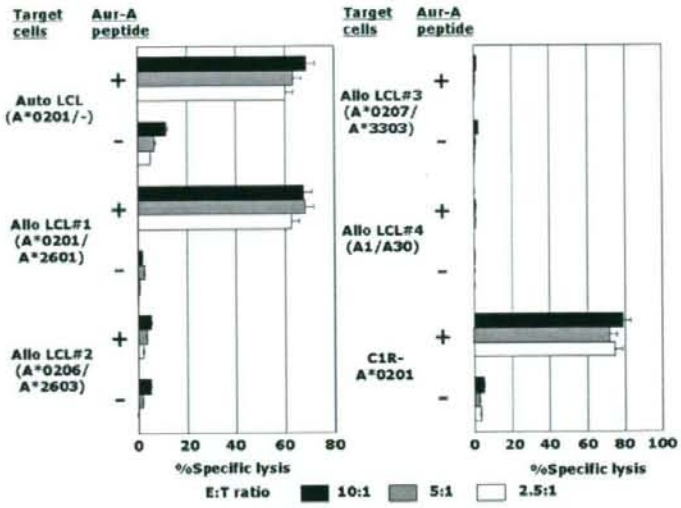


**Figure 1. Establishment of an HLA-A*0201–restricted and Aur-$A_{207-215}$ peptide–specific CTL line, AUR-1.** The cytotoxicity of the CTL line designated AUR-1 against various LCLs and *HLA-A*0201* gene–transfected cells (C1R-A*0201), which were loaded or unloaded with Aur-$A_{207-215}$ peptide, was determined by 4-hour $^{51}$Cr-release assays at effector-to-target (E:T) ratios of 10:1, 5:1, and 2.5:1.

by adding $^{51}$Cr-unlabeled Aur-$A_{207-215}$ peptide–loaded autologous LCL, but not HLA-A*0201–negative allogeneic LCL, indicating that the cytotoxicity of AUR-1 against leukemia cells is Aur-A specific (Figure 2D). These results show that AUR-1 can exert cytotoxicity against leukemia cell lines in an HLA-A*0201–restricted manner through recognition of the Aur-$A_{207-215}$ epitope that is naturally processed from Aur-A protein in leukemia cells and presented on the cell surface in the context of HLA class I molecules.

### Freshly isolated leukemia cells, but not normal PBMCs or normal mitotic cells, express Aur-A abundantly and are lysed by AUR-1

Next, we examined whether Aur-A–specific CTLs can discriminate freshly isolated leukemia cells from normal cells and whether AUR-1 can lyse freshly isolated leukemia cells as well as leukemia cell lines. As shown in Figure 3A, Aur-A appeared to be overexpressed in a wide spectrum of leukemia, including acute lymphoblastic leukemia (ALL), AML, and CML, as reported previously.[17-19] Among the various kinds of leukemia, CML cells express a very high level of *Aur-A* mRNA. In contrast, expression levels of *Aur-A* mRNA in normal PBMCs and phytohemagglutinin (PHA)–stimulated peripheral blood T lymphocytes (normal mitotic cells) were extremely low in comparison with those in freshly isolated leukemia cells.

The cytotoxicity of AUR-1 against freshly isolated leukemia cells was examined by standard $^{51}$Cr-release assay. Because the frequency of HLA-A*0201 in the Japanese population is less than 10%, only 3 HLA-A*0201–positive leukemia samples were available. As expected, all HLA-A*0201–positive freshly isolated leukemia cells were lysed by AUR-1; however, HLA-A*0201–negative freshly isolated leukemia cells, HLA-A*0201–positive normal PBMCs, and HLA-A*0201–positive PHA lymphoblasts were resistant to AUR-1–mediated cytotoxicity (Figure 3B). Taken together, Aur-A–specific CTLs appeared to be capable of discriminating leukemia cells from normal cells in an HLA-restricted manner.

### CD34$^+$ leukemia progenitor cells, but not CD34$^+$ normal hematopoietic progenitor cells, express Aur-A abundantly and are susceptible to AUR-1–mediated cytotoxicity

Because it is important to determine whether Aur-A–specific CTLs can specifically recognize and lyse leukemia progenitors, we further examined Aur-A expression and susceptibility to AUR-1–mediated cytotoxicity of CD34$^+$ fractions in leukemia cells and normal hematopoietic cells. The CD34$^+$ cells were sorted from BMMCs of patients with CML and CBMCs, and their *Aur-A* mRNA expression was examined by QRT-PCR. As shown in Figure 4A, the expression levels of *Aur-A* mRNA in CML CD34$^+$ progenitor cells appeared to be significantly higher than in normal CD34$^+$ hematopoietic progenitor cells. Because it is suggested that leukemia stem cells and normal hematopoietic stem cells are present in the CD34$^+$CD38$^{low}$ fraction, we further examined Aur-A expression in CD34$^+$CD38$^{low}$ cells of CML BMMCs and CBMCs. Consequently, it appeared that *Aur-A* mRNA was abundantly expressed in the CD34$^+$CD38$^{low}$ fraction of CML (Figure 4B); however, the expression level of *Aur-A* mRNA in the CD34$^+$CD38$^{low}$ fraction of normal hematopoietic progenitors was significantly low (Figure 4C).

Since a sufficient number of CD34$^+$CD38$^{low}$ cells could not be obtained, whole CD34$^+$ cells were used as target cells for cytotoxicity assays (Figure 4D). As expected, AUR-1 exerted strong cytotoxicity against CD34$^+$ cells isolated from CML BMMCs of 2 patients. In contrast, AUR-1 did not show any cytotoxicity against normal CD34$^+$ hematopoietic progenitor cells. These data strongly suggest that Aur-A–specific CTLs can discriminate leukemia progenitor cells from normal hematopoietic stem cells and selectively inhibit the growth of leukemia stem cells, and that immunotherapy targeting Aur-A is effective and safe.

### Presence of Aur-$A_{207-215}$–specific CTL precursors in peripheral blood of leukemia patients

When considering the feasibility of cellular immunotherapy for leukemia targeting Aur-A, it seems important to clarify whether Aur-A–specific CTL precursors are present in patients with leukemia. Aur-$A_{207-215}$–specific CTL precursors in HLA-A*0201–positive patients with leukemia including AML in complete remission (CR) after allogeneic hematopoietic stem cell transplantation, ALL in CR after chemotherapy, and CML in the chronic phase before imatinib therapy, and 8 healthy subjects were analyzed by tetramer assay. Because Aur-$A_{207-215}$ peptide can bind to HLA-A*2402 as well as HLA-A*0201, we also examined Aur-$A_{207-215}$–specific CTL precursors in 2 HLA-A*2402–positive

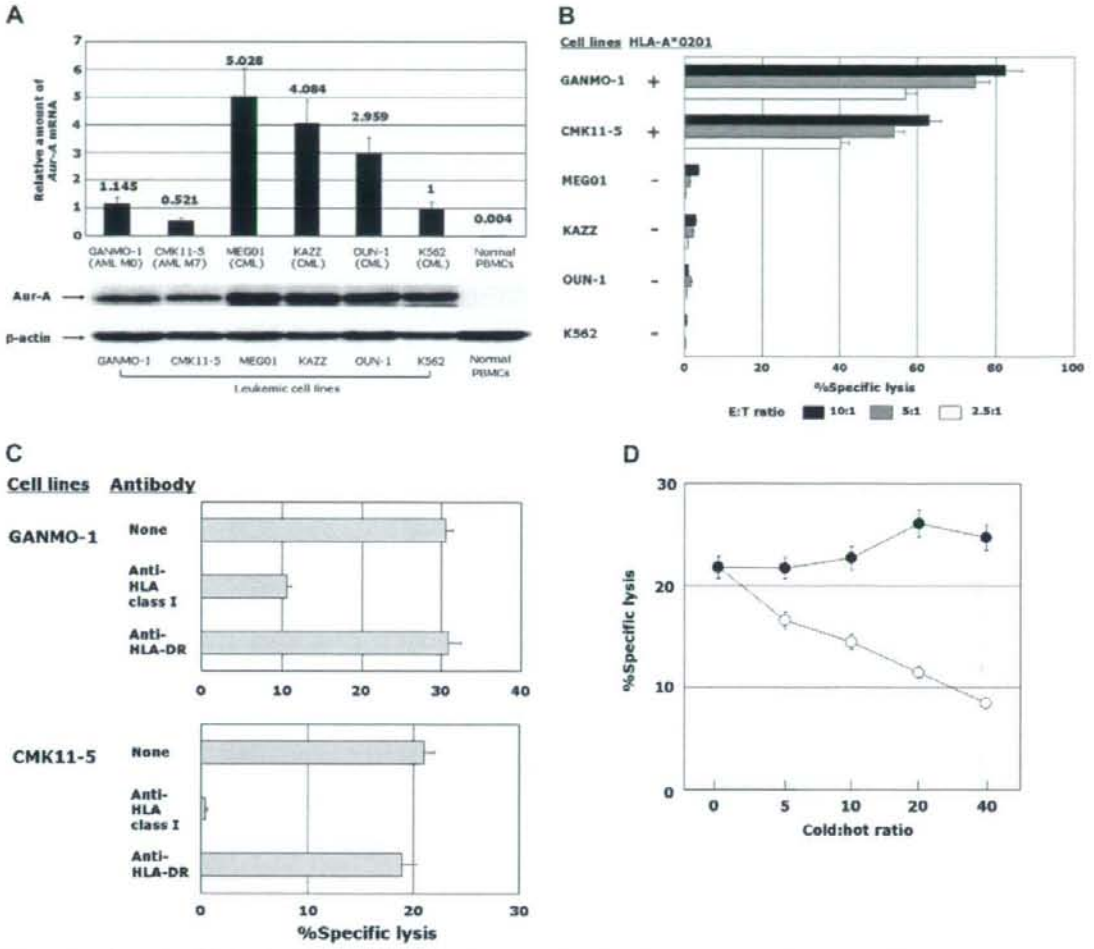


**Figure 2. Expression of Aur-A in leukemia cell lines and the cytotoxicity of AUR-1 against leukemia cell lines.** (A) Expression of *Aur-A* mRNA and protein in leukemia cell lines and normal PBMCs. Expression levels of *Aur-A* mRNA in the cells were determined by QRT-PCR as detailed in "Methods." The level of *Aur-A* mRNA expression in the K562 leukemia cell line, which strongly expresses Aur-A, is shown as 1.0 and the expression levels in the cells were calculated relative to this value. Aur-A protein expression was examined by Western blotting using anti–Aur-A antibody and anti–β-actin antibody as the control. (B) Cytotoxicity of the Aur-$A_{207-215}$–specific CTL line AUR-1 against leukemia cell lines. The cytotoxicity of AUR-1 to HLA-A*0201–positive and HLA-A*0201–negative leukemia cell lines was determined by 4-hour $^{51}$Cr-release assays at E/T ratios of 10:1, 5:1, and 2.5:1. (C) HLA class I restriction of cytotoxicity mediated by AUR-1 against leukemia cells. The cytotoxicity of AUR-1 against leukemia cell lines (GANMO-1 and CMK11-5) was determined by 4-hour $^{51}$Cr-release assays at an E/T ratio of 2.5:1 in the presence or absence of anti–HLA class I MoAb or anti–HLA-DR MoAb. (D) Cold target inhibition assays. $^{51}$Cr-labeled GANMO-1 cells ($5 \times 10^3$ cells) were mixed with various numbers of $^{51}$Cr-unlabeled Aur-$A_{207-215}$ peptide–loaded autologous LCL cells (○) or with $^{51}$Cr-unlabeled Aur-$A_{207-215}$ peptide–loaded HLA-A*0201–negative allogeneic LCL cells (●). The cytotoxicity of AUR-1 to the mixture of $^{51}$Cr-labeled and unlabeled target cells was determined by 4-hour $^{51}$Cr-release assays at an effector-to-$^{51}$Cr-labeled target cell ratio of 10:1.

patients with CML in chronic phase after therapy with interferon or imatinib and 2 healthy individuals. Since we were unable to detect Aur-A–specific CTL precursors when freshly isolated lymphocytes were used for assays, PBMCs were stimulated with Aur-$A_{207-215}$ peptide and then analyzed. Representative data of tetramer assays for HLA-A*0201–positive and HLA-A*2402–positive patients with leukemia are shown in Figure 5A. The frequencies of Aur-$A_{207-215}$–specific CTL precursors in HLA-A*0201–positive and HLA-A*2402–positive patients with leukemia and healthy individuals are summarized in Figure 5B. Consequently, Aur-$A_{207-215}$–specific CTL precursors were apparently detected in both HLA-A*0201–positive and HLA-A*2402–positive patients with leukemia. The frequency of Aur-$A_{207-215}$–specific CTL precursors in leukemia patients appeared to be significantly higher than that in healthy individuals (0.25% ± 0.1% for leukemia patients, and 0.05% ± 0.03% for healthy individuals; $P < .001$). These data strongly suggest that Aur-A–specific CTL precursors are primed in patients with leukemia, and that vaccination with Aur-A peptide may efficiently induce an Aur-A–specific immune response in leukemia patients. To determine whether Aur-$A_{207-215}$–specific CTL precursors detected by tetramer assays are indeed functional, we performed tetramer assays and ELISPOT assays using the same samples simultaneously, and determined the correlation between the 2 sets of data. PBMCs isolated from 7 HLA-A*0201– or HLA-A*2402–positive individuals were used for tetramer assays and ELISPOT assays. Consequently, the frequencies of Aur-$A_{207-215}$ peptide–specific CTL precursors detected by these 2 different assay systems appeared to be closely correlated

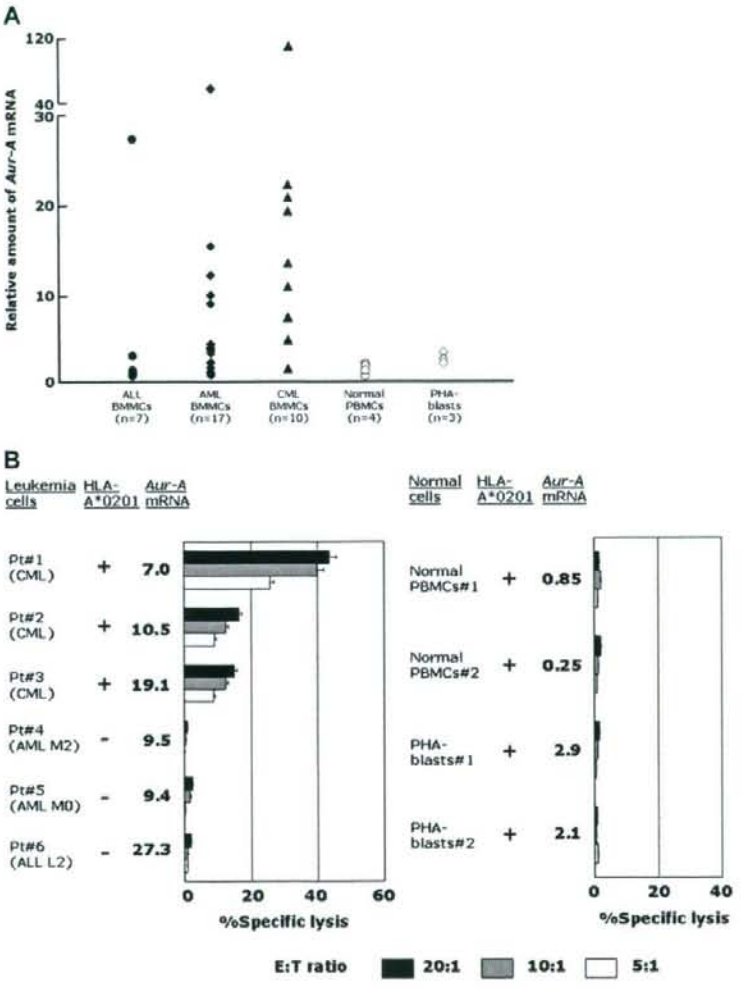


**Figure 3. Expression of Aur-A in freshly isolated leukemia cells and the cytotoxicity of AUR-1 against freshly isolated leukemia cells.** (A) Expression of *Aur-A* mRNA in freshly isolated leukemia cells, normal PBMCs, and PHA-stimulated T lymphocytes. Expression levels of *Aur-A* mRNA in freshly isolated leukemia cells and normal cells were determined using samples obtained from 7 patients with ALL, 17 patients with AML, 10 patients with CML in chronic phase, 4 healthy individuals, and PHA-stimulated T lymphoblasts obtained from 3 healthy individuals. To prepare PHA-stimulated T lymphoblasts, PBMCs were cultured in RPMI 1640 medium supplemented with 10% FCS and PHA at an appropriate concentration for 4 days. The level of *Aur-A* mRNA in normal PBMCs is shown as 1.0 and the expression levels in samples were calculated relative to this value. (B) Cytotoxicity of AUR-1 against freshly isolated leukemia cells and normal cells. The cytotoxicity of AUR-1 against HLA-A*0201–positive and HLA-A*0201–negative freshly isolated leukemia cells, HLA-A*0201–positive normal PBMCs, and HLA-A*0201–positive normal PHA-stimulated T lymphoblasts was determined by $^{51}$Cr-release assays at E:T ratios of 20:1, 10:1, and 5:1. Expression levels of *Aur-A* mRNA in samples are also shown.

(r = 0.817; Figure S1B). These data strongly suggest that Aur-A tetramer–positive cells certainly have a functional response to stimulation with Aur-A.

## Discussion

In the present study, we demonstrated that Aur-A is an ideal target antigen of cellular immunotherapy for leukemia, based on the following findings. First, Aur-A is broadly overexpressed in various types of leukemia but not in normal tissues except for testis, which is negative for HLA expression. Second, an Aur-A–derived peptide, Aur-A$_{207\text{-}215}$, can bind to HLA-A*0201 and HLA*2402 molecules and elicit Aur-A–specific CTLs. Third, Aur-A is efficiently processed in leukemia cells, and leukemia cell lines and freshly isolated leukemia cells, but not normal cells, are lysed by Aur-A–specific CTLs in an HLA class I–restricted manner. Fourth, Aur-A–specific CTL precursors are certainly present in the peripheral blood of patients with leukemia.

One of the important characteristics of proteins that could be used as ideal tumor-associated antigens for cancer immunotherapy is an essential role in tumorigenesis and/or tumor progression. Aur-A is localized mainly at spindle poles and the mitotic spindle during mitosis, where it regulates the functions of centrosomes, spindles, and kinetochores required for proper mitotic progression. Recent studies have revealed that Aur-A is frequently overexpressed in various cancer cells, indicating its involvement in tumorigenesis.[9-14] Overexpression of Aur-A contributes to genetic instability and tumorigenesis by disrupting the proper assembly of the mitotic checkpoint complex at the level of the Cdc20-BubR1 interaction.[34] Its overexpression also causes resistance to apoptosis induced by taxol in human cancer cell lines.[35,36] Moreover, Aur-A is a key regulatory component of the p53 pathway, as its overexpression leads to increased p53 degradation, thus facilitating oncogenic transformation.[37] In addition, Aur-A expression in tumors is often associated with poor histologic differentiation and poor prognosis.[12-14] These characteristics indicate that Aur-A is an ideal target antigen for cancer immunotherapy. Although Aur-A is also expressed in normal cells during mitosis, its expression level in normal tissue is quite low; therefore, normal mitotic cells are resistant to Aur-A–specific CTL-mediated cytotoxicity, as shown in the present study.

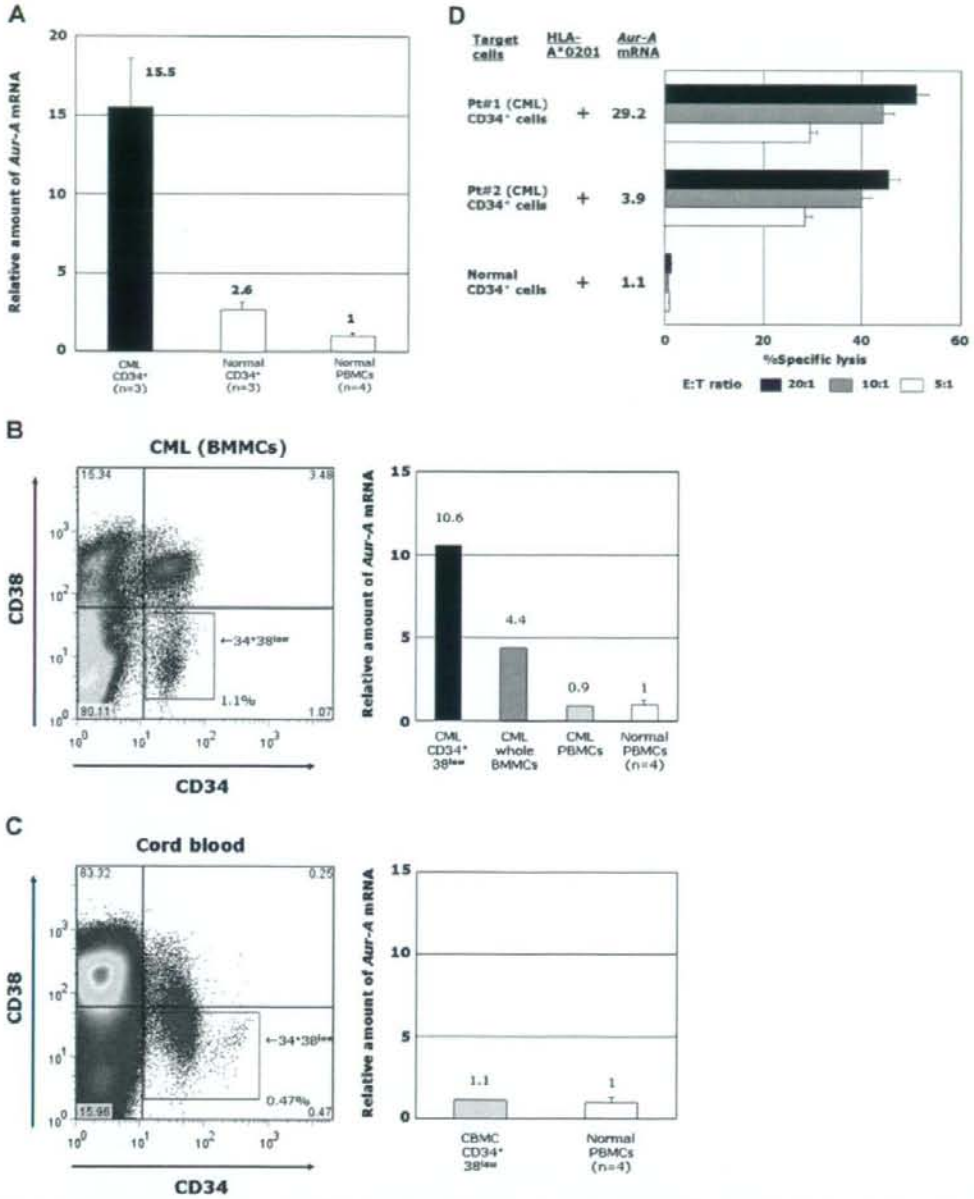


**Figure 4. Expression of Aur-A in CD34$^+$CD38$^{low}$ fractions of CML cells and normal hematopoietic progenitor cells, and cytotoxicity of AUR-1 against CD34$^+$ CML cells and CD34$^+$ normal hematopoietic stem cells.** (A) Expression levels of *Aur-A* mRNA in CD34$^+$ cells isolated from BMMCs of patients with CML, CD34$^+$ cells isolated from normal BMMCs and CBMCs, and normal PBMCs. Expression levels of *Aur-A* mRNA in leukemic CD34$^+$ cells, normal hematopoietic stem cells, and normal PBMCs were determined using 3 samples of CML BMMCs, 1 sample of normal BMMCs, 2 samples of CBMCs, and 4 samples of normal PBMCs. The level of *Aur-A* mRNA in normal PBMCs is shown as 1.0 and the expression levels in samples were calculated relative to this value. (B) Representative data of *Aur-A* mRNA expression in the CD34$^+$CD38$^{low}$ fraction of BMMCs, whole BMMCs, and PBMCs isolated from a patient with CML in chronic phase and PBMCs isolated from 4 healthy individuals. The CD34$^+$CD38$^{low}$ cells were collected using a cell sorter. (C) Representative data of *Aur-A* mRNA expression in the CD34$^+$CD38$^{low}$ fraction of CBMCs isolated from a normal donor and PBMCs isolated from 4 healthy individuals. The CD34$^+$CD38$^{low}$ cells were collected using a cell sorter. (D) Cytotoxicity of AUR-1 against CD34$^+$ leukemia progenitor cells and normal CD34$^+$ hematopoietic progenitor cells. The cytotoxicity of AUR-1 against CD34$^+$ leukemia cells isolated from 2 HLA-A*0201–positive patients with CML and normal CD34$^+$ hematopoietic progenitor cells isolated from an HLA-A*0201–positive cord blood donor was determined by $^{51}$Cr-release assays at E/T ratios of 20:1, 10:1, and 5:1. Expression levels of *Aur-A* mRNA in samples are also shown.

As reported previously,[17-19] the present study demonstrated that Aur-A is overexpressed widely in various types of leukemia including AML, ALL, and CML. Among the leukemias, CML cells appeared to express a large amount of Aur-A. It was also found that Aur-A is abundantly expressed in the CD34$^+$CD38$^{low}$ fraction of CML cells. Previous gene expression profiling analysis has also shown that mitogen-activated protein kinases, which activate mitotic kinases including Aurora kinases, are overexpressed in

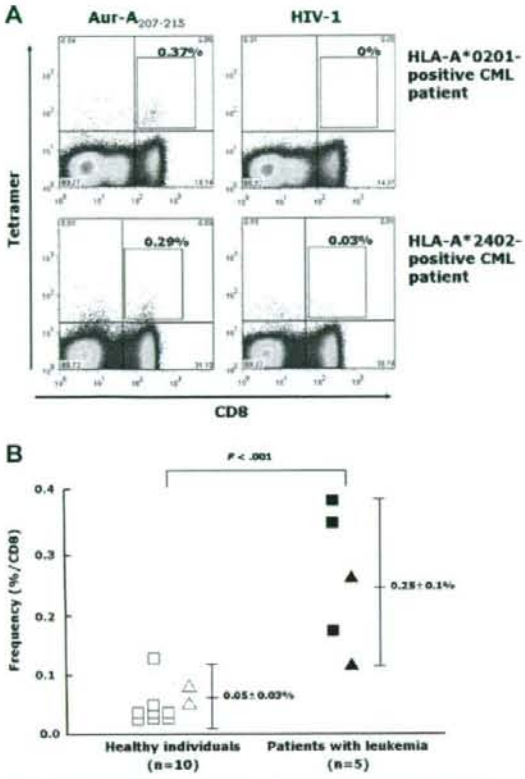


**Figure 5. Detection of Aur-$A_{207-215}$–specific CTL precursors in patients with leukemia.** (A) Representative data of the tetramer assay for Aur-$A_{207-215}$–specific CTL precursors. PBMCs isolated from HLA-A*0201–positive and HLA-A*2402–positive patients with CML in chronic phase were stimulated with Aur-$A_{207-215}$ peptide and then stained with HLA-A*0201/Aur-$A_{207-215}$ tetramer and HLA-A*2402/Aur-$A_{207-215}$ tetramer, respectively. HLA-A*0201/HIV-1 p17 $Gag_{77-85}$ (SLYNTVATL) tetramer and HLA-A*2402/HIV-1 $Env_{584-592}$ (RYLRDQQLL) tetramer were used as negative controls. (B) Summary of tetramer assays for Aur-$A_{207-215}$–specific CTL precursors. PBMCs isolated from 3 HLA-A*0201–positive patients with leukemia (a patient with AML in complete remission after allogeneic stem cell transplantation, a patient with ALL in complete remission after chemotherapy, and a patient with untreated CML in chronic phase; ■), 2 HLA-A*2402–positive patients with leukemia (2 patients with CML in chronic phase after therapy with interferon or imatinib; ▲), 8 HLA-A*0201–positive healthy individuals (□), and 2 HLA-A*2402–positive healthy individuals (△) were stained with HLA-A*0201/Aur-$A_{207-215}$ or HLA-A*2402/Aur-$A_{207-215}$ tetramer. The frequency of Aur-$A_{207-215}$–specific CTL precursors in the patients with leukemia was significantly higher than that in healthy individuals (Student *t* test; $P < .001$).

$CD34^+$ progenitor cells in CML.[38] In contrast to overexpression of Aur-A in the $CD34^+CD38^{low}$ fraction of CML cells, the expression level of Aur-A in the $CD34^+CD38^{low}$ fraction of normal hematopoietic progenitors appeared to be markedly lower than that in leukemic cells. We therefore addressed the question of whether Aur-A–specific CTLs can lyse leukemic progenitors. Because a sufficient number of $CD34^+CD38^{low}$ cells could not be obtained, $CD34^+$ cells were used as target cells. Consequently, in parallel with the expression levels of Aur-A, $CD34^+$ CML cells but not $CD34^+$ normal hematopoietic progenitor cells were efficiently lysed by Aur-A–specific CTLs. Although the detailed characteristics of leukemic stem cells are still obscure, they are considered to be present in the $CD34^+CD38^{low}$ fraction.[39-41] Taken together, targeting of Aur-A may be effective for eradicating leukemic stem cells.

Another interesting finding of this study was that Aur-$A_{207-215}$ peptide is able to bind to HLA-A*2402 as well as to HLA-A*0201. Although AUR-1 could not recognize the complex of Aur-$A_{207-215}$ peptide and HLA-A*0206 or HLA-A*0207, this peptide can bind to the HLA-A*0206 molecule (data not shown; written communication from Dr K. Udaka, Kochi University, Nangoku, Japan, August 5, 2007). Binding of a single peptide to both HLA-A*0201 and HLA-A*2402 has also been reported previously for a WT1-derived peptide ($WT1_{235-243}$; CMTWNQMNL),[30,42] which is now used as a cancer peptide vaccine. Since CTLs recognize a tumor-associated epitope in the context of HLA class I molecules, identification of a peptide that can bind to common HLA types is essential for development of a universal cancer peptide vaccine. Because HLA-A*2402 is the most common HLA type in the Japanese population, Aur-$A_{207-215}$ is a promiscuous peptide and therefore likely useful for development of a cancer vaccine for Asian as well as white patients.

To date, 3 Aurora kinases, Aur-A, Aur-B, and Aur-C, have been identified in mammals. The Aurora kinases show different subcellular localization patterns and perform distinct tasks during cell division.[43] These molecules show a similar domain organization: a N-terminal domain of 39-129 residues, a protein kinase domain, and a short C-terminal domain of 15-20 residues. The N-terminal domain of Aurora kinases shows low sequence conservation, and this determines selectivity during protein-protein interactions.[44] In contrast, the catalytic domain is more highly conserved. Importantly, Aur-$A_{207-215}$ is located in the catalytic domain and the conserved residues of Aur-A, Aur-B, and Aur-C (Figure S2). Interestingly, the Aur-$B_{149-157}$, Aur-$B_{151-159}$, and Aur-$C_{83-91}$ peptides, which are derived from the catalytic domain of Aur-B and Aur-C, can bind to HLA-A*0201 and HLA*2402 molecules (Figure S3), suggesting that these residues could be a universal target epitope for cancer immunotherapy.

In summary, we have demonstrated for the first time that Aur-A is a potentially ideal target of cellular immunotherapy for leukemia. When considering the evidence that Aur-A is overexpressed widely in various kinds of cancer, Aur-A–targeting cancer immunotherapy may be universally applicable. On the basis of our present data, we are now planning a clinical trial of Aur-A peptide vaccination for cancer patients.

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## Authorship

Contribution: T.O. and H.F. designed and performed the research and wrote the paper; K.S., T.A., Y.Y., and T.H. discussed and interpreted the experimental results and provided clinical materials; K.K. made and supplied the tetramer; and M.Y. designed the research, wrote and edited the paper, and provided financial support.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

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# Exploration of the Genetic Basis of GVHD by Genetic Association Studies

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## INTRODUCTION

Graft-versus-host disease (GVHD), as well as graft-versus-leukemia effect (GVL), are essentially allo-immune reactions, which are induced by the engrafted donor T cells that recognize the host-derived allo-antigens presented on their targets (Figure 1). In HLA-matched transplantation, these antigens are called minor histocompatibility antigens (mHags), and are typically defined by the host single nucleotide polymorphisms (SNPs) that are not shared by the donor and therefore considered to be genetically mismatched between the donor and the recipient [1–3]. Thus, the development of both allo-reactions absolutely depends on the presence of 1 or more mismatched mHags, although these reactions could be further modified by other genetic as well as environmental factors, including, cytokine polymorphisms and GVHD prophylaxis. So, in view of better preventing GVHD and specifically targeting allo-immunity to the tumor component, central questions are what mHAgs are responsible for the development of GVHD or GVL and what genetic factors can influence the overall reactions, which are the plausible targets of genome-wide association studies (GWAS) [4-8].

To identify the genetic basis of GVHD, we conducted GWAS by genotyping more than 500,000 SNPs using Affymetrix GeneChip platforms [9,10] in donors and recipients from 1,598 unrelated transplants performed through the Japan Marrow Donor Program (JMDP). All transplants were matched for HLA-A, B, C, DRB1, and DQB1 by high-resolution DNA typing, while 1033 (63%) transplants were mismatched for HLA-DPB1. Six hundred fifty-six (41.7%) and 245 (14.9%) of transplants had developed grade II-IV and grade III-IV of acute GVHD (aGVHD), respectively. Overall SNP call rates exceeded 98% both in donors and in recipients. Unobserved HapMap PhaseII SNPs were rigorously imputed from the genotyped SNPs [11-13]. After excluding those disqualified SNPs showing <95% call rate, deviation from Hardy-Weinberg equilibrium, or <5% minor allele frequency, 1,276,699 SNPs were tested for association with development of aGVHD and chronic GVHD (cGVHD), relapse, and overall survival (OS), by calculating log-rank statistics for each SNP. Statistical thresholds for genome-wide *P* value of .05 were determined empirically by doing 1,000 permutations for each analysis. Association tests were performed with regard to the simple genotype of donor and recipient SNPs. Alternatively, to identify possible mHag loci, GWAS were performed based on the allele-mismatch defined for each SNP locus, rather than simple SNP genotypes in donors and recipients. In the latter setting, associations were tested within the subgroups that shared particular HLA-types based on HLA-restriction. Generally speaking, the sample size of ~1,600 transplants in the current study was relatively small compared to the size of typical GWAS studies, and it was further reduced in the subgroup analysis [8]. Thus, it was likely that we could find only those mHag loci that were restricted to major HLA alleles and whose allele-mismatch conferred strong genetic effects on the development of GvHD [14,15]. However, this did not necessarily preclude conducting the current study, because it was such mHags that are thought to be clinically relevant.

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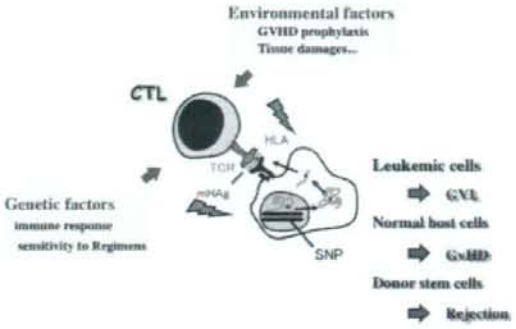


**Figure 1.** Allo-immunity plays central roles in HSCT.

In the analysis regarding genotype mismatch for aGVHD under the assumption of no HLA restriction, SNPs around the HLA-DPB1 locus showed strong association with the development of grade II-IV aGVHD with the maximum $P$ value of $1.81 \times 10^{-9}$ at rs6937034, and thus, the GWAS successfully captured the association of HLA-DPB1 allele mismatch as directly defined by high-resolution DNA typing (hazard ratio [HR] = 1.91, $P = 2.88 \times 10^{-13}$) (Figure 2) [16]. No other loci were identified that were significantly associated with aGVHD under the assumption of no HLA restrictions. To identify the target mHags for aGVHD, we further performed subgroup analyses, in which the analysis were confined to those transplants sharing major HLA types among the Japanese population [17]. Six loci were identified as candidate mHag loci. rs17473423 on chr12 was associated with the A*2402/B*5201/Cw*1201/DRB1*1501/DQB1*0601, which represents the most prevalent HLA haplotype among the Japanese population and shared in ~40% of unrelated transplants in Japanese (grade III-IV aGVHD, with maximum $P = 3.99 \times 10^{-13}$) (Figures 2b and 3b). rs9657655 on chr9 was associated with another common haplotype in Japanese, A*3303/B*4403/Cw*1403 (grade III-IV aGVHD with maximum $P = 8.56 \times 10^{-10}$) (Figures 2c and 3b). We found additional 4 loci that were associated with DQB1*0501, Cw*0102, B*5201, and Cw*1202. We also tested the association of GVHD with simple genotype in either recipients or donors, though which 2 recipient SNPs were found to be associated with aGVHD, rs5998746 on chr22 ($P = 3.41 \times 10^{-8}$) and rs11873016 on chr18 ($P = 1.26 \times 10^{-8}$), whereas no donor SNPs showed significant associations. Similarly, we identified 4 candidate SNPs associated with the development of severe cGVHD or relapse.

Our study provided a unique opportunity, in that a combination of 2 different genotypes, rather than mere genotypes in single individuals, is explored for association with particular disease phenotypes through whole genome association scanning. Although further replication studies and biologic confirmation are

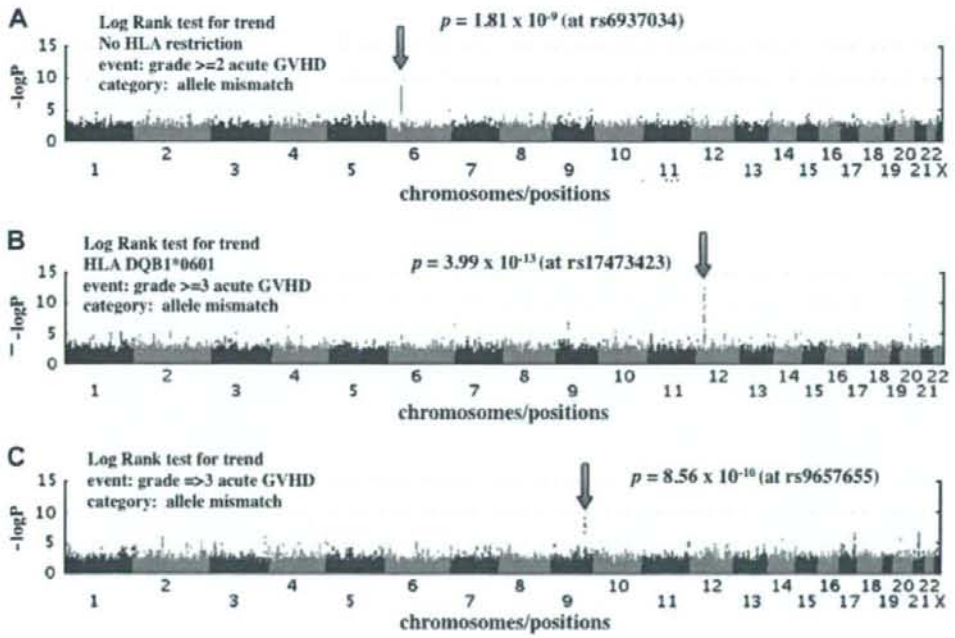


**Figure 2.** Representative results of GWAS based on genotype mismatch. $-\log_{10}P$ values are plotted in genetic order. Results are presented for association tests for genotype mismatch under no HLA restriction (A), and under the restriction to HLA DQB1*0601 (B) and HLA B*4403 (C).

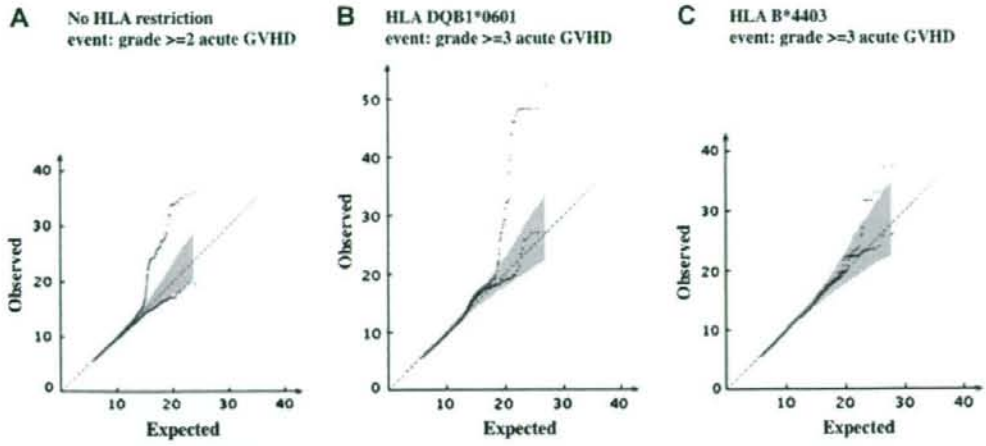


**Figure 3.** QQ-plots of the statistics. QQ-plots of the analysis of genotype mismatch under no restriction on HLA (A), and restriction to HLA DQB1*0601 (B) and HLA B*4403 (C) where observed test statistics values are plotted against expected values from 1000 random permutations (red); 95% confidence intervals are also provided by shadows. Only the plots for the top 20,000 results are presented. The QQ-plots excluding the SNPs that belong to the positive peak are also depicted in blue.

required, our results suggest that whole genome association studies of allo-SCT could provide a novel clue to our understanding of the genetic basis of GVHD.

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# Disease-specific analyses of unrelated cord blood transplantation compared with unrelated bone marrow transplantation in adult patients with acute leukemia

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**We made a disease-specific comparison of unrelated cord blood (CB) recipients and human leukocyte antigen allele–matched unrelated bone marrow (BM) recipients among 484 patients with acute myeloid leukemia (AML; 173 CB and 311 BM) and 336 patients with acute lymphoblastic leukemia (ALL; 114 CB and 222 BM) who received myeloablative transplantations. In multivariate analyses, among AML cases, lower overall survival (hazard ratio [HR] = 1.5; 95% confidence interval [CI], 1.0-2.0, $P = .028$) and leukemia-free survival (HR = 1.5; 95% CI, 1.1-2.0, $P = .012$) were observed in CB recipients. The relapse rate did not differ between the 2 groups of AML (HR = 1.2; 95% CI, 0.8-1.9, $P = .38$); however, the treatment-related mortality rate showed higher trend in CB recipients (HR = 1.5; 95% CI, 1.0-2.3, $P = .085$). In ALL, there was no significant difference between the groups for relapse (HR = 1.4, 95% CI, 0.8-2.4, $P = .19$) and treatment-related mortality (HR = 1.0; 95% CI, 0.6-1.7, $P = .98$), which contributed to similar overall survival (HR = 1.1; 95% CI, 0.7-1.6, $P = .78$) and leukemia-free survival (HR = 1.2; 95% CI, 0.9-1.8, $P = .28$). Matched or mismatched single-unit CB is a favorable alternative stem cell source for patients without a human leukocyte antigen–matched related or unrelated donor. For patients with AML, decreasing mortality, especially in the early phase of transplantation, is required to improve the outcome for CB recipients. (Blood. 2009;113:1631-1638)**

## Introduction

Allogeneic hematopoietic stem cell transplantation (HSCT) with bone marrow (BM) or peripheral blood, the curative treatment of choice for acute leukemia, is limited by the inadequate supply of human leukocyte antigen (HLA)–identical related donors. Bone marrow from HLA-matched unrelated donors has been a major alternative graft source.[1-3] Umbilical cord blood (CB), an alternative stem cell source to BM or peripheral blood stem cells, has been used primarily in children,[4-10] but its use in adults is increasing.[11,12]

Clinical comparison studies of cord blood transplantation (CBT) and bone marrow transplantation (BMT) for leukemia from unrelated donors in adult recipients showed comparable outcomes.[11-13] Recipients of CBT showed delayed neutrophil recovery and lower incidence of acute graft-versus-host disease (GVHD).[11-13] Overall treatment-related mortality (TRM) was reported to be similar[12] or higher[11] compared with HLA-matched BM. Acute myeloid leukemia (AML) and acute lymphoblastic leukemia (ALL) are different disease entities that require different chemotherapy regimens for treatment. However, previous comparison studies have included both diseases because of limitation in the number of CBTs given to adults.

In addition, the study periods of previous studies encompass the pioneering period of CBT, when the general practice was to use these grafts in patients in whom there were no other curative options and when the relevance of cell dose and HLA matching had not yet been recognized.[6,7,14]

Accumulation of a larger number of CBT results enabled us to make a controlled comparison with unrelated BMTs. To avoid the inclusion of the pioneering period of CBT, the subjects were limited to those who received transplantations in and after 2000.

## Methods

### Collection of data and data source

The recipients' clinical data were provided by the Japan Cord Blood Bank Network (JCBBN) and the Japan Marrow Donor Program (JMDP).[15]

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Peripheral blood stem cell donation from unrelated donors is not permitted in Japan. All 11 CB banks in Japan are affiliated to JCBBN. Both JCBBN and JMDP collect recipients' clinical information at 100 days after transplantation. Patients' information on survival, disease status, and long-term complications, including chronic GVHD and second malignancies, are renewed annually by follow-up forms. This study was approved by the data management committees of JMDP and JCBBN.

### Patients

Between January 2000 and December 2005, a total of 1690 adult patients at least 16 years of age with acute leukemia (999 AML, 261 CB and 738 BM; and 691 ALL, 178 CB and 513 BM) received first HSCT with myeloablative conditioning either CB or BM from unrelated donors. Of these, patients who received a single CB unit with 0 to 2 HLA mismatches, or HLA-A, -B, -C, and DRB1 allele-matched BM from unrelated donors were analyzed. HLA matching of CB was performed using low-resolution molecular typing methods for HLA-A and -B, and high-resolution molecular typing for HLA-DRB1. Of 1023 BM recipients with complete HLA high-resolution data, the following recipients with HLA HLA-A, -B, -C, and DRB1 allele mismatches were excluded: 306 recipients with 1 of 8 mismatches (39 for HLA-A, 6 for HLA-B, 137 for HLA-C, and 124 for HLA-DRB1), 150 recipients with 2 of 8 mismatches (36 for 2 class I antigens, and 114 for class I and class II antigens), 33 recipients with 3 of 8 mismatches, and 1 recipient with 4 of 8 mismatches. Of 390 recipients of CB with complete HLA data, 95 recipients with 3 mismatches and 8 patients with 4 mismatches were excluded. A total of 484 patients with AML (173 CBTs and 311 BMTs) and 336 patients with ALL (114 CBTs and 222 BMTs) were the subjects for the analyses. Eighty-five centers performed 287 CBTs analyzed in this study, and 114 centers performed 533 BMTs.

### Definitions

Neutrophil recovery was defined by an absolute neutrophil count of at least 500 cells/mm$^3$ for 3 consecutive points; platelet recovery was defined by a count of at least 50 000 platelets/mm$^3$ without transfusion support. Diagnosis and clinical grading of acute GVHD were performed according to the established criteria.[16] Relapse was defined as a recurrence of underlying hematologic malignant diseases. Treatment-related death was defined as death during a continuous remission. Leukemia-free survival (LFS) was defined as survival in a state of continuous remission.

### Statistical analysis

Separate analyses were performed for AML and ALL. Descriptive statistical analysis was performed to assess patient baseline characteristics, diagnosis, disease classification, disease status at conditioning, donor-patient ABO mismatches, preparative regimen, and GVHD prophylaxis. The 2-sided $\chi^2$ test was used for categorical variables, and the 2-sided Wilcoxon rank sum test was used for continuous variables. Cumulative incidence curves were used in a competing-risks setting to calculate the probability of neutrophil and platelet recovery, acute and chronic GVHD, relapse, and TRM.[17] For neutrophil and platelet recovery, death before neutrophil or platelet recovery was the competing event; for GVHD, death without GVHD and relapse were the competing events; for relapse, death without relapse was the competing event; and, for TRM, relapse was the competing event. Gray test was used for group comparison of cumulative incidence.[18] Overall survival (OS) and LFS were calculated using the Kaplan-Meier method. The log-rank test was used for group comparisons. Adjusted comparison of the stem cell source on OS and LFS was performed with the use of the Cox proportional-hazards regression model. For other outcomes, the Fine and Gray proportional-hazards model for subdistribution of a competing risk was used.[19] Adjusted probabilities of OS and DFS were estimated using the Cox proportional-hazards regression model, with consideration of other significant clinical variables in the final multivariate models. The variables considered were the patient's age at transplantation, patient's sex, donor-patient sex mismatch, donor-patient ABO mismatch, disease status at conditioning, and t(9;22) chromosome abnormality or others for ALL, cytogenetic information and French-American-British (FAB) classification of M5/M6/M7 or others for AML, the conditioning regimen, and the type of prophylaxis against GVHD. Factors differing in distribution between CB and BM recipients ($P < .10$) and factors known to influence outcomes (such as patient age at transplantation and chromosome abnormalities and FAB classification of leukemia) were included in the final models. Variables with more than 2 categories were dichotomized for the final multivariate model. The cutoff points of the variables were chosen to make optimal use of the information, with the proviso that smaller groups contain at least 20% of the patients. Variables were dichotomized as follows: patient age greater or younger than 45 years at transplantation, female donor to male recipient donor-recipient sex mismatch versus others for donor-recipient sex matching, donor-recipient ABO major mismatch versus others for ABO matching, M5/M6/M7 FAB classification versus others for classification of AML, chromosome abnormality other than favorable abnormalities for cytogenetics of AML, cyclophosphamide and total body irradiation (TBI) or busulfan and cyclophosphamide or others for conditioning regimen of AML, cyclophospohamide and TBI, or others for conditioning regimen of ALL, and cyclosporine-based versus tacrolimus-based prophylaxis against GVHD. Disease status at transplantation was categorized as first complete remission (1CR), second or later complete remission (2CR), or more advanced disease; which was included in the final model using dichotomized dummy variables. All $P$ values were 2-sided.

The statistical power to detect hazard ratios (HRs) of 2.0 and 1.5 (a regression coefficient equal to 0.6931 and 0.4055, respectively) on Cox regression of the log hazard ratio at a .05 significance level adjusted for event rate were 99% and 78%, respectively, for 484 patients with AML and 97% and 60%, respectively, for 336 patients with ALL. The levels of statistical power for subgroup analyses were as follows: 54% and 22% for 1CR, 51% and 21% for 2CR, 96% and 58% for more advanced in AML patients, 62% and 26% for 1CR, 47% and 20% for 2CR, and 67% and 29% for more advanced in ALL patients.[20]

## Results

### Patient characteristics

The characteristics of the patients are shown in Table 1. There was no significant difference in recipients' age at transplantation in AML (median age, CB vs BM = 38 vs 38 years, $P = .61$) and in ALL (median age, CB vs BM = 34 vs 32 years, $P = .29$). The female/male ratio was higher (CB vs BM = 54% vs 38% in AML patients, and CB vs BM = 54% vs 38% in ALL patients, $P < .001$ and $P = .005$, respectively) in CB recipients, resulting in the lower donor-patient sex match rate (CB vs BM = 48% vs 69% in AML patients, and CB vs BM = 46% vs 65% in ALL patients, $P < .001$ and $P = .002$, respectively) in CB recipients. The proportion of ALL patients with Philadelphia chromosome abnormality was higher (CB vs BM = 38% vs 23%) in CB recipients. CB recipients were likely to have more advanced disease status at transplantation (relapse or induction failure, CB vs BM = 47% vs 31% in AML patients, and CB vs BM = 26% vs 19% in ALL patients), and the difference was significant in AML ($P = .003$). HLA-A, -B (low-resolution typing), and -DRB1 (high-resolution typing) was mismatched in 93% of both AML and ALL among CB recipients, whereas HLA -A, -B, -C, and -DRB1 were all genotypically matched for BM recipients. The ABO-matched donor-patient pair proportion was consistently lower for CB (CB vs BM = 34% vs 59% in AML patients and CB vs BM = 32% vs 58% in ALL patients).

A preparative regimen with TBI and cyclophosphamide was used in almost all patients, and cytosine arabinoside was supplemented for CB recipients with AML (36%) in addition to TBI and cyclophosphamide. For GVHD prophylaxis, tacrolimus (CB vs BM = 29% vs 56% in AML patients, and CB vs BM = 37% vs 53% in ALL patients) and

**Table 1. Characteristics of recipients of cord blood or bone marrow from unrelated donors in 484 patients with acute myeloid leukemia and 336 patients with acute lymphoblastic leukemia**

| | Acute myeloid leukemia | | | Acute lymphoblastic leukemia | | |
|---|---|---|---|---|---|---|
| **Characteristic** | **U-CBT** | **U-BMT** | ***P*** | **U-CBT** | **U-BMT** | ***P*** |
| No. of transplantations | 173 | 311 | | 114 | 222 | |
| Median patient age at transplantation, y (range) | 38 (16-69) | 38 (16-60) | .61 | 34 (16-56) | 32 (16-59) | .29 |
| **Patient sex, n (%)** | | | | | | |
| Male | 80 (46) | 194 (62) | < .001 | 52 (46) | 137 (62) | .005 |
| Female | 93 (54) | 117 (38) | | 62 (54) | 85 (38) | |
| **Sex matching, n (%)** | | | < .001 | | | .002 |
| Matched | 83 (48) | 216 (69) | | 52 (46) | 145 (65) | |
| Male to female | 44 (25) | 57 (18) | | 35 (31) | 42 (19) | |
| Female to male | 46 (27) | 37 (12) | | 27 (24) | 35 (16) | |
| Unknown | 0 (0) | 1 (0) | | 0 (0) | 0 (0) | |
| **Disease classification** | | | | | | |
| AML (French-American-British) | | | .045 | | | |
| M0 | 17 (10) | 26 (8) | | | | |
| M1 | 30 (17) | 38 (12) | | | | |
| M2 | 52 (30) | 88 (28) | | | | |
| M3 | 4 (2) | 25 (8) | | | | |
| M4 | 27 (16) | 55 (18) | | | | |
| M5 | 23 (13) | 41 (13) | | | | |
| M6 | 3 (2) | 18 (6) | | | | |
| M7 | 2 (1) | 5 (2) | | | | |
| Others/unknown | 15 (9) | 15 (5) | | | | |
| Cytogenetics | | | .042 | | | |
| Favorable* | 19 (11) | 66 (21) | | | | |
| Normal | 74 (43) | 116 (37) | | | | |
| Other | 57 (33) | 95 (31) | | | | |
| Unknown | 23 (13) | 34 (11) | | | | |
| ALL cytogenetics | | | | | | .022 |
| t(9;22) | | | | 43 (38) | 52 (23) | |
| t(4;11) | | | | 2 (2) | 3 (1) | |
| Others | | | | 22 (19) | 51 (23) | |
| Normal | | | | 27 (24) | 85 (38) | |
| Unknown | | | | 20 (18) | 31 (14) | |
| **Disease status** | | | .003 | | | .33 |
| First CR | 50 (29) | 130 (42) | | 63 (55) | 130 (59) | |
| Second or after CR | 39 (23) | 82 (26) | | 21 (18) | 48 (22) | |
| Relapse/induction failure | 81 (47) | 95 (31) | | 30 (26) | 42 (19) | |
| Unknown | 3 (2) | 4 (1) | | 0 (0) | 2 (1) | |
| **HLA matching†** | | | | | | |
| 0 mismatched loci | 12 (7) | | | 8 (7) | | |
| 1 mismatched locus | 35 (20) | | | 25 (22) | | |
| 2 mismatched loci | 126 (73) | | | 81 (71) | | |
| **ABO matching** | | | < .001 | | | < .001 |
| Matched | 59 (34) | 185 (59) | | 37 (32) | 128 (58) | |
| Minor mismatch | 48 (28) | 57 (18) | | 30 (26) | 48 (22) | |
| Major mismatch | 37 (21) | 59 (19) | | 24 (21) | 41 (18) | |
| Bidirectional | 28 (16) | 8 (3) | | 23 (20) | 3 (1) | |
| Unknown | 1 (1) | 2 (1) | | 0 (0) | 2 (1) | |
| Nucleated cells infused per $10^7$/kg, median (range) | 2.44 (1.65-5.49) | 26.3 (2.10-58.8) | < .001 | 2.48 (1.51-4.06) | 28.2 (2.30-79.0) | < .001 |
| **Preparative regimen** | | | < .001 | | | .38 |
| CY + TBI | 43 (25) | 142 (46) | | 42 (37) | 92 (41) | |
| CY + CA + TBI | 62 (36) | 41 (13) | | 31 (27) | 53 (24) | |
| CY + BU + TBI | 7 (4) | 36 (12) | | 3 (3) | 5 (2) | |
| Other TBI regimen | 42 (24) | 33 (11) | | 34 (30) | 54 (24) | |
| BU + CY | 18 (10) | 55 (18) | | 4 (4) | 12 (5) | |
| Other non-TBI regimen | 1 (1) | 4 (1) | | 0 (0) | 6 (3) | |
| **GVHD prophylaxisis** | | | < .001 | | | < .001 |
| Cyclosporine A + sMTX | 103 (60) | 131 (42) | | 65 (57) | 100 (45) | |
| Cyclosporine A ± other | 20 (12) | 4 (1) | | 6 (5) | 3 (1) | |
| Tacrolimus + sMTX | 34 (20) | 168 (54) | | 26 (23) | 106 (48) | |
| Tacrolimus ± other | 15 (9) | 5 (2) | | 16 (14) | 11 (5) | |
| Others | 1 (1) | 3 (1) | | 1 (1) | 2 (1) | |

U-CBT, indicates unrelated cord blood transplantation; U-BMT, unrelated bone marrow transplantation; CR, complete remission; HLA, human leukocyte antigen; CY, cyclophosphamide; CA, cytarabine; BU, oral busulfan; TBI, total body irradiation; and sMTX, short-term methotrexate.

*Favorable abnormal karyotypes are defined as t(8;21), inv16,or t(15;17).

†Number of mismatches was counted among HLA-A, -B (low-resolution typing), and DRB1 (high-resolution typing).

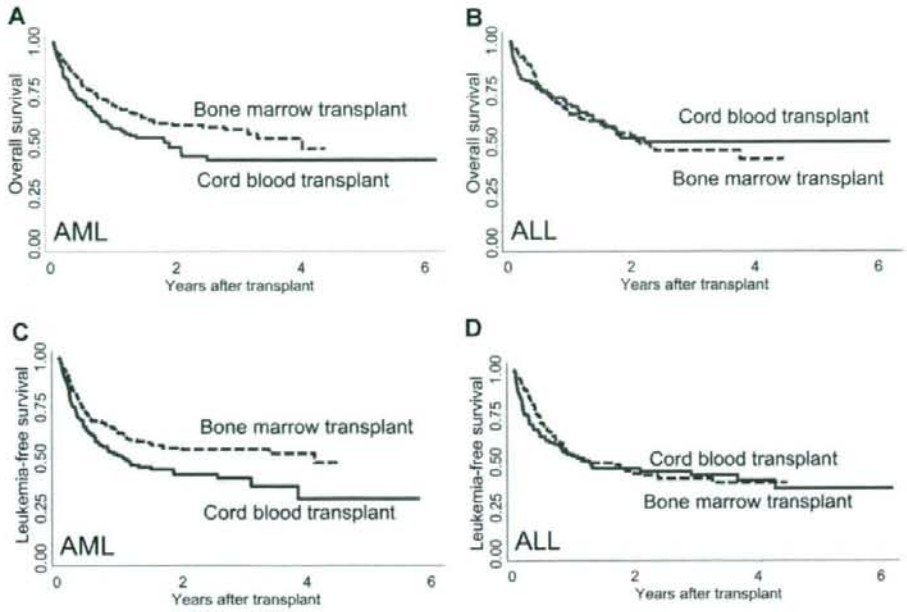


**Figure 1. Adjusted OS and LFS of recipients with AML or ALL of CB or BM from unrelated donors.** For patients with AML, adjusted probabilities of (A) OS (CB vs BM = 48% vs 59% at 2 years, $P = .010$) and (C) LFS (CB vs BM = 42% vs 54% at 2 years, $P = .004$) were both lower in CB recipients. For patients with ALL, the adjusted probabilities of (B) OS (CB vs BM = 52% vs 53% at 2 years, $P = .99$) and (D) LFS (CB vs BM = 46% vs 44% at 2 years, $P = .41$) were similar between CB recipients and BM recipients.

short-term methotrexate (CB vs BM = 80% vs 96% in AML patients, and CB vs BM = 80% vs 93% in ALL patients) were used preferentially in BM recipients. The median follow-up period for survivors was 1.9 years (range, 0.1-6.2 years) for CB recipients and 1.4 years (range, 0.3-4.5 years) for BM recipients.

### Outcome

***OS.*** For patients with AML, the unadjusted probabilities of OS were lower for CB recipients at 1 year (51% vs 69%) and 2 years (43% vs 60%) compared with BM recipients ($P < .001$). For patients with ALL, there were no significant differences between the 2 groups (CB vs BM = 66% vs 66% at 1 year, 49% vs 57% at 2 years, $P = .40$).

Among patients with AML, the use of CB remained a significant risk factor for overall mortality after adjustment for other factors (HR = 1.5; 95% confidence interval [CI], 1.0-2.0; $P = .028$; Table 2). However, in patients with ALL, the use of CB was not a significant factor for overall mortality on multivariate analysis (HR = 1.1; 95% CI, 0.7-1.6; $P = .78$). The adjusted probability of OS was significantly lower for CB recipients (57% vs 69% at 1 year, and 48% vs 59% at 2 years, $P = .010$; Figure 1A) compared with BM recipients for patients with AML, whereas the adjusted probability of OS was similar (69% vs 64% at 1 year, and 52% vs 53% at 2 years, $P = .99$; Figure 1B) between the groups for patients with ALL.

Results of the subgroup analyses showed that the difference in survival among AML patients was prominent in patients demonstrating 1CR at transplantation (RR = 2.9, 95% CI = 1.4-6.2, $P = .005$; Table 3).

***LFS.*** For patients with AML, the unadjusted probabilities of LFS were significantly lower for CB recipients at 1 year (43% vs 62%) and 2 years (36% vs 54%) compared with BM recipients ($P < .001$). For patients with ALL, the unadjusted probabilities of LFS were lower with marginal significance for CB recipients at 1 year (52% vs 58%) and 2 years (45% vs 51%) compared with BM recipients ($P = .06$).

Among patients with AML, the use of CB remained as a significant risk factor for treatment failure (ie, relapse or death) after adjustment for other factors (HR = 1.5; 95% CI, 1.1-2.0; $P = .012$; Table 2). However, in patients with ALL, the use of CB was not a significant factor for treatment failure by multivariate analysis (HR = 1.2; 95% CI, 0.9-1.8; $P = .28$). The adjusted probability of LFS was significantly lower for CB recipients (51% vs 62% at 1 year, and 42% vs 54% at 2 years, $P = .004$; Figure 1C) compared with BM recipients for patients with AML, whereas the adjusted probability of LFS was similar (53% vs 53% at 1 year, and 46% vs 44% at 2 years, $P = .41$; Figure 1D) between the groups for patients with ALL.

### Relapse

On univariate analyses, the cumulative incidence of relapse was higher for CB recipients with marginal significance in both AML (27% vs 20% at 1 year, and 31% vs 24% at 2 years) and ALL (27% vs 19% at 1 year, and 31% vs 24% at 2 years) ($P = .067$, and .085, respectively; Figure 2A,B).

On multivariate analyses adjusted by other factors, there was no significantly higher risk of relapse for CB recipients with either AML (RR = 1.2, 95% CI = 0.8-1.9, $P = .38$) or ALL (RR = 1.4, 95% CI = 0.8-2.4, $P = .19$; Table 2).

### TRM

For patients with AML, the unadjusted cumulative incidence of TRM was significantly higher for CB recipients at 1 year (30% vs 19%) and 2 years (33% vs 22%) compared with those for BM recipients ($P = .004$; Figure 2C). For patients with ALL, the

**Table 2. Results of multivariate analysis of outcomes in 173 recipients of cord blood and 311 recipients of bone marrow with acute myeloid leukemia, and 114 recipients of cord blood and 222 recipients of bone marrow with acute lymphoblastic leukemia**

| | Acute myeloid leukemia | | Acute lymphoblastic leukemia | |
|---|---|---|---|---|
| Outcome | RR (95% CI) | P | RR (95% CI) | P |
| **Overall survival*** | | | | |
| BM | 1.00 | | 1.00 | |
| CB | 1.45 (1.04-2.01) | .028 | 1.06 (0.71-1.57) | .78 |
| **Leukemia-free survival†** | | | | |
| BM | 1.00 | | 1.00 | |
| CB | 1.48 (1.09-2.01) | .012 | 1.22 (0.85-1.76) | .28 |
| **Relapse‡** | | | | |
| BM | 1.00 | | 1.00 | |
| CB | 1.21 (0.79-1.87) | .38 | 1.42 (0.84-2.41) | .19 |
| **TRM§** | | | | |
| BM | 1.00 | | 1.00 | |
| CB | 1.47 (0.95-2.28) | .085 | 1.01 (0.59-1.73) | .98 |
| **Neutrophil recovery‖** | | | | |
| BM | 1.00 | | 1.00 | |
| CB | 0.41 (0.33-0.51) | < .001 | 0.37 (0.29-0.48) | < .001 |
| **Platelet recovery¶** | | | | |
| BM | 1.00 | | 1.00 | |
| CB | 0.34 (0.27-0.44) | < .001 | 0.43 (0.33-0.56) | < .001 |
| **Acute GVHD#** | | | | |
| BM | 1.00 | | 1.00 | |
| CB | 0.80 (0.56-1.15) | .23 | 0.61 (0.39-0.95) | .028 |
| **Chronic GVHD**** | | | | |
| BM | 1.00 | | 1.00 | |
| CB | 0.94 (0.63-1.42) | .79 | 1.08 (0.66-1.77) | .77 |
| **Chronic GVHD, extensive type††** | | | | |
| BM | 1.00 | | 1.00 | |
| CB | 0.36 (0.18-0.72) | .004 | 0.58 (0.28-1.20) | .14 |

RR indicates relative risk; CI, confidence interval; BM, bone marrow; CB, cord blood; and GVHD, graft-versus-host disease.

*For overall survival, other significant variables for AML were patient age more than 45 years at transplantation, more advanced disease status at conditioning, M5/M6/M7 French-American-British classification, and female donor to male recipient donor-recipient sex mismatch; other significant variables for ALL were second or after complete remission disease status, more advanced disease status, and Philadelphia chromosome abnormality.

†For leukemia-free survival, other significant variables for AML were patient age more than 45 years at transplantation, more advanced disease status at conditioning, M5/M6/M7 French-American-British classification, and female donor to male recipient donor-recipient sex mismatch; other significant variables for ALL were second or after complete remission disease status, more advanced disease status, and Philadelphia chromosome abnormality.

‡For relapse, other significant variables for AML were more advanced disease status at conditioning, donor-recipient ABO major mismatch, chromosome abnormality other than favorable abnormalities, and cyclophosphamide and total body irradiation or busulfan and cyclophosphamide conditioning regimen; other significant variables for ALL were second or after complete remission disease status, more advanced disease status, and cyclophosphamide and total body irradiation conditioning.

§For TRM, other significant variables for AML were patient age more than 45 years at transplantation, second or after complete remission disease status, more advanced disease status, and chromosome abnormality other than favorable abnormalities; other significant variables for ALL were patient age more than 45 years at transplantation, more advanced disease status at conditioning, and conditioning other than cyclophosphamide and total body irradiation.

‖For neutrophil recovery, other significant variables for AML were second or after complete remission disease status and more advanced disease status; other significant variables for ALL were more advanced disease status at conditioning and cyclosporine-based GVHD prophylaxis.

¶For platelet recovery; other significant variables for AML were second or after complete remission disease status, more advanced disease status, female donor to male recipient donor-recipient sex mismatch, and tacrolimus-based GVHD prophylaxis; other significant variables for ALL were more advanced disease status at conditioning and conditioning other than cyclophosphamide and total body irradiation.

#For acute GVHD, no other significant variables were identified for both AML and ALL.

**For chronic GVHD, other significant variables for AML were more advanced disease status and conditioning other than cyclophosphamide and total body irradiation or busulfan and cyclophosphamide; there were no other significant variables identified for ALL.

††For extensive chronic GVHD, there were no other significant variables identified for AML; another significant variable for ALL was patient male sex.

cumulative incidence of TRM was similar between the 2 groups (CB vs BM = 21% vs 23% at 1 year, 24% vs 25% at 2 years, $P = .83$; Figure 2D).

On multivariate analyses adjusted by other factors, the risk for TRM was higher for CB recipients compared with that for BM recipients among patients with AML (RR = 1.5, 95% CI = 1.0-2.3, $P = .085$; Table 2) with marginal significance. For patients with ALL, the risk for TRM was similar between CB and BM recipients (RR = 1.0, 95% CI = 0.6-1.7, $P = .98$).

### Cause of death

Recurrence of the primary disease was the leading cause of death in each group (CB vs BM = 37% vs 33% in patients with AML and 36% vs 41% in patients with ALL). The following causes were infection and organ failure in all groups (Table 4).

### Other outcomes of transplantation

***Neutrophil and platelet recovery.*** The unadjusted cumulative incidence of neutrophil recovery or platelet recovery at day 100 was significantly lower in CB recipients for both AML (77% vs 94%) and ALL (80% vs 97%) compared with that among BM recipients ($P < .001$ for both). On multivariate analyses, neutrophil recovery was significantly lower among CB recipients for both AML (RR = 0.4, 95% CI = 0.3-0.5, $P < .001$) and ALL (RR = 0.4, 95% CI = 0.3-0.5, $P < .001$; Table 2).

**Table 3. Results of multivariate analysis of overall survival according to disease status at transplantation**

| | First complete remission | | | Second or after complete remission | | | More advanced | | |
|---|---|---|---|---|---|---|---|---|---|
| Overall survival | n | RR (95% CI) | P | n | RR (95% CI) | P | n | RR (95% CI) | P |
| **AML** | | | | | | | | | |
| UBMT | 130 | 1.00 | | 82 | 1.00 | | 95 | 1.00 | |
| UCBT | 50 | 2.92 (1.38-6.18) | .005 | 39 | 1.24 (0.51-3.04) | .63 | 81 | 1.29 (0.84-1.98) | .25 |
| **ALL** | | | | | | | | | |
| UBMT | 130 | 1.00 | | 48 | 1.00 | | 42 | 1.00 | |
| UCBT | 63 | 1.60 (0.84-3.05) | .16 | 21 | 0.62 (0.22-1.74) | .36 | 30 | 0.80 (0.38-1.69) | .57 |

RR indicates relative risk; CI, confidence interval; UBMT, unrelated bone marrow transplantation; and UCBT, unrelated cord blood transplantation.

The unadjusted cumulative incidence of platelet recovery greater than 50 000/μL at 4 months was significantly lower among CB recipients for both AML (59% vs 85%) and ALL (61% vs 83%) compared with that of BM recipients ($P < .001$ for both). The difference was also significant on multivariate analyses for both AML (RR = 0.3, 95% CI = 0.3-0.4, $P < .001$) and ALL (RR = 0.4, 95% CI = 0.3-0.6, $P < .001$; Table 2).

***Acute GVHD.*** The unadjusted cumulative incidence of grade 2 to 4 acute GVHD was lower among CB recipients compared with that among BM recipients (32% vs 35% in AML, 28% vs 42% in ALL); the difference was significant in patients with ALL ($P = .39$ in AML, $P = .008$ in ALL). The difference was also significant on multivariate analyses in ALL (RR = 0.6, 95% CI = 0.4-1.0, $P = .028$). There was no significant difference in patients with AML (RR = 0.8, 95% CI = 0.6-1.2, $P = .23$; Table 2).

***Chronic GVHD.*** The unadjusted cumulative incidence of chronic GVHD at 1 year after transplantation did not significantly differ between CB recipients and BM recipients in both AML (28% vs 32%, $P = .46$) and ALL (27% vs 30%, $P = .50$). The cumulative incidence of extensive-type chronic GVHD was significantly lower among CB recipients compared with that among BM recipients in both AML (8% vs 20%, $P < .001$) and ALL (10% vs 17%, $P = .034$). On multivariate analyses, the risk of developing chronic GVHD was similar in CB recipients and BM recipients in both AML (RR = 0.9, 95% CI = 0.6-1.4, $P = .79$) and ALL (RR = 1.1, 95% CI = 0.7-1.8, $P = .77$). The risk of developing extensive chronic GVHD was lower in CB recipients compared with BM recipients (RR = 0.4, 95% CI = 0.2-0.7, $P = .004$ in AML, and RR = 0.6, 95% CI, 0.3-1.2, $P = .14$ in ALL) and was significantly different in patients with AML (Table 2).

## Discussion

The objective of our study was to investigate the outcomes of HLA-A, -B, low-resolution, and -DRB1 high-resolution 0 to 2 mismatched single-unit unrelated CBT in adult patients with acute leukemia compared with those of HLA-A, -B, -C, and -DRB1 (8 of 8) allele-matched unrelated BMT. Although AML and ALL are different diseases, previous comparisons of unrelated BMT and

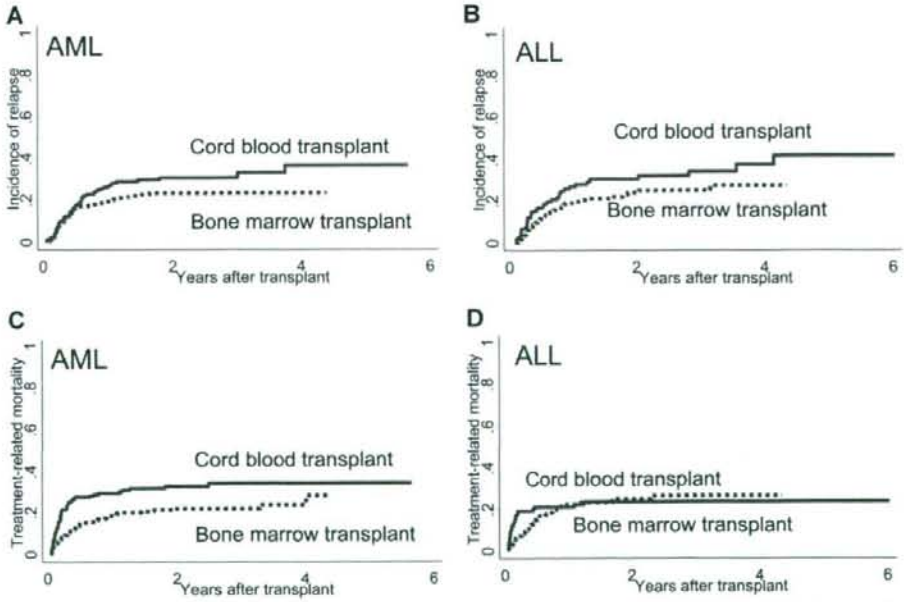


**Figure 2. Cumulative incidence of relapse or TRM of recipients of CB or BM among patients with AML or ALL.** For patients with AML, the cumulative incidence of (A) relapse (CB vs BM = 31% vs 24% at 2 years, $P = .068$) and (C) TRM (CB vs BM = 33% vs 22% at 2 years, $P = .004$) was higher in CB recipients. For patients with ALL, the cumulative incidence of relapse (B) was higher in CB recipients with marginal significance (CB vs BM = 31% vs 24% at 2 years, $P = .085$), but the incidence of TRM (D) was similar in CB and BM recipients (CB vs BM = 24% vs 25% at 2 years, $P = .83$).

**Table 4. Causes of death after transplantation of unrelated cord blood or unrelated bone marrow among patients with acute myeloid leukemia or acute lymphoblastic leukemia**

| | Acute myeloid leukemia | | Acute lymphoblastic leukemia | |
|---|---|---|---|---|
| Cause of death | UCBT | UBMT | UCBT | UBMT |
| Recurrence of disease | 35 (37) | 34 (33) | 18 (36) | 34 (41) |
| Graft failure/rejection | 3 (3) | 4 (4) | 0 (0) | 3 (4) |
| Graft-versus-host disease | 6 (6) | 7 (7) | 3 (6) | 5 (6) |
| Infection | 22 (23) | 19 (18) | 13 (26) | 11 (13) |
| Idiopathic pneumonia | 4 (4) | 4 (4) | 2 (4) | 6 (7) |
| Organ failure | 17 (18) | 17 (16) | 8 (16) | 10 (12) |
| Secondary cancer | 0 (0) | 1 (1) | 0 (0) | 0 (0) |
| Other causes | 5 (5) | 5 (5) | 2 (4) | 4 (5) |
| Unknown/data missing | 2 (2) | 13 (13) | 4 (8) | 10 (12) |
| Total | 94 (100) | 104 (100) | 50 (100) | 83 (100) |

Data are presented as n (%).

UCBT indicates unrelated cord blood transplantation; and UBMT, unrelated bone marrow transplantation.

unrelated CBT did not separate these 2 diseases. Our report is the first to show the result of disease-specific analyses with a sufficient number of patients.

For AML patients, the recipients of CB were more likely to have advanced leukemia at the time of transplantation, as reported previously, suggesting that CB was used as an alternative stem cell source in the later phase of unrelated donor searches, especially in adults.[11,12,14] A larger proportion of CB recipients with ALL had the Philadelphia chromosome abnormality, which correlates with highly aggressive ALL and usually requires urgent transplantation, in which CB has an advantage over BM.[21]

Different outcomes of mortality were found between AML and ALL in a controlled comparison using multivariate analyses. Whereas significantly lower OS and LFS rates were observed in CB recipients with AML, rates of overall mortality and treatment failure were similar between CB and BM recipients with ALL. The relapse rate was not different between CBT and BMT in patients with both AML and ALL, which was consistent with previous reports.[11-13] In adult patients with ALL, a previous report showed no difference in the outcome of related compared with unrelated BM or peripheral blood transplantation in 1CR.[22] Favorable disease status at transplantation could be a more important factor affecting outcome rather than the type of stem cell source or donor type in patients with ALL. It is notable that TRM in HLA allele-matched unrelated BM recipients with AML was quite low in our study. This is probably associated with the low incidence of acute and chronic GVHD in the Japanese population, which is thought to be the result of genetic homogeneity.[23-26] Among patients with AML, although the difference was not statistically significant, a higher trend of TRM observed in CB recipients might be associated with higher overall and TRM rates in CB recipients. Reasons for higher TRM could include the graft source and delayed neutrophil recovery. Better supportive care is required after CBT for patients going through a prolonged neutropenic period. Development of better graft engineering or better conditioning regimens would help to decrease the TRM rate in CB recipients. Because relapse was the major cause of death in all groups, any attempt to decrease TRM should preserve the antileukemia effect to improve OS and LFS. Another reason for the higher TRM could be a higher risk patient population, higher risk for both disease status and comorbid conditions, requiring rapid transplantation. Searching for unrelated donors earlier and providing transplantation earlier in the disease course could help to decrease TRM in CB recipients.

Neutrophil and platelet recovery was slower in CB recipients with either AML or ALL, consistent with the results of previous reports.[11,12,27] Multiple studies have reported lower incidence of acute GVHD in CB recipients.[8-10,12,13] In our study, particularly in patients with ALL, the risk of developing grade 2 to 4 acute GVHD in CB recipients was lower compared with BM recipients, which was reported to be lower compared with the incidence reported from Western countries.[23-25] The risk of developing chronic GVHD was similar between CB and BM recipient with either disease, but the risk of developing extensive-type chronic GVHD was lower in CB recipients; the difference was significant in patients with AML. It is notable that there was no increase in the incidence of acute or chronic GVHD in CB recipients among patients with either AML or ALL, despite HLA disparity.

For differences in outcomes between AML and ALL, one possibility is a difference of treatment before conditioning therapy. Most AML patients received a more intense treatment for induction and consolidation therapy compared with that for ALL. There was no adjustment made for previous treatment, and this could be the reason for higher mortality in CBT, which requires a longer time for neutrophil recovery. Another possible cause of the difference in outcomes is the difference in conditioning regimens. Preparative regimens were similar between CB and BM recipients among ALL patients. However, in patients with AML, the proportion of standard regimens, such as cyclophosphamide and TBI or busulfan and cyclophosphamide, was smaller among CB recipients. These differences in the distribution of preparative regimens were also seen in a previous report.[11] Although the final model was adjusted for conditioning regimens, we cannot rule out the possibility of an effect that larger CB recipients received additional or different chemotherapeutic agents compared with BM recipients among patients with AML. Although the difference was small, the median age of CB recipients with AML was 4 years older than CB recipients with ALL (median age, 38 vs 34 years, $P = .021$), which might have affected the higher mortality rate among CB recipients with AML. It is also possible that some unknown biologic aspects have contributed to these differences, and this would require further evaluation in future studies.

Further subgroup analyses indicated that the superiority of HLA allele-matched BM versus CB for OS was mostly found in patients with AML showing 1CR at conditioning. However, because of the limited numbers of patients in these subgroup analyses and the possibility of an unidentified bias in stem cell source selection, our findings should be verified by further analysis in a larger population.

In conclusion, we found different outcomes between patients with AML and ALL, indicating the importance of disease-specific analyses in alternative donor studies. HLA-A, -B low-resolution, and -DRB1 high-resolution 0 to 2 mismatched single-unit CB is a favorable alternative stem cell source for patients without a suitable related or 8 of 8 matched unrelated BM donor. In the absence of a suitable donor, unrelated CBT should be planned promptly to transplant the patient while in a better disease status and better clinical condition. For patients with AML, decreasing mortality, especially in the early phase of transplantation, is required to improve the outcome for CB recipients.

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## Authorship

Contribution: Y.A. and R.S. designed the study and wrote the paper; Y.A. analyzed results and made the figures; S. Kato and Y.M. designed the research; T.-N.I., H.A., and M. Takanashi reviewed and cleaned the Japan Cord Blood Bank Network data and reviewed the results; S. Taniguchi, S. Takahashi, S. Kai, H.S., Y. Kouzai, M.K., and T.F. submitted and cleaned the data; and S.O., M. Tsuchida, K.K., Y.M., and Y. Kodera reviewed and cleaned the Japan Marrow Donor Program data and reviewed the results.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

A complete list of members from the Japan Marrow Donor Program and the Japan Cord Blood Bank Network can be found in the Supplemental Appendix (available on the *Blood* website; see the Supplemental Materials link at the top of the online article).

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ORIGINAL ARTICLE

# CD16$^+$ CD56$^-$ NK cells in the peripheral blood of cord blood transplant recipients: a unique subset of NK cells possibly associated with graft-versus-leukemia effect

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**Abstract**

A marked increase in CD16$^+$ CD56$^-$ NK cells in the peripheral blood (PB) was observed in a cord blood transplant (CBT) recipient with refractory acute myeloid leukaemia (AML) in association with attaining molecular remission. CD16$^+$ CD56$^-$ NK cells isolated from the patient became CD16$^+$CD56$^+$NKG2D$^+$ when they were cultured in the presence of IL-2. Although cultured CD16$^+$CD56$^-$ NK cells retained the killer-cell immunoglobulin receptor (KIR)-ligand (KIR-L) specificity and the patient's leukemic cells expressed corresponding KIR ligands, they killed patient's leukemic cells expressing ULBP2. The cytotoxicity by cultured CD16$^+$CD56$^-$ NK cells was abrogated by anti-ULBP2 antibodies. When leukemic cells obtained at relapse after CBT were examined, both the ULBP2 expression and susceptibility to the cultured NK cells decreased in comparison to leukemic cells obtained before CBT. An increase in the CD16$^+$CD56$^-$ NK cell count ($0.5 \times 10^9$/L or more) in PB was observed in seven of 11 (64%) CBT recipients but in none of 13 bone marrow (BM) and eight peripheral blood stem cell (PBSC) transplant recipients examined during the similar period after transplantation. These findings suggest an increase in CD16$^+$CD56$^-$ NK cells to be a phenomenon unique to CBT recipients and that mature NK cells derived from this NK cell subset may contribute to the killing of leukemic cells expressing NKG2D ligands *in vivo*.

**Key words** CD56$^+$CD16$^-$ NK cell; NKG2D; graft-versus-leukemia; cord blood transplantation

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Cord blood transplantation (CBT) is being increasingly used for treatment of hematologic malignancies because its efficacy in the treatment of adult patients has been proven based on the findings of recent studies (1–4). One possible drawback of CBT is the less potent graft-versus-leukemia (GVL) effect than that of bone marrow transplantation (BMT) or peripheral blood stem cell transplantation (PBSCT) due to the immaturity of T cells contained in the cord blood (CB) graft (5). However, a recent study has shown the relapse rate after CBT to be comparable to that after BMT or PBSCT from human leukocyte antigen (HLA) matched sibling donors (1). Moreover, an analysis on the outcome of CBT for adult patients with acute myeloid leukaemia (AML) in Japan revealed that the rate of leukemic relapse after HLA-mismatched CBT was lower than that after HLA-matched CBT despite the fact that the incidence of graft-versus-host disease (GVHD) was similar between the two groups (Cord Blood Bank Network of Japan; unpublished observation). These clinical findings suggest that immunocompetent cells other than T cells may mediate the GVL effect after CBT.

Natural killer (NK) cells play a major role in the development of GVL effect after an HLA-mismatched stem cell transplantation (SCT) (6, 7). The GVL effect by NK cells depends on the presence of

HLA-mismatches and T cell recovery after SCT (8). Because CBT is often carried out from HLA-mismatched donors and is also associated with delayed T cell recovery (9–11), NK cells may be more likely to contribute to the development of GVL effect after CBT than after BMT or PBSCT. Few studies, however, have previously focused on the GVL effect by NK cells after CBT.

CB has a unique subset of NK cells characterized by a phenotype CD16$^+$CD56$^-$ (12–14). This NK cell subset is thought to be immature NK cells capable of differentiating into CD16$^+$CD56$^+$ NK cells (15). We recently observed an apparent increase in this NK cell subset in a patient who underwent reduced-intensity CBT for the treatment of relapsed AML after PBSCT from an HLA-compatible sibling donor. The patient achieved a molecular remission of AML in association with the NK cell increase. This observation prompted the characterization of CD16$^+$CD56$^-$ NK cells of this patient and other patients after allogeneic SCT. The present study revealed that CD16$^+$CD56$^-$ NK cells may potentially play a role in the development of the GVL effect in patients whose leukemic cells express NKG2D ligands.

## Materials and methods

### Patients

Peripheral blood (PB) was obtained from 11 CBT, 13 BMT (10 from related and three from unrelated donors), and eight PBSCT patients 2–135 months after transplantation. None of the patients had active graft-versus-host disease requiring corticosteroids at time of sampling or signs of infection. The original diseases of the CBT recipients included AML in four, non-Hodgkin's lymphoma (NHL) in four, myelodysplastic syndromes (MDS) in two and renal cell carcinoma in one. In the BMT recipients, those were AML in four, acute lymphoblastic leukemia (ALL) in four, MDS in three, chronic myeloid leukaemia (CML) in one, and aplastic anaemia (AA) in one while in the PBSCT recipients, those were AML in four, ALL in one, biphenotypic leukemia in two and NHL in one. All CBT recipients received an HLA-mismatched graft; the number of HLA mismatches between donor and recipient were two in seven, three in three and four in one. No HLA mismatch was observed between each donor and the BMT or PBSCT recipient except for six PBSCT recipients whose mismatches with their donors was one in two, two in one and three in one. This study was approved by our institutional review board and all patients gave their informed consent for the phenotypic and functional analyses of their peripheral blood mononuclear cells (PBMCs).

### Phenotype analysis of PBMC after SCT and leukemia cells

The cell surface phenotype was determined by three-color flow cytometry. The cells were stained with various monoclonal antibodies (mAbs) specific to cell surface proteins including CD3, CD56, CD16, CD158a, CD158b (Becton Dickinson Pharmingen), NKG2A, NKG2D, NKp30, NKp44 and NKp46 (Beckman Coulter, Marseille, France). The expression of NKG2D ligands on leukemic cells from a CBT recipient was determined using mAbs specific to MICA/B (Becton Dickinson Pharmingen), ULBP1, ULBP2 and ULBP3 (R&D Systems, Minneapolis, MN).

### Cell separation

PBMCs were isolated using density gradient centrifugation. NK cells were enriched by negative selection using immunomagnetic beads (Dynal NK cell isolation kit; Dynal Biotech, Lake success, NY) according to the manufacturer's recommendation (16). NK cell purity was confirmed by flow cytometry. CD16$^+$CD56$^+$ and CD16$^+$CD56$^-$ NK cells were separated from the enriched NK cells with anti-CD56-coated microBeads (MACS) by passing them through two sequential large-scale columns (Milteny Biotec, Gladbach, Germany) according to the manufacturer's instructions. CD158b$^+$ and CD158b$^-$ NK cells were separated with anti-CD158b-FITC Abs and anti-FITC microbeads.

### NK cell culture

Isolated $2 \times 10^6$ CD16$^+$CD56$^+$ and CD16$^+$CD56$^-$ subsets were cultured with or without $2 \times 10^5$ irradiated (45 Gy) K562 cells transfected with the membrane-bound form of IL-15 and human 4-1BBL (K562-mb15-41BBL) kindly provided by Dr. Dario Campana of University of Tennessee College of Medicine (17) in RPMI1640 containing 10% fetal bovine serum (FBS), 50 U/mL penicillin, 50 μg/mL streptomycin and 100 IU/mL IL-2 for 14 d. The cultured NK cells were washed with RPMI1640 and then were used for the cytotoxicity assay.

### Transfection of 721-221 cells with retroviral vector

An HLA class I-negative B cell line 721–221 was transfected with retrovirus vectors containing HLA-C*0301 (.221-Cw3) or HLA-C*0401 (.221-Cw4) as described previously (18). Transfectants were selected in the presence of 0.1 mg/mL neomycin and 0.1 mg/mL puromycin. The surface expression of HLA-C molecules was confirmed by flow cytometry using a mAb HLA-ABC (Immunotech, Marseille, France). A clone exhibiting the highest

level of HLA-C expression was used as a target in the cytotoxicity assay.

### Cytotoxicity assay

NK cell cytotoxicity was assessed using the standard chromium release assay, as described previously (19). In blocking experiments, anti-ULBP Abs were added at 10 μg/mL to the $^{51}$Cr labeled target cells and target cells were incubated at 37°C for 30 min before the addition of NK cells. The percentage of specific lysis was calculated using the formula: 100 × (count per minute [cpm] released from test sample-cpm spontaneous release)/(cpm maximum release − cpm spontaneous release).

### Statistical analysis

The significance of difference in the PB CD16⁺CD56⁻ cell count between CBT recipients and recipients of BM, PBSCT, or healthy individual was assessed by Student's *t*-test. The significance of difference in the time of sampling after SCT between CBT, BMT and PBSCT was assessed by Mann-Whitney test. *P*-values $< 0.05$ were considered to be significant.

## Results

### An increase in the number of CD16⁺CD56⁻ NK cells in a CBT recipient

A 56-yr-old male (Patient 1) who relapsed with AML M0 after PBSCT from a sibling donor underwent CBT following preconditioning with fludarabin 125 mg/m$^2$, melphalan 80 mg/m$^2$, and 4 Gy TBI. The patient's leukemia was refractory to chemotherapy and there were 18% leukemic blasts in the PB at the time of preconditioning. He achieved complete chimerism in PB on day 22 after CBT. The WT1 copy number in BM RNA decreased from 13 000 copies/μg RNA before the start of preconditioning to 140 copies/μg RNA on day 60 (20). However, it rose to 1500 copies/μg RNA on day 80 after CBT. Although a molecular relapse was suspected, the WT1 copy number spontaneously decreased to 230 on day 172. Surface phenotype analysis of PB leukocytes on day 84 showed an increase in the count of CD3⁻CD16⁺CD56⁻ NK cells (Fig. 1). The CD16⁺CD56⁻ NK cell count remained as high as 3.2–4.5 × 10$^9$/L for the following 11 months during which he remained in remission. The patient eventually relapsed with AML and died 16 months after CBT. The unexpected long term remission after reduced-intensity CBT associated with an increase in the CD16⁺CD56⁻ NK cell count prompted the characterization of the CD16⁺CD56⁻ NK cells of this patient and other patients who underwent allogeneic SCT.

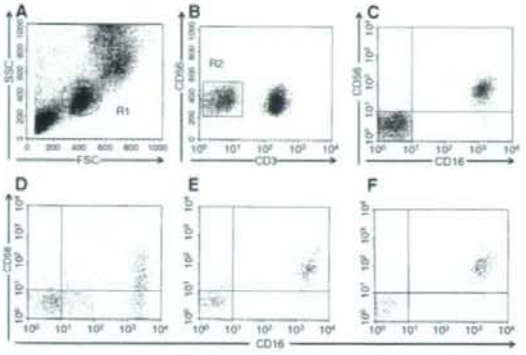


**Figure 1** Phenotype of the CD16⁺ NK cells in the peripheral blood. Representative results of flow cytometry on CD3⁻ lymphocytes from SCT recipients and healthy individuals are shown. Gates were set up to exclude any CD3⁺ lymphocytes as shown in (A) and (B); (C) a healthy individual; (D) a CBT recipient (Patient 1); (E) a BMT recipient; (F) a PBSCT recipient.

### CD16⁺CD56⁻ NK cells in PB of allogeneic SCT recipients

Because the presence of CD16⁺CD56⁻ NK cells has been reported to be characteristics of CB, the proportion of PB CD16⁺CD56⁻ NK cells as well as their absolute count was determined for other recipients of CB and the other stem cell grafts. An increase in the CD16⁺CD56⁻ NK cell count greater than 0.5 × 10$^9$/L was seen in seven of 11 CBT recipients but in none of 13 BMT and eight PBSCT recipients (Figs 1 and 2). There was no significant difference in the time of sampling after SCT between CBT recipients and BMT recipients ($P > 0.772$) or CBT recipients and PBSCT recipients ($P > 0.265$). Both the CD16⁺CD56⁻ NK cell proportion and the absolute count were significantly higher in CBT recipients than in other SCT recipients or in healthy individuals. In contrast, there were no significant differences in the count of other NK cell subsets including CD56$^{dim}$CD16⁺ and CD56$^{bright}$CD16⁻ cells among these three SCT recipient groups (data not shown). A CD16⁺CD56⁻ NK cell increase greater than 1.5 × 10$^9$/L was restricted to Patient 1 and another CBT recipient with NHL (Patient 2). The CD16⁺CD56⁻ NK cell counts of Patient 2, 5 months and 15 months after CBT were 1.5 × 10$^9$/L and 1.8 × 10$^9$/L, respectively.

### Surface phenotype of CD16⁺CD56⁻ NK cells and leukemic cells

To characterize this unusual NK cell subset, the surface phenotype was compared between CD16⁺CD56⁻ and CD16⁺CD56⁺ NK cells from Patient 1 and Patient 2