ALL cells and (b) primary recipients of CD34+CD38-CD19+ human B-ALL cells.

Primary human B-ALL CD34+CD38+CD19+ cells possess self-renewal capacity

As the ALL-initiating capacity and the ability to infiltrate recipient organs were confirmed in the CD34+CD38+CD19+ as well as the CD34+CD38−CD19+ population, we examined the self-renewal capacity of CD34+CD38+CD19+ and CD34+CD38−CD19+ populations by serial transplantation. From CD34+CD38+CD19+ recipients, we intravenously transplanted 10^4–10^6 sorted CD45+CD34+CD38+CD19+ cells or enriched CD34+ cells from the primary (all three cases) and secondary (Cases 2 and 3) recipients into secondary and tertiary newborn NOD/SCID/

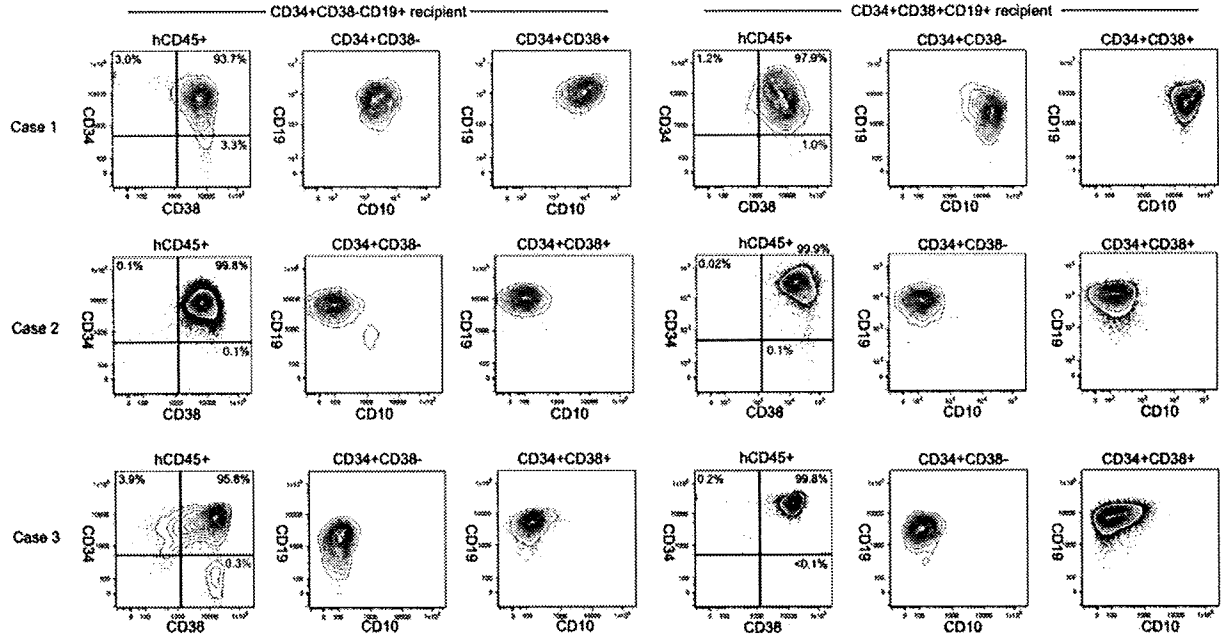

Figure 2 Both CD34+CD38−CD19+ and CD34+CD38+CD19+ B-ALL cells have the ability to reconstitute B-ALL *in vivo*. Flow cytometric analyses of BM from representative recipients of each cell fraction from each case are shown. Left panels: gated on hCD45+ cells; middle panels: gated on hCD34+CD38− cells; right panels: gated on hCD34+CD38+ cells. For Case 1, 6.5×10^4 CD34+CD38+CD19+ and CD34+CD38−CD19+ were transplanted. For Case 2, 5.0×10^4 CD34+CD38+CD19+ and 4.0×10^4 CD34+CD38−CD19+ cells were transplanted. For Case 3, 2.5×10^4 CD34+CD38+CD19+ and 6.5×10^4 CD34+CD38−CD19+ cells were transplanted. ALL, acute lymphocytic leukemia; BM, bone marrow.

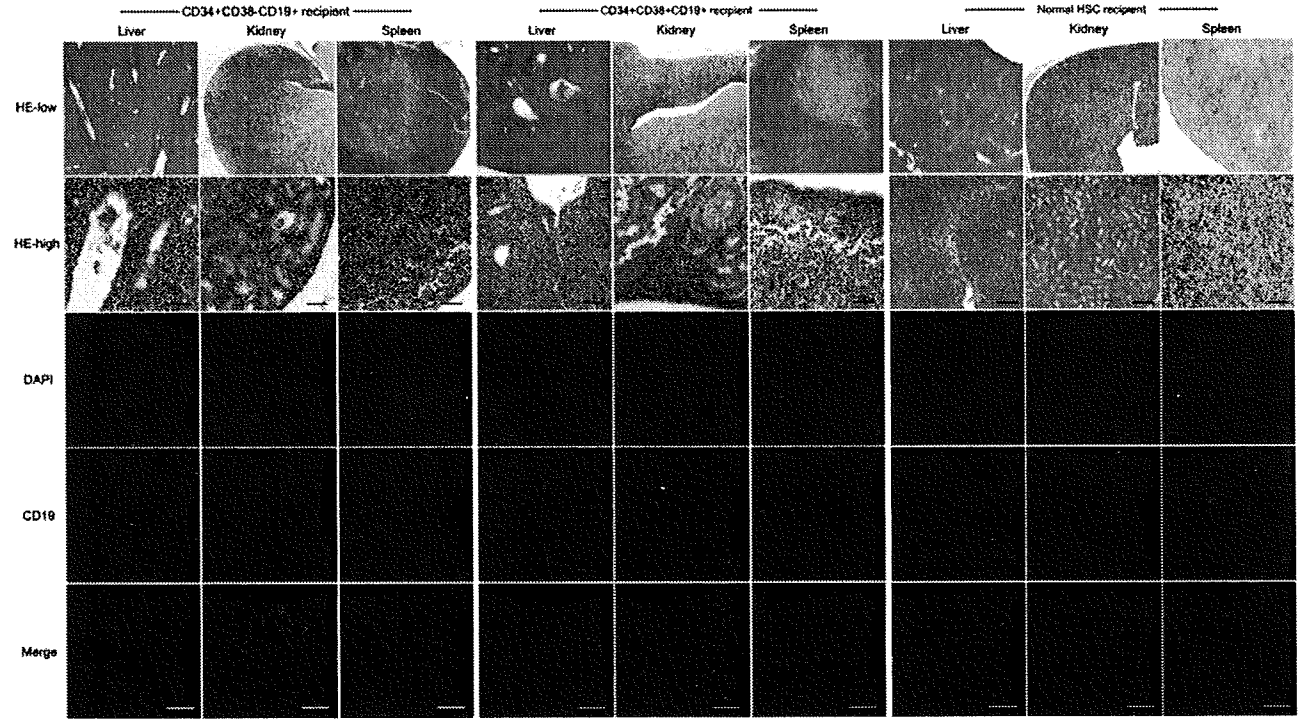

Figure 3 Both CD34+CD38−CD19+ and CD34+CD38+CD19+ B-ALL cells infiltrate into recipient organs. Hematoxylin-eosin staining and anti-human CD19 antibody labeling of the liver, kidney and spleen of a recipient transplanted with CD34+CD38+CD19+ cells and a recipient transplanted with CD34+CD38−CD19+ cells. Similarly stained and labeled liver, kidney and spleen sections from a recipient of normal human cord blood CD34+CD38− cells are included as controls. Nuclei were stained with DAPI. ALL, acute lymphocytic leukemia; DAPI, 4,6-diamidino-2-phenylindole. Scale bars represent 20 μm in HE staining and 50 μm in CD19 immunostaining.

IL2rγ^{null} recipients. In all the secondary and tertiary recipients, high levels of hCD45+CD19+ engraftment were seen (Table 1a). Similarly, when 2×10^5–2.8×10^6 enriched CD34+ cells from CD34+CD38−CD19+ primary recipients (Cases 1 and 2) were transplanted into secondary recipients, high levels of hCD45+CD19+ engraftment were found (Table 1b). These findings suggest that CD34+CD38+CD19+ and CD34+CD38−CD19+ cells not only initiate leukemia, but also possess self-renewal capacity.

CD34+CD38−CD10−CD19− B-ALL cells exhibit normal multilineage differentiation capacity

In normal hematopoiesis and in AML, the CD34+CD38− population is highly enriched for self-renewing stem cells. Transplantation of either CD34+CD38−CD19+ or CD34+CD38−CD10−CD19− cells derived from B-ALL BM resulted in the development of hCD19+ cells in the recipient PB. However, 7/7 recipients transplanted with CD34+CD38−CD19+ cells died of leukemia, whereas 0/3 transplanted with CD34+CD38−CD10−CD19− cells developed disease. While almost all the engrafted CD19+ cells in the PB of recipients transplanted with CD34+CD38−CD19+ cells express CD34 on their surface and lack CD20 and surface IgM expression, the engrafted CD19+ cells in the PB of recipients transplanted with CD34+CD38−CD10−CD19− cells express CD20 and surface IgM, not CD34, on their surface (Figure 4). In addition, human myeloid and platelet development is also detected only in the recipients transplanted with CD34+CD38−CD10−CD19− cells (Figure 4). CD34+CD38−CD10−CD19− cells derived from B-ALL BM cells are highly enriched with normal HSCs, suggesting that the expression of CD19 distinguish LICs from normal HSCs within the CD34+CD38− population.

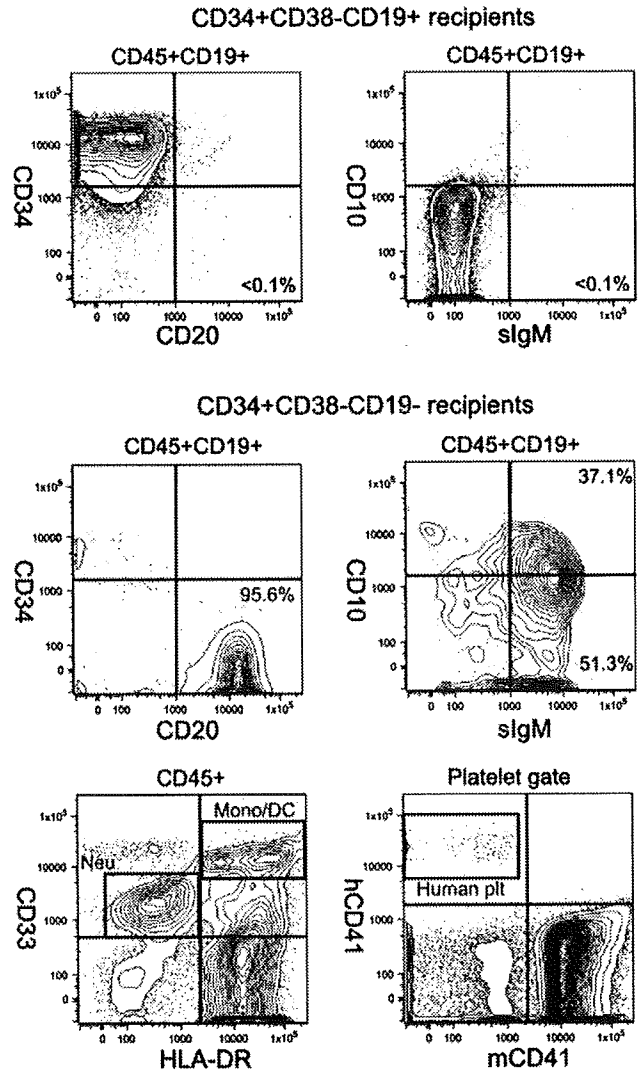

Figure 4 CD34+CD38−CD10−CD19− B-ALL cells show multilineage normal hematopoietic differentiation *in vivo*. Representative flow cytometric analysis of PB mononuclear cells demonstrating that the majority of human CD45+CD19+ cells in a representative recipient of CD34+CD38−CD19+ B-ALL cells express CD34 but lack CD20, CD10 and surface IgM expression. In contrast, mature human B cells, myeloid cells and platelets can be detected in a representative recipient transplanted with 2.0×10^3 CD34+CD38−CD10−CD19− cells at 4 months post-transplantation. ALL, acute lymphocytic leukemia; Mono/DC, monocytes and dendritic cells; Neu, neutrophils; PB, peripheral blood; plt, platelets.

Discussion

We have recently established the newborn NOD/SCID/IL2rγ^{null} mouse transplantation model that supports significantly higher engraftment levels of human normal HSCs and primary AML stem cells compared with the NOD/SCID/β2m^{null} mice.[12,13] In this study, we describe the efficient engraftment of primary human pediatric B-precursor ALL using the newborn NOD/SCID/IL2rγ^{null} mouse transplantation model. This is the first report that purified CD34+CD38+CD19+ ALL cells efficiently engraft, initiate leukemia, and self-renew *in vivo*. Although only three individual patient samples were analyzed, the consistent engraftment in primary and secondary recipients by CD34+CD38+CD19+ cells provides new insights into the leukemogenesis of ALL.

Previous studies have described the engraftment of purified primary B-ALL cells in adult NOD/SCID recipients. Cobaleda *et al*.[10] demonstrated that CD34+CD38− Ph+ ALL cells, but not CD34+CD38+ cells, engrafted and initiated leukemia in adult NOD/SCID recipients. In contrast, in pediatric B-precursor ALL, we found that the CD34+CD38+CD19+ ALL cells, as well as CD34+CD38−CD19+ cells, are able to efficiently engraft, infiltrate into non-hematopoietic organs and self-renew *in vivo*. B-precursor ALL and Ph+ ALL have distinct biological and clinical characteristics, as the former is the most common leukemia in the pediatric population and the latter is more common in adults. The finding that the hierarchical structure of B-precursor ALL, as defined by the surface phenotype of LSCs, is distinct from that of Ph+ ALL may reflect the biological differences between these two types of lymphoid malignancy. In particular, CD34+CD38+CD19+ cells predominantly reproduced themselves, rather than giving rise to heterogenous cell fraction such as CD34+CD38−CD19+ or CD34− ALL cells. As FACS discrimination gate border between CD38− and CD38+ cells within CD34+ fraction is not clearly delineated, it is not possible to totally exclude the possibility of contamination even with high levels of purity achieved with cell sorting. Additionally, limiting dilution and serial transplantation studies are required to fully define the differences between these CD34+CD38−CD19+ and CD34+CD38+CD19+ populations in B-precursor ALL. In addition, whether there are differences in the LIC phenotype and the function between these two subtypes of B-ALL remains to be determined. LICs in

other subtypes of ALL (for example, Ph+ ALL and mature B-ALL) and various subtypes of B-precursor ALL, based on genetic abnormalities, need to be examined in the NOD/SCID/IL2rγ^{null} newborn transplantation model to address this question.

In this study, we have successfully demonstrated that in human primary B-ALL, CD38 expression is irrelevant in defining a leukemogenic population, but rather the presence or absence of CD19 segregates the populations with malignant or normal repopulating capacity within the CD34+CD38− cell population. The role of CD19 as a marker to identify the clonogenic B-ALL precursor cells has been examined in the past. In t(4;11)-positive and t(9;22)-positive high-risk pediatric ALL, the leukemia-specific translocations have been identified in CD34+CD19− as well as CD34+CD19+ ALL cells.[13] The reconstitution of the myeloid lineage occurring from the purified CD19−, but not CD19+, cells has been reported in TEL–AML1 fusion-positive and Ph+ ALL.[15] Similarly, only CD34+CD19− and CD34+CD10− cells were found to engraft when sorted populations from t(9;22) and t(4;11)-negative B-precursor ALL were transplanted into NOD/SCID recipients.[8] Here, we report the reconstitution of mature human B cell and human platelets in addition to myeloid cells from CD34+CD38−CD10−CD19− primary human B-ALL cells. The discrepancy in the engraftment and proliferation of normal myeloid as well as lymphoid lineages in these studies, compared with that in our findings, may be due to the superior sensitivity of engraftment and capacity for normal human hematopoietic development in the newborn NOD/SCID/IL2rγ^{null} transplantation system. Although a previous publication has raised a concern for the possibility of LSC contamination in autologous stem cell graft in ALL,[16] our finding suggests that by CD34+CD38−CD10−CD19− purification, the potential contamination of autologous stem cell graft by B-ALL LSCs may be avoidable.

The finding that it is possible for both CD34+CD38+CD19+ and CD34+CD38−CD19+ cells to act as the LICs in B-precursor ALL may be an important pathophysiological difference between AML and ALL. Delineation of the molecular basis of these differences between CD34+CD38− and CD34+CD38+ B-ALL-initiating cells may allow us to develop targeted therapy specific for primitive LICs that elude current antileukemia treatment strategies.

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Allogeneic Bone Marrow Transplantation from Unrelated Human T-Cell Leukemia Virus-I–negative Donors for Adult T-Cell Leukemia/Lymphoma: Retrospective Analysis of Data from the Japan Marrow Donor Program

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ABSTRACT

Allogeneic hematopoietic stem cell transplantation (allo-HSCT) from an HLA-matched related donor has been suggested to improve the poor prognosis of adult T-cell leukemia/lymphoma (ATLL). However, the infusion of HTLV-I–infected cells from HTLV-I–positive related donors could lead to the development of donor-derived ATLL under immunosuppressive conditions. Although most ATLL patients lack a suitable HLA-matched related donor and require an HTLV-I–negative unrelated donor, little information is currently available regarding the outcome of unrelated bone marrow transplantation (UBMT) for ATLL. To evaluate the role of UBMT in treating ATLL, we retrospectively analyzed data from 33 patients with ATLL treated by UBMT through the Japan Marrow Donor Program (JMDP). Overall survival (OS), progression-free survival, and cumulative incidence of disease progression and progression-free mortality at 1 year after UBMT were 49.5%, 49.2%, 18.6%, and 32.3%, respectively. Multivariate analysis identified recipient age as an independent prognostic factor for OS (P = .044). Patients age ≥50 years who showed nonremission at transplantation tended to have higher rates of treatment-related mortality. Our observations suggest that UBMT could represent a feasible treatment option for ATLL patients and warrant further investigation based on these risk factors.

KEY WORDS

Adult T-cell leukemia/lymphoma • Allogeneic hematopoietic stem cell transplantation • Unrelated donor • Graft-versus-adult T-cell leukemia/lymphoma

INTRODUCTION

Adult T-cell leukemia/lymphoma (ATLL) is a peripheral T-cell neoplasm caused by human T-cell leukemia virus type I (HTLV-I) [1,2]. ATLL is generally classified into 4 clinical subtypes based on clinical and laboratory features: acute, chronic, smoldering, and lymphoma type. Clinically, acute- and lymphoma-type ATLL show an aggressive course, with tumor

burden, severe hypercalcemia, multiorgan failure, and poor performance status. ATLL has an extremely poor prognosis, with a median survival of about 6 months for the acute type and about 10 months for the lymphoma type; these patients are usually highly immunocompromised and develop various opportunistic infections. [3] Furthermore, their tumor cells are usually resistant to conventional chemotherapies, because overexpression of multidrug-resistance genes leads to intrinsic drug resistance. [4,5] Intensified chemotherapy [6,7] and autologous stem cell transplantation [8] likewise have failed to improve the prognosis. Thus, alternative treatment strategies for ATLL are needed.

Some cases of successful treatment with allogeneic stem cell transplantation (allo-HSCT) from an HLA-matched related donor have been reported, and a graft-versus-ATLL (GvATLL) effect has been implicated for improving treatments outcomes in transplant patients undergoing transplantation for ATLL. [9–11] However, more than 2/3 of patients with ATLL lack HLA-matched related donors. Furthermore, approximately 2/3 of the siblings of patients with ATLL are HTLV-I carriers [12], and allo-HSCT from an HTLV-I–positive donor may carry a risk of promoting the development of ATLL through the addition of a new HTLV-I load on the immunocompromised host. [13,14] Although most ATLL patients lack a suitable HLA-matched related donor and require an unrelated donor to benefit from allo-HSCT, few reports are available concerning the results of unrelated donor bone marrow transplantation (UBMT) for ATLL [9,11,15–18], and the number of patients in these few reports has been too small on which to base any solid conclusions. Therefore, to clarify the feasibility and efficacy of UBMT from an HTLV-I-negative donor for ATLL, we retrospectively analyzed registered data and clinical outcomes of UBMT for ATLL through the Japan Marrow Donor Program (JMDP).

PATIENTS AND METHODS

Patients and Transplantation Procedure

The subjects of this retrospective study consisted of 33 patients with ATLL (acute type, n = 20; lymphoma type, n = 7; not described, n = 6) who received UBMT from a donor mediated and recruited through the JMDP between September 1999 and January 2004. The clinical indications for UBMT were determined by each individual institution. The median time from diagnosis of ATLL to UBMT was 8 months (range, 5–28 months). At the time of transplantation, 13 patients were in complete remission (CR), 2 patients were in partial remission (PR), and 14 patients were in nonremission (NR); disease status at the time of transplantation was not described in 4 patients. CR status was reported in detail for 13 patients, with 11 patients in first CR (CR1) and 2 patients in second CR (CR2) (Table 1). All unrelated donors were HTLV-I antibody–negative. Serologic typing for HLA-A, -B, and -DR was performed using a standard 2-stage complement-dependent test of microcytotoxicity. [19] Alleles at the HLA-A, -B, and -DRB1 loci were identified by high-resolution DNA typing as described previously. [20] Serologic typing revealed that 22 patients were matched at the HLA-A, -B, and -DR loci. Four patients were mismatched at 1 HLA-DR locus, and 1 patient was mismatched at 2 loci of HLA-A and -DR. DNA typing revealed that 13 patients were matched at HLA-A, -B and -DRB1 loci. Ten patients were mismatched at 1 locus; 9 patients were mismatched at the HLA-DRB1 locus, and the remaining patient was mismatched at 1 HLA-A locus. Another 4 patients were mismatched at 2 loci. HLA typing data were not described in 6 patients. Patient and donor characteristics are summarized in Table 2.

Table 1. *Patient characteristics*

Characteristic	Value
Median age at transplantation, years (range)	49 (range, 24–59)
Sex, n	
Male	18
Female	15
Performance status, n	
0–1	21
2–4	4
ND	8
Subtypes of ATLL, n	
Acute	20
Lymphoma	7
ND	6
Disease status at transplantation, n	
CR or PR	15
NR	14
ND	4
Duration from diagnosis to UBMT, n	
Within 1 year	21
Beyond 1 year	11
ND	1
Conditioning, n	(TBI-containing, 22; non–TBI-containing, 11)
CST	27
RIST	6
Cell dose, n	
$< 3.0 \times 10^8$/kg	16
$\geq 3.0 \times 10^8$/kg	14
ND	3
GVHD prophylaxis, n	
CsA + MTX	13
TCR + MTX	20

ND indicates not described; CR, complete remission; PR, partial remission; NR, nonremission; UBMT, unrelated bone marrow transplantation; TBI, total body irradiation; CST, conventional stem cell transplantation; RIST, reduced-intensity stem cell transplantation; GVHD, graft-versus-host disease; CsA, cyclosporine; MTX, methotrexate; TCR, tacrolimus.

Table 2. *Patient and donor characteristics*

Characteristic	Value
HLA-A, -B, and -DRB1 allele mismatches, n	
0	13
1	10
2	4
ND	6
Sex of donor/patient, n	
Male/male	13
Female/female	8
Female/male	5
Male/female	7
Extent of ABO match, n	
Match	19
Minor mismatch	4
Major mismatch	7
Major/minor	2
ND	1

ND indicates not described.

Transplantation was performed according to the protocol of each institution; therefore, conditioning regimens and prophylaxis against graft-versus-host disease (GVHD) differed among patients. Conditioning regimens were myeloablative in 27 patients; total body irradiation (TBI) was incorporated in 22 patients. Reduced-intensity conditioning regimens were used in 6 patients. GVHD prophylaxis included cyclosporine (n = 13) and tacrolimus (n = 20) combined with methotrexate. All recipients received bone marrow transplantation, which was not manipulated.

Assessment of Engraftment, GVHD, Survival, and Progression-Free Mortality

The day of sustained engraftment was defined as the first of 3 consecutive days with an absolute neutrophil count exceeding 0.5×10^9/L. Acute GVHD was diagnosed and graded according to the standard criteria described previously. [21,22] Chronic GVHD was evaluated according to standard criteria [23] in patients who survived more than 100 days after transplantation. Overall survival (OS) was defined as the duration (in days) from transplantation to death from any cause. Progression-free survival (PFS) was defined as days from transplantation to disease progression or death from any cause. Progression-free mortality was defined as death without disease progression.

Data Management and Statistical Considerations

Data were collected by the JMDP using a standardized report form. Follow-up reports were submitted at 100 days, 1 year, and every subsequent year after transplantation. The cumulative incidence of disease progression and progression-free mortality were evaluated using Gray's method, [24] considering each other risk as a competing risk. OS and PFS were estimated using the Kaplan-Meier method. Potential confounding factors considered in the analysis were age, sex, disease status, duration from diagnosis to transplantation, Eastern Cooperative Oncology Group (ECOG) performance status, [25] conditioning regimen, number of bone marrow cells transplanted, and presence of grade II–IV acute GVHD. Proportional hazard modeling was used to evaluate any influence of these factors on OS, treating development of acute GVHD as a time-dependent covariate. Factors associated with at least borderline significance ($P < .05$) in univariate analyses were subjected to multivariate analyses using backward-stepwise proportional hazards modeling. P values $P < .10$ were considered statistically significant.

RESULTS

Engraftment and GVHD

Transplantation outcomes are summarized in Table 3. The median number of cells transplanted was 2.44×10^8 nucleated cells/kg of recipient body weight (range, $0.58–3.58 \times 10^8$ nucleated cells/kg of recipient body weight). Five patients (15%) died within 20 days. Neutrophil engraftment was achieved in 28 patients. Late graft failure occurred in 1 of these 28 patients, although the patient showed engraftment on

Table 3. *Transplantation outcome*

	Value
Alive/dead, n	19/14
Median follow-up for survivors, days (range)	139 (87–600)
Cause of death	
Progression, n	2
Death without progression, n	9
Median days after transplantation (range)	32 (10–71)
Late graft failure, n	1
GVHD, n	1
Infection, n	3
TMA, n	2
VOD, n	1
Arrhythmia, n	1
Not described, n	3
Disease progression, n	5
Median days after transplantation (range)	122 (61–223)
Engraftment, n	
Engraftment	28
Death within 20 days	5
Late graft failure	1
Acute GVHD, n	
None	3
Grade I	8
Grade II	12
Grade III	3
Grade IV	2
Chronic GVHD, n	
None	14
Limited	1
Extensive	3

GVHD indicates graft-versus-host disease; TMA, thrombotic microangiopathy; VOD, venooculusive disease.

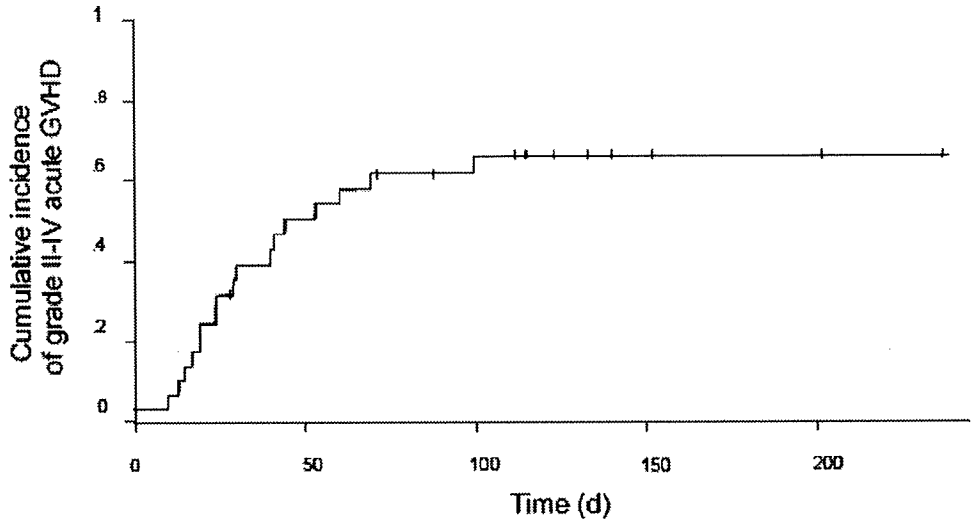

Figure 1. Cumulative incidence of grade II–IV acute GVHD in patients who achieved neutrophil engraftment.

day 14. Acute GVHD developed in 25 of the 28 patients who achieved engraftment (89%): grade I GVHD in 8 patients, grade II in 12 patients, grade III in 3 patients, and grade IV in 2 patients. The cumulative incidence of grade II–IV acute GVHD was 61% (Figure 1). Chronic GVHD developed in 4 of 18 patients, with limited disease in 1 patient and extensive disease in the other 3 patients.

Survival and disease progression

The 1-year OS and PFS were 49.5% (95% confidence interval [CI], 31.2%–78.5%) and 49.2% (95% CI, 33.6%–72.1%), respectively (Figure 2). Disease progression was observed in 5 patients, and the median number of days from transplantation to disease progression was 122 (range, 61–223 days). As of the last follow-up, 14 deaths had been reported. Primary cause of death was disease progression in 2 patients and was not described in 3 patients, but the other 9 deaths were not due to disease progression (see Table 3). Primary causes of transplantation-related death within 100 days after transplantation were late graft failure in 1 patient, GVHD in 1 patient, infection in 3 patients (with methicillin-resistant *Staphylococcus aureus*–positive sepsis in 1 patient and pulmonary infection in 2 patients), thrombotic microangiopathy (TMA) in 2 patients, veno-occlusive disease (VOD) in 1 patient, and arrhythmia in 1 patient.

Univariate and Multivariate Analyses for OS

Pretransplantation and posttransplant factors were calculated for OS (Table 4). In univariate analyses, OS was not significantly associated with sex, duration from diagnosis to transplantation, ECOG performance status, conditioning regimen, number of bone marrow cells transplanted, or presence of grade II–IV acute GVHD. On the other hand, patient age and disease status at transplantation were identified as significant independent risk factors. In multivariate analyses, only patient age at transplantation was identified as exerting a significant independent risk impact on OS (≥50 years vs <50 years; relative risk, 3.47; 95% CI, 1.03–11.6; $P = .044$). Disease status at transplantation exerted a marginally significant impact on OS (NR vs CR or PR; relative risk, 3.17; 95% CI, 0.96–10.5; $P = .059$) (Figure 3).

Influence of Pretransplantation Factors on Disease Progression and Progression-Free Mortality

The cumulative incidence of disease progression and progression-free mortality at 1 year were 18.6% and 32.3%, respectively (Figure 4). To clarify how age and disease status at transplantation affected OS, we evaluated the relationship between these factors and the incidence of progression-free mortality. The cumulative incidence of progression-free mortality was significantly higher in patients age ≥50 years at transplantation (50% vs 18%; $P = .048$; Figure 5A). NR at transplantation exerted a marginally significant effect on increased progression-free mortality (54% vs 20%; $P = .070$; Figure 5B).

DISCUSSION

This study analyzed the data and evaluated treatment outcomes for 33 patients with ATLL who received UBMT. Two important findings were identified regarding UBMT for ATLL. First, UBMT from HTLV-I–negative donors for ATLL represents a feasible treatment. Second, recipient age (≥50 years) and NR disease status at transplantation were independent risk factors for OS, and patients with ATLL displaying these risk factors tended to exhibit higher frequencies of treatment-related mortality.

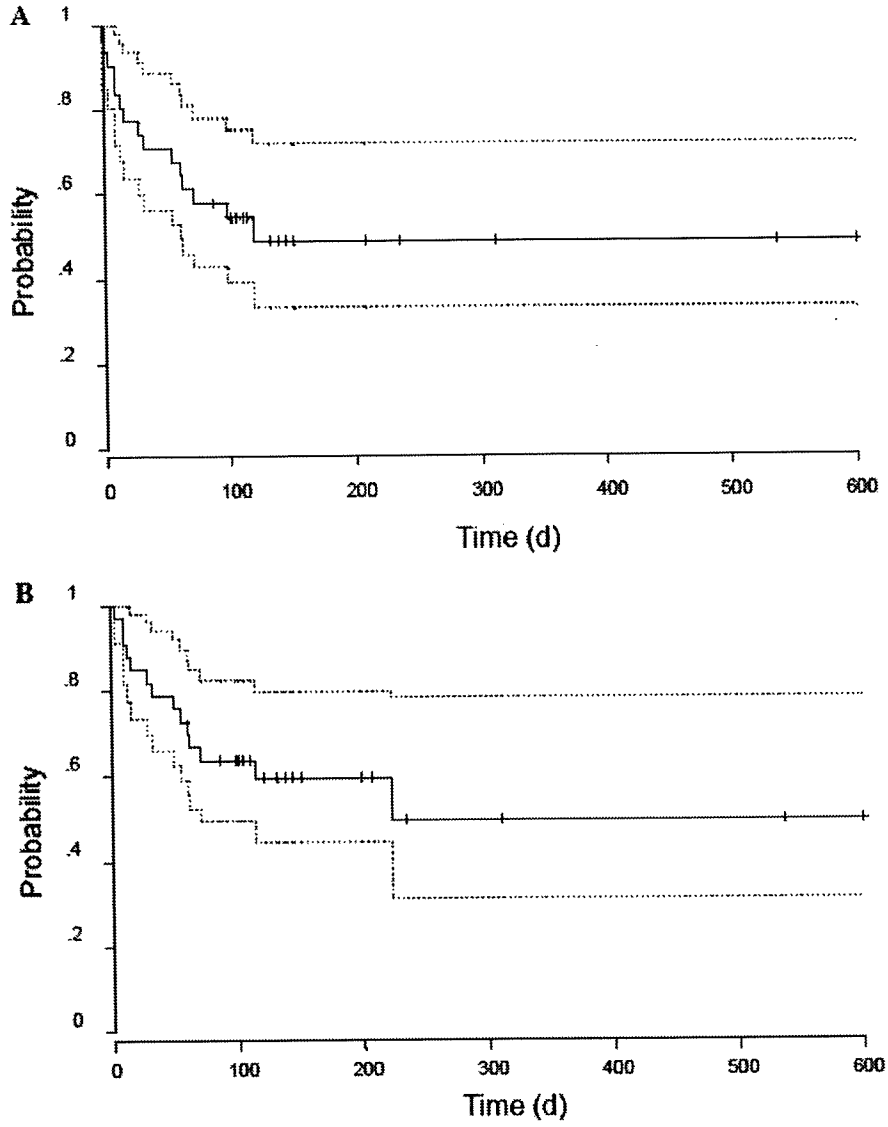

Figure 2. Probability of progression-free survival (A) and overall survival (B) after unrelated bone marrow transplantation for adult T-cell leukemia/lymphoma. Dashed lines represent 95% confidence intervals.

Table 4. *Prognosis factors in univariate and multivariate analyses*

	Univariate		Multivariate	
	Relative risk (95% CI)	P	Relative risk (95% CI)	P
Age ≥50 versus <50 years	4.03 (1.23–13.3)	.022	4.03 (1.23–13.3)	.022
Male versus female	0.97 (0.34–2.80)	.95		
PS 0–1 versus 2–4	0.44 (0.11–1.70)	.23		
NR versus CR or PR	3.37 (1.03–11.0)	.044		.059
UBMT within 1 year versus beyond 1 year	0.54 (0.15–2.00)	.35		
RIST versus CST	0.71 (0.19–2.59)	.60		
TBI versus non-TBI	1.35 (0.45–4.04)	.59		
Cell dose < 3.0 × 10^8/kg versus ≥ 3.0 × 10^8/kg	0.98 (0.31–3.05)	.97		
GVHD II–IV present versus absent	1.91 (0.50–7.26)	.34		

CI indicates confidence interval; PS, performance status; NR, nonremission; CR, complete remission; PR, partial remission; UBMT, unrelated bone marrow transplantation; RIST, reduced-intensity stem cell transplantation; CST, conventional stem cell transplantation; TBI, total body irradiation; GVHD, graft-versus-host disease.

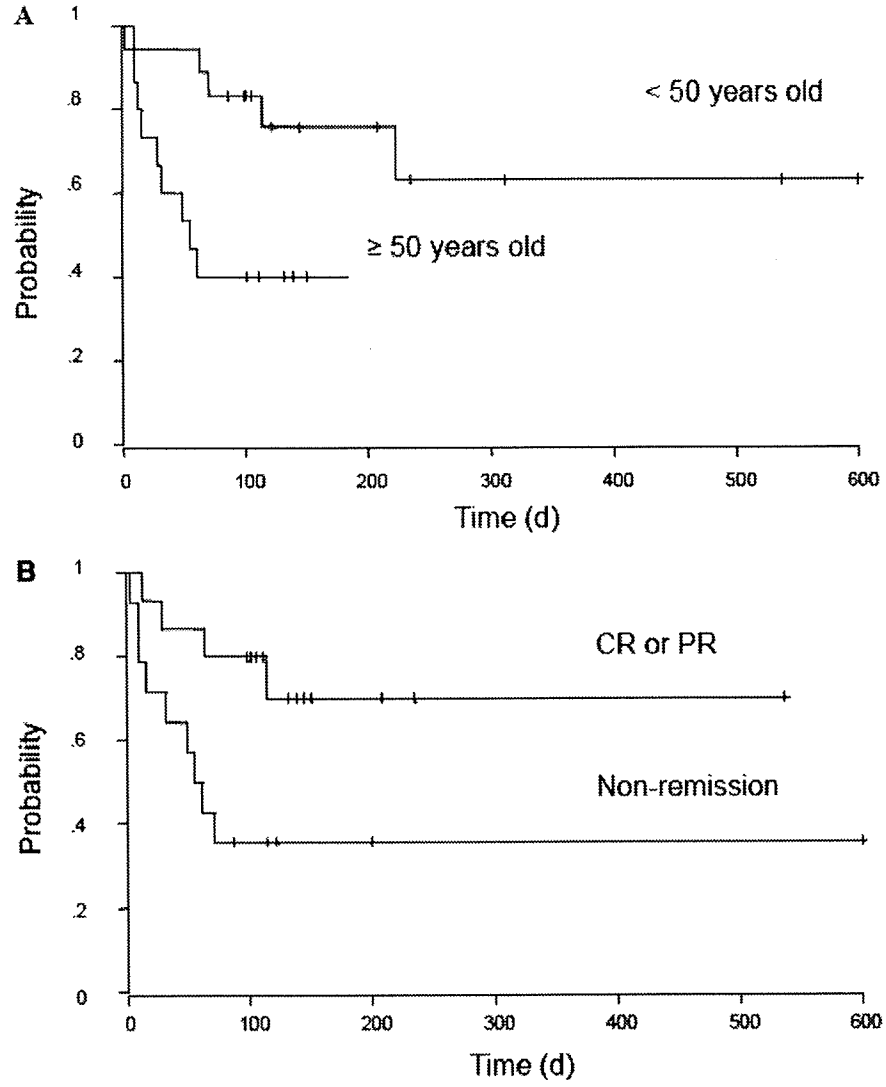

Figure 3. Overall survival according to pretransplantation factors, age (A) and disease status at transplantation (B).

ATLL has an extremely poor prognosis, with projected 2- and 4-year survival rates of 16.7% and 5.0% for the acute type and 21.3% and 5.7% for the lymphoma type, respectively. [3] Neither intensified chemotherapy nor autologous stem cell transplantation have improved the prognosis. Encouraging results for allo-HSCT for ATLL from HLA-matched related donors have been reported by several groups; thus, allo-HSCT may improve the poor prognosis of ATLL. However, the number of patients in most reports has been too small to allow evaluation of the efficacy of allo-HSCT for ATLL. The present results were derived from a large number of patients who underwent transplantation (33 patients) performed through the JMDP. Longer follow-up is, of course, needed to confirm the curative potential of allo-HSCT for ATLL. However, the good survival rates noted here suggest that allo-HSCT is an effective treatment for ATLL, and that patients with ATLL will benefit from allo-HSCT through HTLV-I–negative unrelated donors, because the OS and PFS rates at 1 year after UBMT were 49.5% and 49.2%, respectively. Compared with the results for patients with non-Hodgkin's lymphoma in the National Marrow Donor Program, the incidence of grade III–IV acute GVHD in the present study was low (18% vs 30%). [26] The outcome in the present study appears to be favorable, possible due to the lower incidence of grade III–IV acute GVHD. This observation is compatible with previous studies showing a lower incidence of acute GVHD in Japanese patients compared with Western patients, which might reflect the less diverse genetic background of in the Japanese population. [27,28]

Frequency of relapse after transplantation differs between autologous and allo-HSCT for ATLL. The use of high-dose chemotherapy with autologous HSCT has been reported in only 9 patients, all of whom relapsed or died from transplantation-related mortality. [8] In contrast, the cumulative incidence of

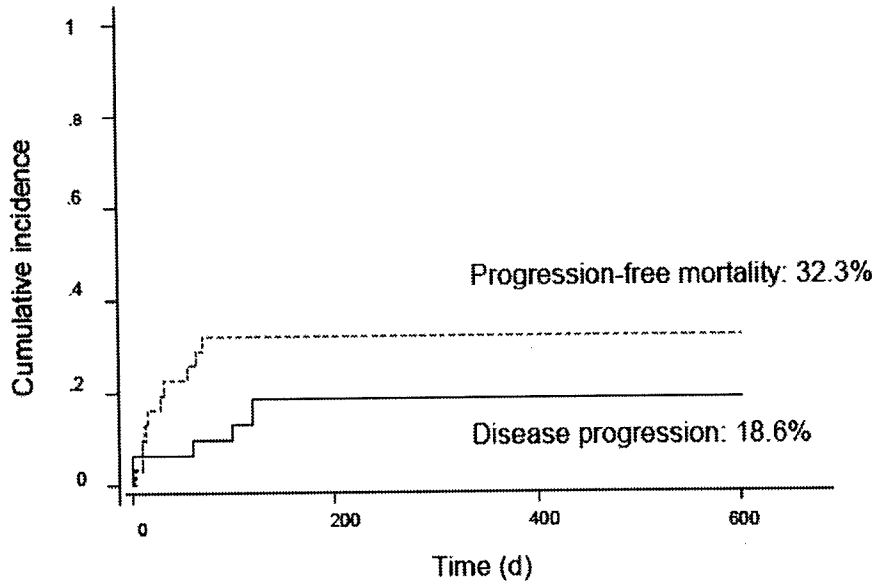

Figure 4. Cumulative incidence of disease progression (—) and progression-free mortality (---) after transplantation.

disease progression was lower after UBMT in this study. Interestingly, patients with ATLL displaying acute or chronic GVHD reportedly did not relapse. [9] In another report, patients with ATLL who relapsed after allo-HSCT reachieved CR after tapering or discontinuation of immunosuppressive agents and donor lymphocyte infusions. [10,11] Reactivation in tax-specific CD8-positive cytotoxic T lymphocytes (CTLs), which has been recently shown in patients with ATLL after allo-HSCT, may indicate a potential contribution of CTLs to anti-ATLL immunity and induction of a GvATLL effect. [29] These results strongly suggest that a GvATLL effect could work on some patients with ATLL to prevent relapse after allo-HSCT. In the present study, neither univariate nor multivariate analysis showed a survival benefit for acute GVHD. We were unable to analyze the relationship between chronic GVHD and relapse, because of the low number of patients with chronic GVHD. In fact, the number of patients may have been insufficient to confirm GvATLL in this study. On the other hand, the absence of benefit from GVHD in preventing relapse suggests that a GvATLL effect could occur in patients with ATLL after allo-HSCT without clinically obvious GVHD. [11]

Transplantation-related mortality was a significant problem in this study. Five patients (15%) died within 20 days, from infection in 3 patients and TMA in 2 patients. Nine patients (27%) died within 100 days, due to infection in 3 patients, TMA in 2 patients, and VOD in 1 patient. Patients with ATLL might have an increased risk of frequent opportunistic infection, because they have an associated T-cell immunodeficiency. Furthermore, ATLL is usually systemic in distribution, and the accumulated organ damages as a result of repeated cytotoxic chemotherapy seen in patients before transplantation may have contributed to the onset of TMA. In univariate and multivariate analysis, recipient age (≥50 years) and NR disease status at transplantation represented significant risk factors for OS. The multivariate analyses were limited by the small number of patients in each subgroup; however, patients displaying these risk factors tended to have a higher rate of treatment-related mortality than patients without these factors, and it can be assumed that these risk factors have a significant relationship with outcome clinically. In this study, mostly myeloablative conditioning regimens were used before transplantation. Given that conventional allo-HSCT is designed to eradicate tumor cells with myeloablative intensity using maximally tolerated doses of high-dose chemotherapy and radiotherapy, the desirable effects often may be offset by overwhelming toxicity in patients age ≥50 years. Moreover, the number of patients with ATLL who are eligible for allo-HSCT with myeloablative conditioning is limited, because the typical patient with ATLL has a relatively advanced age at presentation (about 60 years). To reduce treatment-related mortality, allo-HSCT with reduced-intensity conditioning offers a new treatment option for patients with ATLL who are ineligible for allo-HSCT with myeloablative conditioning due to advanced age or medical infirmity. [30,31] Okamura et al [32] reported on 16 patients age > 50 years with ATLL who underwent allo-HSCT with reduced-intensity conditioning from HLA-matched related donors and found that treatment-related mortality was acceptable and that allo-HSCT with reduced-intensity conditioning was a feasible treatment for ATLL. Given these findings, UBMT

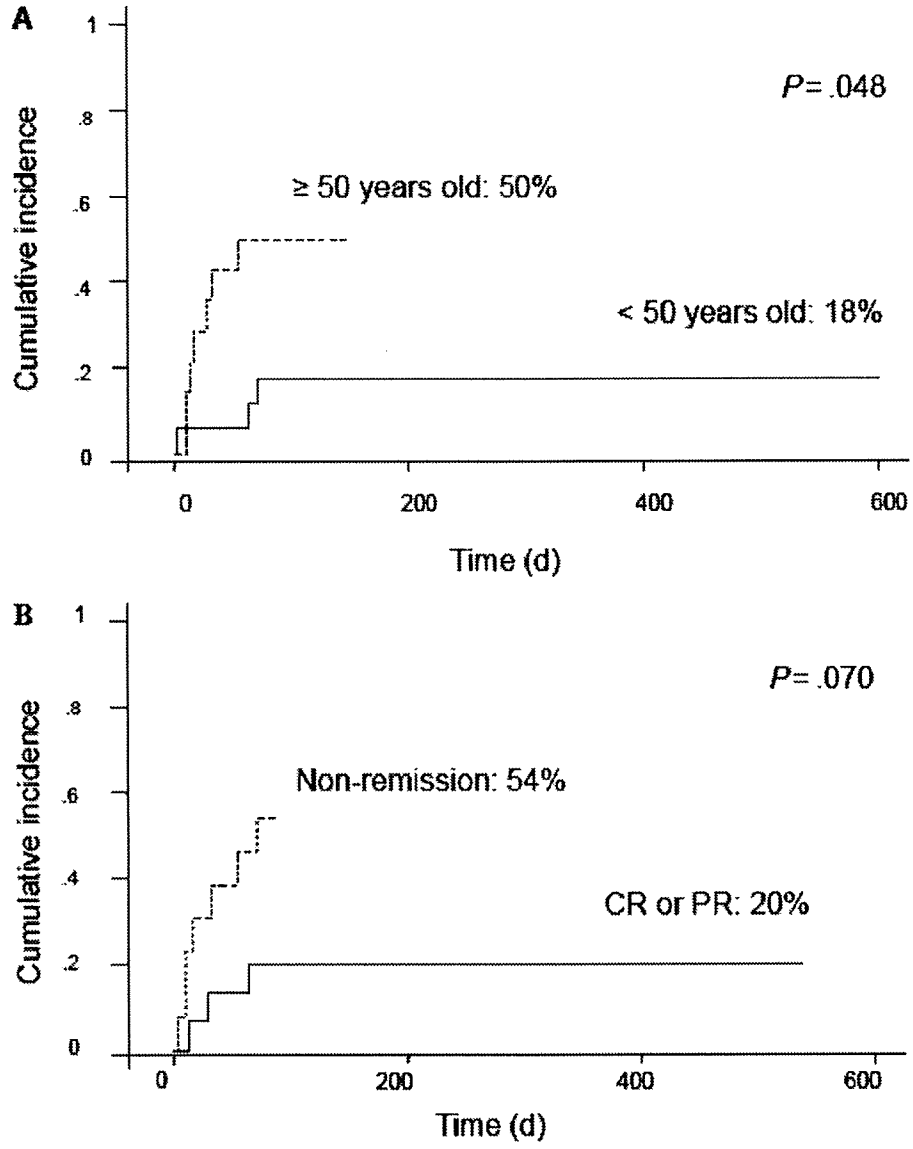

Figure 5. Cumulative incidence of progression-free mortality grouped according to pretransplantation factors, age (A) and disease status at transplantation (B).

with reduced-intensity conditioning should be considered for elderly patients with ATLL.

Another concern related to allo-HSCT for ATLL involves the use of HTLV-1–positive carrier donors. About 2/3 of siblings of patients with ATLL are HTLV-I carriers. From the perspective of HTLV-I–positive donor risk, granulocyte colony–stimulating factor (G-CSF) can reportedly stimulate the proliferation of ATLL cells [33], and HTLV-I–positive donors may be at increased risk of developing ATLL due to the administration of G-CSF in the setting of allogeneic peripheral blood stem cell transplantation. From the perspective of patients with ATLL, allo-HSCT from an HTLV-I–positive donor may carry a risk of HTLV-I–associated disease after allo-HSCT [34] or a risk of promoting the future development of ATLL due to the new HTLV-I load on immunocompromised recipients [13,14]. On the other hand, to date there is no evidence in the JMDP or the literature that ATLL can develop from infected HTLV-I–negative donor cells due to the HTLV-I load of the recipient. The HTLV-I proviral load dramatically decreased to an undetectable level after transplantation, especially after transplantation from HTLV-I–negative donors. [18, 32] This decreased HTLV-I proviral load was observed after both myeloablative and reduced-intensity conditioning. Transplantation from an HTLV-I–positive donor is reportedly associated with a higher frequency of relapse compared with transplantation from an HTLV-I–negative donor. [11] Therefore, the uninfected normal donor T cells might overwhelm infected HTLV-I recipient T cells due to a GvATLL response and might act as an antiviral therapy. However, an HTLV-I–positive do-

nor might avoid clonal expansion of HTLV-I–infected T lymphocytes after allo-HSCT through the provision of cytotoxic T cells. Thus, it is currently difficult to determine whether an HTLV-I–positive or–negative donor should be selected. Longer follow-up is needed to resolve this issue. In the meantime, a prudent clinical attitude toward both HTLV-I–positive donors and recipients with ATLL is warranted.

In conclusion, allo-HSCT from an HTLV-I–negative unrelated donor appears to be an feasible alternative treatment for patients with ATLL for whom an HLA-matched related donor is unavailable. Further prospective controlled studies are needed to assess the efficacy of allo-HSCT for ATLL and to define the clinical indications of allo-HSCT for ATLL, taking into account donor selection, the conditioning regimen, and the prognostic factors identified in this study.

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APPENDIX: PARTICIPATING INSTITUTIONS

The following centers in Japan participated in this study: Hokkaido University Hospital, Sapporo University Hospital, Sapporo Hokuyu Hospital, Japanese Red Cross Asahikawa Hospital, Asahikawa Medical College Hospital, Hirosaki University Hospital, Tohoku University Hospital, Yamagata University Hospital, Akita University Hospital, Fukushima Medical College, National Cancer Center Central Hospital, Institute of Medical Science at the University of Tokyo, Toho University Hospital, Omori Hospital, Tokyo Metropolitan Komagome Hospital, Nihon University Hospital, Itabashi Hospital, Jikei University Hospital, Keio University Hospital, Tokyo Medical College Hospital, Tokyo Medical and Dental University Hospital, Tokyo University Hospital, Yokohama City University Hospital, Kanagawa Children's Medical Center, Kanagawa Cancer Center, Tokai University Hospital, St Marianna University Hospital, Chiba University Hospital, Chiba Children's Hospital, Matsudo Municipal Hospital, Kameda General Hospital, Saitama Children's Medical Center, Saitama Cancer Center Hospital, Saitama Medical School Hospital, Ibaraki Children's Hospital, Jichi Medical School Hospital, Dokkyo University Hospital, Fukaya Red Cross Hospital, Saiseikai Maebashi Hospital, Gunma University Hospital, Niigata University Hospital, Niigata Cancer Center Hospital, Shinshu University Hospital, Saku Central Hospital, Hamamatsu University Hospital, Hamamatsu Medical Center, Shizuoka General Hospital, Shizuoka Children's Hospital, Japanese Red Cross Nagoya First Hospital, Nagoya Daini Red Cross Hospital, Meitetsu Hospital, Nagoya University Hospital, Nagoya Ekisaikai Hospital, National Nagoya Hospital, Aichi Medical School Hospital, Nagoya City University Hospital, Showa Hospital, Anjo Kousei Hospital, Fujita Health University Hospital, Mie University Hospital, Kanazawa University Hospital, Kanazawa Medical University Hospital, Toyama Prefectural Central Hospital, Fukui Medical School Hospital, Shiga University of Medical Science, Center for Adult Disease in Osaka, Kinki University Hospital, Osaka University Hospital, Osaka Medical Center and Research Institute for Maternal and Child Health, Matsushita Memorial Hospital, Hyogo College of Medicine Hospital, Hyogo Medical Center for Adults, Kobe City General Hospital, Kobe University Hospital, Kyoto University Hospital, Kyoto Prefectural University of Medicine Hospital, Social Insurance Kyoto Hospital, Tottori Prefectural Central Hospital, Tottori University Hospital, Hiroshima Red Cross Hospital and Atomic-Bomb Survivors Hospital, Yamaguchi University Hospital, Ehime Prefectural Central Hospital, Okayama National Hospital, Kurashiki Central Hospital, Kyushu University Hospital, Harasanshin General Hospital, Hamanomachi General Hospital, National Kyushu Cancer Center, St Mary's Hospital, Kokura Memorial Hospital, Saga Prefectural Hospital, Nagasaki University Hospital, Miyazaki Prefectural Hospital, Kumamoto National Hospital, Kumamoto University Hospital, Oita Medical University Hospital, Kagoshima University Hospital, and Imamura Bun-in Hospital.

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

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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

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[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