Table 2 Univariate and multivariate analysis of prognostic factors for overall mortality and relapse

	Overall mortality				Relapse			
	Univariate analysis		Multivariate analysis		Univariate analysis		Multivariate analysis	
	HR (95 % CI)	*P*	HR (95 % CI)	*P*	HR (95 % CI)	*P*	HR (95 % CI)	*P*
Graft source								
Related BMT/PBSCT	1		1		1		1	
Unrelated BMT	0.67(0.21–2.17)	0.51	0.45(0.10–1.87)	0.27	0.58(0.15–2.18)	0.43	0.82(0.20–3.27)	0.79
Unrelated CBT	0.32(0.11–0.89)	0.03	2.23(0.55–9.09)	0.26	0.36(0.13–1.03)	0.05	1.07(0.31–3.72)	0.91
Age at transplantation								
<30 years	1		1		1		1	
≥30 years	0.30(0.12–0.74)	<0.01	0.62(0.16–2.34)	0.48	0.53(0.21–1.34)	0.18	1.97(0.50–7.78)	0.33
WBC count at diagnosis								
$<30\times10^9$/L	1		1		1		1	
$\geq30\times10^9$/L	1.98(0.81–4.81)	0.12	2.07(0.65–6.58)	0.21	1.62(0.61–4.26)	0.33	1.50(0.46–4.88)	0.49
Use of TKI before transplantation								
Yes	1		1		1		1	
No	8.54(2.48–29.31)	<0.01	5.71(0.48–67.23)	0.16	4.36(1.43–13.30)	<0.01	6.23(1.35–28.67)	0.01
Disease status at transplantation								
CR1	1		1		1		1	
Beyond CR1	8.32(2.97–23.33)	<0.01	7.07(2.07–24.14)	<0.01	5.18(1.93–13.88)	<0.01	3.83(1.01–14.49)	0.04
Years of transplantation								
2001 to 2012	1		1		1		1	
1990 to 2000	4.95(1.94–12.6)	<0.01	2.63(0.55–12.46)	0.22	2.34(0.90–6.03)	0.07	0.52(0.17–1.55)	0.25

BMT indicates bone marrow transplantation; PBSCT, peripheral blood stem cell transplantation; CBT, cord blood transplantation; WBC, white blood cell; TKI, tyrosine kinase inhibitor; CR, complete remission; HR, hazard ratio; CI, confidence interval.

duration of TKI administration was 8.5 months (range, 1 to 71 months). Imatinib was used in 10 patients and dasatinib in 2 patients. Among five patients with hematological relapse, four patients died from disease progression with transient or no response, but one patient achieved and sustained CR after a second CBT. One of four patients with molecular detection of BCR-ABL developed hematological relapse at 9 months after TKI administration, and two patients were discontinued TKI administration due to continuous molecular remission for 54 and 57 months. The remaining one patient sustained CR under TKI administration for 32 months. On the other hand, one of three patients receiving maintenance therapy also developed hematological relapse at 45 months after TKI administration. The remaining two patients were discontinued TKI administration due to gastrointestinal toxicity or continuous molecular remission for 71 months. Compared with patients without administration of TKI after transplantation, which included five patients who received TKI only at the time of hematological relapse after transplantation, TKI administration after transplantation did not significantly impact survival (P=0.12) or relapse (P=0.31).

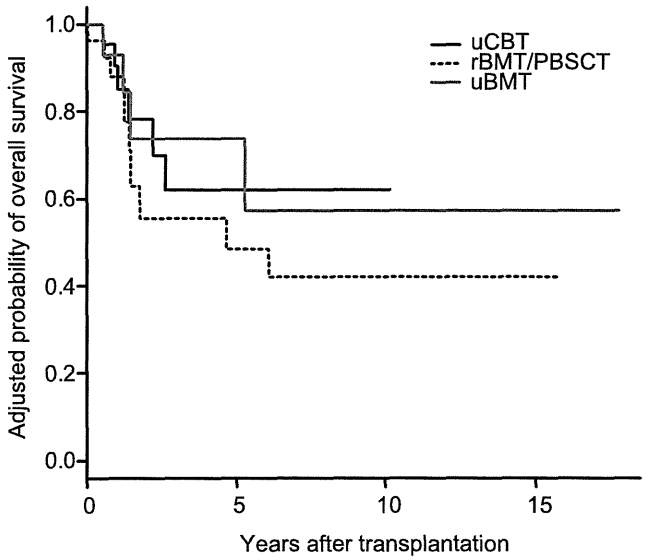

Fig. 1 Adjusted probability of overall survival in 40 patients with Ph+ ALL according to the graft source

Discussion

The objective of this study was to compare the transplant outcomes after CBT, uBMT, and rBMT/PBSCT following

myeloablative conditioning in patients with Ph+ALL. Since allo-HSCT is the only potentially curative therapy for Ph+ ALL, it is quite important to clarify the role of graft sources on allo-HSCT for patients with Ph+ALL. Although there were numerous potential confounding differences between the three groups of recipients in our cohorts, we showed there were no significant differences in overall mortality or relapse between the three groups of recipients after adjusting for confounding variables. On the other hand, similar to previous reports [12–14], we found disease status at transplantation to be one of the most important prognostic factors for outcome.

Comparative studies of CBT with other graft sources in allo-HSCT for adult ALL demonstrated that survival rates in CBT recipients were comparable to those in BMT/PBSCT recipients from related or unrelated donors [15–18]. Although these studies included various proportions of patients with Ph+ALL, the details of outcomes of Ph+ALL were not described. However, encouraging reports have shown the role of CBT for Ph+ALL. Onishi et al. compared CBT (n=8) and uBMT (n=12) in adult patients with Ph+ALL. The CBT group had significantly better OS than the uBMT group (P= 0.02) [19]. Piñana et al. reported the results of 45 patients with Ph+ALL who received CBT after myeloablative busulfan-based conditioning. They showed that the 5-year OS was 44 % [20]. In our study, the 5-year OS was 62 % for recipients of CBT. Yanada et al. demonstrated that a TBI-based myeloablative conditioning regimen showed significantly better survival compared with non-TBI myeloablative conditioning for Ph+ALL undergoing allo-HSCT [12]. In fact, all but one recipient of CBT received the TBI-based myeloablative conditioning regimen, which might explain the favorable results in our study. Recently, Bachnova et al. reported similar survival for myeloablative conditioning and reduced-intensity conditioning (RIC) for Ph+ALL undergoing allo-HSCT [21]. However, this study did not include CBT. Since the risk of graft failure might have remained higher after RIC compared with myeloablative conditioning [22], myeloablative conditioning should be considered if CB is used as the graft source. In addition, in comparison with other sources from unrelated donors, the rapid availability of the graft may be one of the major advantages of CBT because all patients with Ph+ALL require urgent allo-HSCT after achieving CR with induction chemotherapy. In our study, the median time from diagnosis to transplantation was slightly shorter among CBT or rBMT/PBSCT recipients than uBMT recipients (P=0.11), although the difference was not significant. The quick availability of CB grafts might also contribute to improved outcome in patients with Ph+ALL.

Although TKI have shown higher rates of CR in combination with conventional induction chemotherapy, it is unknown whether use of TKI before transplantation improves survival after allo-HSCT. Several studies have shown improved survival and decreased relapse incidences after allo-HSCT for patients who received imatinib before or after transplantation compared with control patients who never received imatinib [23–26], whereas another study did not [14]. Recently, Fielding et al. demonstrated that imatinib in combination with induction chemotherapy increased the rate of allo-HSCT, resulting in improved OS after allo-HSCT [4]. In our study, use of TKI before transplantation was not associated with survival, but was with relapse incidences. Nevertheless, disease status at transplantation was the most important factor for both survival and relapse. In fact, use of TKI before transplantation was associated with CR1 status at transplantation (P= 0.01). Therefore, use of TKI before transplantation may also have strongly affected the outcome in our study. Moreover, to reduce disease relapse, which is the most common cause of death after allo-HSCT for patients with Ph+ALL, the role of maintenance therapy with TKI after transplantation has been examined. Recently, Pfeifer et al. reported on a prospective randomized comparison of imatinib administration prophylactically or following detection of minimal residual disease (MRD) after transplantation. Prophylactic imatinib administration did not significantly impact survival [27]. In our study, TKI administration after transplantation did not have a significant impact on the outcomes for Ph+ALL, either. Further studies are warranted to evaluate the role of maintenance therapy with TKI on the outcome of allo-HSCT for Ph+ALL.

In conclusion, our data showed that graft source did not have a significant effect on the survival or relapse incidence after allo-HSCT for Ph+ALL. Disease status at transplantation rather than graft source was the most important factor in the outcome. However, these results should be interpreted with caution because this study was a retrospective single-institute analysis which only included a relatively small number of Japanese patients. Although these findings should be confirmed in larger prospective studies, unrelated CBT should be considered early in the course of patients with Ph+ALL when HLA-compatible related and unrelated donors are not available.

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Conflict of interest The authors declare no conflict of interest.

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Granulocyte colony-stimulating factor combined regimen in cord blood transplantation for acute myeloid leukemia: a nationwide retrospective analysis in Japan

Cord blood transplantation (CBT) from an unrelated donor has been increasingly used as an alternative transplant method for adult patients without human leukocyte antigen (HLA)-compatible related or unrelated donors.[1-4] However, the main disadvantage of CBT is still the limited cell dose, especially in adults, and this might contribute to a higher incidence of graft failure and delayed hematopoietic recovery, leading to higher transplant-related mortality (TRM) or overall mortality after CBT.

The purpose of a conditioning regimen prior to allogeneic hematopoietic stem cell transplantation (allo-HSCT) for hematologic malignancies is disease eradication and immunosuppression to overcome graft rejection. Although the standard myeloablative conditioning regimen prior to allo-HSCT has been total body irradiation (TBI) or busulfan combined with cyclophosphamide (CY) for patients with adult acute myeloid leukemia (AML), the role of an intensified conditioning regimen has been analyzed extensively in order to reduce the rate of post-transplant relapse and improve survival.[5-7] However, the majority of these studies analyzed patients receiving allo-HSCT using bone marrow (BM) or mobilized peripheral blood (PB) as a stem cell source. Therefore, an optimal myeloablative conditioning regimen prior to CBT for adult AML still has to be determined.

Granulocyte colony-stimulating factor (G-CSF) stimulates proliferation, differentiation, and functional activation of neutrophils. In clinical use, G-CSF is most commonly used for reducing the duration of neutropenia after chemotherapy and HSCT, and for the mobilization of hematopoietic stem/progenitor cells from the BM into PB

Table 1. Characteristics of patients, cord blood units, and transplantation.

	Total	TBI≥10Gy+Ara-C +CY	TBI≥10Gy+Ara-C /G-CSF+CY	TBI≥10Gy+other	TBI<10Gy+other or non-TBI	P
Number of patients	438	163	80	156	39	
Age						0.61
16-39 years	226(52 %)	74(45 %)	40(50 %)	81(52 %)	17(44 %)	
40-55 years	212(48 %)	89(54 %)	40(50 %)	75(48 %)	22(56 %)	
Sex						0.82
Male	217(50 %)	83(50 %)	42(53 %)	73(47 %)	19(49 %)	
Female	221(50 %)	80(49 %)	38(48 %)	83(56 %)	20(51 %)	
Disease status at CBT *						0.32
Standard risk	214 (49 %)	74(45 %)	45(56 %)	79(51 %)	16(41 %)	
High risk	221(50 %)	87(53 %)	35(44 %)	76(49 %)	23(59 %)	
Unknown	3(<1 %)	2(1 %)	0	1(<1 %)	0	
GVHD prophylaxis						<0.001
Cyclosporine A+methotrexate	304(69 %)	107(66 %)	74(93 %)	100(64 %)	23(59 %)	
Tacrolimus+methotrexate	134(31 %)	56(34 %)	6(8 %)	56(39 %)	16(41 %)	
Number of nucleated cells						0.71
$<2.5\times10^7$/kg	204(47 %)	70(43 %)	40(50 %)	75(48 %)	19(49 %)	
$\geq2.5\times10^7$/kg	200(46 %)	79(48 %)	33(41 %)	70(45 %)	18(37 %)	
Unknown	34(8 %)	14(9 %)	7(9 %)	11(7 %)	2(5 %)	
Number of $CD34^+$cells						0.23
$<1\times10^5$/kg	279(64 %)	110(67 %)	43(54 %)	101(64 %)	25(64 %)	
$\geq1\times10^5$/kg	144(33 %)	52(32 %)	34(43 %)	46(29 %)	12(31 %)	
Unknown	15(3 %)	1(<1 %)	3(4 %)	9(6 %)	2(5 %)	
HLA disparities†						0.24
0	40(9 %)	9(6 %)	7(9 %)	19(1 %)	5(1 %)	
1	148(34 %)	64(39 %)	22(28 %)	50(1 %)	12(1 %)	
≥2	250(57 %)	90(55 %)	51(64 %)	87(1 %)	22(1 %)	
ABO incompatibility						0.11
Match	152(35 %)	67(41 %)	29(36 %)	42(27 %)	14(39 %)	
Major/bidirectional mismatch	175(25 %)	30(18 %)	22(28 %)	47(30 %)	11(28 %)	
Minor mismatch	110(40 %)	66(40 %)	29(36 %)	66(42 %)	14(39 %)	
Unknown	1(<1 %)	0	0	1(<1 %)	0	
Year of CBT						<0.001
1998-2002	56(13 %)	12(7 %)	16(20 %)	24(15 %)	4(10 %)	
2003-2005	158(36 %)	40(25 %)	32(40 %)	64(41 %)	22(56 %)	
2006-2008	224(51 %)	111(68 %)	32(40 %)	68(44 %)	13(33 %)	

Disease status at CBT was classified as standard risk or high risk; complete remission without poor prognostic karyotype according to the MRC10 criteria was classified as standard risk, whereas patients in all other situations were classified as high risk. †The number of HLA disparities was defined as low resolution for HLA-A, -B, and –DR in graft-versus-host direction. Ara-C: cytosine arabinoside; CBT: cord blood transplantation; CY: cyclophosphamide; G-CSF: granulocyte colony-stimulating factor; GvHD: graft-versus-host disease; HLA: human leukocyte antigen; TBI: total body irradiation.

for HSCT. Furthermore, since administration of G-CSF increases the susceptibility to cytarabine arabinoside (Ara-C) through induction of cell cycle entry of dormant leukemia cells,[8,9] the efficacy of concomitant use of G-CSF and chemotherapy has been analyzed.[10,11] Several studies, as well as our own single institute studies, have demonstrated that G-CSF combined with myeloablative conditioning prior to allo-HSCT could be safely and effectively used for patients with myeloid malignancies in a single arm trial.[9,12,13] However, there has been no comparative study of transplant outcomes for AML after allo-HSCT following a conditioning regimen with or without G-CSF. This retrospective study is the first to assess the effect of a G-CSF combination in a myeloablative conditioning regimen for CBT on the transplant outcome in adult AML patients in Japan. Patients and study methods are described in the *Online Supplementary Appendix*.

Characteristics of patients and cord blood units are shown in Table 1. There was a significant difference in cumulative incidence of neutrophil recovery among the four groups in univariate analysis (P<0.001) (Figure 1A). In the multivariate analysis, the hazard risk of neutrophil engraftment was significantly higher in the TBI≥10Gy+Ara-C/G-CSF+CY group (P<0.001) and lower in the TBI≥10Gy+other group (P=0.03) and TBI<10Gy+other or non-TBI group (P<0.001) compared with the TBI≥10Gy+Ara-C+CY group (Table 2). Among patients achieving neutrophil engraftment, neutrophil recovery times were significantly shorter in the TBI≥10Gy+Ara-C/G-CSF+CY group compared with the TBI≥10Gy+Ara-C+CY group (P<0.001). There was a significant difference in cumulative incidence of platelet recovery among the four groups in univariate analysis (P<0.001) (Figure 1B). Multivariate analysis showed no significant difference between the TBI≥10Gy+Ara-C+CY group and TBI≥10Gy+Ara-C/G-CSF+CY group (P=0.14). However, the hazard risk of platelet engraftment was significantly lower in the TBI≥10Gy+other group (P<0.001) and TBI<10Gy+other or non-TBI group (P<0.001) compared with the TBI≥10Gy+Ara-C+CY group (Table 2). Among patients achieving platelet engraftment, there was no significant difference in platelet recovery times among the four groups (P=0.32).

Among patients in the entire cohort, the cumulative incidence of TRM at 100 days and at one year was 17% (95%CI: 13%-20%) and 22% (95%CI: 18%-26%), respectively. There was no significant difference in cumulative incidence of TRM at one year among the four groups in univariate analysis (P=0.19) (Figure 1C). Multivariate analysis of TRM, adjusting for other variables, showed no significant difference between the TBI≥10Gy+Ara-C+CY group and the TBI≥10Gy+Ara-C/G-CSF+CY group (P=0.67), TBI≥10Gy+other group (P=0.25), or TBI<10Gy+other or non-TBI group (P=0.95) (Table 2). The cumulative incidence of relapse at three years was 30% (95%CI: 25%-35%) in the entire cohort. There was no significant difference in cumulative incidence of relapse at three years among the four groups (P=0.05) (Figure 1D). In multivariate analysis, the hazard risk of relapse was lower in the TBI≥10Gy+Ara-C/G-CSF+CY group (P=0.03), but not in the TBI≥10Gy+other group (P=0.94) and TBI<10Gy+other or non-TBI group (P=0.73) compared with the TBI≥10Gy+Ara-C+CY group (Table 2).

Among the entire cohort, the probability of disease-free survival (DFS) and overall survival (OS) at three years was 44% (95%CI: 39%-49%) and 52% (95%CI: 46%-57%), respectively. There was a significant difference in the probability of DFS at three years among the four groups in univariate analysis (P=0.001) (Figure 1E). The probability of

Table 2. Multivariate analysis of transplant outcomes.

Outcomes	N. of patients	HR (95 % CI)	P
Neutrophil engraftment			
Conditioning regimen			
TBI≥10Gy+Ara-C+CY	163	1	Reference
TBI≥10Gy+Ara-C/G-CSF+CY	80	1.57(1.17-2.11)	0.002
TBI≥10Gy+other	156	0.76(0.58-0.98)	0.03
TBI<10Gy+other or non-TBI	39	0.46(0.27-0.78)	0.004
Number of CD34$^+$cells			
<1×10^5/kg	279	1	Reference
≥1×10^5/kg	144	1.56(1.23-1.98)	<0.001
Platelet engraftment			
Conditioning regimen			
TBI≥10Gy+Ara-C+CY	163	1	Reference
TBI≥10Gy+Ara-C/G-CSF+CY	80	1.25(0.92-1.71)	0.14
TBI≥10Gy+other	156	0.54(0.39-0.73)	<0.001
TBI<10Gy+other or non-TBI	39	0.40(0.23-0.67)	<0.001
Number of CD34$^+$cells			
<1×10^5/kg	279	1	Reference
≥1×10^5/kg	144	1.58(1.22-2.06)	<0.001
Transplant-related mortality			
Conditioning regimen			
TBI≥10Gy+Ara-C+CY	163	1	Reference
TBI≥10Gy+Ara-C/G-CSF+CY	80	0.86(0.44-1.68)	0.67
TBI≥10Gy+other	156	1.31(0.82-2.10)	0.25
TBI<10Gy+other or non-TBI	39	1.02(0.46-2.25)	0.95
Age			
<40 years	226	1	Reference
≥40 years	212	1.64(1.08-2.49)	0.01
Disease status at CBT			
Standard risk	214	1	Reference
High risk	221	1.81(1.20-2.72)	0.004
Relapse			
Conditioning regimen			
TBI≥10Gy+Ara-C+CY	163	1	Reference
TBI≥10Gy+Ara-C/G-CSF+CY	80	0.45(0.21-0.95)	0.03
TBI≥10Gy+other	156	0.98(0.61-1.57)	0.94
TBI<10Gy+other or non-TBI	39	1.14(0.53-2.44)	0.73
Disease status at CBT			
Standard risk	214	1	Reference
High risk	221	3.28(2.16-4.98)	<0.001
Treatment failure			
Conditioning regimen			
TBI≥10Gy+Ara-C+CY	163	1	Reference
TBI≥10Gy+Ara-C/G-CSF+CY	80	0.57(0.36-0.91)	0.01
TBI≥10Gy+other	156	1.24(0.90-1.70)	0.17
TBI<10Gy+other or non-TBI	39	1.24(0.75-2.02)	0.39
Disease status at CBT			
Standard risk	214	1	Reference
High risk	221	3.10(2.29-4.19)	<0.001
Overall mortality			
Conditioning regimen			
TBI≥10Gy+Ara-C+CY	163	1	Reference
TBI≥10Gy+Ara-C/G-CSF+CY	80	0.52(0.31-0.87)	0.01
TBI≥10Gy+other	156	1.19(0.84-1.69)	0.31
TBI<10Gy+other or non-TBI	39	1.25(0.74-2.12)	0.39
Disease status at CBT			
Standard risk	214	1	Reference
High risk	221	2.68(1.93-3.71)	<0.001

The only significant variables other than conditioning regimen were described in each end point. Variables considered in multivariate analysis were conditioning regimen (TBI≥10Gy+Ara-C+CY vs. TBI≥10Gy+Ara-C/G-CSF+CY vs. TBI≥10Gy+other vs. TBI<10Gy+other or non-TBI), age (<40 vs. ≥40 years), patients' gender (male vs. female), disease status at CBT (standard risk vs. high risk), GVHD prophylaxis (cyclosporine A with methotrexate vs. tacrolimus with methotrexate), cord blood nucleated cell count (<2.5×10^7/kg vs. ≥2.5×10^7/kg), cord blood CD34$^+$ cell count (<1×10^5/kg vs. ≥1×10^5/kg), HLA disparities (0 vs. 1 vs. ≥2), donor–recipient ABO compatibility (match vs. major/bidirectional mismatch vs. minor mismatch), and year of CBT (1998-2002 vs. 2003-2005 vs. 2006-2008). Ara-C: cytosine arabinoside; CBT: cord blood transplantation; CI: confidence interval; CY: cyclophosphamide; G-CSF: granulocyte colony-stimulating factor; HR: hazard ratio; TBI: total body irradiation.

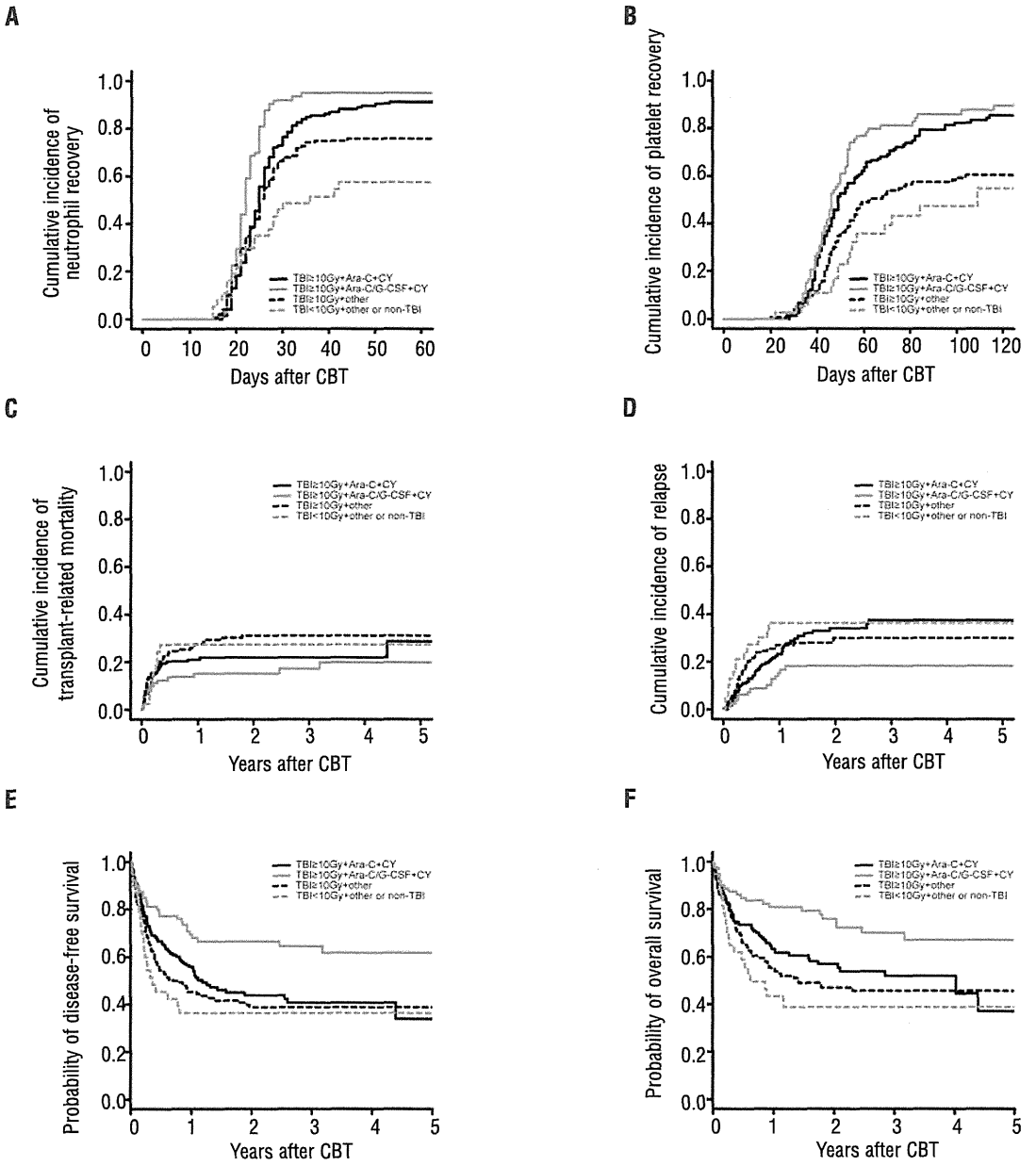

Figure 1. Cumulative incidences of neutrophil (A) and platelet (B) recovery, transplant-related mortality (TRM) (C) and relapse (D), probabilities of disease-free survival (E) and overall survival (F) after cord blood transplantation according to conditioning regimen. (A) Cumulative incidence of neutrophil recovery 42 days after CBT was 88% [95% confidence interval (CI): 81%-92%] in the TBI≥10Gy+Ara-C+CY group, 95% (95%CI: 85%-98%) in the TBI≥10Gy+Ara-C/G-CSF+CY group, 74% (95%CI: 66%-81%) in the TBI≥10Gy+other group, and 57% (95%CI: 37%-70%) in the TBI<10Gy+other or non-TBI group. Median times to neutrophil recovery were 24 days (range 17-53 days) in the TBI≥10Gy+Ara-C+CY group, 22 days (range 16-34 days) in the TBI≥10Gy+Ara-C/G-CSF+CY group, 23 days (range 15-65 days) in the TBI≥10Gy+other group, and 22 days (range 15-42 days) in the TBI<10Gy+other or non-TBI group. (B) Cumulative incidence of platelet recovery 100 days after CBT was 82% (95%CI: 74%-87%) in the TBI≥10Gy+Ara-C+CY group, 85% (95%CI: 74%-92%) in the TBI≥10Gy+Ara-C/G-CSF+CY group, 58% (95%CI: 48%-66%) in the TBI≥10Gy+other group, and 47% (95%CI: 25%-62%) in the TBI<10Gy+other or non-TBI group. Median times to platelet recovery were 46 days (range 28-168 days) in the TBI≥10Gy+Ara-C+CY group, 45.5 days (range 27-263 days) in the TBI≥10Gy+Ara-C/G-CSF+CY group, 48 days (range 20-249 days) in the TBI≥10Gy+other group, and 51 days (range 22-109 days) in the TBI<10Gy+other or non-TBI group. (C) Cumulative incidence of TRM at one year was 21% (95%CI: 15%-27%) in the TBI≥10Gy+Ara-C+CY group, 15% (95%CI: 8%-23%) in the TBI≥10Gy+Ara-C/G-CSF+CY group, 27% (95%CI: 20%-35%) in the TBI≥10Gy+other group, and 27% (95%CI: 14%-42%) in the TBI<10Gy+other or non-TBI group. (D) Cumulative incidence of relapse at three years was 37% (95%CI: 28%-46%) in the TBI≥10Gy+Ara-C+CY group, 18% (95%CI: 10%-27%) in the TBI≥10Gy+Ara-C/G-CSF+CY group, 30% (95%CI: 22%-37%) in the TBI≥10Gy+other group, and 36% (95%CI: 20%-52%) in the TBI<10Gy+other or non-TBI group. (E) Probability of disease-free survival at three years was 40% (95%CI: 31%-49%) for the TBI≥10Gy+Ara-C+CY group, 64% (95%CI: 52-74%) for the TBI≥10Gy+Ara-C/G-CSF+CY group, 38% (95%CI: 30%-47%) for the TBI≥10Gy+other group, and 36% (95%CI: 20%-51%) for the TBI<10Gy+other or non-TBI group. (F) Probability of overall survival was 52% (95%CI: 42%-60%) for the TBI≥10Gy+Ara-C+CY group, 70% (95%CI: 57%-79%) for the TBI≥10Gy+Ara-C/G-CSF+CY group, 45% (95%CI: 36%-54%) for the TBI≥10Gy+other group, and 39% (95%CI: 22%-55%) for the TBI<10Gy+other or non-TBI group. Median period of follow up for survivors (n=261) in the entire cohort was 24 months (range 1-122 months) after CBT.

DFS at three years was significantly better in the TBI≥10Gy+Ara-C/G-CSF+CY group compared with the TBI≥10Gy+Ara-C+CY group (P=0.02), the TBI≥10Gy+other group (P=0.002) and TBI<10Gy+other or non-TBI group (P=0.006). Multivariate analysis showed significantly decreased rates of treatment failure in the TBI≥10Gy+Ara-C/G-CSF+CY group compared with the TBI≥10Gy+Ara-C+CY group (P=0.01) (Table 2). In univariate analysis, there was a significant difference in the probability of OS at three years among the four groups (P=0.001) (Figure 1F). Multivariate analysis showed significantly decreased overall mortality in the TBI≥10Gy+Ara-C/G-CSF+CY group compared with the TBI≥10Gy+Ara-C+CY group (P=0.01) (Table 2). We also analyzed a subgroup of patients with standard risk (n=214) or high risk (n=221) at CBT. In standard-risk patients, the hazard risk of overall mortality (P=0.04), treatment failure (P=0.01) and relapse (P=0.002) was significantly lower in the TBI≥10Gy+Ara-C/G-CSF+CY group compared with the TBI≥10Gy+Ara-C+CY group, while that of high-risk patients was not (*Online Supplementary Table S1 and Figures S1 and S2*).

Anti-leukemia effects of allo-HSCT consist of leukemia eradication by both a conditioning regimen of chemotherapy with or without radiation and the graft-versus-leukemia (GvL) effect. Since relapse is the most common cause of death after allo-HSCT, an intensified conditioning regimen or enhancement of GvL effects is needed to reduce the incidence of relapse. Because of the difficulty in controlling the degree of GvL effects, an intensified conditioning regimen has been extensively analyzed. The several improvements to a typical conditioning regimen have included the addition of other agents to a standard myeloablative regimen, a dose escalation of drugs or TBI, or administration of drugs other than CY. Among these, the addition of other agents to a standard myeloablative regimen has been the most commonly used.[5,6] In fact, several studies have reported a decrease in the incidence of relapse following intensified conditioning, but with a higher TRM, and no improvement in survival was achieved.[5,7] Furthermore, the effect of adding high-dose Ara-C to a TBI/CY myeloablative conditioning regimen is controversial.[7] However, all of these studies analyzed patients receiving BM or mobilized PB stem cell transplantation from related or unrelated donors. This finding was not confirmed in CBT. In our study, neutrophil and platelet engraftment was significantly higher in the TBI≥10Gy+Ara-C+CY group compared with the TBI≥10Gy+other group, suggesting that the addition of Ara-C to TBI/CY was beneficial in terms of stable engraftment, but not for survival in CBT for AML.

Granulocyte colony-stimulating factor was originally identified as an agent for stimulation of neutrophil production. Although G-CSF is most commonly used to reduce the duration of neutropenia after chemotherapy, it is also commonly used for hematopoietic stem cell (HSC) mobilization for HSCT. Although the mechanism of HSC mobilization is not clearly understood, G-CSF could disrupt the contact between HSC in a BM niche, leading to HSC migration. In a mouse bone marrow transplantation (BMT) model, G-CSF prior to low-dose irradiation enhanced donor HSC engraftment.[14] This effect might be mainly due to the migration of recipient HSC from a BM niche by G-CSF treatment before transplantation. In fact, our data showed that neutrophil engraftment was significantly higher in the TBI≥10Gy+Ara-C/G-CSF+CY group compared with the TBI≥10Gy+Ara-C+CY group. These data suggest that the effect of the addition of G-CSF to a conditioning regimen could enhance neutrophil engraftment after CBT.

It has been reported that the administration of G-CSF increased the susceptibility of the cell-cycle-specific agent Ara-C in leukemia cells *in vitro* and in a xenograft model.[8,9,15] In clinical studies, several regimens have attempted to demonstrate the efficacy of concomitant use of G-CSF with chemotherapy for newly diagnosed AML.[10,11] We hypothesized that the addition of G-CSF to a conditioning regimen might improve outcome in an allo-HSCT setting. In our study, relapse was significantly lower in the TBI≥10Gy+Ara-C/G-CSF+CY group compared with the TBI≥10Gy+Ara-C+CY group. In a subgroup analysis, the effect of a G-CSF combination regimen for reduced relapse was significant in standard-risk but not high-risk patients. This is similar to a previous prospective randomized study of concomitant use of G-CSF with chemotherapy by Löwenberg *et al.*[11] Further studies are required to confirm which subgroup of patients with AML could benefit from a G-CSF combination regimen in CBT to reduce the incidence of relapse.

In conclusion, our data show that the addition of G-CSF-combined Ara-C to a TBI+CY conditioning regimen resulted in a significantly higher incidence of neutrophil engraftment and significantly better DFS and OS, and a reduced relapse rate in CBT for AML. Although these findings should be confirmed in prospective studies, a G-CSF-combined myeloablative conditioning regimen promotes better engraftment and survival results in CBT for AML.

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Effective treatment against severe graft-versus-host disease with allele-specific anti-HLA monoclonal antibody in a humanized mouse model

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Graft-versus-host disease (GVHD), mediated by donor-derived alloreactive T cells, is a major cause of nonrelapse mortality in allogeneic hematopoietic stem cell transplantation. Its therapy is not well-defined. We established allele-specific anti-human leukocyte antigen (HLA) monoclonal antibodies (ASHmAbs) that specifically target HLA molecules, with steady death of target-expressing cells. One such ASHmAb, against HLA-A*02:01 (A2-kASHmAb), was examined in a xenogeneic GVHD mouse model. To induce fatal GVHD, non-irradiated NOD/Shi-scid/IL-2Rγ^{null} mice were injected with healthy donor human peripheral blood mononuclear cells, some expressing HLA-A*02:01, some not. Administration of A2-kASHmAb promoted the survival of mice injected with HLA-A*02:01-expressing peripheral blood mononuclear cells ($p < 0.0001$) and, in humanized NOD/Shi-scid/IL-2Rγ^{null} mice, immediately cleared HLA-A*02:01-expressing human blood cells from mouse peripheral blood. Human peripheral blood mononuclear cells were again detectable in mouse blood 2 to 4 weeks after A2-kASHmAb administration, suggesting that kASHmAb may be safely administered to GVHD patients without permanently ablating the graft. This approach, different from those in existing GVHD pharmacotherapy, may open a new door for treatment of GVHD in HLA-mismatched allogeneic hematopoietic stem cell transplantation.

Allogeneic hematopoietic stem cell transplantation (allo-HSCT) can cure hematologic disorders like leukemia [1]. Cord blood or haplo-identical-donor HSCT holds promise for patients without a human leukocyte antigen (HLA)-matched donor. However, HLA-mismatched allo-HSCT may be complicated by alloreactive T cell-mediated graft-versus-host disease (GVHD), a major cause of mortality (after recurrent original disease) in allo-HSCT [2]. Although moderate GVHD induces a graft-versus-tumor effect and reduces disease relapse [3–6], severe GVHD confers a poor prognosis, as it is difficult to control. Agents used to treat GVHD include steroids [7], calcineurin inhibitors [8], and anti-thymocyte globulin (ATG) [9,10]. While these work well, they have many side effects (opportunistic infection, anaphylaxis-like reactions, etc.), which clinicians must carefully monitor.

Antithymocyte globulin endures as an antibody-based drug for GVHD treatment; however, its polyclonality and nonspecified target molecule cause it to react not only with T cells but also with other cells [9,10]. Current alternative therapies include monoclonal antibodies such as anti-CD3 [11] and anti-CD52 [12,13]. These antibodies have target molecules far more specific than those of ATG, but they cannot discriminate host cells from donor cells, resulting in opportunistic infection and other serious adverse effects.

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One major shortcoming of all current GVHD therapies is that they affect every cell without distinction between donor and recipient cells. Damage to cells of both the donor and the recipient causes side effects that impair long-term prognosis such as organ failure and immune compromise. Therefore, we suggest a novel therapeutic approach to GVHD using an antibody that specifically recognizes a donor HLA molecule and damages only donor-derived cells.

Flow cytometry using anti-HLA antibodies is useful for determination of chimerism and minimal residual disease after HLA-mismatched HSCT [14]. Allele-specific anti-HLA monoclonal antibodies (ASHmAbs) are notoriously more difficult to generate than are non-allele-specific anti-HLA antibodies. However, we established a rapid and efficient strategy to generate ASHmAb using HLA-transgenic mice [15]. Allele-specific antibodies thus generated can damage target cells quickly and selectively; we assessed whether or not ASHmAb can treat GVHD in a mouse model. This report describes the success of a novel therapeutic approach to GVHD with ASHmAb that recognizes donor and recipient cells allele-specifically, damaging only donor-derived cells.

Methods

Mice

NOD/Shi-Scid Jic (NOD/SCID) mice were purchased from Sankyo Laboratory (Ibaraki, Japan). NOD/Shi-scid/IL-2Rγ^{null} (NOG) mice were purchased from the Central Institute for Experimental Animals (Kanagawa, Japan). HLA-B51 (B*5101) transgenic mice were all bred and maintained in the Animal Research Facility of the Institute of Medical Science, University of Tokyo. Animal care in our laboratory was in accordance with the guidance of the University of Tokyo for animal and recombinant DNA experiments.

Human samples

Human peripheral blood mononuclear cells (PBMCs) were collected from healthy donors at the University of Tokyo, and human cord blood was obtained from the Japanese Red Cross Kanto-Koshinetsu Cord Blood Bank, according to protocols approved by the institutional review board of the Institute of Medical Science, University of Tokyo. Mononuclear cells were isolated by centrifugation of human PBMCs on Lymphosepar I (Immuno-Biological Laboratories, Gunma, Japan). The isolated cells were washed once with ice-cold phosphate-buffered saline, suspended in a small volume of phosphate-buffered saline containing 5% mouse serum (Dako, Glostrup, Denmark) to block nonspecific fluorescence-labeled antibody binding to immunoglobulin Fc receptors, and kept on ice until staining.

Flow cytometric analysis

Studies of human-derived cells in humanized mice or GVHD model mice were performed using fluorescein isothiocyanate-conjugated anti-HLA-A9 (OneLambda, Canoga Park, CA, USA) and anti-human lineage CD3, 14, 16, 19, 20, and 56 (BioLegend, San Diego, CA, USA); phycoerythrin (PE)-conjugated anti-HLA A2 (BD Biosciences, San Jose, CA) and anti-CD56 (BioLegend); PE/cyanine (Cy) 5-conjugated anti-CD235ab (BD Biosciences); peridinin chlorophyll/Cy5.5-conjugated anti-CD8 (BioLegend); PE/Cy7-conjugated anti-CD3, anti-CD33 (BD Biosciences) and anti-CD38 (BD Biosciences); allophycocyanin (APC)-conjugated anti-CD3 (BioLegend) and anti-CD34 (BD Biosciences); APC/Cy7-conjugated anti-CD19 (BioLegend); Pacific blue-conjugated anti-CD4 and anti-CD45 (BioLegend); and Alexa Fluor 405- or 647-conjugated anti-CD45 (BioLegend). Propidium iodide (PI, 1 μg/mL; Sigma–Aldrich, St. Louis, MO, USA) was added to samples to stain dead cells just before flow cytometric analysis. Becton-Dickinson AriaII & CantoII fluorescence-activated cell sorters were used for all multicolor FACS analysis and sorting. Flow cytometry standard data were analyzed using FlowJo software (Treestar, Ashland, OR, USA).

Establishment of ASHmAb

We followed the method of Yamazaki et al. to generate ASHmAb [15] using PE-conjugated anti-HLA A2 tetramer loaded with NLVPMVATV peptide (HLA-A*02:01-restricted human cytomegalovirus-specific epitope pp65) and PE-conjugated anti-HLA A24 tetramer loaded with QYDPVAALF peptide (HLA-A*24:02-restricted human cytomegalovirus-specific epitope pp65), which were purchased from Medical and Biological Laboratories (Nagoya, Japan). FlowPRA screening (OneLambda) was performed according to the manufacturer's instructions. The isotypes of kASHmAb selected were IgM (HLA-A9) and IgG2b (HLA-A2) by Rodent Monoclonal Isotyping Strips (AbD Serotec, Kidlington, UK). We used an Alexa Fluor 647 monoclonal antibody labeling kit (Life Technologies, Carlsbad, CA, USA) to label kASHmAbs.

Examinations of peripheral blood

Analyses of mouse retro-orbital venous plexus blood samples were performed using a Celltac α (Nihon Kohden, Tokyo, Japan) to obtain complete blood counts and a Dri-Chem 3000 (Fujifilm, Tokyo, Japan) to measure serum total bilirubin and lactate dehydrogenase, alanine aminotransferase, and aspartate aminotransferase activities.

Xenogeneic model of GVHD

Non-irradiated female NOG mice 9 to 10 weeks old were injected intravenously with 1.0×10^7 human PBMCs (day 0) and were treated with ASHmAb (3 μg/g/day) on days 3 and 4 (total dose: 120 μg/mouse).

Purification of human CD34-positive cells and xenogeneic transplantation

Human CD34-positive cells from cord blood mononuclear cells were enriched using anti-human CD34 Micro-beads (Miltenyi Biotec, Bergisch Gladbach, Germany). For transplantation, female NOD/SCID mice or NOG mice 6 to 8 weeks old were irradiated (1.5 to 2.0 Gy) before transplantation, and 1.0 to 2.0×10^5 live CD34-positive cells were injected by tail vein. Live cells were identified by microscopy as those able to exclude trypan blue.

Systemic assessment of GVHD

To evaluate GVHD symptoms, we chose objective measurements (loss of weight, change of body temperature, and changes in biomarker values). Body weights and body temperatures of all mice were determined twice weekly. The liver, gut, kidneys, and

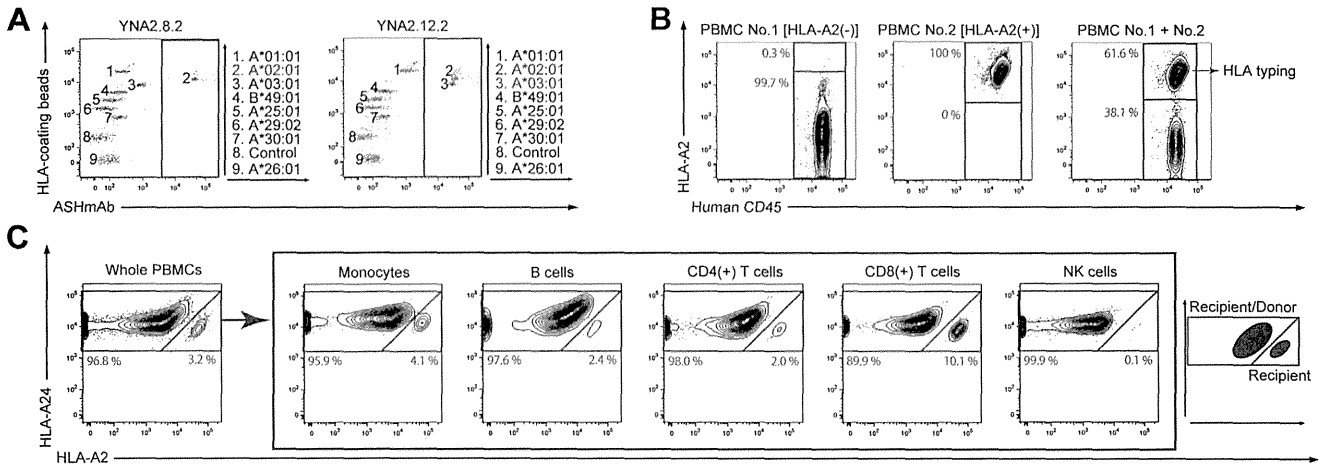

Figure 1. Assessment of allele specificity of kASHmAbs. (**A**) To establish ASHmAb-producing clones, on day 28 hybridomas (Supplementary Fig. E1, online only, available at www.exphem.org) selected as producing anti-HLA antibody at initial screening were secondarily screened with FlowPRA, using flow cytometry (YNA2.8.2 and YNA2.12.2). 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*01:01, A*02:01, A*03:01, B*49:01, A*25:01, A*29:02, A*30:01, and control antigen or A*26:01. (**B**) Flow cytometric analysis of A2-ASHmAb with healthy donor PBMCs. PBMCs No. 2 express HLA-A*02:01; PBMCs No. 1 do not. We stained three types of PBMCs (PBMCs No. 1 only, PBMCs No. 2 only, and PBMCs No. 1 + No. 2) with Alexa Fluor 647-conjugated ASHmAb to assess this antibody's specificity. Cells from PBMCs No. 1 + No. 2 sorted as HLA-A2(+) were subjected to sequencing-based HLA typing. (**C**) Chimerism analysis of clinical samples using ASHmAb. In a 46-year-old man who underwent cord blood transplantation more than 12 years earlier for acute myeloid leukemia, we analyzed chimerism of whole PBMCs, B cells, T cells, natural killer cells, and monocytes using anti-HLA A2 (recipient), anti-HLA A24 (recipient and donor), and anti-human CD3, CD4, CD8, CD19, and CD56. Doublets and dead cells were excluded from flow cytometry data. ASHmAbs = allele-specific anti-human leukocyte antigen monoclonal antibodies; HLA = human leukocyte antigen; kASHmAbs = ASHmAbs; PBMCs = peripheral blood mononuclear cells.

lungs were collected from euthanized mice and analyzed by light microscopy of immunostained sections. Animal cages were maintained at room air temperature of 22°C to 23°C with a humidity of 55%.

Immunostaining of organ sections

Liver, lungs, guts, and kidneys from mice transplanted with human PBMCs were fixed with 10% buffered formalin and embedded in paraffin. Sections 5 μm thick were placed on glass slides (Matsunami Glass, Osaka, Japan), deparaffinized, incubated with anti-human CD3 and CD45 monoclonal antibodies (Dako) overnight at 4°C, and serially incubated with peroxidase-labeled polymer conjugated goat anti-mouse antibody (Nichirei, Tokyo, Japan) for 30 min at room temperature. These sections then were incubated with 0.02% 3,3-diaminobenzidine (Dojindo, Kumamoto, Japan) substrate solution containing 0.006% H_2O_2 and counterstained with hematoxylin for visualization of nuclei. TdT-mediated dUTP nick end labeling (TUNEL) staining (Medical and Biological Laboratories) was performed according to the manufacturer's instructions to permit evaluation of apoptosis.

Statistics

Mouse survival data are presented as Kaplan–Meier survival curves. Differences between groups were analyzed by log-rank testing with GraphPad Prism (GraphPad Software, San Diego, CA, USA). Differences between group means were tested using Student's *t* test, also with GraphPad Prism; values for which $p < 0.05$ were considered significant.

Results

Generation of ASHmAbs and their specificities

Using our established method [15], we developed a panel of ASHmAbs that recognize HLA alleles specifically. First, we immunized HLA class I transgenic mice with tetramers specific for HLA-A or HLA-B alleles [16]. After alloimmunity was confirmed by assay of sera from immunized mice, the mice were sacrificed and lymph node-derived cells and splenic cells were fused with SP2/0 myeloma cells to yield hybridoma cells (Supplementary Figure 1E, online only, available at www.exphem.org). We screened supernatants of hybridomas in single-clone culture by enzyme-linked immunosorbent assay to detect reactivity against the HLA tetramer (data not shown). Selected clones were expanded for panel-reactive antibody (PRA) screening, performed using FlowPRA (Fig. 1A). We chose several monoclonal antibodies for further work, one binding specifically to HLA-A*02:01 (YNA2.8.2) and one binding to both HLA-A*02:01 and HLA-A*03:01 (YNA2.12.2). We also established the antibodies that bind to HLA-A*23:01, HLA-A*24:02, and HLA-A*32:01 (Clone YNA24.3.2 and YNA24.19.2). Focusing on YNA2.8.2, we purified it and subjected it to biotinylation or to conjugation with the fluorescent chromophore AF647. Using this biotinylated ASHmAb or the ASHmAb's fluorescent conjugate, we analyzed

whether it could, in flow cytometry, distinguish members of two different HLA-positive healthy donor PBMC sets. We mixed PBMCs from two donors, one HLA-A2-positive and the other HLA-A2-negative, and stained the pool with biotinylated ASHmAb. Flow cytometry results revealed that we could distinctly separate the two types of cells (Fig. 1B). With sequencing-based typing (Special Reference Laboratories, Tokyo, Japan), we confirmed that cells sorted as HLA-A*02:01 positive were absolutely, by genetic criteria, HLA-A*02:01 expressing (data not shown). To confirm this antibody's specificity in vivo further, we analyzed PBMCs of a patient who underwent allo-HSCT to examine whether the antibody enabled separation of donor (HLA-A2-negative/A24-positive) cells from recipient (HLA-A2/A24-positive) cells (Fig. 1C). The patient was a 46-year-old man who 12 years earlier had undergone cord blood transplantation for acute myeloid leukemia and who suffered from chronic GVHD. Using YNA2.8.2, we could successfully separate the patient's PBMCs into donor- and recipient-derived cells. Chimerism analysis unexpectedly revealed that T cells, B cells, natural killer (NK) cells, and monocytes of native origin persisted in this patient after HSCT. These results confirm the reported utility of ASHmAbs as a diagnostic tool [14].

Allele-specific cytotoxicity of ASHmAb

To evaluate the killing ability of ASHmAb, we first analyzed in vitro cytotoxicity (Fig. 2A). HLA-A2-negative or -positive cells (1.0×10^6 cells/well) were cultured with ASHmAb hybridoma supernatant, Dulbecco's modified Eagle medium (Sigma–Aldrich), and 10% fetal bovine serum, with or without 30% baby rabbit complement, and were incubated for 24 hours (37°C, 5%CO_2). Percentages of dead cells were determined by PI staining of cells using a flow cytometer. On average, 61.9% of HLA-A2-positive cells were PI positive, and on average, only 14.2% of HLA-A2-negative cells were PI positive. These results indicate that ASHmAb can bind with HLA-A2-positive cells in vitro, a phenomenon resulting in cell death, presumably via complement-dependent cytotoxicity. We designated this ASHmAb that kills only HLA-A*02:01-positive cells as HLA-A2 killing ASHmAb (A2-kASHmAb). A2-kASHmAb was capable of killing target cells quickly: when we examined cell death after 3 hours of incubation, we found 30% to 40% mortality manifest as aggregation and failure of trypan blue exclusion (data not shown).

To analyze the cytotoxicity of A2-kASHmAb in vivo, we transplanted 1.0×10^5 cells/mouse cord blood-derived HLA-A2-negative or -positive mononuclear cells into CB17-Prkdc^{scid}/J (NOD/SCID) mice previously subjected to 2.0 Gy of irradiation and made human–mouse bone marrow chimeric mice (Fig. 2B). Before A2-kASHmAb injection (0.5 mg/mouse, intravenously), chimerism of cord blood-derived HLA-A2-positive cells was 12.0%; 1 day after injection, chimerism fell to 0.3%. Conversely, chimerism of cord blood-derived HLA-A2-negative cells before A2-kASHmAb injection was 2.3% and 1 day after injection, chimerism increased to 5.9% (Fig. 2C), an effect significantly different on statistical analysis (Fig. 2D, E). These experiments indicate that kASHmAb can damage cells allele-specifically in vitro and in vivo. We also found that kASHmAb administered intraperitoneally could damage target cells as efficiently as kASHmAb administered intravenously (Supplementary Figure E2A–C, online only, available at www.exphem.org).

Xenogeneic model of GVHD treatment with kASHmAb

We focused on kASHmAb's cytotoxicity and its ability to discriminate between donor and recipient cells. ATG, a representative clinical GVHD molecular-targeted agent, is used both as a treatment for and as a prophylactic against GVHD. However, ATG reacts against cells without distinguishing between donor and recipient cells and causes many side effects, such as allergic reaction and increased susceptibility to infection, as a result of repression of T cells. Moreover, the exact molecular target of ATG is unknown, because ATG is polyclonal. As kASHmAb induces donor-specific cell death in GVHD treatment and is directed against a clear molecular target, we thought that kASHmAb might be useful as a molecule-targeted drug in GVHD treatment as an alternative to ATG.

Ito et al. reported a novel xeno-GVHD animal model using NOG mice in which, thanks to intravenous transfer of human PBMCs, GVHD symptoms were of early onset [17]. We employed this model to analyze the therapeutic effect of kASHmAb in GVHD. We transplanted 1.0×10^7 cells/mouse HLA-A2-negative or -positive PBMCs into NOG mice without irradiation (day 0). To judge whether GVHD had developed or not, blood tests, determinations of body temperature and weight, and assays of skin damage were used in previous studies [18–20]. In this study, to ensure as objective a determination of the onset of GVHD as possible, we decided to define GVHD onset based on minor changes in biomarker values, body temperature, and body weight, as in clinical settings (Fig. 3C, Supplementary Figure E3, online only, available at www.exphem.org). Determinations of complete blood counts (CBC) and biomarkers in peripheral blood established that the onset of GVHD—as indicated by significant changes in complete blood count values—was 3 days after PBMC injection. We thus decided to start administration of A2-kASHmAb from this point with A2-kASHmAb injection (days 3 and 4; 60 μg/day × 2 days) coupled with analysis of PBMCs by flow cytometry (Fig. 3A); complete blood count and biomarker determinations; records of body weight, body temperature, and calculated survival rate; and histopathologic analysis. First, using flow cytometry, we analyzed the frequency of human-derived cells in peripheral blood of GVHD model mice before and after

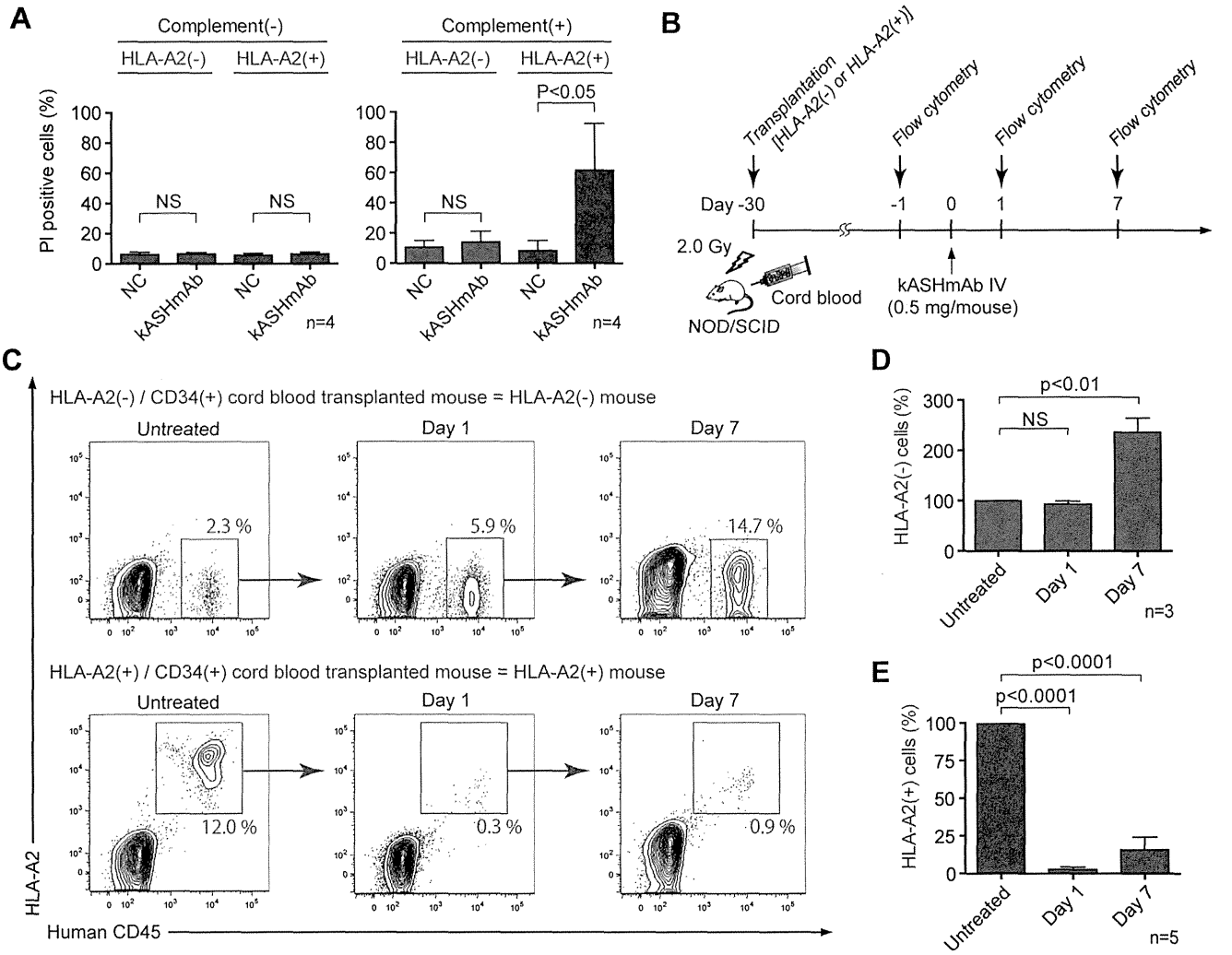

Figure 2. Allele-specific cytotoxicity of ASHmAbs. (**A**) Statistical analysis of in vitro killing assay using A2-ASHmAb with and without baby rabbit complement (right and left, respectively). HLA-A2(−) cells (*red bar*) and HLA-A2(+) cells (*blue bar*) from healthy donors were each cultured with isotype control or A2-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 ± SD ($n = 4$, $p < 0.05$ by Student's t test). (**B**) Schedule of in vivo assay of killing ASHmAbs. Cord blood-derived CD34(+)/HLA-A2(−) or CD34(+)/HLA-A2(+) cells were transplanted into NOD/SCID mice with 2 Gy of irradiation to create respectively HLA-A2(−) and HLA-A2(+) human–mouse bone marrow chimeric mice. One month after transplantation, peripheral blood was analyzed by flow cytometry just before kASHmAb injection and on days 1 and 7 thereafter. (**C**) Representative flow cytometric analyses. Top: HLA-A2(−)/CD45(+) cells (%) among HLA-A2(−) mouse peripheral blood mononuclear cells in marked gate. Bottom: HLA-A2(+)/CD45(+) cells (%) among HLA-A2(+) mouse PBMCs in marked gate. Doublets and dead cells were excluded from flow cytometry data. (**D, E**) Statistical analysis of (**C**). *Red bars* and *blue bars* respectively represent HLA-A2(−) and HLA-A2(+) cells. Data shown as mean ± SD ($n = 3$ and 5, respectively; $p < 0.05$ by Student's t test). HLA = human leukocyte antigen; kASHmAbs = killing allele-specific anti-human leukocyte antigen monoclonal antibodies; NS = not significant; PBMCs = peripheral blood mononuclear cells.

A2-kASHmAb administration (Fig. 3B). Chimerism of the HLA-A2-positive group was 5.4% before A2-kASHmAb injection; on day 8 after PBMC injection, it was 0%. Chimerism of the HLA-A2-negative group was 8.8% immediately before A2-kASHmAb injection; on day 8 after PBMC injection, it was 27.8%. The survival rate of the HLA-A2-positive group was 100%, with a mean survival of more than 6 months; however, all members of the HLA-A2-negative group died within 2 months (Fig. 3D). To confirm that GVHD had caused death, we histopathologically examined liver, lungs, intestine, and kidneys of these mice. Immunostaining revealed intensive tissue infiltration by CD3-positive/CD45-positive cells, especially in lungs and intestine (Fig. 3E, Supplementary Figure E4, online only, available at www.exphem.org), and TdT-mediated dUTP nick end labeling studies revealed apoptosis (Supplementary Figure E5, online only, available at www.exphem.org). These results verified experimentally that kASHmAb could be a novel drug to damage target cells in GVHD safely, quickly, and selectively.

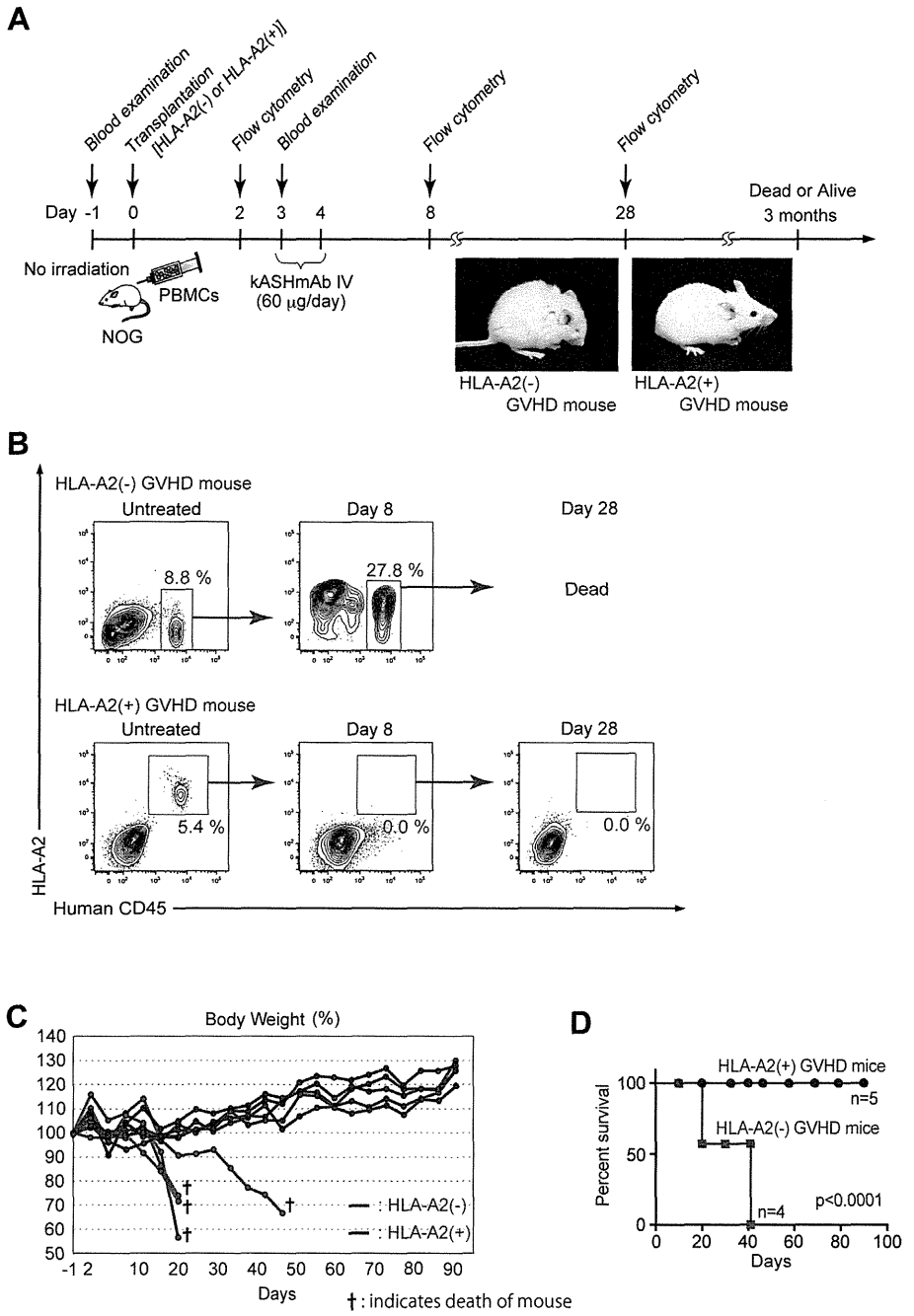

Figure 3. GVHD kASHmAb treatment model. (**A**) Schedule, GVHD kASHmAb treatment model. Day −1: Blood examination. Day 0: HLA-A2(−) or HLA-A2(+) PBMCs transplanted into non-irradiated NOG mice. Day 3: Blood examination immediately before first kASHmAb dose; first kASHmAb dose. Day 4: Second kASHmAb dose. Days 2, 8, and 28: Flow cytometric analyses. Day 90: Survival assessment. (**B**) Representative flow cytometric analyses. Human-derived cells (%) among GVHD model mouse PBMCs, days 3, 8, and 28. Top: HLA-A2(−)/CD45(+) cells, HLA-A2(−) GVHD mouse. Bottom: HLA-A2(+)/CD45(+) cells in HLA-A2(+) GVHD mouse. Forward-scatter, side-scatter, and propidium iodide gatings excluded residual erythrocytes, debris, doublets, and dead cells. (**C**) Body weight 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(−)]. Weight was determined twice weekly between 1500 and 1900 hours at room temperature (22°C) and ambient humidity (55%). Percentage change from initial weight is illustrated. (**D**) Survival of GVHD model mice (kASHmAb-treated). *Red* and *blue lines* respectively represent HLA-A2(−) GVHD mice and HLA-A2(+) GVHD mice. Data shown as Kaplan–Meier estimates (n = 4 and 5, respectively; $p < 0.05$).

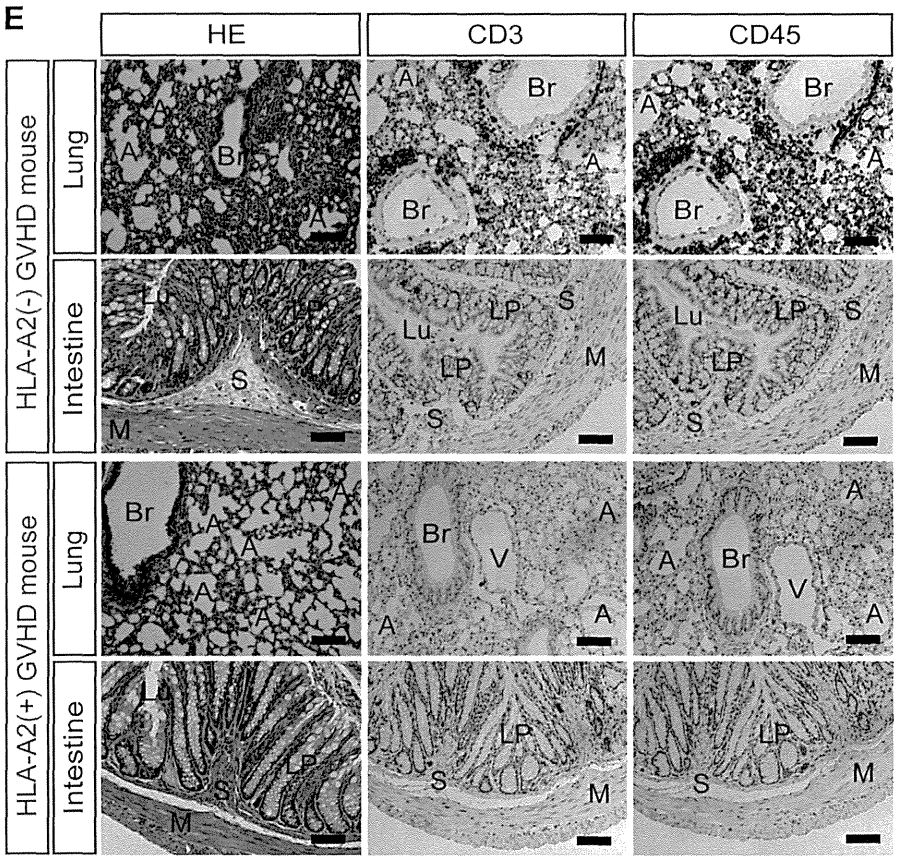

Figure 3. (*continued*) (**E**) Lung and intestine, day 21, GVHD model mice (NOG mice intravenously transplanted with human PBMCs (HLA-A2(−) or HLA-A2(+)); anti-human CD3 and CD45 developed with 3,3′-diaminobenzidine, hematoxylin nuclear counterstaining. × 200. Bar = 400 μm. A = alveoli; Br = bronchiole; GVHD = graft-versus-host disease; HE = hematoxylin and eosin; HLA = human leukocyte antigen; kASHmAbs = allele-specific anti-HLA monoclonal antibodies against HLA-A*02:01; LP = lamina propria; Lu = lumen; M = muscularis; NOG = NOD/Shi-scid/IL-2Rγ^{null}; PBMCs = peripheral blood mononuclear cells; S = submucosa.

kASHmAb administration did not cause graft failure

We confirmed that A2-kASHmAb specifically damages cells that express HLA-A2 in human–mouse bone marrow chimera mice (Fig. 2C, D). A potential risk of anti-GVHD therapy with kASHmAb is damage to donor hematopoietic stem cells (HSCs) that necessitates another transplantation. Unexpectedly, we discovered that in many mice (14 of 16) in the HLA-A2-positive group chimerism rebounded to high levels 1 to 2 months after A2-kASHmAb injection. The dose of kASHmAb used to treat GVHD was, at greatest, equivalent to that of the polyclonal ATG preparation usually given for acute GVHD in actual clinical settings (3 mg/kg/day × 5 days). The clinical risk might be substantial if kASHmAbs were administered to GVHD patients, because if donor-derived HSCs are ablated, patients may have to undergo HSCT again. The observation that chimerism returns quickly after A2-kASHmAb administration let us hypothesize that HSCs resist antibody-mediated killing and that adjusting the dose of kASHmAb may circumvent damage to HSCs while yet treating GVHD.

We used humanized mice, generated by transplanting 2.0 × 10^5 cells/mouse cord blood-derived HLA-A2-positive/CD34-positive cells into NOG mice after 1.5 Gy irradiation, to track numbers of cord blood-derived HLA-A2-positive/CD45-positive human cells among mouse PBMCs and bone marrow cells after high-dose A2-kASHmAb (60 μg/day × 2 days) administration (Fig. 4A). PBMC human cell chimerism in humanized mice immediately before A2-kASHmAb injection was 72.2%; 2 days after A2-kASHmAb injection, it was 12.9%. Although most CD34-positive cord blood-derived cells disappeared after A2-kASHmAb injection, human cell high chimerism (70.7%) returned in the same mice within 1 month (Fig. 4B, C). We determined numbers of human cells in mouse PBMCs and bone marrow cells before and after A2-kASHmAb injection. Although many human PBMCs disappeared, the proportion of human-derived cells in bone marrow continued to be substantial (Fig. 4D). HLA resides on the surface of all nucleated cells, including HSCs [21,22]; these results unexpectedly indicated that the killing ability of kASHmAb is selective,

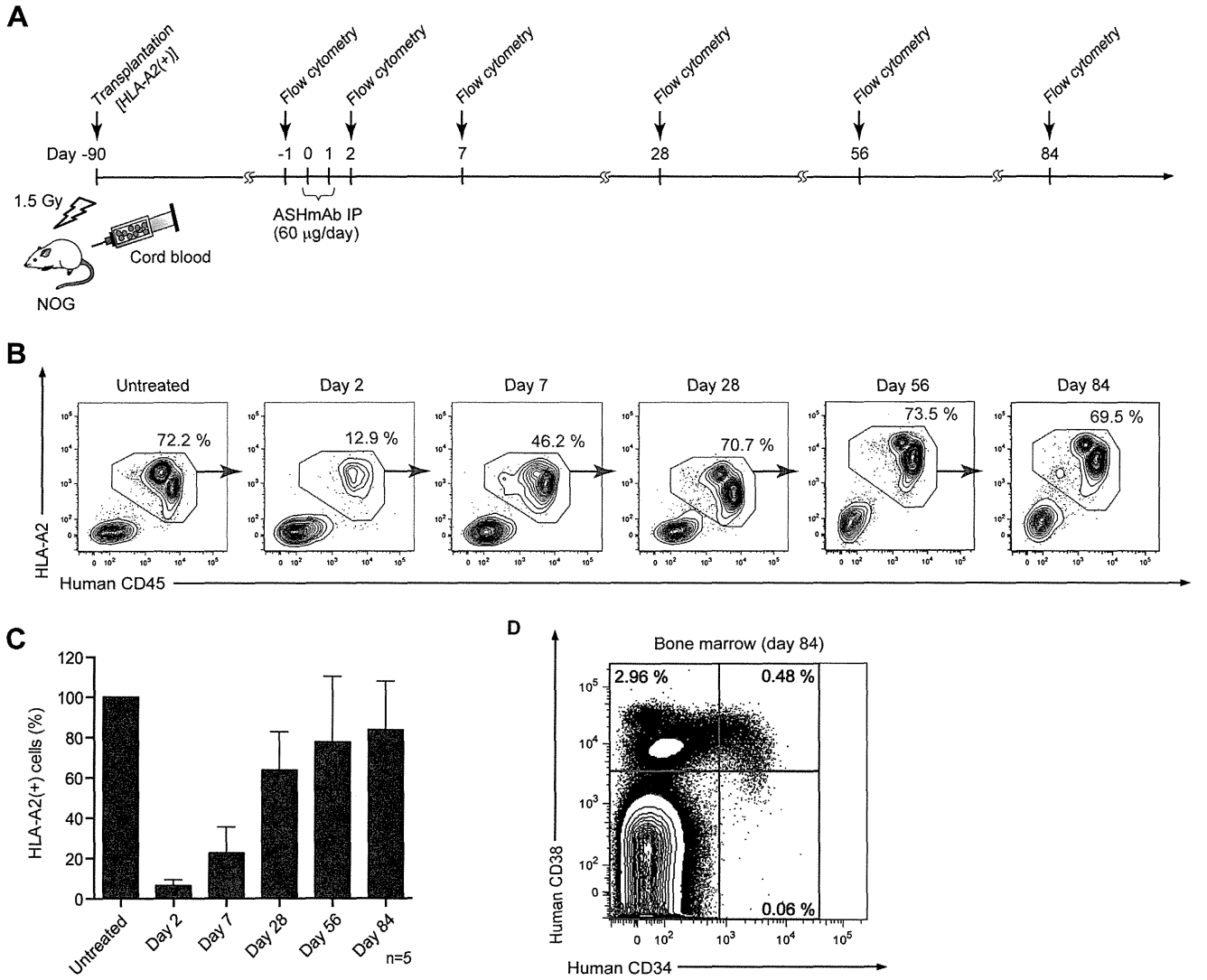

Fig. 4. Humanized mice: kASHmAb treatment without graft failure. (**A**) Schedule. (**B**) Representative flow cytometric analyses. Cord blood-derived human cells (%) among PBMCs of irradiated NOD/Shi-scid/IL-2Rγ^{null} (NOG) mice given HLA-A2(+)/CD34(+) cord blood cells; "humanized mice," day −1 (day before intraperitoneal kASHmAb injection) and days 2, 7, 28, 56, and 84. Abscissa: Human CD45; ordinate: HLA-A2. Forward-scatter, side-scatter, and propidium iodide gatings excluded residual erythrocytes, debris, doublets, and dead cells. (**C**) Bars: HLA-A2(+)/CD45(+) cells (%) among humanized mouse PBMCs. The data are normalized against the baseline percentage of human cells. Data shown as mean ± SD (n = 5). (**D**) Representative flow cytometric analyses of cord blood-derived human cells (%) among bone marrow cells, say 84. Mouse bone marrow cells were stained with anti-human CD34 and anti-human CD38 antibodies. Forward-scatter, side-scatter, and propidium iodide gatings excluded residual erythrocytes, debris, doublets, and dead cells. HLA = human leukocyte antigen; kASHmAbs = killing allele-specific anti-HLA monoclonal antibodies against HLA-A*02:01; NOG = NOD/Shi-scid/IL-2Rγ^{null}; PBMCs = peripheral blood mononuclear cells.

damaging cells in peripheral blood more efficiently than those in bone marrow. These findings indicated that at the correct dose, kASHmAb likely could be administered to GVHD patients safely, without causing graft failure and necessitating repeat HSCT.

Discussion

Emergence of antibodies to HLA antigens is often associated with disorders such as graft failure and resistance to transfusion [23–27]. This study is the first to find that anti-HLA antibodies also can be used therapeutically. Confronted with growing numbers of HLA-mismatched cord blood or haplo-identical HSCT, we thought about generating kASHmAbs that can recognize donor HLA molecules in an allele-specific manner and damage them while sparing host cells. Because they act specifically against donor cells, these kASHmAbs, in principle, should constitute novel anti-GVHD drugs with minimum side effects. Indeed, unlike ATG and anti-CD3 or anti-CD52 antibodies, ASHmAbs destroyed donor cells specifically and quickly and thus treated acute GVHD efficiently, as shown in our

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.

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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.

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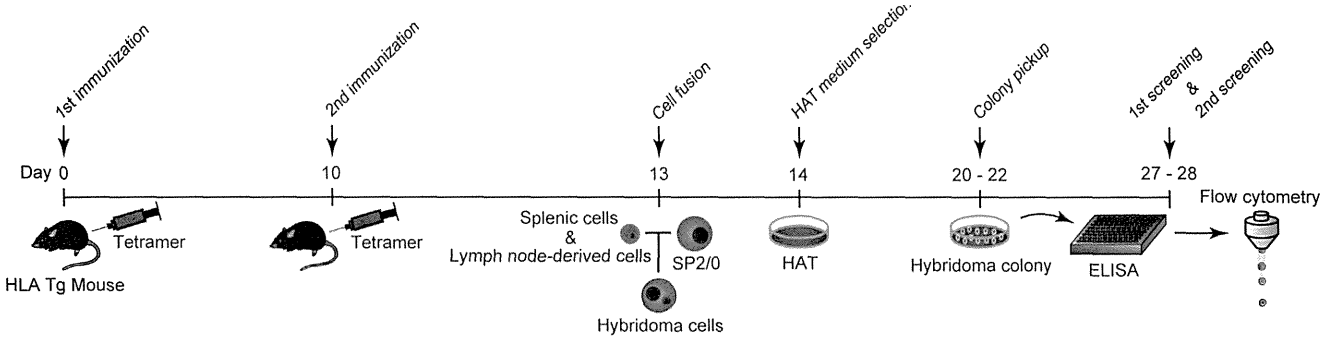
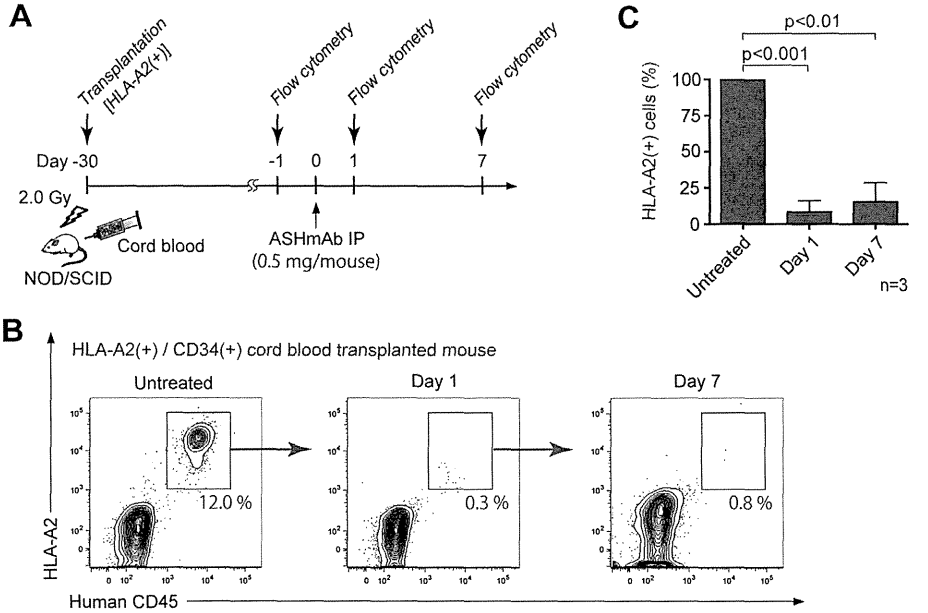


Supplementary Figure E1. Schedule for ASHmAb generation and screening. Day 0: First immunization of HLA-transgenic (Tg) mouse with HLA tetramer. Day 9: Mouse serum assessed for antibodies. Day 10: Second immunization (boost injection) of HLA-Tg mouse with HLA tetramer. Day 13: Fusion of spleen- and lymph 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.