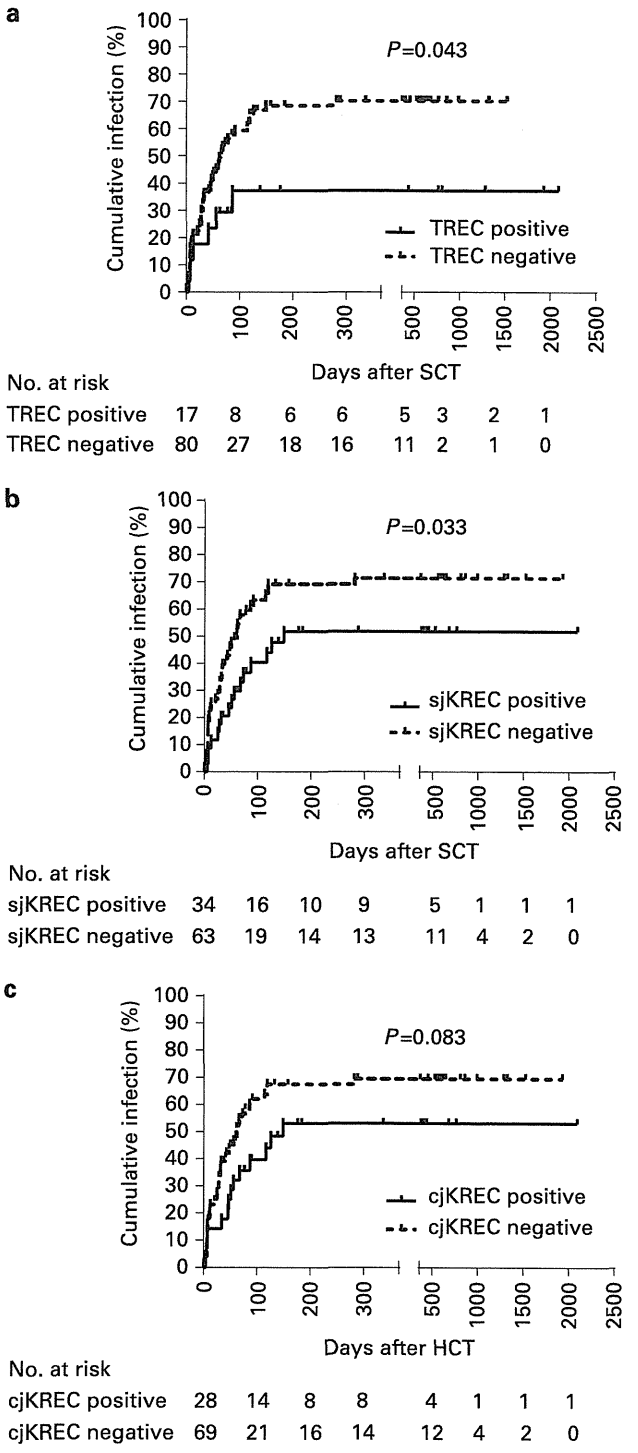

Figure 5. Cumulative incidence of infection after HCT. Cumulative incidence of infection and the levels of TRECs (**a**), sjKRECs (**b**) and cjKRECs (**c**) 1 month after HCT. Solid lines indicate the positive levels of TRECs (**a**), sjKRECs (**b**) and cjKRECs (**c**), and the dotted lines indicate their negative levels.

not correlate with negative TRECs, sjKRECs or cjKRECs at 3 months after HCT (data not shown).

DISCUSSION

In this study, we examined TRECs and sjKRECs/cjKRECs in post-transplantation patients with malignancies or PID. Our data showed the following. (1) The levels of sjKRECs and cjKRECs increase earlier than those of TRECs. (2) A younger recipient age is favorable for better recovery of sjKRECs and cjKRECs post HCT. (3) The use of CB achieves rapid recovery of sjKRECs and cjKRECs compared with that of BM or PB as a graft source. (4) Detectable sjKRECs 1 month after HCT is related to a decreased frequency of infectious episodes.

Patients with positive sjKRECs at 1 month had increased levels of sjKRECs at 3 and 6 months, suggesting that positivity can predict sound B-cell immune reconstitution. In addition, the levels of sjKRECs and cjKRECs increased earlier than those of TRECs (Figure 1, Supplementary Figures 1 and 2).

There have been no reports of the factors that contribute to better KREC reconstitution. Compared with BM and PB, we found that the levels of sjKRECs and cjKRECs recovered rapidly in patients who received CB. Faster B-cell reconstitution after CBT has been reported previously.[14,25] CB itself does not have high sjKREC/cjKREC levels. Our results suggest that rapid B-cell recovery by CBT is because of B-cell neogenesis and not B-cell expansion in the periphery.

A previous study has demonstrated that sjKREC levels are the highest in < 1-year-olds and then declines with age in healthy children.[17] Thus, it is likely that younger donors have an advantage in terms of B-cell reconstitution. Our results indicated that a younger recipient age also contributed to increased levels of sjKRECs and cjKRECs.

In addition, our data showed that acute 0–2 GVHD, no steroid use and no ATG use were associated with positive sjKRECs and cjKRECs (Supplementary Tables III and IV). These data indicate that steroid or ATG use affects not only T-cell recovery but also B-cell immune reconstitution.

As expected, patients with chronic GVHD showed significantly lower levels of TRECs at 6 months and 1 year. On the other hand, and in contrast to our expectation, we observed lower sjKRECs and cjKRECs from 3 months to 2 years in patients with chronic GVHD (Supplementary Figure 5). This observation does not support the data of Allen *et al.*,[26] which revealed increased numbers of B cells and expression of BAFF (B-cell-activating factor belonging to the TNF family) in patients with chronic GVHD. This discrepancy may be because the patients with chronic GVHD were on more active immunosuppressants compared with those without chronic GVHD. Additionally, there may be relatively high levels of KRECs in patients with the severe extensive type of chronic GVHD. However, we would need more patients and additional analyses of B-cell numbers and activation to reach a conclusion.

Our study suggests that patients with positivity for TRECs or sjKRECs at 1 month are less likely to develop post-transplant infections. The contribution of earlier B-cell recovery to overall immunity, especially anti-microbial immunity, needs further investigation. Patients with early B-cell neogenesis may attain early myeloid recovery. B-cells may also serve as antigen-presenting cells in addition to antibody-producing cells.

A correlation between KREC levels and prognosis has not been addressed previously. Although there was a tendency toward better survival for the KREC-positive group at 1 month, we observed no statistical significance. Further study with a larger cohort is required to determine whether the difference can be significant.

It is still unclear whether TREC levels are lower in patients post CBT than in those receiving BMT.[25] Our data focusing on adult patients showed that T-cell recovery was at least not inferior and appeared to be similar in CB and BM recipients (data not shown). On the other hand, compared with BM and PB, CB was superior for B-cell recovery. This observation suggests quantitative superiority of B-cell recovery following CBT. Further study should investigate the repertoire diversity and somatic hypermutation of B-cell receptors to evaluate qualitative differences and determine whether rapid qualitative maturation has an effect on improved

outcomes. In combination with *in vitro* immunological data and clinical data such as long-term infection, autoimmunity and immunological findings, KRECs and TRECs may serve as useful tools for immunological monitoring after HCT.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Experimental Hematology

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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

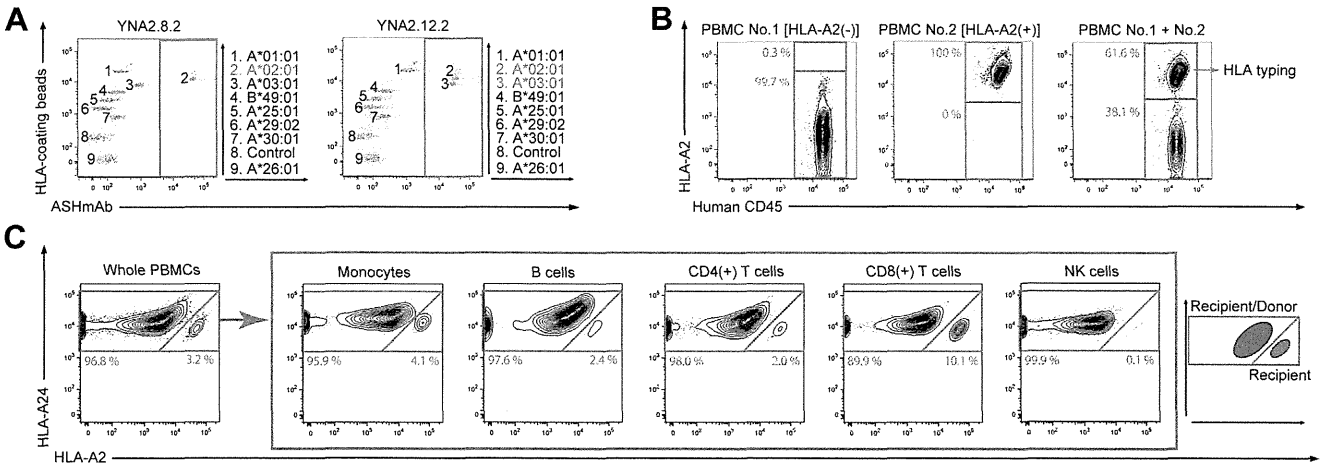

Figure 1. Assessment of allele specificity of kASHmAbs. (**A**) To establish ASHmAb-producing clones, on day 28 hybridomas (Supplementary Fig. E1, online only, available at www.exphem.org) selected as producing anti-HLA antibody at initial screening were secondarily screened with FlowPRA, using flow 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

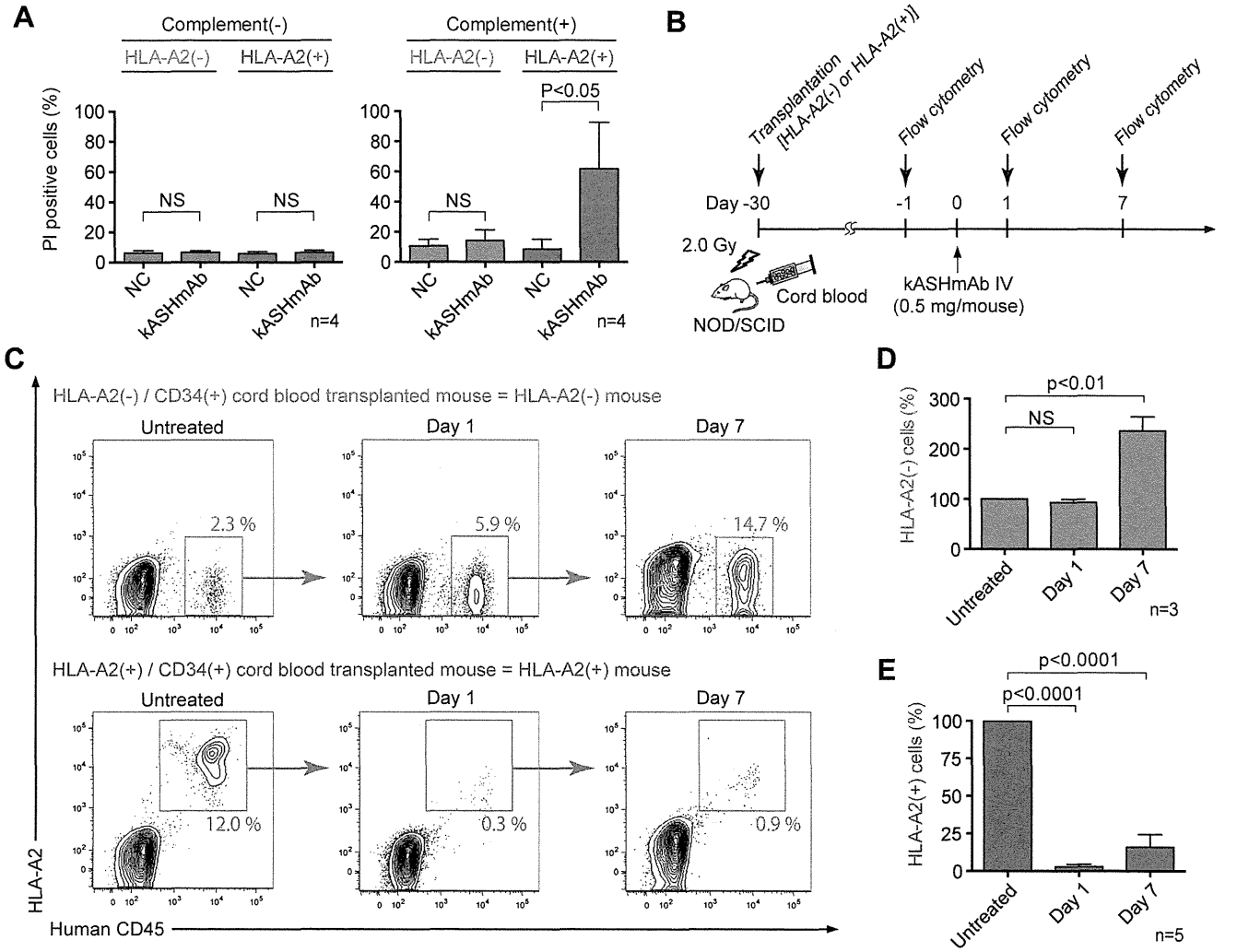

Figure 2. Allele-specific cytotoxicity of ASHmAbs. (**A**) Statistical analysis of in vitro killing assay using A2-ASHmAb with and without baby rabbit complement (right and left, respectively). HLA-A2(−) cells (*red bar*) and HLA-A2(+) cells (*blue bar*) from healthy donors were each cultured with isotype 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.

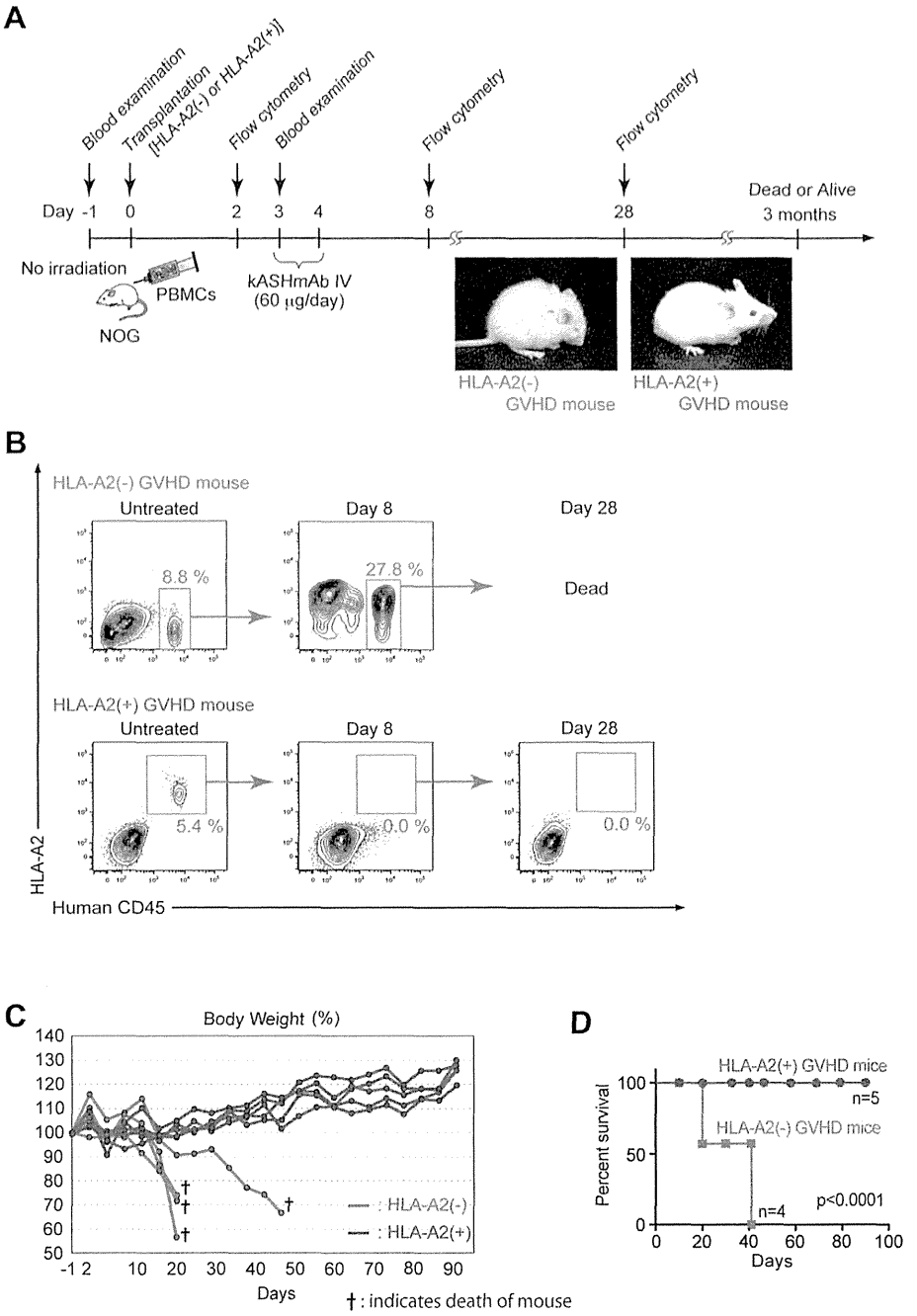

Figure 3. GVHD kASHmAb treatment model. (**A**) Schedule, GVHD kASHmAb treatment model. Day −1: Blood examination. Day 0: HLA-A2(−) or HLA-A2(+) PBMCs transplanted into non-irradiated NOG mice. Day 3: Blood examination immediately before first kASHmAb dose; first kASHmAb dose. Day 4: Second kASHmAb dose. 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; $p < 0.05$).

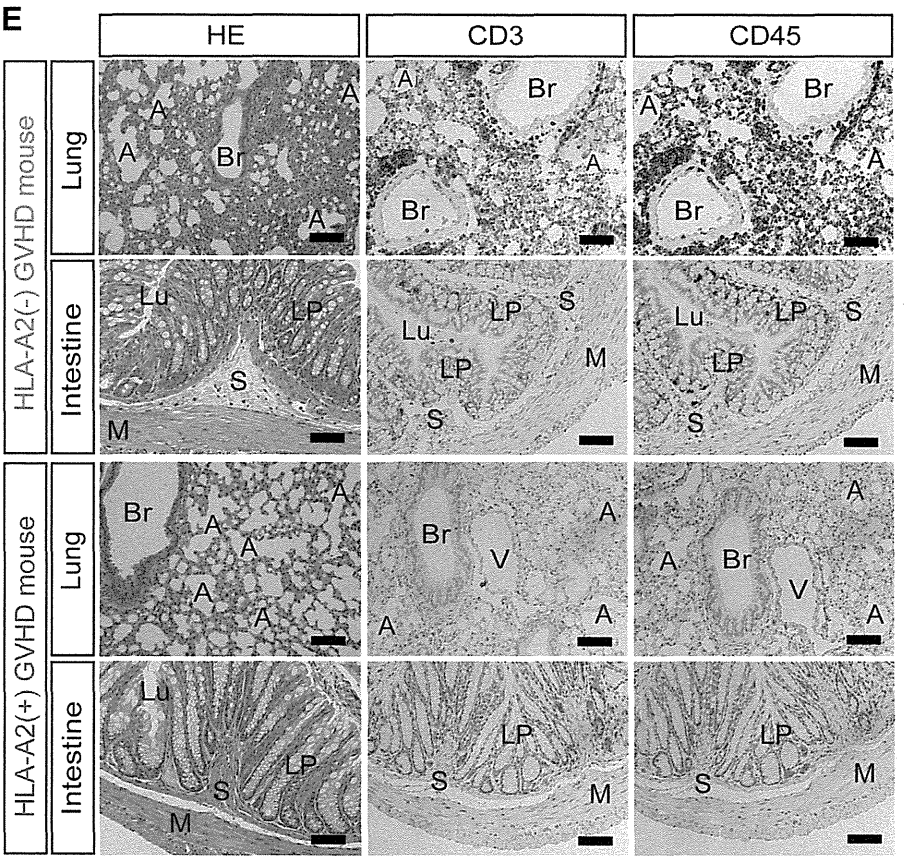

Figure 3. (*continued*) (**E**) Lung and intestine, day 21, GVHD model mice (NOG mice intravenously transplanted with human PBMCs (HLA-A2(−) or HLA-A2(+)); anti-human CD3 and CD45 developed with 3,3′-diaminobenzidine, hematoxylin nuclear counterstaining. × 200. Bar = 400 μm. A = alveoli; Br = bronchiole; 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,

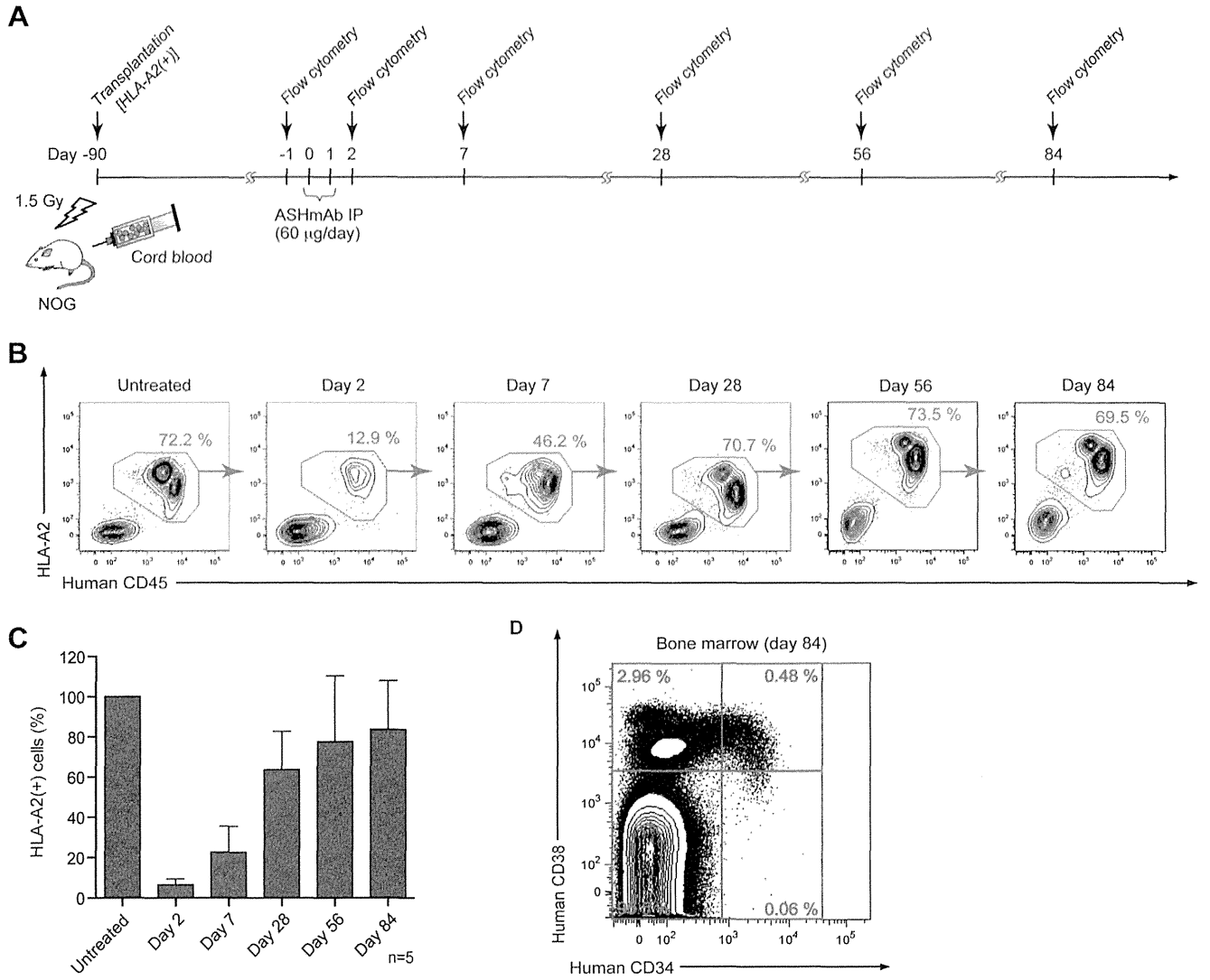

Fig. 4. Humanized mice: kASHmAb treatment without graft failure. (**A**) Schedule. (**B**) Representative flow cytometric analyses. Cord blood-derived human cells (%) among PBMCs of irradiated NOD/Shi-scid/IL-2Rγ^{null} (NOG) mice given HLA-A2(+)/CD34(+) cord blood cells; "humanized mice," day −1 (day 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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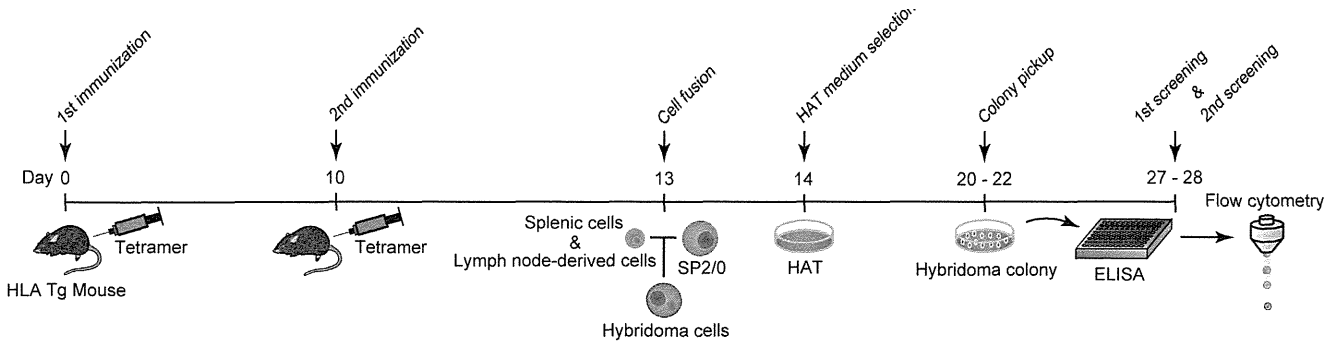
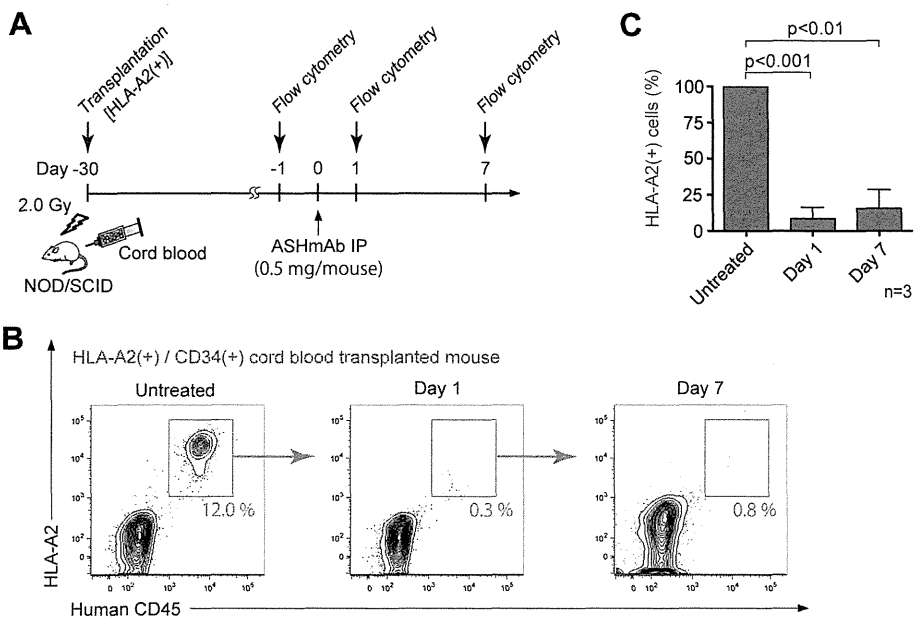
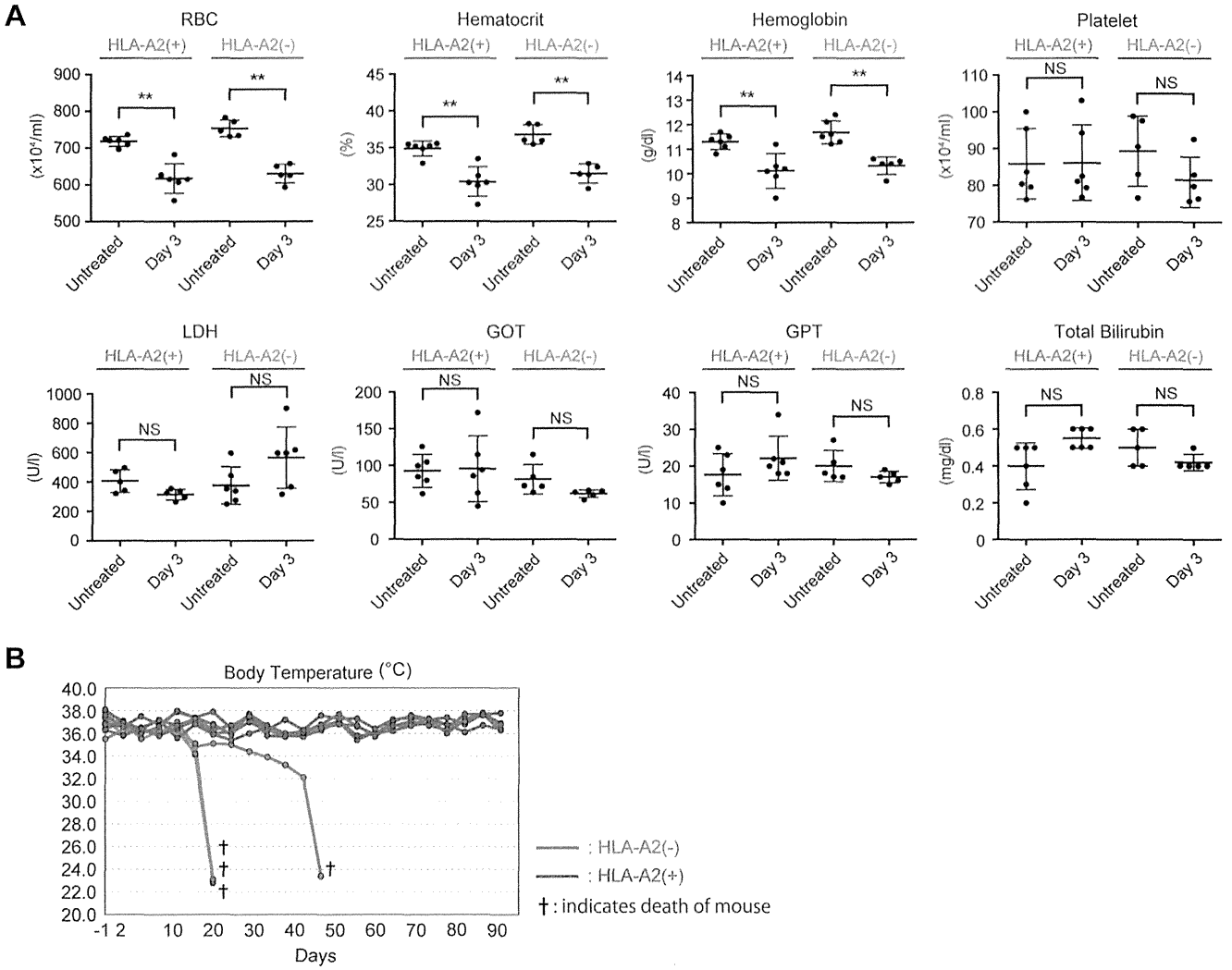


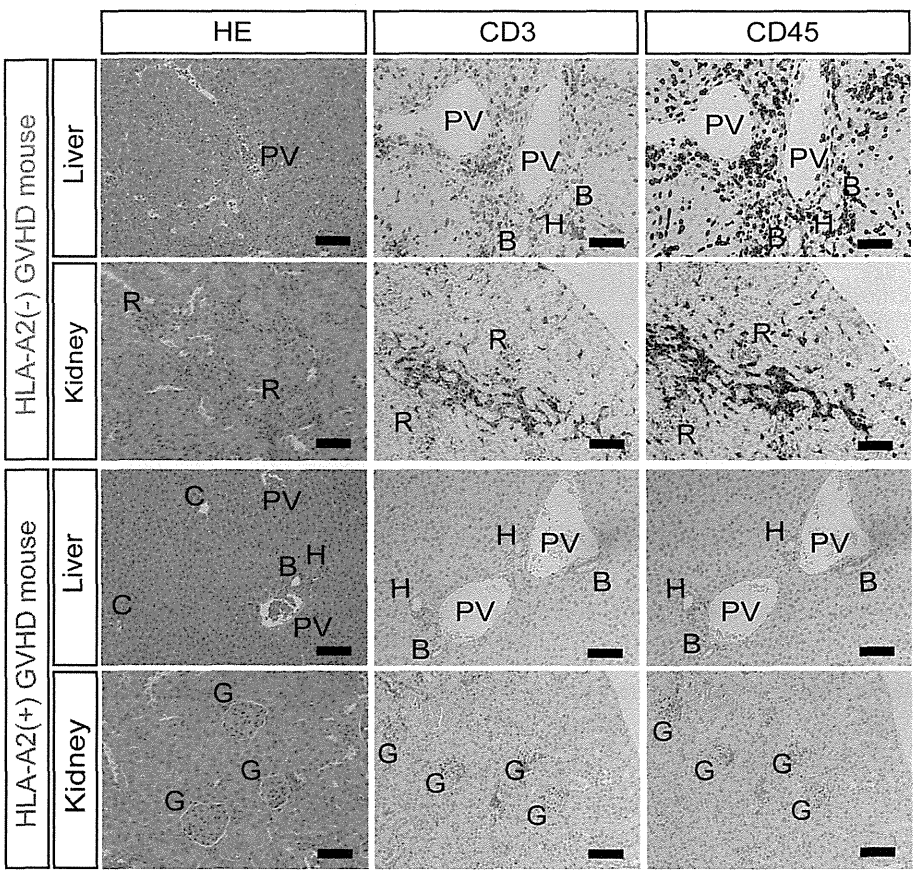
Supplementary Figure E1. Schedule for ASHmAb generation and screening. Day 0: First immunization of HLA-transgenic (Tg) mouse with HLA tetramer. Day 9: Mouse serum assessed for antibodies. Day 10: Second immunization (boost injection) of HLA-Tg mouse with HLA tetramer. Day 13: Fusion of spleen- and lymph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



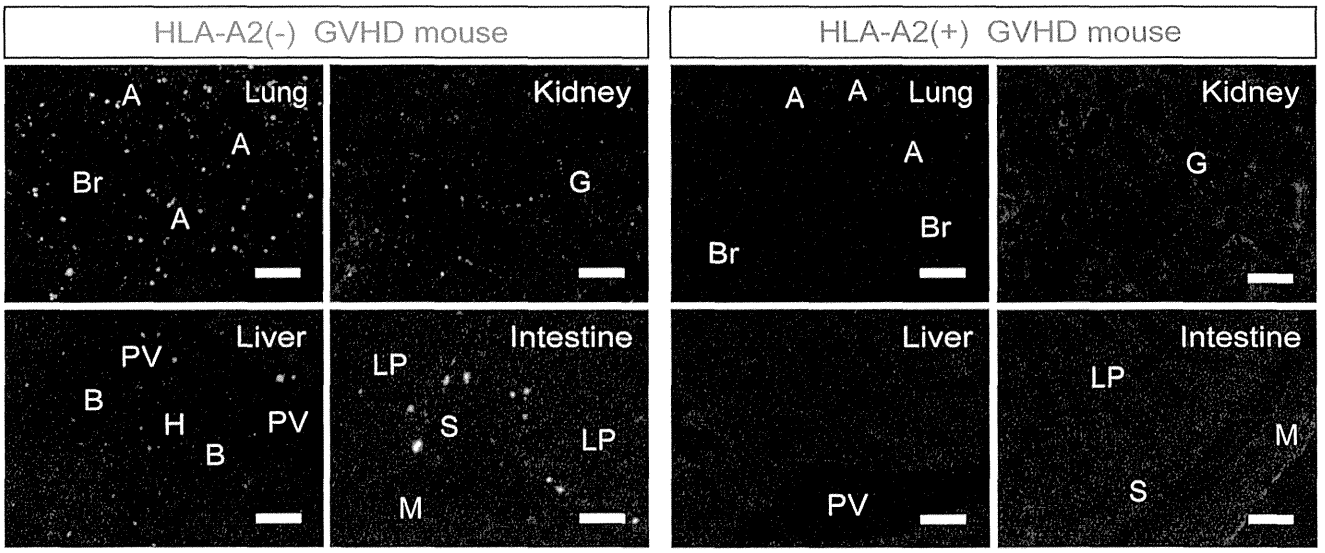
Supplementary Figure E2. Allele-specific cytotoxicity of HLA-A2-killing ASHmAb (kASHmAb) when injected intraperitoneally (IP). (A, B) Schedule and representative examples of in vivo assay of IP-administered HLA-A2 kASHmAb. Cord blood-derived CD34(+) / HLA-A2(+) cells were transplanted into irradiated 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.



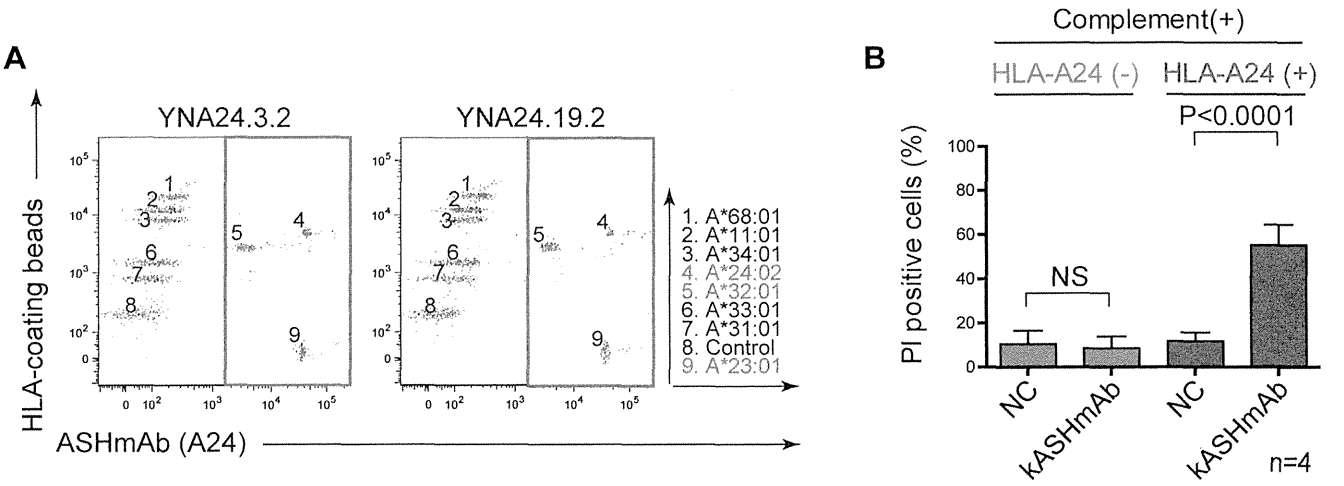
Supplementary Figure E3. Results of hematologic and biomarker studies of GVHD-model mice before and after kASHmAb treatment. (A) Blood was sampled from the retroorbital venous plexus and examined on days -1 (just before PBMC transplantation) and 3 (just before kASHmAb injection). We determined complete 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.

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Comparable Long-Term Outcome of Unrelated Cord Blood Transplantation with Related Bone Marrow or Peripheral Blood Stem Cell Transplantation in Patients Aged 45 Years or Older with Hematologic Malignancies after Myeloablative Conditioning

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A B S T R A C T

We investigated whether bone marrow or peripheral blood stem cells from older sibling donors or cord blood from unrelated donors provided a better outcome in allogeneic hematopoietic stem cell transplantation for relatively older patients who were candidates for myeloablative conditioning. Clinical outcomes of 97 patients aged 45 years or older with hematologic malignancies who received unrelated cord blood transplantation (CBT) (n = 66) or bone marrow transplantation (BMT) or peripheral blood stem cell transplantation (PBSCT) from related donors (n = 31) were compared. The cumulative incidences of grades III to IV acute and extensive chronic graft-versus-host diseases were similar between both groups. Although transplant-related mortality was significantly lower after CBT compared with BMT/PBSCT from related donors (hazard ratio [HR], .29, $P = .04$), overall mortality (HR, .72, $P = .47$) and relapse (HR, 2.02, $P = .23$) were not significantly different after CBT and BMT/PBSCT from related donors. These data suggest that CBT could be as safe and effective as BMT/PBSCT from older related donors for relatively older patients when it is used as a primary unrelated stem cell source.

INTRODUCTION

Donor age has been associated with transplant outcomes in allogeneic hematopoietic stem cell transplantation (allo-HSCT) after myeloablative conditioning or reduced-intensity conditioning (RIC) [1-5]. Older donor age resulted in an increased incidence of severe graft-versus-host disease (GVHD), which led to higher transplant-related mortality (TRM) or overall mortality after allo-HSCT from unrelated adult donors [1,2]. In contrast, it is difficult to determine the exact effect of the age of related donors, because increasing recipient age is frequently accompanied by increased donor age after allo-HSCT from related donors. However, older donor age of related donors may also be associated with adverse outcomes [3-5].

Several studies, including ours, comparing both cord blood transplantation (CBT) and bone marrow transplantation (BMT)/peripheral blood stem cell transplantation (PBSCT) from unrelated donors after myeloablative conditioning in adult patients demonstrated that the incidence of severe GVHD was significantly lower after CBT than after unrelated BMT/PBSCT. The survival rate and relapse incidence in CBT recipients were comparable with those in unrelated BMT/PBSCT recipients [6-9]. Moreover, we also demonstrated similar survival, relapse, and TRM between unrelated CBT and related BMT/PBSCT (rBMT/PBSCT) recipients [10]. The incidences of grades III to IV acute GVHD (aGVHD) and extensive chronic GVHD (cGVHD) among CBT recipients were also significantly lower than those among rBMT/PBSCT recipients. Because the lower risk of severe GVHD is one of the most attractive advantages of CBT, the use of cord blood instead of bone marrow or mobilized peripheral blood as a stem cell source might offer the possibility of decreasing severe GVHD in older patients. However, there has been no comparative study between CBT and BMT/PBSCT from older related donors after myeloablative conditioning in relatively older patients.

We previously reported that unrelated CBT after myeloablative conditioning is feasible in patients over the age of 45 years [11,12]. In this retrospective study, we report on a clinical comparison of CBT from unrelated donors and BMT/PBSCT from older related donors in patients older than 45 years of age with hematologic malignancies who were candidates for a myeloablative conditioning.

METHODS

Patients and Transplant Procedures

This retrospective study included 97 consecutive patients, 45 years of age or older, who received CBT (n = 66) from unrelated donors or BMT (n = 26) or PBSCT (n = 5) from related donors for acute myeloid leukemia (AML), myelodysplastic syndrome (MDS), chronic myeloid leukemia (CML), acute lymphoblastic leukemia (ALL), and non-Hodgkin lymphoma (NHL) at the Institute of Medical Science, University of Tokyo between May 1992 and July 2013. Nineteen patients who received rBMT/PBSCT and 32 patients who received CBT were included from our previous study with extended

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Table 1
Characteristics of Patients, Grafts, and Transplantation

Characteristic	rBMT/PBSCT	CBT	*P*
Number of patients	31	66	
Recipient age, yr, median (range)	48 (45-58)	49 (45-55)	.60
Recipient sex, n (%)			.51
Male	20 (64)	37 (56)	
Female	11 (35)	29 (43)	
Recipient CMV serostatus, n (%)			.18
Positive	28 (90)	64 (96)	
Negative	0 (0)	2 (3)	
Unknown	3 (9)	0 (0)	
Disease type, n (%)			.08
AML	16 (51)	44 (66)	
MDS	2 (6)	8 (12)	
CML	6 (19)	3 (4)	
ALL	3 (9)	8 (12)	
NHL	4 (12)	3 (4)	
Disease status at transplantation,* n (%)			.48
Standard	8 (25)	23 (34)	
High	23 (74)	43 (65)	
Conditioning regimen, n (%)			<.01
TBI12Gy+Ara-C/G-CSF	21 (64)	0 (0)	
TBI12Gy+Ara-C/G-CSF+CY	2 (6)	52 (78)	
TBI12Gy+Ara-C/G-CSF+Flu	0 (0)	3 (4)	
TBI12Gy+CY	3 (9)	3 (4)	
TBI12Gy+Ara-C+CY	1 (3)	8 (12)	
TBI12Gy+VP16	4 (12)	0 (0)	
GVHD prophylaxis, n (%)			.23
Cyclosporine A+methotrexate	29 (93)	65 (98)	
Cyclosporine A	2 (6)	1 (1)	
Number of nucleated cells, $\times 10^7$/kg, median (range)	26.6 (3.13-50.0)‡	2.39 (1.72-5.07)	<.01
Number of CD34$^+$ cells, $\times 10^5$/kg, median (range)	40.5 (20.6-75.0)§	1.04 (.17-3.15)	<.01
Donor age, yr, median (range)	46.5 (38-58)	—	—
Sex compatibility, n (%)			.81
Female donor to male recipient	8 (25)	20 (30)	
Other	23 (74)	46 (69)	
HLA disparities,† n (%)			<.01
0	28 (90)	1 (1)	
1	2 (6)	13 (19)	
2	1 (3)	52 (78)	
ABO incompatibility, n (%)			.04
Match	19 (61)	20 (30)	
Major mismatch	4 (12)	17 (25)	
Minor mismatch	5 (16)	18 (27)	
Bidirectional mismatch	3 (9)	11 (16)	
Time from diagnosis to transplantation, days, median (range)	521 (59-2501)	390.5 (55-6783)	.84
<365 d, n (%)	12 (38)	31 (46)	.51
≥365 d, n (%)	19 (61)	35 (53)	
Year of transplantation, n (%)			<.01
1992-2002	27 (87)	17 (25)	
2003-2013	4 (12)	49 (74)	
Follow-up for survivors, mo, median (range)	185 (32-258)	87 (4-175)	<.01

CMV indicates cytomegalovirus; CY, cyclophosphamide; Flu, fludarabine; VP-16, etoposide.

* Disease status at transplantation was classified as standard risk or high risk; CR1 or CR2 without poor prognostic karyotype for AML and ALL, refractory anemia for MDS, chronic phase for CML, and CR1 or CR2 for NHL were classified as standard risk, whereas patients in all other situations were classified as high risk.

† Number of HLA disparities defined as low resolution for HLA-A, -B, and -DR.

‡ Number of nucleated cells was only for BMT recipients.

§ Number of CD34$^+$ cells was only for PBSCT recipients.

follow-up [10]. For disease status at transplantation, patients in first complete remission (CR1) or second complete remission (CR2) without poor prognostic karyotype for AML and ALL, refractory anemia for MDS, chronic phase for CML, and CR1 or CR2 for NHL were classified as standard risk, whereas patients in all other situations were classified as high risk.

Although bone marrow or mobilized peripheral blood from HLA-compatible related donors within immediate families is a frontline graft source, patients without a suitable closely HLA-compatible related donor were eligible for CBT as an alternative first treatment option, unless they had any type of anti-HLA antibody. Cord blood units were obtained from the Japan Cord Blood Bank Network and were selected as reported previously [9,10]. All patients received 12 Gy total body irradiation (TBI)-based myeloablative conditioning regimens, and cyclosporine-based GVHD prophylaxis regimens, as previously reported [9,10]. For myeloid disease, granulocyte colony-stimulating factor (G-CSF) was added to the conditioning regimen to increase the susceptibility to cytosine arabinoside (Ara-C) through induction of cell cycle entry of dormant leukemia cells, as previously reported [10]. Almost all patients received some supportive care, such as antibacterial, antifungal and antiviral agents, as previously reported [9,10]. The institutional review board of the Institute of Medical Science, University of Tokyo approved this study, which was conducted in accordance with the Declaration of Helsinki.

End Points and Definitions

The primary study end point was overall survival (OS), which was defined as the time from the date of transplantation to the date of death or last contact. Secondary end points were relapse, TRM, GVHD, and neutrophil and platelet recovery. Relapse was defined by morphologic evidence of disease in peripheral blood, bone marrow, or extramedullary sites. TRM was defined as death during a remission. Both aGVHD and cGVHD were graded according to previously published criteria [13,14]. 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. 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 defined as the first of 7 consecutive days with a platelet count of 20 × 10^9/L or higher without platelet transfusion.

Statistical Analysis

Baseline patient and transplant characteristics were compared using the chi-square test for categorical variables and the Mann-Whitney U test for continuous variables. The probability of OS was estimated according to the Kaplan-Meier method, and groups were compared using Cox regression models or the log-rank test. The probabilities of relapse, TRM, aGVHD and cGVHD, and neutrophil and platelet engraftment were estimated based on a cumulative incidence method to accommodate competing risks. Multivariate analysis was performed with a Cox proportional hazard model adjusted for OS and a Fine and Gray proportional hazards model for the others. In addition to the stem cell source (CBT versus rBMT/PBSCT), the following variables were considered: disease type (myeloid [AML, MDS, CML] versus lymphoid [ALL, NHL] disease), disease status at transplantation (standard risk versus high risk), time from diagnosis to transplantation (<365 days versus ≥365 days), sex compatibility between donor and recipient (female donor to male recipient versus other), ABO compatibility between donor and recipient (match versus mismatch), and year of transplantation (1992 to 2002 versus 2003 to 2013).

All statistical analyses were performed with EZR (Saitama Medical Center, Jichi Medical University, Saitama, Japan), a graphic user interface for R 3.0.2 (R Foundation for Statistical Computing, Vienna, Austria) [15]. $P < .05$ was considered significant. Analysis of data was performed in December 2013.

RESULTS

Characteristics of Patients and Grafts

The characteristics of patients, grafts, and transplant procedures are summarized in Table 1. Recipients' age, sex, cytomegalovirus serostatus, disease type, disease status at transplantation, GVHD prophylaxis, sex incompatibility between donors and recipients, and time from diagnosis to transplantation were almost the same between the CBT and rBMT/PBSCT recipients. On the other hand, there were significant differences in the following variables (Table 1). The conditioning regimen significantly differed between the CBT and rBMT/PBSCT recipients ($P < .01$). The most common conditioning regimen was TBI12Gy+Ara-C/G-CSF+cyclophosphamide (78%) for CBT and TBI12Gy+Ara-C/G-CSF (64%) for rBMT/PBSCT. The number of nucleated cells or $CD34^+$ cells for CBT recipients was 1 log lower than in rBMT or rPBSCT recipients, respectively. The proportion of HLA disparity and ABO incompatibility was higher among CBT recipients than rBMT/PBSCT recipients. CBT was more frequently performed in recent years, resulting in the significantly shorter follow-up period for CBT compared with that for rBMT/PBSCT. Median follow-up was 185 months (range, 32 to 258 months) for rBMT/PBSCT recipients and 87 months (range, 4 to 175 months) for CBT recipients ($P < .01$).

Table 2
Univariate and Multivariate Analysis of Transplant Outcomes after rBMT/PBSCT and CBT in Patients Aged 45 Years or Older

	Univariate Analysis		Multivariate Analysis*	
	HR (95% CI)	P	HR (95% CI)	P
Neutrophil engraftment				
rBMT/PBSCT vs. CBT	.69 (.38-1.04)	.07	.46 (.26-.81)	<.01
Platelet engraftment				
rBMT/PBSCT vs. CBT	.30 (.14-.61)	<.01	.24 (.12-.50)	<.01
Grades III-IV aGVHD				
rBMT/PBSCT vs. CBT	.57 (.18-1.85)	.36	.53 (.15-1.90)	.34
Extensive cGVHD				
rBMT/PBSCT vs. CBT	1.01 (.53-1.91)	.97	1.08 (.49-2.35)	.84
Overall mortality				
rBMT/PBSCT vs. CBT	.69 (.36-1.32)	.26	.72 (.30-1.73)	.47
Relapse				
rBMT/PBSCT vs. CBT	1.42 (.52-3.87)	.49	2.02 (.63-6.42)	.23
TRM				
rBMT/PBSCT vs. CBT	.38 (.16-.93)	.03	.29 (.08-.99)	.04

* For neutrophil engraftment, lymphoid disease was also a significant variable (HR, 2.40; 95% CI, 1.52 to 3.79; $P < .01$). For platelet engraftment, lymphoid disease was also a significant variable (HR, 1.74; 95% CI, 1.17 to 2.59; $P < .01$). For grades III-IV aGVHD, ABO incompatibility was a significant variable (HR, 4.41; 95% CI, 1.06 to 18.24; $P = .04$). For extensive cGVHD, high risk of disease status at transplantation was a significant variable (HR, 3.14; 95% CI, 1.39 to 7.09; $P < .01$). For overall mortality, high risk of disease status at transplantation (HR, 3.33; 95% CI, 1.36 to 8.11; $P < .01$) and ABO incompatibility (HR, 3.14; 95% CI, 1.44 to 6.87; $P < .01$) were significant variables. For relapse, high risk of disease status at transplantation was a significant variable (HR, 4.55; 95% CI, 1.08 to 19.23; $P = .03$). For TRM, female donor to male recipient (HR, 2.89; 95% CI, 1.11 to 7.52; $P = .02$) and ABO incompatibility (HR, 5.20; 95% CI, 1.56 to 17.33; $P < .01$) were also significant variables.

Neutrophil and Platelet Engraftment

One patient in the CBT group died on day 21 due to encephalitis, and 1 patient in the rBMT/PBSCT group died on day 7 due to organ failure. Primary graft failure occurred in 3 of the surviving 65 patients in the CBT group, but there was no primary graft failure in the rBMT/PBSCT group. As expected, neutrophil recovery was significantly delayed after CBT as compared with rBMT/PBSCT. Median times to neutrophil recovery were 22 days (range, 18 to 34 days) after CBT, as compared with 18 days (range, 11 to 40 days) after rBMT/PBSCT ($P < .01$). The cumulative incidence of neutrophil recovery on day 60 was slightly lower after CBT (93.9%; 95% confidence interval [CI], 83.5% to 97.9%) compared with rBMT/PBSCT (96.8%; 95% CI, 57.8% to 99.8%) ($P = .07$). In the multivariate analysis, the hazard risk of neutrophil engraftment was significantly lower after CBT as compared with rBMT/PBSCT (hazard ratio [HR], .46; 95% CI, .26 to .81; $P < .01$, Table 2).

Platelet recovery was also significantly delayed after CBT as compared with rBMT/PBSCT. Median times to platelet recovery were 42 days (range, 13 to 104 days) after CBT, as compared with 24 days (range, 15 to 300 days) after rBMT/PBSCT ($P < .01$). The cumulative incidence of platelet recovery on day 100 was significantly lower after CBT (90.8%; 95% CI, 80.0% to 95.9%) compared with rBMT/PBSCT (93.5%; 95% CI, 71.5% to 98.7%) in the univariate analysis ($P < .01$); the difference was also significant in multivariate analyses (HR, .24; 95% CI, .12 to .50; $P < .01$, Table 2).

Acute and Chronic GVHD

The cumulative incidences of grades II to IV (HR, .90; 95% CI, .49 to 1.64; $P = .76$) and grades III to IV aGVHD (HR, .53; 95% CI, .15 to 1.90; $P = .34$) were similar between CBT and rBMT/PBSCT recipients in multivariate analyses (Table 2). The unadjusted cumulative incidence of grades III to IV aGVHD at 100 days was 9.2% (95% CI, 3.7% to 17.8%) in CBT recipients and 16.1% (95% CI, 5.7% to 31.2%) in rBMT/PBSCT recipients ($P = .35$). Extensive cGVHD developed in 27 of 58 CBT recipients and in 13 of 27 rBMT/PBSCT recipients surviving more than 100 days. In a multivariate analysis, the cumulative incidences of cGVHD (HR, .94; 95% CI, .55 to 1.62; $P = .84$) and extensive cGVHD (HR, 1.08; 95% CI, .49 to 2.35; $P = .84$) were similar between CBT and rBMT/PBSCT recipients in multivariate analysis (Table 2).

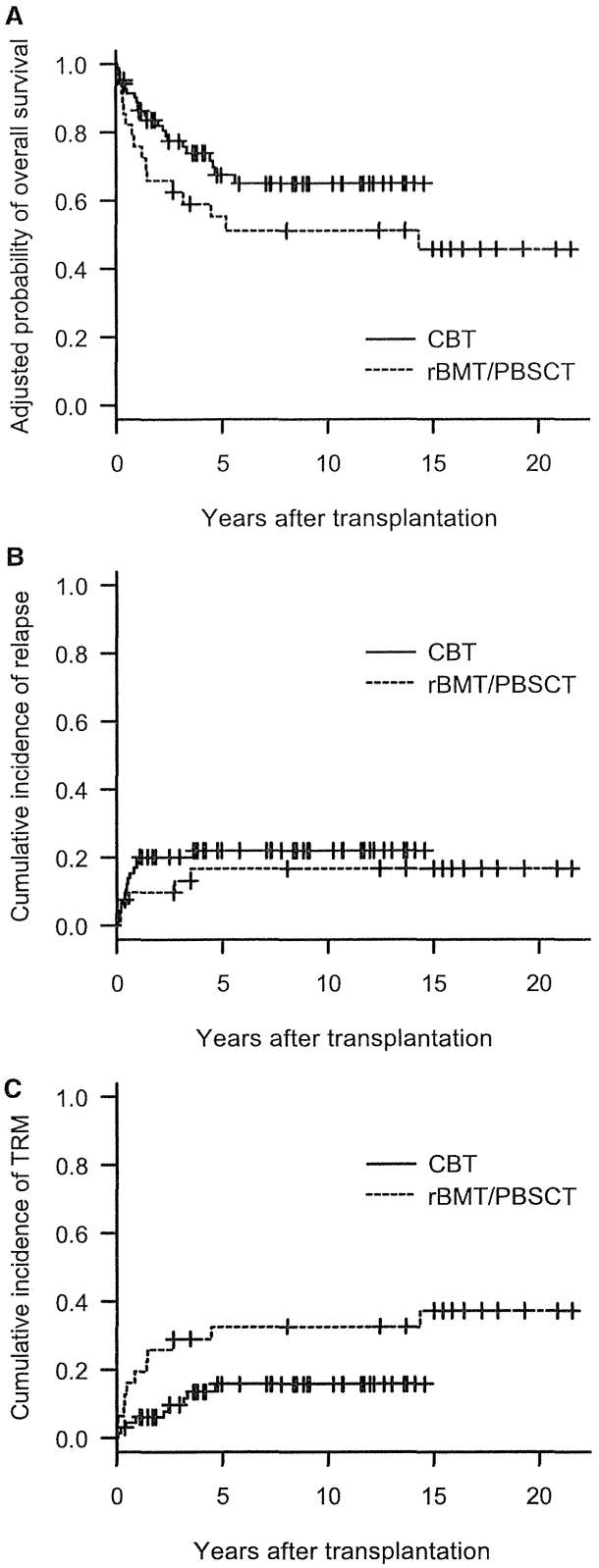

Figure 1. Outcomes after CBT or BMT/PBSCT from related donors in patients aged 45 years or older with hematologic malignancies after a myeloablative conditioning regimen. Adjusted probability of OS (A), unadjusted cumulative incidence of relapse (B), and TRM (C).

Table 3
Cause of Death

	Death before 100 Days		Death after 100 Days	
	rBMT/PBSCT (n = 2)	CBT (n = 4)	rBMT/PBSCT (n = 14)	CBT (n = 19)
Primary disease	0	2	5	12
GVHD	0	0	5	5
Infection	1	2	4	2
Organ failure	1	0	0	0

Survival, Relapse, and TRM

The adjusted probabilities of OS at 5 years were 67.4% (95% CI, 55.7% to 81.6%) for recipients of CBT and 55.2% (95% CI, 39.4% to 77.4%) for recipients of rBMT/PBSCT (Figure 1A). In multivariate analysis, the hazard risk of overall mortality was similar between CBT and rBMT/PBSCT recipients (HR, .72; 95% CI, .30 to 1.73; $P = .47$; Table 2). We also compared OS of both groups for each disease risk. However, OS of both recipient groups was also equivalent in standard-risk patients (n = 31) and high-risk patients (n = 66) (data not shown). The unadjusted cumulative incidence of relapse at 5 years was 22.0% (95% CI, 12.7% to 33.0%) in CBT recipients and 16.7% (95% CI, 5.9% to 32.3%) in rBMT/PBSCT recipients ($P = .48$) (Figure 1B). In multivariate analysis, the hazard risk for relapse was similar between CBT and rBMT/PBSCT recipients (HR, 2.02; 95% CI, .63 to 6.42; $P = .23$; Table 2). The unadjusted cumulative incidence of TRM was significantly lower after CBT at 100 days (3.0%; 95% CI, .6% to 9.4%) and 5 years (15.8%; 95% CI, 7.6% to 26.6%) compared with rBMT/PBSCT at 100 days (6.5%; 95% CI, 1.1% to 18.9%) and 5 years (32.7%; 95% CI, 16.8% to 49.6%) ($P = .04$) (Figure 1C). In multivariate analysis, the hazard risk of TRM was significantly lower after CBT as compared with rBMT/PBSCT (HR, .29; 95% CI, .08 to .99; $P = .04$; Table 2).

We also analyzed a subgroup of patients aged 50 years or older after CBT (n = 29) and rBMT/PBSCT (n = 11). In multivariate analysis, the hazard risk of overall mortality (HR, .36, $P = .10$) and relapse (HR, 2.73, $P = .41$) after CBT was comparable with that after rBMT/PBSCT, respectively. However, the hazard risk of TRM was lower after CBT than after rBMT/PBSCT (HR, .16; 95% CI, .04 to .56; $P < .01$).

The causes of death before and after 100 days after transplantation by donor type are summarized in Table 3. The major cause of death in both recipient groups was primary disease. However, GVHD and infection as a primary cause of late mortality were more common after rBMT/PBSCT compared with CBT.

DISCUSSION

The objective of this study was to compare the transplant outcomes after CBT and rBMT/PBSCT in relatively older patients who were candidates for myeloablative conditioning. Unexpectedly, there were no significant differences in aGVHD and cGVHD between CBT and rBMT/PBSCT recipients. However, TRM was higher after rBMT/PBSCT compared with CBT. The reduced TRM in CBT might be in part due to improved supportive care, because CBT was more frequently performed in recent years. However, year of transplantation did not affect any clinical results in our multivariate analysis. On the other hand, we used almost the same 12-Gy TBI-based myeloablative conditioning and cyclosporine-based GVHD prophylaxis regimens during the period for both recipients of CBT and rBMT/PBSCT. Among relatively older