GVHD-model mice. Many models have been developed to manipulate, and to clarify, human T-cell-mediated acute GVHD in vivo [28]. The model that we used is optimal for evaluation of the killing ability of ASHmAbs, because it is very simple; it is reproducible; and most of all, the immune response is typically more robust to xenografts than to allografts.

We thought that graft failure requiring second transplantation was a potential adverse effect of kASHmAb administration, because the antibody might target for elimination all donor-type HLA-expressing cells, in particular, HSCs (which are known to express high levels of class I major histocompatibility complex) [21,22]. Unexpectedly, human PBMCs reappeared in mouse peripheral blood 2 to 3 weeks after kASHmAb administration at very high doses. This suggests that the cytotoxic effect of kASHmAb may preferentially injure mature PBMCs, sparing hematopoietic stem progenitor cells. The mechanism of this preference is not clear at present. It could be due to the fact that most HSCs in the bone marrow niche are in a quiescent state and resistant to cell damage such as apoptosis [29,30]. Although the concern persists that patients given kASHmAb are at risk of graft failure, optimization of kASHmAb dosages may solve this issue.

So far, we have generated kASHmAbs against HLA-A2 and HLA-A24 (Supplementary Figure E6, online only, available at www.exphem.org). These two kASHmAbs can cover approximately 23.1% of HLA-mismatched transplants (data from our institute). More kASHmAbs are needed to cover other HLA-mismatched transplants. For example, if we establish 12 more kASHmAbs (HLA-A*02:03, HLA-A*02:06, HLA-A*02:07, HLA-A*24:02, HLA-A*24:20, HLA-A*11:01, HLA-A*26:02, HLA-A*31:01, HLA-A*33:03, HLA-B*35:01, HLA-B*40:02, and HLA-B*51:01), at least 72.3% of GVHD cases (n = 199) in our institute can be treated. As an alternative to class I kASHmAbs, we have succeeded in generating several HLA class II ASHmAbs. These mAbs can potentially target activated T cells and antigen presenting cells that play a central role in eliciting GVHD. Administration of them alone or in combination with class I kASHmAbs may further enhance the anti-GVHD effect but minimize side effects.

Furthermore, if the diagnosis of GVHD can be made earlier using recently discovered biomarkers of early GVHD [31], for example, we may be able to treat GVHD safely with low doses of kASHmAb. Should target cells evade kASHmAbs by internalizing HLA molecules [32], kASHmAbs can be labeled with cell-damaging agents such as anti-cancer drugs and radio-isotopes [33,34], thereby inducing cell death. If HLA expression is downregulated [35,36], cell surface expression of HLA can be induced with drugs such as interferon-γ [37]. An additional concern in treatment of GVHD with kASHmAbs is their influence on the graft-versus-tumor (GVT) effect. Although little is known about how GVT and GVHD effects differ, clearly in most cases there will be no GVT effect without GVHD. To observe the GVT effect even after treatment of GVHD with either class I or class II kASHmAbs will pose intriguing questions.

In conclusion, kASHmAb is an antibody that accurately discriminates between donor and recipient cells and induces target cell death. As use of HLA-mismatched cord blood transplantation and haplo-identical HSCT increases, so may the incidence of GVHD. ASHmAbs may provide an effective treatment for GVHD, favorably influencing the outcome of allo-HSCT. We believe that kASHmAbs, ready-made agents created through well-designed antibody-processing technologies, have great potential in the clinical treatment of GVHD.

Acknowledgments

We thank all members of the Nakauchi laboratory for critical assessment of this work. We appreciate all the patients and healthy donors who volunteered to participate in this study as well as Eri Watanabe and Yuji Yamazaki (University of Tokyo) for expert technical assistance in FACS operation; Haruo Onoda (University of Tokyo) for assistance with histologic studies; Takaaki Konuma, Seiko Kato, Yasuhiro Ebihara (University of Tokyo) for clinical samples; and Shuji Matsuoka (Juntendo University), Shin Kaneko (Kyoto University), Tomohiro Ishigaki, Toshiharu Kimura, Keiichi Ito, Ryo Yamamoto, Hiroshi Watarai, Makoto Otsu, Masataka Kasai, and Motoo Watanabe (University of Tokyo) for technical support and helpful discussions.

Author contributions

YN and SY designed the study. YN conducted animal experiments, contributed to data analysis and wrote the first draft of the manuscript. JU and YO were responsible for histopathologic analysis of the mice. YN, SY, SCN, NW, ST, and HN contributed to the writing of the article. This work is part of the doctoral thesis of YN.

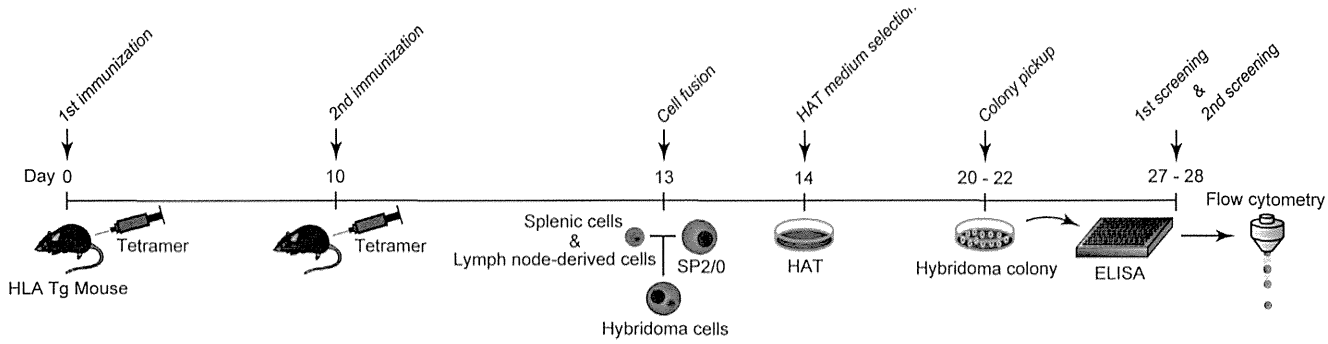
Conflict of interest disclosure

No financial interest/relationships with financial interest relating to the topic of this article have been declared.

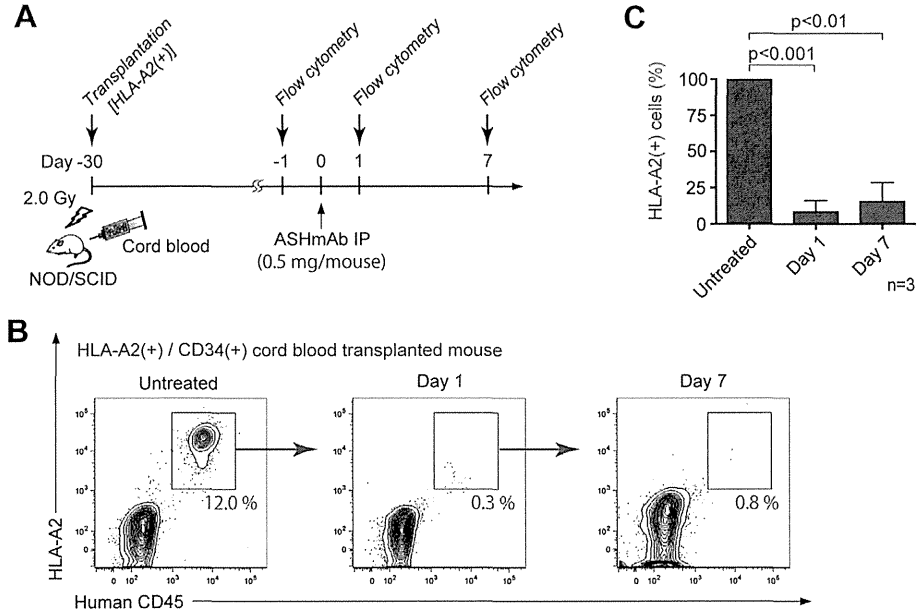
References

1. Storb R. Edward Donnall Thomas (1920–2012). Nature. 2012;491:334.
2. Ferrara JLM, Levine JE, Reddy P, Holler E. Graft-versus-host disease. Lancet. 2009;373:1550–1561.
3. Weiden PL, Flournoy N, Thomas ED, et al. Antileukemic effect of graft-versus-host disease in human recipients of allogeneic-marrow grafts. N Engl J Med. 1979;300:1068–1073.
4. Ho VT, Soiffer RJ. The history and future of T-cell depletion as graft-versus-host disease prophylaxis for allogeneic hematopoietic stem cell transplantation. Blood. 2001;98:3192–3204.

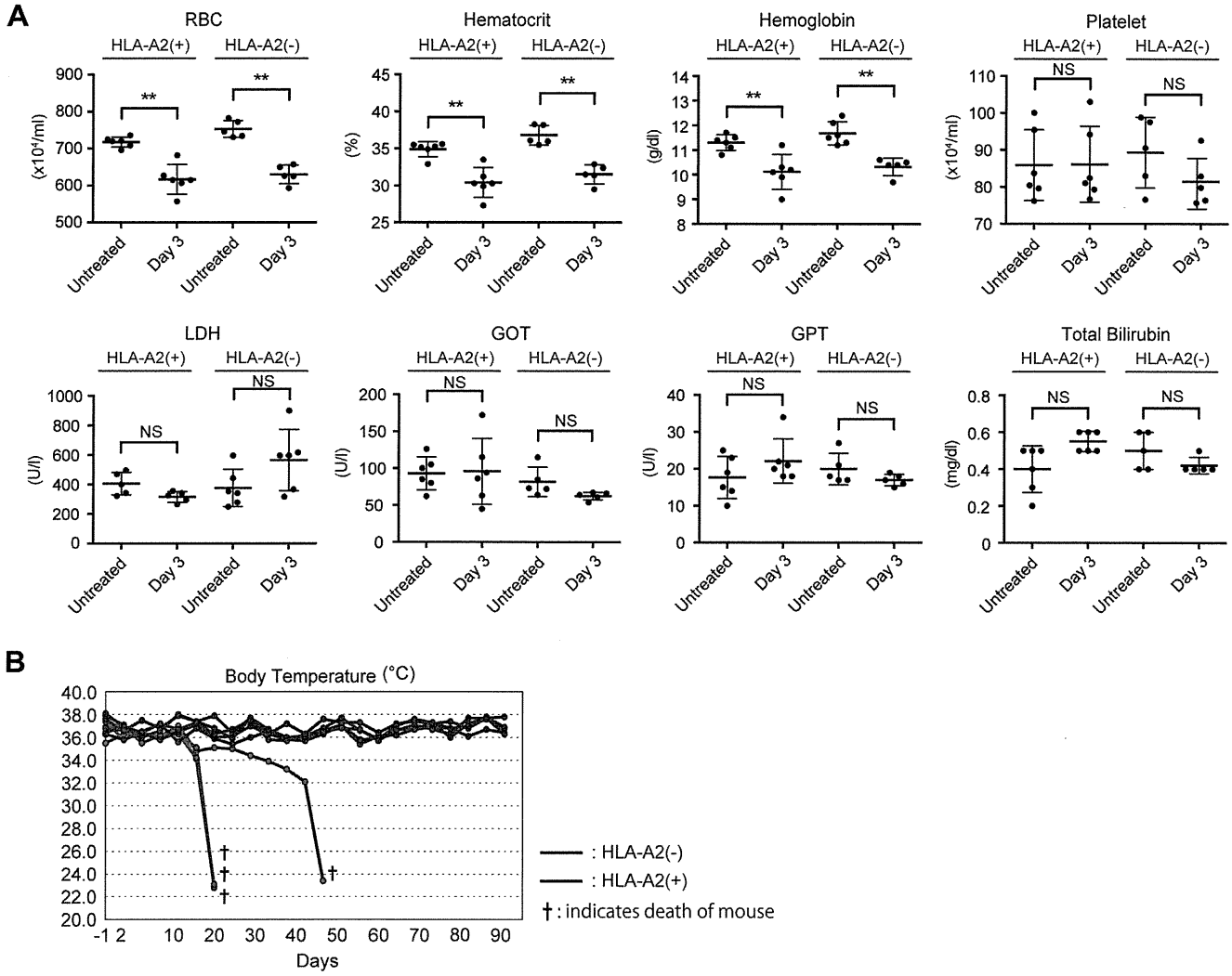
5. Storb R, Gyurkocza B, Storer BE, et al. Graft-versus-host disease and graft-versus-tumor effects after allogeneic hematopoietic cell transplantation. J Clin Oncol. 2013;31:1530–1538.
6. Warren EH, Deeg HJ. Dissecting graft-versus-leukemia from graft-versus-host-disease using novel strategies. Tissue Antigens. 2013;81: 183–193.
7. Martin PJ, Rizzo JD, Wingard JR, et al. First- and second-line systemic treatment of acute graft-versus-host disease: Recommendations of the American Society of Blood and Marrow Transplantation. Biol Blood Marrow Transplant. 2012;18:1150–1163.
8. Jørgensen KA, Koefoed-Nielsen PB, Karamperis N. Calcineurin phosphatase activity and immunosuppression. A review on the role of calcineurin phosphatase activity and the immunosuppressive effect of cyclosporin A and tacrolimus. Scand J Immunol. 2003;57:93–98.
9. Ikuya Tsuge SK. Comparison of antibody specificities of four anti-thymocyte/anti-lymphocyte globulin products. Curr Ther Res. 1995; 56:671–677.
10. Mueller TF. Mechanisms of action of thymoglobulin. Transplantation. 2007;84:S5–S10.
11. Knop S, Hebart H, Gratwohl A, et al. Treatment of steroid-resistant acute GVHD with OKT3 and high-dose steroids results in better disease control and lower incidence of infectious complications when compared to high-dose steroids alone: A randomized multicenter trial by the EBMT Chronic Leukemia Working Party. Leukemia. 2007;21: 1830–1833.
12. Martínez C, Solano C, Ferrá C, Sampol A, Valcárcel D, Pérez-Simón JA. Alemtuzumab as treatment of steroid-refractory acute graft-versus-host disease: Results of a phase II study. Biol Blood Marrow Transplant. 2009;15:639–642.
13. Kanda Y, Oshima K, Kako S, et al. In vivo T-cell depletion with alemtuzumab in allogeneic hematopoietic stem cell transplantation: Combined results of two studies on aplastic anemia and HLA-mismatched haploidentical transplantation. Am J Hematol. 2013;88:294–300.
14. Watanabe N, Takahashi S, Ishige M, et al. Recipient-derived cells after cord blood transplantation: Dynamics elucidated by multicolor FACS, reflecting graft failure and relapse. Biol Blood Marrow Transplant. 2008;14:693–701.
15. Yamazaki S, Suzuki N, Saito T, et al. A rapid and efficient strategy to generate allele-specific anti-HLA monoclonal antibodies. J Immunol Methods. 2009;343:56–60.
16. Karaki S, Kariyone A, Kato N, Kano K, Iwakura Y, Takiguchi M. HLA-B51 transgenic mice as recipients for production of polymorphic HLA-A, B-specific antibodies. Immunogenetics. 1993;37:139–142.
17. Ito R, Katano I, Kawai K, et al. Highly sensitive model for xenogenic GVHD using severe immunodeficient NOG mice. Transplantation. 2009;87:1654–1658.
18. Nishimori H, Maeda Y, Teshima T, et al. Synthetic retinoid Am80 ameliorates chronic graft-versus-host disease by down-regulating Th1 and Th17. Blood. 2012;119:285–295.
19. Nervi B, Rettig MP, Ritchey JK, et al. Factors affecting human T cell engraftment, trafficking, and associated xenogeneic graft-vs-host disease in NOD/SCID beta2mnull mice. Exp Hematol. 2007;35:1823–1838.
20. Anderson BE, McNiff J, Yan J, et al. Memory CD4+ T cells do not induce graft-versus-host disease. J Clin Invest. 2003;112:101–108.
21. Visser JW, Bauman JG, Mulder AH, Eliason JF, de Leeuw AM. Isolation of murine pluripotent hemopoietic stem cells. J Exp Med. 1984; 159:1576–1590.
22. Sabir HJ, Nehlin JO, Qanie D, et al. Separate developmental programs for HLA-A and -B cell surface expression during differentiation from embryonic stem cells to lymphocytes, adipocytes and osteoblasts. PLoS One. 2013;8:e54366.
23. Hod E, Schwartz J. Platelet transfusion refractoriness. Br J Haematol. 2008;142:348–360.
24. Takanashi M, Atsuta Y, Fujiwara K, et al. The impact of anti-HLA antibodies on unrelated cord blood transplantations. Blood. 2010;116: 2839–2846.
25. Shaz BH, Stowell SR, Hillyer CD. Transfusion-related acute lung injury: From bedside to bench and back. Blood. 2011; 117:1463–1471.
26. Loupy A, Hill GS, Jordan SC. The impact of donor-specific anti-HLA antibodies on late kidney allograft failure. Nat Rev Nephrol. 2012;8: 348–357.
27. Taniguchi K, Taniguchi K, Yoshihara S, et al. Donor-derived HLA antibody production in patients undergoing SCT from HLA antibody-positive donors. Bone Marrow Transplant. 2012;47:1338–1342.
28. Schroeder MA, DiPersio JF. Mouse models of graft-versus-host disease: Advances and limitations. Dis Model Mech. 2011;4: 318–333.
29. Yamazaki S, Ema H, Karlsson G, et al. Nonmyelinating Schwann cells maintain hematopoietic stem cell hibernation in the bone marrow niche. Cell. 2011;147:1146–1158.
30. Li L, Bhatia R. Stem cell quiescence. Clin Cancer Res. 2011;17:4936–4941.
31. Harris AC, Ferrara JLM, Levine JE. Advances in predicting acute GVHD. Br J Haematol. 2013;160:288–302.
32. Chatenoud L, Bach JF. Antigenic modulation: A major mechanism of antibody action. Immunol Today. 1984;5:20–25.
33. Carter P. Improving the efficacy of antibody-based cancer therapies. Nat Rev Cancer. 2001;1:118–129.
34. Gerber HP, Koehn FE, Abraham RT. The antibody–drug conjugate: An enabling modality for natural product-based cancer therapeutics. Nat Prod Rep. 2013;30:625–639.
35. Zachary AA, Kopchaliiska D, Jackson AM, Leffell MS. Immunogenetics and immunology in transplantation. Immunol Res. 2010;47: 232–239.
36. Del Campo AB, Carretero J, Aptsiauri N, Garrido F. Targeting HLA class I expression to increase tumor immunogenicity. Tissue Antigens. 2012;79:147–154.
37. Lippitz BE. Cytokine patterns in patients with cancer: A systematic review. Lancet Oncol. 2013;14:e218–e228.



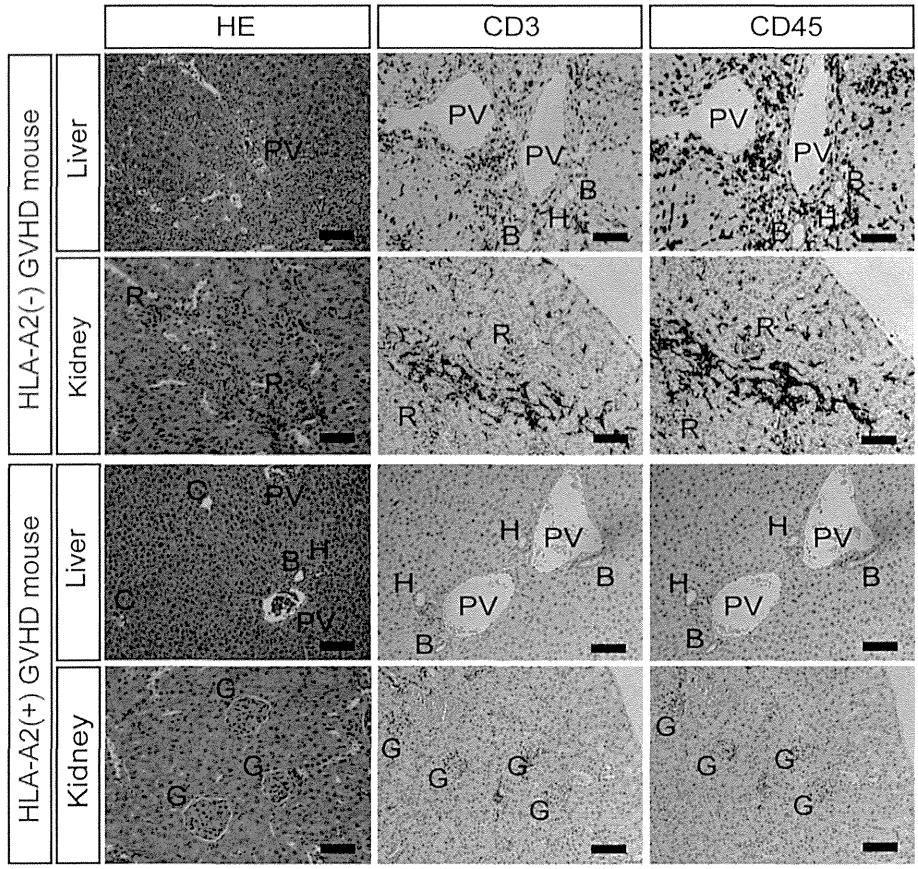
Supplementary Figure E1. Schedule for ASHmAb generation and screening. Day 0: First immunization of HLA-transgenic (Tg) mouse with HLA tetramer. Day 9: Mouse serum assessed for antibodies. Day 10: Second immunization (boost injection) of HLA-Tg mouse with HLA tetramer. Day 13: Fusion of spleen- and lymph node-derived B cells with SP2/0 myeloma cells. Fused cells were cultured in medium without aminopterin for 24 hours. Day 14: Suspension of fused cell-daughter cell candidates in methylcellulose-based HAT medium in 10-cm dishes for culture pending selection. Days 20-22: Selection of hybridoma colonies replating into 96-well plates filled with Dulbecco's modified Eagle medium. Days 27-28: Screening of supernatants from hybridoma culture; initially by enzyme-linked immunosorbent assay, secondarily by flow cytometry. ELISA = enzyme-linked immunosorbent assay; HAT = hypoxanthine-aminopterin-thymidine; HLA = human leukocyte antigen.



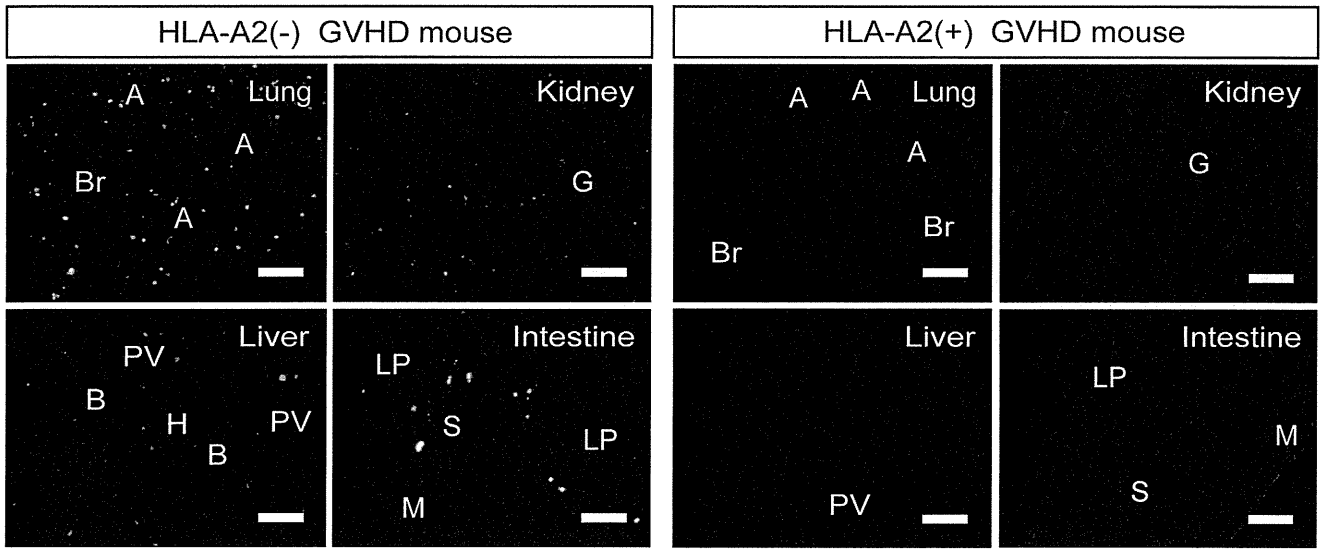
Supplementary Figure E2. Allele-specific cytotoxicity of HLA-A2-killing ASHmAb (kASHmAb) when injected intraperitoneally (IP). (A, B) Schedule and representative examples of in vivo assay of IP-administered HLA-A2 kASHmAb. Cord blood-derived CD34(+) / HLA-A2(+) cells were transplanted into irradiated NOD/SCID mice to create human-mouse bone marrow chimeric mice. One month after transplantation, flow-cytometric peripheral blood analysis was performed just before IP kASHmAb injection (0.5 mg, single dose) and on days 1 and 7 thereafter. Flow-cytometric analysis results show percentages of HLA-A2(+) / human CD45(+) cells in the marked gate. Doublets and dead cells were excluded. (C) Results of statistical analysis of (B). Blue bars represent HLA-A2(+) cells among humanized-mouse peripheral blood mononuclear cells (PBMC). Data shown as mean ± s.d (n=3, $p < 0.05$ by Student's t-test). ASHmAb = allele-specific anti-HLA monoclonal antibody; HLA = human leukocyte antigen.



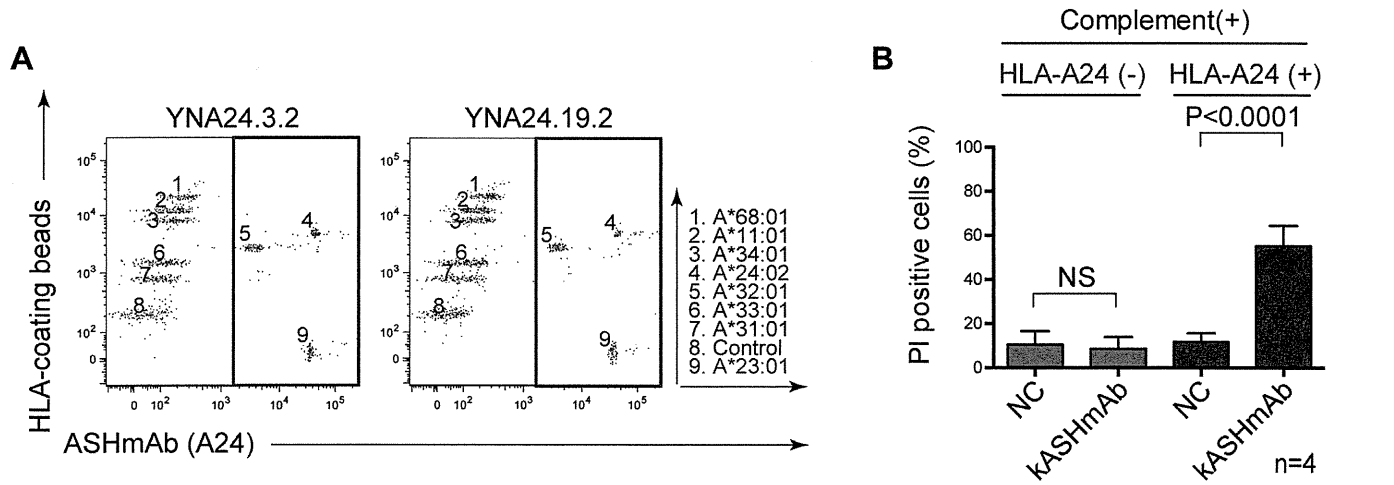
Supplementary Figure E3. Results of hematologic and biomarker studies of GVHD-model mice before and after kASHmAb treatment. (A) Blood was sampled from the retroorbital venous plexus and examined on days -1 (just before PBMC transplantation) and 3 (just before kASHmAb injection). We determined complete blood counts (CBC; hematocrit with red blood cell, hemoglobin, and platelet concentrations), serum total bilirubin concentration, LDH, GOT, and GPT activities in 5 HLA-A2(-) PBMC-transplanted mice and 6 HLA-A2(+) PBMC-transplanted mice. All mice were 7-8 weeks old. Paired t-test, $*P<0.05$; $**P<0.01$; NS: not significant. (B) Body temperature of GVHD-model mice before and after kASHmAb treatment [Red line; HLA-A2(-) PBMC-transplanted mice = GVHD(+), Blue line; HLA-A2(+) PBMC-transplanted mice = GVHD(-)]. Body temperature was determined twice weekly between 1500h and 1900h at room temperature 22°C and ambient humidity 55%. Percentage change from initial weight is shown. HLA = human leukocyte antigen; RBC = red blood cells; LDH = lactate dehydrogenase; GOT = glutamic oxaloacetic transaminase; GPT = glutamic pyruvic transaminase; GVHD = graft-versus-host disease; PBMC = peripheral blood mononuclear cell.



Supplementary Figure E4. Tissues from GVHD-model mice immunostained for human antigens (kidney and liver). Three weeks after transplantation, liver and kidneys were obtained from NOD/Shi-scid/IL-2Rγnull mice intravenously transplanted with human PBMCs [HLA-A2(-) or HLA-A2(+)]. Sections of routinely processed formalin-fixed, paraffin-embedded material were stained with anti-human CD3 and CD45 antibodies with 3,3'-diamino-benzidine (DAB) substrate as chromogen and hematoxylin as nuclear counterstain. While HLA-A2(+) GVHD mouse shows no human derived-cells, HLA-A2(-) GVHD mouse organs show human CD3 or CD45 cells; magnification, original image, x200, liver; x400, kidney). Scale bar 400 μm (kidney: 200 μm). B = Bile duct; C = Central vein; G = Glomerulus; GVHD = graft-versus-host disease; H = Hepatic artery; HE = hematoxylin and eosin; HLA = human leukocyte antigen; PBMC = peripheral blood mononuclear cell ; PV = Portal vein; R = Renal corpuscle.



Supplementary Figure E5. Apoptosis in sections of organs of HLA-A2(-) and HLA-A2(+) GVHD-model mice after TdT-mediated dUTP nick end-labeling (TUNEL). Using routinely processed formalin-fixed, paraffin-embedded material, we evaluated apoptosis in sections of liver, lungs, intestine, and kidneys of HLA-A2(-) and HLA-A2(+) GVHD-model mice, obtained 3 weeks after transplantation, by light microscopy after TdT-mediated dUTP nick end-labeling. Green dots indicate apoptotic cells. Magnification, original images, x200. Scale bars 400 μm. A = Alveoli; B = Bile duct; Br = Bronchiole; G = Glomerulus; GVHD = graft-versus-host disease; H = Hepatic artery; HLA = human leukocyte antigen; LP = Lamina propria; M = Muscularis; PV = Portal vein; S = submucosa.



Supplementary Figure E6. Allele-specific cytotoxicity of HLA-A24-killing ASHmAb (in vitro). (A) To establish ASHmAb-producing clones, on day 28 hybridomas (Supplementary Fig. 1) selected as producing anti-HLA antibody at initial screening were secondarily screened with FlowPRA, using flow cytometry (YNA24.3.2 and YNA24.19.2). Hybridoma-culture supernatants were incubated with HLA-coated beads. After incubation, the beads were washed and stained with secondary antibodies. Bead fluorescence intensities were measured using a flow cytometer and specificities of anti-HLA monoclonal antibodies were determined. Representative flow cytometry data are shown for a combination of FlowPRA beads coated with HLA-A*68:01, A*11:01, A*34:01, A*24:02, A*32:01, A*33:01, A*31:01, and control antigen or A*23:01. (B) Statistical analysis of in vitro killing assay using A24 ASHmAb with baby-rabbit complement. HLA-A24(-) cells (red bar) and HLA-A2(+) cells (blue bar) from healthy donors were each cultured with isotype control or A24 ASHmAb hybridoma supernatant. Percentages of dead cells were determined by propidium iodide staining and flow cytometry. Doublets and dead cells were excluded from flow-cytometry data. NC; Negative control. NS; Not significant. Data shown as mean ± s.d (n=4, $p < 0.05$ by Student's t-test). ASHmAb = allele-specific anti-HLA monoclonal antibody; HLA = human leukocyte antigen; kASHmAb = killing ASHmAb; NC = negative control; NS = nonsignificant; PI = propidium iodide.

Biol Blood Marrow Transplant 20 (2014) 396–401

Single-Unit Cord Blood Transplantation after Granulocyte Colony-Stimulating Factor–Combined Myeloablative Conditioning for Myeloid Malignancies Not in Remission

Takaaki Konuma [1,*], Seiko Kato [1], Jun Ooi [2], Maki Oiwa-Monna [1], Yasuhiro Ebihara [1], Shinji Mochizuki [1], Koichiro Yuji [1], Nobuhiro Ohno [1], Toyotaka Kawamata [1], Norihide Jo [1], Kazuaki Yokoyama [1], Kaoru Uchimaru [1], Shigetaka Asano [3], Arinobu Tojo [1], Satoshi Takahashi [1]

[1] *Department of Hematology/Oncology, Institute of Medical Science, University of Tokyo, Tokyo, Japan*
[2] *Department of Hematology/Oncology, Teikyo University School of Medicine, Tokyo, Japan*
[3] *System Medical Biology Laboratory, School of Advanced Science and Engineering, Waseda University, Tokyo, Japan*

Article history:
Received 9 November 2013
Accepted 4 December 2013

Key Words:
Cord blood transplantation
Granulocyte colony-stimulating factor
Acute myelogenous leukemia
Myelodysplastic syndrome
not in remission

A B S T R A C T

High disease burden in myeloablative allogeneic hematopoietic stem cell transplantation is associated with adverse outcomes in patients with acute myelogenous leukemia (AML) and myelodysplastic syndrome (MDS). Quiescent leukemia stem cells could be induced to enter cell cycle by granulocyte colony-stimulating factor (G-CSF) administration and become more susceptible to chemotherapy. We report on the outcome of unrelated cord blood transplantation (CBT) using a conditioning regimen of 12 Gy total body irradiation, G-CSF–combined high-dose cytarabine, and cyclophosphamide in 61 adult patients with AML or advanced MDS not in remission. With a median follow-up of 97 months, the probability of overall survival and cumulative incidence of relapse at 7 years were 61.4% and 30.5%, respectively. In multivariate analysis, poor-risk cytogenetics and high lactate dehydrogenase values at CBT were independently associated with inferior survival. These data demonstrate that CBT after G-CSF–combined myeloablative conditioning is a promising curative option for patients with myeloid malignancies not in remission.

INTRODUCTION

The prognoses of patients with acute myelogenous leukemia (AML) and advanced myelodysplastic syndrome (MDS) who have not achieved remission after chemotherapy have been poor. Although allogeneic hematopoietic stem cell transplantation (allo-HSCT) is the only potentially curative therapy for such patients, high disease burden has been reported to be associated with increased relapse or poor survival rate after allo-HSCT [1-9]. Recently, cord blood (CB) has been considered an acceptable alternative as a source of hematopoietic stem cells in unrelated allo-HSCT for adult patients without HLA-identical related or unrelated donors [9-16]. In comparison with other sources of allo-HSCT, one of the main advantages of using CB for patients with a high disease burden who require urgent transplantation is its rapid and convenient availability. Because it was shown that administration of granulocyte colony-stimulating factor (G-CSF) increased the susceptibility of cell-cycle–specific agent cytarabine in leukemia cells in vitro [17], we administered G-CSF–combined high-dose cytarabine in myeloablative conditioning for allo-HSCT [18,19] and reported that a G-CSF–combined conditioning regimen provided better engraftment and survival results in cord blood transplantation (CBT) for myeloid malignancies [13-16]. The objective of this retrospective study was to confirm the effects of CBT after G-CSF–combined myeloablative conditioning in adult patients with myeloid malignancies not in remission and to identify variables influencing long-term outcomes.

PATIENTS AND METHODS

Patients and Transplantation Procedures

This retrospective study included 61 consecutive adult patients who underwent unrelated transplantation using single-unit CB for AML or advanced MDS not in remission at our institute between 1998 and 2013. Thirty-two patients were included in our previous study [15,16] and extended the follow-up. The diagnoses of AML and MDS were made according to the World Health Organization classification. Advanced MDS was defined as having refractory anemia with excess blasts type 1 or refractory anemia with excess blasts type 2 by World Health Organization classification. Myeloid malignancies not in remission were defined as more than 5% blasts in the bone marrow (BM), or circulating blasts in peripheral blood (PB) or central nervous system. The cytogenetic subgroups were defined according to the Southwest Oncology Group/Eastern Cooperative Oncology Group criteria for AML [20] and International Prognostic Scoring System criteria for MDS [21]. All patients received 12 Gy total body irradiation (TBI) in 4 divided fractions on days −8 and −7, cytarabine on days −5 and −4 (total dose 12 g/m2, and 3 g/m2 every 12 hours for 2 days) with 5 μg/kg G-CSF (lenograstim) from 12 hours before the first dose of cytarabine to the end of cytarabine dosing, and cyclophosphamide (total dose 120 mg/kg) on days −3 and −2 [15,16]. Fifty-eight patients received cyclosporine (CSP) (3 mg/kg/day) with a short course of methotrexate (15 mg/m2 on day +1 and 10 mg/m2 on days +3 and +6), and 3 patients received CSP only as graft-versus-host disease (GVHD) prophylaxis. CB units were obtained from the Japanese Cord Blood Bank Network. Donor-recipient HLA-matching status was based on antigen level HLA-A and -B and on allele level HLA-DRB1 typing. All patients received similar supportive care and CB units were

Financial disclosure: See Acknowledgments on page 400.

* Correspondence and reprint requests: Takaaki Konuma, Department of Hematology/Oncology, Institute of Medical Science, University of Tokyo, 4-6-1, Shirokanedai, Minato-ku, Tokyo 108-8639, Japan.
E-mail address: tkonuma@ims.u-tokyo.ac.jp (T. Konuma).

1083-8791/$ – see front matter © 2014 American Society for Blood and Marrow Transplantation.
http://dx.doi.org/10.1016/j.bbmt.2013.12.555

Table 1
Characteristics of Patients, Cord Blood Units, and Transplantation

Characteristic	Value
No. of patients	61
Sex	
Male	36 (59)
Female	25 (41)
Age, median (range), yr	41 (18-55)
CMV serostatus	
Positive	54 (86)
Negative	7 (11)
Disease type	
De novo AML	24 (39)
AML secondary to MDS	24 (39)
Advanced MDS*	13 (21)
Cytogenetics†	
Good	1 (2)
Intermediate	27 (44)
Poor	30 (49)
Unknown	3 (5)
Bone marrow blasts at CBT, median (range), %	17.7 (1.4-86.0)¶
< 25%	39
≥ 25%	22
Peripheral blood blasts at CBT, median (range), %	6.5 (0-68.5)
Absent	12
Present	49
LDH at CBT	
≤ ULN	41 (67)
> ULN	20 (33)
Disease status at CBT‡	
Untreated	31 (51)
Primary refractory	14 (23)
Refractory relapse	16 (26)
Time from diagnosis to CBT, median (range), mo	7 (1-219)
Conditioning regimen	
TBI12Gy+Ara-C/G-CSF+CY	61
GVHD prophylaxis	
CyclosporineA+methotrexate	58 (95)
CyclosporineA	3 (5)
Number of nucleated cells, median (range), $\times 10^7$/kg	2.43 (1.32-5.50)
Number of $CD34^+$cells, median (range), $\times 10^5$/kg	1.03 (.21-2.27)
HLA disparities§	
1	13 (21)
2	32 (52)
3	14 (22)
4	2 (3)

CMV indicates cytomegalovirus; AML, acute myelogenous leukemia; MDS, myelodysplastic syndrome; CBT, cord blood transplantation; LDH, lactate dehydrogenase; ULN, upper limit of normal; TBI, total body irradiation; Ara-C, cytosine arabinoside; G-CSF, granulocyte colony-stimulating factor; CY, cyclophosphamide; GVHD, graft-versus-host disease; HLA, human leukocyte antigen.
Data presented are n (%) unless otherwise indicated.
* Advanced MDS are defined as having refractory anemia with excess blasts-1 (RAEB-1) or RAEB-2 by WHO criteria.
† The cytogenetic subgroups according to the Southwest Oncology Group/Eastern Cooperative Oncology Group criteria for AML and International Prognostic Scoring System criteria for MDS.
‡ Untreated was defined as no treatment before conditioning regimen, indicating that the majority of patients with AML secondary to MDS or advanced MDS received CBT as an up-front treatment. Primary refractory was defined as failure to achieve complete remission with induction chemotherapy. Refractory relapse was defined as failure to achieve complete remission with salvage chemotherapy after first or subsequent relapse.
§ The number of HLA disparities, defined as the low resolution for HLA-A and -B and the high resolution for HLA-DRB1.
¶ The 5 patients with less than 5% blasts in the bone marrow included circulating blasts in peripheral blood (n = 3) or central nervous system (n = 2).

selected, as previously reported [15,16]. The institutional review board of the Institute of Medical Science, University of Tokyo approved this study. This study was conducted in accordance with the Declaration of Helsinki.

End Points and Statistical Analysis

The primary study end point was overall survival (OS), defined as time from the date of transplantation to the date of death or last contact. Secondary end points were relapse, including disease progression before engraftment; transplantation-related mortality (TRM); neutrophil and platelet engraftment; acute graft-versus-host disease (aGVHD); and chronic GVHD (cGVHD). Relapse was defined as morphologic evidence of disease in PB, BM, or extramedullary sites. TRM was defined as death during remission. Neutrophil engraftment was defined as the first of 3 consecutive days during which the absolute neutrophil count was at least .5 × 10^9/L. Platelet engraftment was achieved on the first of 3 days when the platelet count was higher than 50 × 10^9/L without transfusion support. Both aGVHD and cGVHD were graded according to the previously published criteria [22,23]. The incidence of aGVHD was evaluated in all engrafted patients, whereas the incidence of cGVHD was evaluated in engrafted patients surviving more than 100 days.

The probability of OS was estimated according to the Kaplan-Meier method, and the groups were compared using the log-rank test. The probabilities of relapse, TRM, neutrophil and platelet engraftment, and acute and chronic GVHD were estimated based on a cumulative incidence method to accommodate competing risks [24]. Multivariate analysis was performed with a Cox proportional hazard model adjusted for OS and Fine and Gray proportional hazards model for relapse [25]. The following variables were considered: age (< 45 versus ≥ 45 years), disease type (de novo AML versus AML secondary to MDS versus advanced MDS), cytogenetic risk (other than poor versus poor), proportion of blasts in BM (< 25 versus ≥ 25%), the presence of blasts in PB (absent versus present), lactate dehydrogenase (LDH) at CBT (≤ upper limit of normal versus > upper limit of normal), disease status at CBT (untreated versus primary refractory versus refractory relapse), cord blood nucleated cell count (< 2.5 versus ≥ 2.5 × 10^7/kg), and HLA disparities based on antigen level HLA-A and -B and allele level

HLA-DRB1 (≤ 2 versus ≥ 3). All statistical analyses were performed with EZR (Saitama Medical Center, Jichi Medical University, Saitama, Japan), a graphical user interface for R 2.13.0 (R Foundation for Statistical Computing, Vienna, Austria) [26]. $P < .05$ was considered significant. Analysis of data was performed in August 2013.

RESULTS

Patient and CB unit characteristics are shown in Table 1. The median age was 41 years (range, 18 to 55 years), the median number of nucleated cells was 2.43 $\times$ 10^7/kg (range, 1.32 to 5.50 $\times$ 10^7/kg), and the median number of CD34+ cells was 1.03 $\times$ 10^5/kg (range, .21 to 2.27 $\times$ 10^5/kg). Disease types were de novo AML in 24 patients, AML secondary to MDS in 24, and advanced MDS in 13. The majority of patients with de novo AML with multilineage dysplasia (n = 2), AML secondary to MDS (n = 19), or advanced MDS (n = 10) received CBT as an up-front treatment, which was classified as untreated group (n = 31). Among patients with primary refractory status (n = 14), 3 patients received CBT after the first cycle of induction chemotherapy. The median number of prior chemotherapy treatments before CBT for primary refractory status was 3 (range, 1 to 5). The median time from diagnosis to CBT was 7 months (range, 1 to 219 months), and the median period of follow-up for survivors after CBT was 97 months (range, 5 to 181 months).

The cumulative incidence of neutrophil recovery was 93.4% (95% confidence interval [CI], 81.0% to 97.8%) at 60 days after CBT with a median time to achieve greater than .5 $\times$ 10^9/L neutrophils of 22 days (range, 18 to 41 days). Disease progression before engraftment occurred in 2 patients. The cumulative incidence of platelet recovery was 78.7% (95% CI, 65.7% to 87.2%) at 100 days after CBT with a median time to an untransfused platelet count greater than 50 $\times$ 10^9/L of 50 days (range, 30 to 179 days). The cumulative incidences of grade II to IV acute GVHD and extensive chronic GVHD were 62.3% (95% CI, 48.7% to 73.2%) at 100 days and 32.9% (95% CI, 21.4% to 44.9%) at 3 years after CBT, respectively. The probability of OS at 7 years was 61.4% (95% CI, 47.1% to 72.9%). The cumulative incidence of relapse at 7 years was 30.5% (95% CI, 19.2% to 42.6%). The cumulative incidence of TRM at 100 days and at 1 year was 6.6% (95% CI, 2.1% to 14.7%) and 8.2% (95% CI, 3.0% to 16.9%), respectively (Figure 1).

In multivariate analysis, poor-risk cytogenetics (hazard ratio [HR], 7.14; 95% CI, 2.33 to 21.80; $P < .001$) and high LDH value (HR, 4.00; 95% CI, 1.33 to 12.07; $P = .013$) were associated with inferior survival (Figure 2, Table 2). De novo AML (HR, 9.66; 95% CI, 1.06 to 87.75; $P = .044$), primary refractory status at CBT (HR, 6.47; 95% CI, 1.86 to 22.51; $P = .003$), and high LDH value (HR, 3.75; 95% CI, 1.11 to 12.57; $P = .032$) were associated with an increased relapse incidence (Table 3, Supplemental Figure 1). In contrast, the proportion of blasts in BM and the presence of blasts in PB did not show any impact on survival and relapse incidence.

DISCUSSION

Previous reports have suggested that the only potentially curative therapy for patients with myeloid malignancies not in remission is allo-HSCT. However, the incidence of relapse has been reported to be high, and several reports showed long-term survival rates of only 10% to 30% [1-6]. Several factors, including blasts in BM or PB, cytogenetics, and donor availability, have been associated with outcome. In this study, poor-risk cytogenetics and high LDH value were significantly associated with inferior OS. De novo AML, primary refractory status, and high LDH value were associated with increased relapse. However, we found no impact of disease burden on survival and relapse. In fact, several retrospective studies did not show any advantage of induction chemotherapy before allo-HSCT to reduce the disease burden for patients with advanced MDS or AML secondary to MDS [27-29]. Therefore, the majority of patients with advanced MDS or AML secondary to MDS received G-CSF–combined myeloablative conditioning followed by CBT without prior induction chemotherapy in our institute.

After physicians have decided that allo-HSCT is appropriate for patients with myeloid malignancy not in remission, the elective timing of the transplantation is the main advantage of CBT. In fact, CBT timing is decided depending on the patient's conditions, such as control of infection and disease burden. Such elective timing of CBT might have contributed to disease burden not being shown to influence outcome in our study. On the other hand, the use of CB as a source of hematopoietic stem cells could offer the opportunity for patients to receive allo-HSCT without related or unrelated donors. Moreover, the lower incidence of severe GVHD without compromising graft-versus-leukemia effects in CBT may also have contributed to long-term survival in our study.

Relapse is the most important cause of treatment failure after allo-HSCT, particularly in patients with myeloid malignancies not in remission. This is mainly due to the residual leukemic cells that have escaped the cytotoxic effect of conditioning before transplantation. To reduce disease relapse, the role of a more intense conditioning regimen has been analyzed extensively [30]. Since chemosensitization of leukemia cells with G-CSF enhances the cytotoxicity of the cell-cycle–specific agent cytarabine [17], we administered G-CSF–combined high-dose cytarabine in the standard conditioning regimen of TBI/cyclophosphamide. The clinical efficacy of concomitant use of G-CSF with chemotherapy has remained controversial in newly diagnosed or relapsed refractory AML and MDS [31,32]. Recently, Pabst et al. reported

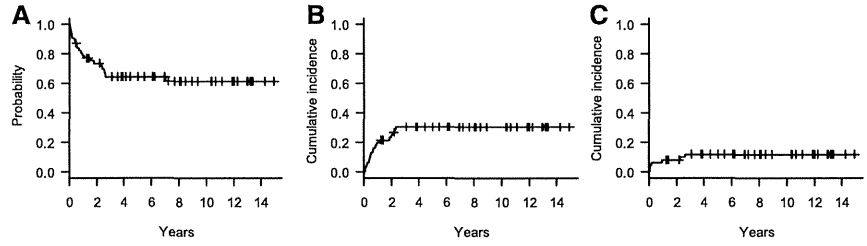


Figure 1. Probability of overall survival and cumulative incidences of relapse and transplant-related mortality after G-CSF–combined myeloablative CBT. Overall survival (A), relapse (B), and transplantation-related mortality (C) in 61 patients with AML or advanced MDS not in remission after G-CSF–combined myeloablative CBT.

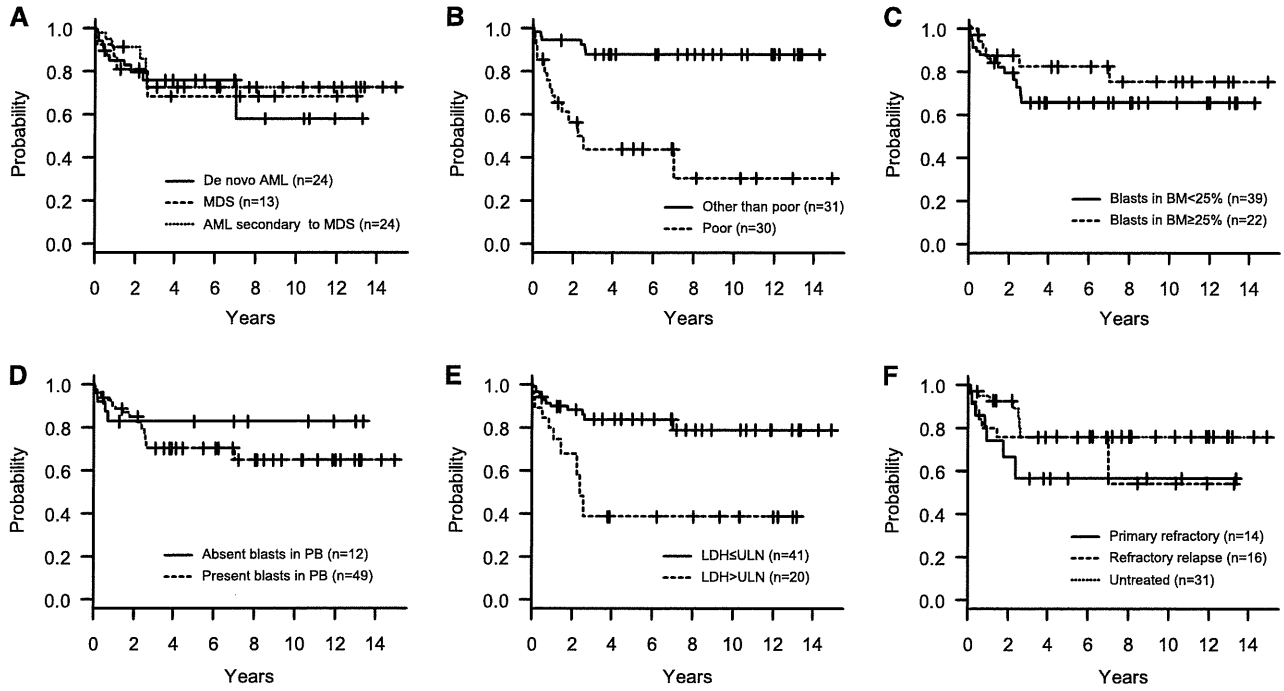


Figure 2. Adjusted probabilities of overall survival in 61 patients with AML and advanced MDS not in remission after G-CSF–combined myeloablative CBT. The adjusted probabilities of overall survival grouped according to the disease type (A), cytogenetic risk (B), the proportion of blasts in bone marrow (BM) (C), the presence of blasts in peripheral blood (PB) (D), the lactate dehydrogenase (LDH) value at cord blood transplantation (CBT) (E), and disease status at CBT (F). Multivariate analysis for overall survival is shown in Table 2.

Table 2
Univariate and Multivariate Analysis of Prognostic Factors for Survival

Variable	Univariate Analysis			Multivariate Analysis		
	Number	7-year OS (95% CI)	*P*	Hazard Ratio*	95% CI	*P*
Age						
< 45 years	36	63.5 (44.1-77.7)		1		
≥ 45	25	58.7 (36.7-75.4)	.555	.69	.25-1.86	.464
Disease type						
Advanced MDS	13	59.3 (27.5-81.0)		1		
AML secondary to MDS	24	74.4 (51.6-87.6)		.58	.13-2.54	.471
De novo AML	24	47.4 (23.0-68.4)	.234	.97	.18-5.16	.978
Cytogenetics†						
Other than poor	31	80.3 (61.3-90.6)		1		
Poor	30	38.9 (18.8-58.6)	.002	7.14	2.33-21.80	<.001
Bone marrow blasts at CBT, %						
< 25	39	58.0 (40.8-71.8)		1		
≥ 25	22	68.2 (41.2-84.7)	.297	.59	.16-2.09	.418
Peripheral blood blasts at CBT						
Absent	12	66.7 (33.7-86.0)		1		
Present	49	60.2 (44.0-73.1)	.983	1.18	.34-4.10	.787
LDH value at CBT						
≤ ULN	41	67.4 (48.9-80.4)		1		
> ULN	20	50.0 (27.1-69.2)	.147	4.00	1.33-12.07	.013
Disease status at CBT						
Untreated	31	71.1 (50.1-84.5)		1		
Primary refractory	14	50.0 (22.9-72.2)		2.76	.78-9.77	.114
Refractory relapse	16	50.0 (20.2-74.1)	.234	1.75	.30-10.22	.530
Number of nucleated cells, $\times 10^7$/kg						
≥ 2.5	29	59.2 (37.9-75.3)		1		
< 2.5	32	64.1 (44.3-78.4)	.989	.99	.38-2.58	.989
HLA disparities‡						
≤ 2	45	60.3 (43.7-73.4)		1		
≥ 3	16	65.0 (35.1-83.7)	.597	.98	.30-3.18	.975

MDS indicates myelodysplastic syndrome; AML, acute myelogenous leukemia; CBT, cord blood transplantation; LDH, lactate dehydrogenase; ULN, upper limit of normal; HLA, human leukocyte antigen; OS, overall survival; CI, confidence interval.

* Hazards ratio for overall mortality.

† The cytogenetic subgroups according to the Southwest Oncology Group/Eastern Cooperative Oncology Group criteria for AML and International Prognostic Scoring System criteria for MDS.

‡ The number of HLA disparities defined as the low resolution for HLA-A and -B and the high resolution for HLA–DRB1.

Table 3
Univariate and Multivariate Analysis of Prognostic Factors for Relapse

Variable	Univariate Analysis			Multivariate Analysis		
	Number	7-year Relapse (95% CI)	*P*	Hazard Ratio	95% CI	*P*
Age						
< 45	36	29.3 (15.0-45.2)		1		
≥ 45	25	32.0 (14.9-50.6)	.567	1.62	.50-5.17	.420
Disease type						
Advanced MDS	13	7.7 (.4-30.5)		1		
AML secondary to MDS	24	29.8 (12.9-49.0)		4.37	.38-49.80	.230
De novo AML	24	43.4 (22.4-62.7)	.096	9.66	1.06-87.75	.044
Cytogenetics*						
Other than poor	31	23.0 (9.9-39.2)		1		
Poor	30	38.2 (20.5-55.7)	.163	2.33	.90-5.97	.078
Bone marrow blasts at CBT, %						
< 25	39	26.0 (13.3-40.6)		1		
≥ 25	22	39.2 (18.0-59.9)	.397	1.72	.57-5.16	.330
Peripheral blood blasts at CBT						
Absent	12	16.7 (2.3-42.8)		1		
Present	49	33.8 (20.6-47.4)	.309	3.08	.40-23.70	.280
LDH value at CBT						
≤ ULN	41	25.6 (13.1-40.1)		1		
> ULN	20	40.0 (18.5-60.8)	.240	3.75	1.11-12.57	.032
Disease status at CBT						
Untreated	31	17.8 (6.3-34.1)		1		
Primary refractory	14	50.0 (21.4-73.3)		6.47	1.86-22.51	.003
Refractory relapse	16	37.5 (14.5-60.7)	.043	1.36	.26-7.05	.71
Number of nucleated cells, $\times 10^7$/kg						
≥ 2.5	29	35.5 (18.3-53.1)		1		
< 2.5	32	25.3 (11.7-41.5)	.525	.54	.14-2.12	.380
HLA disparities†						
≤ 2	45	34.0 (20.4-48.1)		1		
≥ 3	16	20.3 (4.5-43.9)	.306	.53	.11-2.49	.420

MDS indicates myelodysplastic syndrome; AML, acute myelogenous leukemia; CBT, cord blood transplantation; LDH, lactate dehydrogenase; ULN, upper limit of normal; HLA, human leukocyte antigen; CI, confidence interval.

* The cytogenetic subgroups according to the Southwest Oncology Group/Eastern Cooperative Oncology Group criteria for AML and International Prognostic Scoring System criteria for MDS.

† The number of HLA disparities defined as the low resolution for HLA-A and -B and the high resolution for HLA–DRB1.

significantly improved survival with concomitant use of G-CSF with escalated-dose, but not with conventional-dose cytarabine [31]. In the setting of allo-HSCT, the conditioning regimen consisting of G-CSF–combined high-dose cytarabine and TBI 12 Gy was feasible and might reduce post-transplantation relapse in patients with AML [18,19]. The presence of quiescent leukemia stem cells (LSCs), which are thought to be resistant to chemotherapy, might contribute to relapse after treatment. Recently, a xenograft model demonstrated that cytarabine with G-CSF recruited quiescent LSCs into a phase of the cell cycle, leading to enhanced elimination of LSCs within the niche [33]. This effect might have contributed to reduced relapse in our study. Although these findings should be confirmed in prospective studies, the combination of G-CSF–combined myeloablative conditioning with CBT offered a promising curative option for patients with myeloid malignancies not in remission.

ACKNOWLEDGMENTS

The authors thank all of the physicians and staff at the hospitals and the 8 cord blood banks in Japan for their help in this study. This work was supported in part by The Kobayashi Foundation.

Conflict of interest statement: There are no conflicts of interest to report.

Financial Disclosure: The authors have nothing to disclose.

SUPPLEMENTARY DATA

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbmt.2013.12.555.

REFERENCES

1. Duval M, Klein JP, He W, et al. Hematopoietic stem-cell transplantation for acute leukemia in relapse or primary induction failure. *J Clin Oncol.* 2010;28:3730-3738.
2. Craddock C, Labopin M, Pillai S, et al. Factors predicting outcome after unrelated donor stem cell transplantation in primary refractory acute myeloid leukaemia. *Leukemia.* 2011;25:808-813.
3. Todisco E, Ciceri F, Oldani E, et al. The CIBMTR score predicts survival of AML patients undergoing allogeneic transplantation with active disease after a myeloablative or reduced intensity conditioning: a retrospective analysis of the Gruppo Italiano Trapianto Di Midollo Osseo (GITMO). *Leukemia.* 2013;27:2086-2091.
4. Blum W, Bolwell BJ, Phillips G, et al. High disease burden is associated with poor outcomes for patients with acute myeloid leukemia not in remission who undergo unrelated donor cell transplantation. *Biol Blood Marrow Transplant.* 2006;12:61-67.
5. Fung HC, Stein A, Slovak ML, et al. A long-term follow-up report on allogeneic stem cell transplantation for patients with primary refractory acute myelogenous leukemia: impact of cytogenetic characteristics on transplantation outcome. *Biol Blood Marrow Transplant.* 2003;9:766-771.
6. Wong R, Shahjahan M, Wang X, et al. Prognostic factors for outcomes of patients with refractory or relapsed acute myelogenous leukemia or myelodysplastic syndromes undergoing allogeneic progenitor cell transplantation. *Biol Blood Marrow Transplant.* 2005;11:108-114.
7. Lim Z, Brand R, Martino R, et al. Allogeneic hematopoietic stem-cell transplantation for patients 50 years or older with myelodysplastic syndromes or secondary acute myeloid leukemia. *J Clin Oncol.* 2010;28:405-411.
8. Warlick ED, Cioc A, Defor T, et al. Allogeneic stem cell transplantation for adults with myelodysplastic syndromes: importance of pretransplant disease burden. *Biol Blood Marrow Transplant.* 2009;15:30-38.
9. Robin M, Sanz GF, Ionescu I, et al. Unrelated cord blood transplantation in adults with myelodysplasia or secondary acute myeloblastic leukemia: a survey on behalf of Eurocord and CLWP of EBMT. *Leukemia.* 2011;25:75-81.
10. Ballen KK, Gluckman E, Broxmeyer HE. Umbilical cord blood transplantation: the first 25 years and beyond. *Blood.* 2013;122:491-498.

11. Eapen M, Rocha V, Sanz G, et al. Effect of graft source on unrelated donor haemopoietic stem-cell transplantation in adults with acute leukaemia: a retrospective analysis. *Lancet Oncol.* 2010;11:653-660.
12. Mori T, Tanaka M, Kobayashi T, et al. Prospective multicenter study of single-unit cord blood transplantation with myeloablative conditioning for adult patients with high-risk hematologic malignancies. *Biol Blood Marrow Transplant.* 2013;19:486-491.
13. Sato A, Ooi J, Takahashi S, et al. Unrelated cord blood transplantation after myeloablative conditioning in adults with advanced myelodysplastic syndromes. *Bone Marrow Transplant.* 2011;46:257-261.
14. Ooi J, Takahashi S, Tomonari A, et al. Unrelated cord blood transplantation after myeloablative conditioning in adults with acute myelogenous leukemia. *Biol Blood Marrow Transplant.* 2008;14: 1341-1347.
15. Takahashi S, Ooi J, Tomonari A, et al. Comparative single-institute analysis of cord blood transplantation from unrelated donors with bone marrow or peripheral blood stem-cell transplants from related donors in adult patients with hematologic malignancies after myeloablative conditioning regimen. *Blood.* 2007;109:1322-1330.
16. Takahashi S, Iseki T, Ooi J, et al. Single-institute comparative analysis of unrelated bone marrow transplantation and cord blood transplantation for adult patients with hematologic malignancies. *Blood.* 2004;104:3813-3820.
17. Beekman R, Touw IP. G-CSF and its receptor in myeloid malignancy. *Blood.* 2010;115:5131-5136.
18. Takahashi S, Okamoto SI, Shirafuji N, et al. Recombinant human glycosylated granulocyte colony-stimulating factor (rhG-CSF)-combined regimen for allogeneic bone marrow transplantation in refractory acute myeloid leukemia. *Bone Marrow Transplant.* 1994;13:239-245.
19. Takahashi S, Oshima Y, Okamoto S, et al. Recombinant human granulocyte colony-stimulating factor (G-CSF) combined conditioning regimen for allogeneic bone marrow transplantation (BMT) in standard-risk myeloid leukemia. *Am J Hematol.* 1998;57:303-308.
20. Slovak ML, Kopecky KJ, Cassileth PA, et al. Karyotypic analysis predicts outcome of preremission and postremission therapy in adult acute myeloid leukemia: a Southwest Oncology Group/Eastern Cooperative Oncology Group Study. *Blood.* 2000;96:4075-4083.
21. Greenberg P, Cox C, LeBeau MM, et al. International scoring system for evaluating prognosis in myelodysplastic syndromes. *Blood.* 1997;89: 2079-2088.
22. Glucksberg H, Storb R, Fefer A, et al. Clinical manifestations of graft-versus-host disease in human recipients of marrow from HL-A-matched sibling donors. *Transplantation.* 1974;18:295-304.
23. Shulman HM, Sullivan KM, Weiden PL, et al. Chronic graft-versus-host syndrome in man. A long-term clinicopathologic study of 20 Seattle patients. *Am J Med.* 1980;69:204-217.
24. Gooley TA, Leisenring W, Crowley J, et al. Estimation of failure probabilities in the presence of competing risks: new representations of old estimators. *Stat Med.* 1999;18:695-706.
25. Fine JP, Gray RJ. A proportional hazards model for the subdistribution of a competing risk. *J Am Stat Assoc.* 1999;94:496-509.
26. Kanda Y. Investigation of the freely-available easy-to-use software "EZR" (Easy R) for medical statistics. *Bone Marrow Transplant.* 2013;48: 452-458.
27. Scott BL, Storer B, Loken MR, Storb R, Appelbaum FR, Deeg HJ. Pretransplantation induction chemotherapy and posttransplantation relapse in patients with advanced myelodysplastic syndrome. *Biol Blood Marrow Transplant.* 2005;11:65-73.
28. Nakai K, Kanda Y, Fukuhara S, et al. Value of chemotherapy before allogeneic hematopoietic stem cell transplantation from an HLA-identical sibling donor for myelodysplastic syndrome. *Leukemia.* 2005;19:396-401.
29. Anderson JE, Gooley TA, Schoch G, et al. Stem cell transplantation for secondary acute myeloid leukemia: evaluation of transplantation as initial therapy or following induction chemotherapy. *Blood.* 1997;89: 2578-2585.
30. Bensinger WI. High-dose Preparatory Regimens. In: Appelbaum FR, Forman SJ, Negrin RS, editors. *Thomas' Hematopoietic Cell Transplantation: Stem Cell Transplantation*, 4th ed. Cambridge, MA: Oxford: Blackwell Scientific Publications; 2009. p. 316-332.
31. Löwenberg B, van Putten W, Theobald M, et al. Effect of priming with granulocyte colony-stimulating factor on the outcome of chemotherapy for acute myeloid leukemia. *N Engl J Med.* 2003;349:743-752.
32. Pabst T, Vellenga E, van Putten W, et al. Favorable effect of priming with granulocyte colony-stimulating factor in remission induction of acute myeloid leukemia restricted to dose escalation of cytarabine. *Blood.* 2012;119:5367-5373.
33. Saito Y, Uchida N, Tanaka S, et al. Induction of cell cycle entry eliminates human leukemia stem cells in a mouse model of AML. *Nat Biotechnol.* 2010;28:275-280.