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Intrabone Marrow Transplantation of Unwashed Cord Blood Using Reduced-Intensity Conditioning Treatment: A Phase I Study

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The outcome of cord blood transplantation following reduced-intensity conditioning is suboptimal because of fatal infection triggered by prolonged neutropenia and graft-versus-host disease (GVHD) in addition to graft rejection. Intrabone marrow injection (IBMI) may improve the outcome by providing better hematopoietic engraftment and less GVHD. We therefore evaluated IBMI safety in reduced-intensity stem cell transplantation. Furthermore, we used unwashed cord blood to avoid stem cell loss. Ten patients (median age = 61 years old) were enrolled. Cord blood cells were thawed at the bedside and injected into 4 iliac bone sites (2 at each hemipelvis). The procedure was well tolerated with no injection-related complications. Nine patients achieved donor engraftment. The median time to neutrophil recovery ($>0.5 \times 10^9$/L) was 17 days, and platelet recovery was achieved in 8 patients. Early full donor chimerism was achieved (median of 15 and 20 days in T cells and myeloid cells, respectively). Three of 9 evaluable patients developed grade II to III GVHD, and 5 of 10 patients died of treatment-related toxicities. The probability of survival at 1 year was 46.7%. IBMI of unwashed cord blood following reduced-intensity conditioning is safe, well tolerated, and may lead to an increased donor engraftment rate.

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KEY WORDS: Cord blood transplantation, Reduced-intensity conditioning, Intrabone marrow injection, Unwashed cord blood

INTRODUCTION

In recent years, umbilical cord blood transplantation (CBT) has been increasingly used for adult patients with hematologic malignancies. The use of reduced-intensity conditioning (RIC) markedly broadened the indication of CBT to elderly patients lacking an HLA-matched sibling donor; however, although the outcome of CBT for younger patients who have received myeloablative conditioning is reportedly similar to that of patients who have undergone unrelated bone marrow transplantation [1-5], the outcome of RIC CBT (RI-CBT) for elderly patients remains suboptimal [6-9]. In particular, the major drawbacks of CBT are the high incidence of graft failure and delayed hematopoietic recovery, both of which are mainly attributable to the small number of progenitors infused.

Studies using animal models have suggested that intrabone marrow injection (IBMI) of hematopoietic stem cells promotes engraftment [10-12] and reduces the risk of graft-versus-host disease (GVHD) [11]. The improvement of engraftment conferred by this technique is likely attributable to the advantage of the seeding efficiency of progenitor cells, which is superior to that of intervenous (i.v.) infusion, where only a small proportion of progenitor cells engraft bone marrow (BM) [12-14].

Thus, we planned to perform a clinical study of IBMI of cord blood after RIC treatment. Moreover, most institutes in Japan, including our transplant team, routinely use unwashed cord blood for i.v.

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CBT in an attempt to avoid the loss of progenitor cells and to reduce the risks associated with the cell-washing procedure, such as bacterial contamination, and have not experienced particular problems. Based on this previous experience, we planned to use unwashed cord blood for IBMI. Because the safety of IBMI of unwashed cord blood containing dimethyl sulfoxide (DMSO) as a cryoprotectant has not been established to date, we conducted a phase I study to evaluate the safety of this procedure.

METHODS

Patients

The major objective in this study was to evaluate the safety of reduced-intensity intrabone marrow CBT using unwashed cord blood units. It was planned to enroll 10 patients in this phase I study. The subjects were adult patients age 55 to 70 years, or younger patients who were considered ineligible for myeloablative preparative regimens because of comorbidities. Patients were eligible to enroll in this study if they required allogeneic stem cell transplantation and could not be paired with human leukocyte antigen (HLA)-matched or 1 antigen-mismatched related donors, or unrelated donors (genotypically matching or 1 locus-mismatching in HLA-A, B, and DRB1 loci) within a clinically useful period of time. If even 1 patient had life-threatening adverse effects after the infusion of unwashed cord blood, the study would be discontinued. This protocol was approved by the institutional review board of Hyogo College of Medicine and the ethics committee of the Japan Cord Blood Bank Network. All patients signed an approved consent form before participating in the study.

Cord Blood Grafts

A cord blood search was performed through the Japan Cord Blood Bank Network. All cord blood units were processed (ie, red blood cells and plasma removed) and cryopreserved at each of the 11 cord blood banks in Japan according to the New York Blood Center method. The final volume of each unit was uniformly 25 mL. HLA-A, -B, and -DR antigens were identified by low-molecular and then high-resolution molecular typing using polymerase chain reaction (PCR) sequence-specific primers. A cord blood unit was chosen for patients after considering the HLA disparity between the donor and recipient, and the cell dose. Cord blood units had $\geq$4 of 6 matches regarding HLA-A, -B, and -DR antigens, and had a cell dose of $\geq 2.0 \times 10^7$ nucleated cells per kilogram of the recipient's body weight before freezing.

IBMI Procedure

Each cord blood unit was thawed in a 37°C water bath at the bedside in a clean room, and was collected into a 50-mL syringe with a Luer lock just before injection. Patients were premedicated with hydrocortisone 100 mg and hydroxyzine 30 minutes before starting the IBMI procedure. After local anesthesia with 1% lidocaine, standard BM aspiration needles (16-gauge) were inserted into the superior–posterior iliac crest of the patient in the prone position. Two needles were inserted into each hemipelvis at a distance of 4 to 5 cm, except for 1 patient (No. 8) who received cord blood at 2 sites on 1 side of the pelvis. The stylet was removed from the needle, a small volume of BM fluid was aspirated to confirm that the needle was inserted into the BM space, and approximately 6 mL of the collected cord blood aliquot corresponding to one-fourth of the total volume was slowly injected into the BM space. Saline (0.5-1 mL) was subsequently injected to push the small number of cord blood cells left in the needle into the BM space. The stylet was reattached to the needle in order to avoid the backflow of cord blood into the needle. This procedure was repeated for the remaining aliquots. The patient who received cord blood on 1 side of the pelvis received a 12-mL aliquot at 1 site. The entire IBMI procedure took 20 to 30 minutes from the start of local anesthesia to the end of injection.

Preparative Conditioning and GVHD Prophylaxis

The conditioning regimen consisted of fludarabine (40 mg/m^2) for 5 days (day –6 to day –2), cyclophosphamide (50 mg/kg) on day –6, and total body irradiation 3 Gy on day –1 [8]. GVHD prophylaxis consisted of cyclosporine A (CsA) and mycophenolate mofetil (MMF): A continuous infusion of CsA was started at a dose of 3 mg/kg on day –3 with target serum concentration of 250 to 450 ng/mL, and MMF was started at a dose of 15 mg/kg/day (divided into 2 or 3 doses) on day –3 for the first 3 patients and at a dose of 30 mg/kg/day for the next 7 patients because 2 of the first 3 patients developed severe GVHD. When patients did not have acute GVHD (aGVHD) by day 30, tapering of CsA and MMF was started: CsA was tapered over months (approximately 5%-10% per week) and MMF was tapered over 2 weeks.

Supportive Care

Supportive care was performed as previously described [15]. In brief, the enrolled patients were hospitalized in a single room ventilated with a high-efficiency particulate air filtration system. Each patient received fluoroquinolones and azoles (fluconazole, itraconazole, or voriconazole) from the beginning of the preparative regimen until the discontinuation of immunosuppressants. In addition, acyclovir was administered at a dose of 1000 mg/day for 5 weeks after transplantation, and was subsequently continued at

a dose of 200 mg/day until discontinuation of the immunosuppressants. Patients seropositive for cytomegalovirus received prophylactic ganciclovir (5 mg/kg) for 5 days during the preparative treatment. Cytomegalovirus pp65 antigenemia was monitored weekly. In addition, the reactivation of human herpesvirus-6 was monitored biweekly by PCR analysis of the virus DNA, and was also checked when patients developed a preengraftment immune reaction. Granulocyte colony-stimulating factor (300 $\mu g/m^2$) was started from day 1 and was continued until the neutrophil count was $>2.5 \times 10^9$/L for 2 consecutive tests.

Chimerism Analysis

The chimerism status of the donor/recipient was monitored using the T cell– or granulocyte-enriched cell fraction of the peripheral blood. The methodology used for cell separation and chimerism analysis has been detailed elsewhere [15,16]. T cells were enriched by a negative selection system (RosetteSep; StemCell, Vancouver, Canada) to a purity of >95%, and granulocytes were recovered from the Ficoll–red blood cell interface with a purity of >99%. Chimerism analysis involved quantitative PCR of informative short tandem repeats in the recipient and donor.

Definitions and Statistical Analysis

Engraftment was defined as the first of 3 consecutive days of absolute neutrophil count $>0.5 \times 10^9$/L. Mixed chimerism was defined as between 5% and 95% donor cells, and full donor chimerism was defined as the presence of >95% donor cells [17]. Primary graft failure was defined as the lack of even partial donor engraftment (mixed chimeric status). Secondary graft failure was defined as a decreased percentage of donor cells to <5% after achievement of at least mixed chimerism. Diagnosis of aGVHD and chronic GVHD (cGVHD) was based on standard clinical criteria [18], and was pathologically confirmed if possible. Cumulative incidence was used to estimate the rates of neutrophil engraftment and platelet recovery, treating nonevent deaths as a competing risk. Overall survival (OS) was calculated using the Kaplan-Meier method.

The data were "locked" for analysis on December 31, 2010.

RESULTS

Patient and Graft Characteristics

Ten patients were enrolled in this clinical study between June 2008 and June 2010. The patient and graft characteristics are summarized in Table 1. The median age of the patients was 61 years (range: 51-66 years), and their median weight was 57.1 kg (range: 43.0-86.3 kg). The diagnoses included myelodysplastic syndrome or acute myeloid leukemia evolved from myelodysplastic syndrome (n = 4), acute myeloid leukemia (n = 2), acute lymphoblastic leukemia (n = 2), and non-Hodgkin lymphoma (n = 2). At the time of transplantation, 5 patients were in a nonremission status, and the remaining 5 were hematologically in complete remission but 4 had minimal residual disease detected by real-time PCR analysis using the *WT1* gene [19] or chimeric genes. Patients received cord blood cells containing a median of 2.72×10^7 (range: $2.04\text{-}3.50 \times 10^7$/kg) nucleated cells/kg and a median of 0.60×10^5 (range: $0.30\text{-}0.99 \times 10^5$/kg) $CD34^+$ cells/kg.

Safety and Tolerability of IBMI Procedure

No particular adverse events were observed in the IBMI of cord blood. Injection of cord blood into the BM space could be performed without resistance. During injection, the patients did not experience pain except for some patients who experienced mild pain during the injection of saline to push cord blood cells in the needle into the BM space. Moreover, no abnormalities were observed in general clinical examinations, including blood pressure, heart rate, and oxygen saturation.

For the first 7 patients who received 6-mL injections of cord blood at each IBMI site, a distinct odor derived from DMSO was imperceptible by the staff in the individual clean rooms during and after the injection of cord blood. In contrast, for the eighth patient who received 12-mL injections of cord blood at each IBMI site, the DMSO-derived odor was clearly perceptible in the room. These observations suggest that a considerable proportion of cord blood cells injected into the BM came out into the circulation soon after the 12-mL injection at each BM site; therefore, we used 6-mL injections for the remaining 2 patients, and noted that the DMSO-derived odor was again imperceptible in their rooms.

Engraftment and Chimerism

One patient (No. 9) showed no signs of neutrophil recovery, with 100% recipient chimerism on day 24, and was diagnosed with primary graft failure. The remaining 9 patients achieved engraftment: the median time to reach a neutrophil count of $\geq 0.5 \times 10^9$/L was day 17 (range: 7-33 days), as shown in Figure 1A. Platelet recovery was achieved in 8 patients, and the median time to reach a nontransfused platelet count of $\geq 20 \times 10^9$/L and $\geq 50 \times 10^9$/L was 41 days (range: 12-55 days) and 43 days (range: 36-103 days), respectively (Table 1, Figure 1A). In the majority of patients achieving donor engraftment, donor T cell recovery preceded donor neutrophil recovery (Figure 1B). Neutrophils increasing in the early period of transplantation were mostly of recipient origin, which

Table 1. Patient Characteristics and Transplant Outcomes

					HLA Disparity		Cord Blood Unit		Time to recovery (days)			Acute GVHD				Chronic			
Patient No.	Age/ Sex	Weight (kg)	Diagnosis	Disease Status	GVH Vector	HVG Vector	NCC ($\times 10^7$/kg)	CD34 ($\times 10^5$/kg)	Neutrophil $\geq 0.5 \times 10^9$/L	Platelet $\geq 20 \times 10^9$/L	Platelet $\geq 50 \times 10^9$/L	Skin	Gut	Liver	Grade	GVHD	Relapse	Current Status (Day)	Cause of Death
1	64/M	48.4	MDS-AML	Untreated	4/6	4/6	3.05	0.88	7	12	39	2	1	0	II	Ex	—	dead (214)†	MRSA sepsis
2	58/F	43.0	MDS-AML	CR5, MRD+	4/6	4/6	3.50	0.69	9	—	—	3	3	0	III	NA	—	dead (81)	TMA
3	60/M	56.5	ALL, Ph+	CR1, MRD+	5/6	6/6	3.27	0.99	9	42	45	2	0	0	I	—	—	alive (>809)	—
4	66/F	47.1	AML, M4	Refractory	5/6	5/6	2.05	0.45	24	55	103	2	0	0	I	—	—	alive (>505)	—
5	51/M	62.4	MDS	Refractory	5/6	5/6	2.58	0.45	17	35	40	3	1	0	II	—	—	dead (111)	CMV pneumonia
6	62/M	66.0	ALL	CR1	5/6	5/6	2.71	0.30	33	49	55	2	0	0	I	—	—	dead (291)	*Aspergillus* pneumonia
7	57/M	86.3	NHL (FL)	RR-Auto	4/6	4/6	2.04	0.63	17	40	40	2	0	0	I	—	—	alive (>330)	—
8	63/M	70.0	AML, M4	CR2, MRD+	4/6	4/6	2.24	0.56	21	36	36	1	0	0	I	—	+ (166)*	alive (>239)	—
9	61/M	57.3	MDS-AML	CR1, MRD+	4/6	4/6	3.00	0.70	—	—	—	NA	NA	NA	NA	—	—	dead (143)	GVHD after second SCT
10	57/M	56.8	NHL (PTCL)	Refractory	5/6	5/6	2.72	0.57	17	47	47	3	0	0	II	—	—	alive (>141)	—

M indicates male; F, female; AML, acute myeloid leukemia; MDS-AML, AML evolved from myelodysplastic syndrome; ALL, acute lymphoid leukemia; Ph, Philadelphia chromosome; M4, myelomonocytic leukemia; NHL, non-Hodgkin lymphoma; FL, follicular lymphoma; PTCL, peripheral T cell lymphoma; MRD, minimal residual disease; CR, complete remission; RR-Auto, refractory relapse after autologous stem cell transplantation; GVH, graft-versus-host; HVG, host-versus-graft; NCC, nucleated cell dose; NA, not applicable; Ex, extensive type; MRSA, methicillin-resistant *Staphylococus aureus*; TMA, thrombotic microangiopathy.
Figures in the columns of skin, gut, and liver of acute GVHD indicate the stage of GVHD in each organ.
As GVHD prophylaxis, patient Nos. 1, 2, and 3 received CsA and MMF 15 mg/kg, and the next 7 patients received CsA and MMF 30 mg/kg.
*The number in parentheses indicates the day when the patient had a relapse.
†The number in parentheses indicates survival days after transplantation.

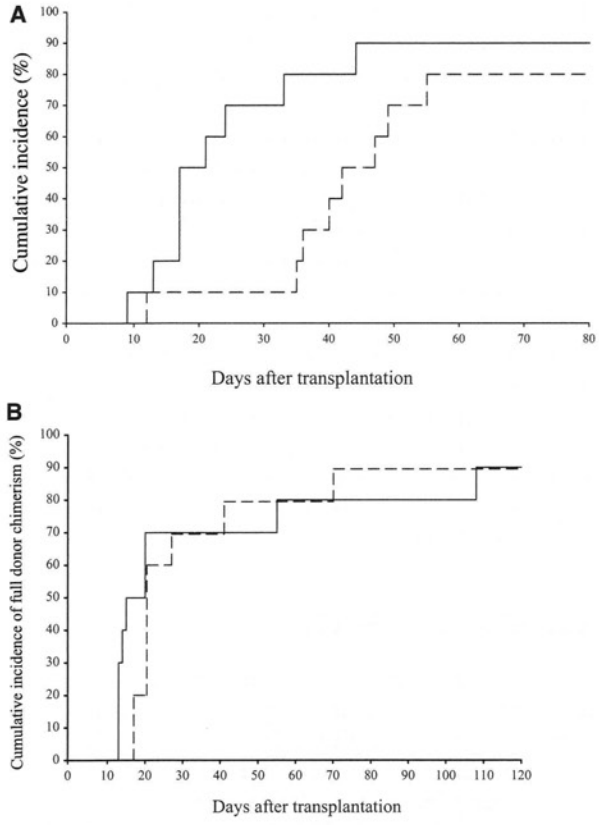

Figure 1. (A) Hematopietic recovery. Solid and broken lines indicate cumulative incidence of neutrophil recovery $\geq 0.5 \times 10^9$/L and unsupported platelet recovery $\geq 20 \times 10^9$/L, respectively. (B) Cumulative incidence of achieving full donor chimerism in T (solid line) and myeloid (broken line) cells of the peripheral blood, respectively.

may have contributed to the prevention of infection to some extent: recipient neutrophil counts peaked 0.15 to 1.30×10^9/L between days 7 and 11, then decreased, and recipient neutrophils were rapidly displaced with donor neutrophils. It took a median of 15 days (range: 13-108 days) to achieve full donor T cell chimerism and a median of 20 days (range: 17-70 days) to achieve full donor myeloid chimerism.

Outcomes

Patient outcomes are also summarized in Table 1. The majority of patients achieving donor engraftment developed preengraftment immune reactions; however, no particular treatments were needed. Two (Nos. 1 and 2) of the 3 patients who received CsA and MMF 15 mg/kg as GVHD prophylaxis developed grade II and III GVHD, respectively, ultimately resulting in fatal complications of methicillin-resistant *Staphylococcus aureus* sepsis or thrombotic microangiopathy. Among the remaining 6 evaluable patients who received CsA and MMF 30 mg/kg, 4 had grade I GVHD and 2 had grade II GVHD. All patients achieving donor engraftment developed skin GVHD, 4 gut GVHD, and none hepatic GVHD. Of note, all of these patients responded to a small dose of prednisolone. No patients developed chronic GVHD except for 1 patient (No. 1).

Overall, 5 of the 10 patients died from treatment-related toxicities: 3 from infection, 1 from thrombotic microangiopathy, and 1 from GVHD after a second transplantation. Although 1 patient (No. 8) relapsed 166 days after transplantation, he achieved complete remission again by receiving salvage chemotherapy. Five of the 9 patients who achieved donor engraftment are alive at a median follow-up of 392 days (range: 203-871 days). The probability of OS at 1 year after transplantation was 46.7%.

DISCUSSION

In the present study, we demonstrated that IBMI using unwashed cord blood was safe and well tolerated. In addition, the fact that 9 of the 10 recipients achieved donor-type engraftment suggested that this IBMI procedure coupled with a RIC treatment had sufficient ability to obtain donor engraftment. Recently, Frassoni et al. [20,21] reported the outcome of 32 patients treated by IBM-CBT, the majority of whom received a myeloablative conditioning treatment (including 2 patients receiving RIC). Their study also showed that the IBMI procedure was safe, with the observation of a high engraftment rate, early and robust platelet recovery, and a low incidence of aGVHD. More recently, Brunstein et al. [22,23] reported the outcome of 10 patients who underwent double-unit CBT after myeloablative conditioning, in which 1 unit was injected into the BM and the other into the vein. Although Brunstein et al. [22,23] also showed the safety of the IBMI procedure, they could not observe any superiority of IBMI over i.v. infusion.

In the present study, first, we demonstrated that unwashed cord blood was safely used in the IBMI procedure. In many transplant centers, cord blood units are washed before transplantation, according to a method previously reported by Rubinstein et al. [24]. The 2 aforementioned IBMI studies of cord blood also used washed cord blood. Removal of DMSO may reduce the incidence of adverse reactions associated with the infusion of DMSO [25,26]; however, the clinical implications of cell washing are arguable because a much greater amount of DMSO is routinely infused with autologous peripheral blood stem cell products and, more important, cell washing may lead to the loss of cells to be transplanted. Of note, Barker et al. [27] reported a high engraftment rate in CBT using a simplified method of diluting thawed cord blood with albumin-dextran. Our procedure of IBMI using unwashed cord blood is a particularly simple method that could be conveniently completed at the bedside, although the method might

not be applied to cord blood units of much larger volume, such as cord blood units without plasma reduction.

Even though the aim of the present study was not to evaluate the efficacy of IBMI, better donor engraftment may be expected with this transplant procedure because 9 of the 10 patients achieved donor engraftment with early achievement of full donor chimerism; however, because of the small number of patients treated, we have not yet determined whether this IBMI procedure using unwashed cord blood produces early hematopoietic recovery or a high rate of donor engraftment compared with the usual i.v. infusion methods. Several RI-CBT studies using i.v. infusion methods showed that the platelet recovery rate (platelet count $\geq 20 \times 10^9$/L or $\geq 50 \times 10^9$/L) varied from 42% to 80% [6,8,9,28]. In the present study, platelet recovery occurred in 80% of the patients. This favorable platelet recovery after IBMI is consistent with the results reported by Frassoni et al. [20], but not by Brunstein et al. [22,23], in which only 5 of 10 patients achieved platelet recovery. Regarding the volume of cord blood injected at each BM site, we and Frassoni et al. [20] injected a small aliquot (4-6 mL) of cord blood at each site, whereas Brunstein et al. [22,23] injected a large volume of 20 mL into each site. In order to assess the efficacy of IBMI with cord blood accurately, as many cells as possible should be retained in the BM after injection; however, there is no accurate method at present to assess how many cells are retained. Interestingly, as described by Brunstein et al. [22] who used washed cord blood, we also noticed the distinct odor of DMSO in 1 patient who received a 12-mL injection at each site, which strongly suggested that a substantial number of cord blood cells immediately entered the bloodstream with DMSO and that DMSO was noticed in expiratory air from the lungs; however, with other patients who received a 6-mL injection at 1 injection site, the DMSO-derived odor was imperceptible in their rooms. This observation suggests that the volume of cord blood injected at each BM site may be important in IBM-CBT, which may explain the difference in hematopoietic recovery between the studies by Frassoni et al. [20] and Brunstein et al. [22], although a difference in the strength of preparative regimens can affect engraftment, as shown by Barker et al. [6]; however, delivering hematopietic stem cells to the BM site might not be sufficient to explain any observed advantages of intrabone transplantation if the BM and circulatory system are closely connected. In this regard, Feng at al. [29] reported that intrabone injection facilitated BM homing and redistribution to noninjection-side BM cavities through enhanced expression of cell adhesion molecules on donor cells.

Regarding the occurrence of GVHD, 2 of the first 3 patients in our study who received MMF 15 mg/kg/day as GVHD prophylaxis developed severe aGVHD that ultimately led to transplant-related deaths. The dose of MMF 15 mg/kg may have been too low to obtain a clinically effective blood concentration of the agent. The subsequent 6 evaluable patients who received MMF at an increased dose of 30 mg/kg daily, which was the same dosage as used by Frassoni et al. [20,21], still developed preengraftment immune reactions, and 2 had grade II GVHD; however, it is of note that GVHD occurring in these patients was easily controlled with a small dose of steroid, and none developed cGVHD.

In conclusion, the present study demonstrated the safety of IBMI using unwashed cord blood in a transplant setting of RIC. A larger-scale clinical study is required to determine whether IBMI has advantages in hematopoietic recovery or the risk of GVHD. We are planning to proceed to a multicenter, phase II study to evaluate the efficacy of IBM-CBT.

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Progressive hearing loss following acquired cytomegalovirus infection in an immunocompromised child

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Abstract We report a rare case of progressive hearing loss after acquired CMV infection in a child with Langerhans cell histiocytosis (LCH). A 5-month-old female was diagnosed as having LCH. When she was 14 months old, she received an unrelated donor umbilical cord blood transfusion for the treatment of intractable LCH. CMV infection was confirmed after the blood transfusion. Because her own umbilical cord had no CMV, the CMV infection was not congenital. When she was 7 years old, mixed hearing loss was noted with bilateral otitis media with effusion. After that time, the sensorineural hearing loss progressed to bilateral profound hearing loss over 3 years. Three-dimensional fluid-attenuated inversion recovery magnetic resonance imaging with gadolinium contrast enhancement revealed a high intensity area in the inner ear that suggested bilateral labyrinthitis. This case demonstrates the possibility that, under the immunodeficiency, the acquired CMV infection causes progressive sensorineural hearing loss.

1. Introduction

Cytomegalovirus (CMV) is a DNA-containing herpes virus. Its scientific name is human herpes virus 5 and it causes a range of symptoms after the first infection or after reactivation. CMV is well known as the cause of congenital sensorineural hearing loss or the opportunistic pathogen. Congenital CMV infection, which is caused by transplacental infection, also has various symptoms including low birth weight, microcephaly, hepatosplenomegaly, meningitis, and sensorineural hearing loss. Congenital CMV infection is the most widespread cause of sensorineural hearing loss other than inherited disease [1–6]. However, few reports have described sensorineural hearing loss caused by acquired CMV infection. We report progressive sensorineural hearing loss caused by acquired CMV infection in an immunocompromised child.

2. Case report

A 5-month-old female was referred to the Department of Pediatrics at Nagoya University Hospital because of fever, lymphadenopathy and purpura. Based on histological examination of neck lymph nodes, she was diagnosed with disseminate Langerhans cell histiocytosis (LCH). Then she received chemotherapy. First, one course of DAL-HX 83 study group protocol (etoposide (VP-16)+vinblastine (VBL)+prednisolone (PDN)) as initial chemotherapy and secondly, one course of next chemotherapy (VP-16+VBL+cyclophosphamide (CPA)) were performed. But the LCH did not response completely. When she was 9 months old, her splenomegaly became worse and caused C-reactive protein

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(CRP) elevation and anemia had progressed. Although she was given blood transfusions many times, her anemia did not improve. Finally, splenectomy was performed. After the operation, two courses of CHOP therapy (CPA+VP-16+vincristine (VCR)+PDN) and then, one course of next chemotherapy (CPA+VP-16+VCR) were performed but her symptom such as fever elevation, anemia, thrombocytopenia, and liver dysfunction became worse.

When she was 14 months old, finally, a cord stem cell transplant from an unrelated donor was performed and her LCH improved. However, CMV infection that was recognized before the cord stem cell transplantation was continuing. Because CMV antigen was not detected in her own cord blood, it was considered that the CMV infection was not congenital infection but acquired during treatment of the LCH. Due to her immunodeficiency with few CD4+ T cells (< 100/μl–400/μl), CMV infection continued including retinitis. CMV retinitis started when she was 18 months old. She was administered the antiviral drugs ganciclovir and foscarnet. During treatment, CMV antigen became negative, but when the antiviral drugs were discontinued, it became positive again, and she underwent repeated hospitalization and discharge. The retinitis did not become worse during this period. Fig. 1 shows changes of CMV-DNA amount that was investigated from the age of seven. When she was 7 years old, CMV DNA increased to 3913 copies/ml, and then decreased to zero or hundreds of copies/ml. When she was 9 years old, CMV DNA increased to 8705 copies/ml, but again decreased to zero or hundreds of copies/ml. Together with antiviral drug therapy, she was transfused with activated CD4+ T cells. Her blood CD4+ T cells increased to within the range of 500–900/μl, but her immunodeficiency did not improve. Her CMV antigen periodically became positive, at which times it was necessary to administer foscarnet.

When she was 6 years old, she consulted the Department of Otorhinolaryngology in our hospital because of suspicion of otitis media. At that time her mother did not notice her hearing impairment. However, three months after her first visit to the Department of Otorhinolaryngology, mixed hearing impairment with otitis media (OME) with effusion was recognized bilaterally (first audiogram in Fig. 2). A half year later, bilateral tympanostomy and ventilation tube insertion were performed because OME did not improve. Her bone-conduction hearing levels deteriorated to profound hearing loss (Fig. 2). When she was 8 years old, she complained of dizziness transiently. Three-dimensional fluid-attenuated inversion recovery (3D-FLAIR) MRI before and after enhancement showed a high intensity lesion in the internal auditory canal and in the cochlea without inner ear malformation. When she was 11 years old, 3D-FLAIR MRI after enhancement was performed again. The signal intensity of internal auditory canal was much stronger than that taken when she was 8 years old (Fig. 3). These findings suggested the exacerbation of meningitis close to the inner ear. When she was 11 years old, she had inflammation of the ventricles of the brain and a large amount of CMV-DNA was detected from her cerebrospinal fluid.

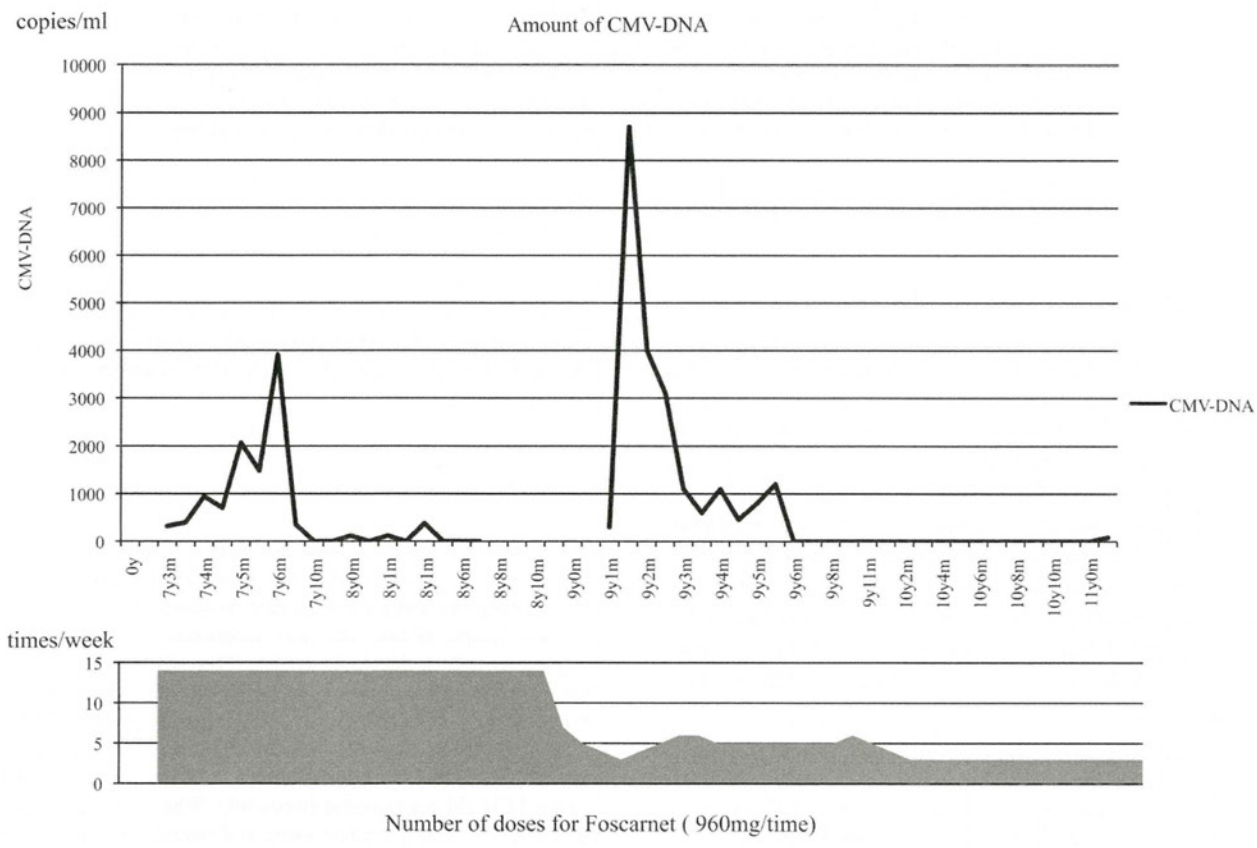

Fig. 1. The upper panel shows the changes in CMV DNA numbers from when she was 7 years old through when she was 11 years old. The lower panel shows the frequency of foscarnet administration during this period.

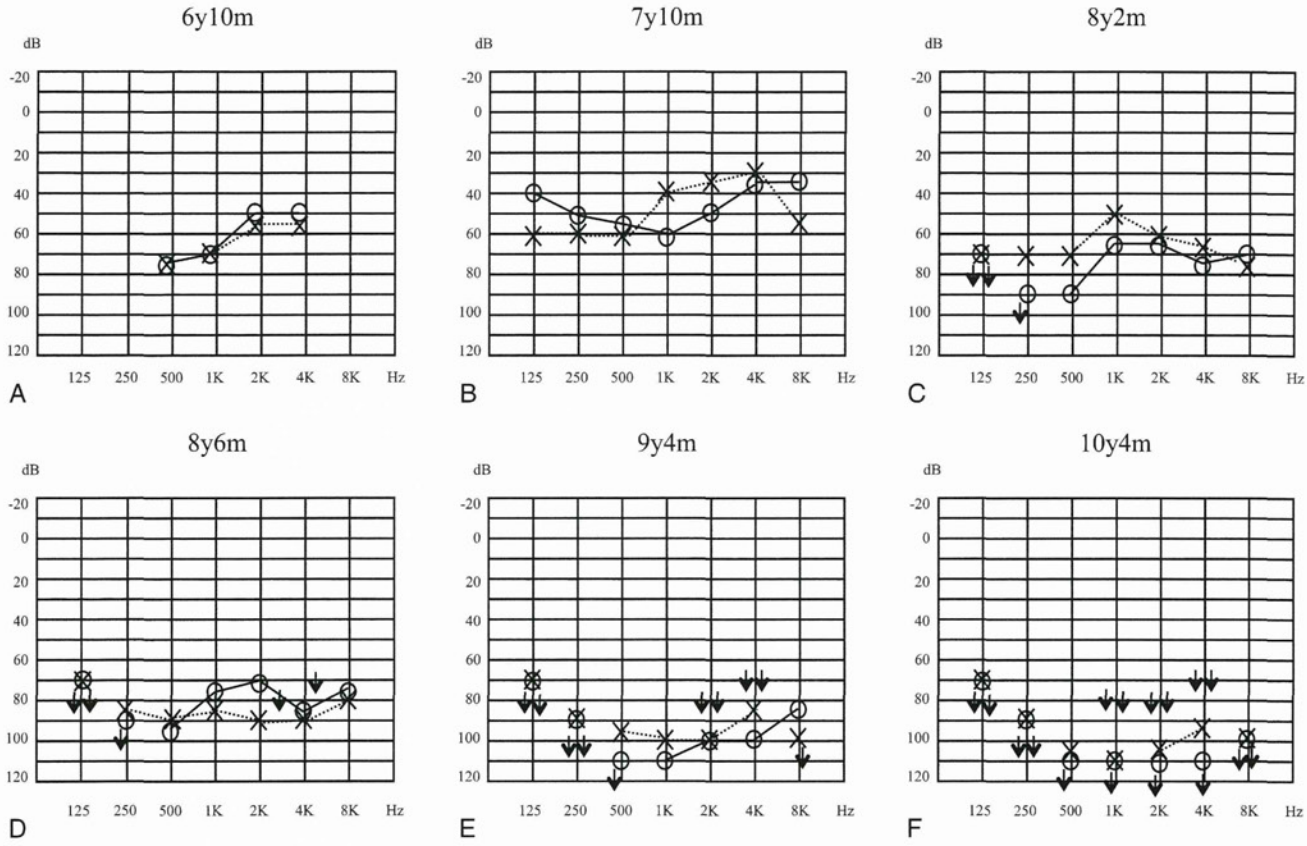

Fig. 2. From January 2007 to the present, the patient's hearing level has deteriorated. In particular, her bony conductive hearing level became worse. This showed that her sensorineural hearing loss was progressing.

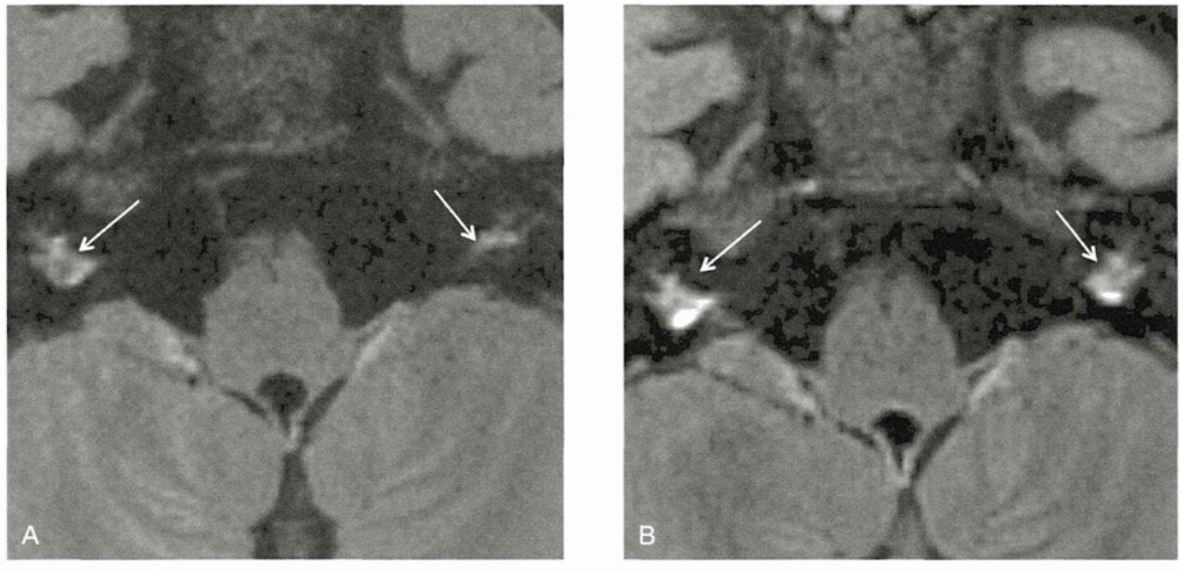

Fig. 3. 3D-FLAIR MRI after intervenous gadolinium administration. A was taken when she was 8 years old, and B was when she was 11 years old. The gadolinium enhancement of the internal auditory canal indicated by arrows was stronger in B than in A.

3. Comments

Because congenital CMV infection is transplacental infection, CMV is detected from the dried umbilical cord. On the contrary, in acquired CMV infection, CMV is not detected from the umbilical cord [7,8]. We diagnosed this case as acquired CMV infection since CMV was not detected from dried umbilical cord blood, we diagnosed that it was acquired CMV infection. There were few papers that described progressive sensorineural hearing loss in patients with acquired CMV infection except for the two cases reported by Meynard et al. [9]. These patients were also infected with human immunodeficiency virus (HIV) and were immunodeficient. Our patient is also suffering from immunodeficiency because of her allogeneic cord stem cell transplantation. Thus, her condition is similar to these cases where it was presumed that sensorineural hearing loss was caused by opportunistic infection.

In animal studies, CMV-infected cells have been detected in the perilymph area and spinal ganglion, but not in the endolymph area or hair cells [4,10,11]. Katano et al. [4] and Schraff et al. [11] injected CMV into the inner ear of guinea pigs and observed severe inflammation and bleeding in the scala tympani and spiral ganglion with progressive hearing loss. Virally encoded macrophage inflammatory proteins play the important role of inflammation in the scala tympani and CMV-related hearing loss [11]. Sugiura et al. [12] and Nardo et al. [13] succeeded in detecting CMV DNA in the inner ear fluid of congenitally or acquired CMV-infected patients who received cochlear implants. In these patients, CMV DNA was not detected in peripheral blood mononuclear cell. These articles are indirect evidence of CMV affinity and activity in the inner ear. In the temporal bone of patients congenitally infected with CMV, many pathologic findings such as endolabyrinthitis, endolymphatic hydrops, loss of cochlear hair cells and CMV infecting many parts of the cochlea and the vestibular system are observed [5,6]. Bachor et al. [14] reported their findings in the temporal bone of patients with acquired CMV infection. In our patient, the existence of asymptomatic meningitis and labyrinthitis was recognized by 3D-FLAIR MRI.

4. Conclusion

We experienced a case with progressive sensorineural hearing loss following acquired CMV infection in an immunocompromised child with Langerhans cell histiocytosis (LCH). To detect labyrinthitis, 3D-FLAIR MRI was very useful.

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Rabbit antithymocyte globulin and cyclosporine as first-line therapy for children with acquired aplastic anemia

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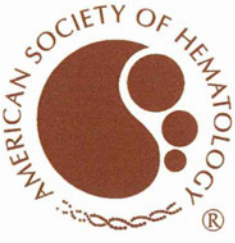

To the editor:

Rabbit antithymocyte globulin and cyclosporine as first-line therapy for children with acquired aplastic anemia

Horse antithymocyte globulin (hATG) and cyclosporine have been used as standard therapy for children with acquired aplastic anemia (AA) for whom an HLA-matched family donor is unavailable. However, in 2009, hATG (lymphoglobulin; Genzyme) was withdrawn and replaced by rabbit ATG (rATG; thymoglobulin; Genzyme) in Japan. Many other countries in Europe and Asia are facing the same situation.[1] Marsh et al recently reported outcomes for 35 adult patients with AA who were treated with rATG and cyclosporine as a first-line therapy.[2] Although the hematologic response rate was 40% at 6 months, several patients subsequently achieved late responses. The best response rate was 60% compared with 67% in a matched-pair control group of 105 patients treated with hATG. The overall and transplantation-free survival rates appeared to be significantly inferior with rATG compared with hATG at 68% versus 86% ($P = .009$) and 52% versus 76% ($P = .002$), respectively. These results are comparable to those from a prospective randomized study reported by Scheinberg et al comparing hATG and rATG.[3] Both studies showed the superiority of hATG over rATG.[2,3]

We recently analyzed outcomes for 40 Japanese children (median age, 9 years; range, 1-15) with AA treated using rATG and cyclosporine. The median interval from diagnosis to treatment was 22 days (range, 1-203). The numbers of patients with very severe, severe, and nonsevere disease were 14, 10, and 16, respectively. The ATG dose was 3.5 mg/kg/day for 5 days. The median follow-up time for all patients was 22 months (range, 6-38). At 3 months, no patients had achieved a complete response (CR) and partial response (PR) was seen in only 8 patients (20.0%). At 6 months, the numbers of patients with CR and PR were 2 (5.0%) and 17 (42.5%), respectively. After 6 months, 5 patients with PR at 6 months had achieved CR and 4 patients with no response at 6 months had achieved PR, offering a total best response rate of 57.5%. Two patients relapsed at 16 and 19 months without receiving any second-line treatments. Two patients with no response received a second course of rATG at 13 and 17 months, but neither responded. Sixteen patients underwent hematopoietic stem cell transplantation (HSCT) from alternative donors (HLA-matched unrelated donors, n = 13; HLA-mismatched family donors, n = 3). Two deaths occurred after rATG therapy, but no patients died after HSCT. Causes of death were intracranial hemorrhage at 6 months and acute respiratory distress syndrome at 17 months. The overall 2-year survival rate was 93.8% and the 2-year transplantation-free survival rate was 50.3% (Figure 1).

In our previous prospective studies with hATG, the response rates after 6 months were 68% and 70%, respectively, with no increases in response rates observed after 6 months.[4,5] Our results support the notion that rATG is inferior to hATG for the treatment of AA in children. First-line HSCT from an alternative donor may be justified, considering the excellent outcomes in children who received salvage therapies using alternative donor HSCT.

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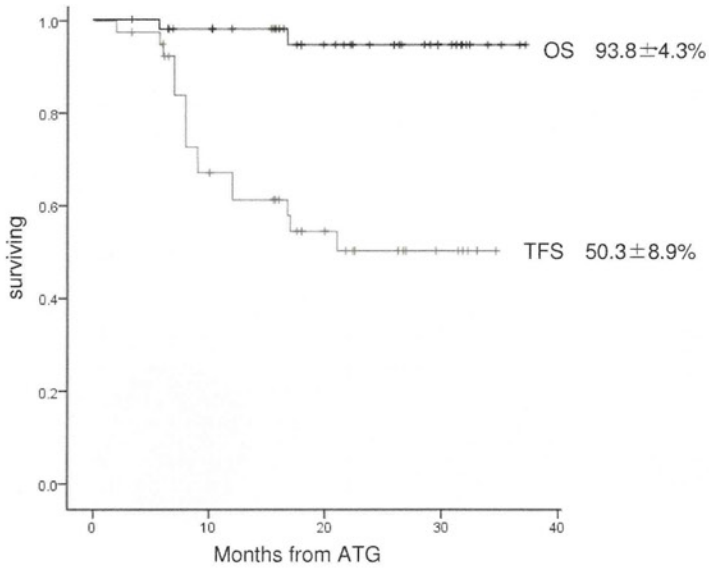

Figure 1. Kaplan-Meier estimates of overall survival (OS) and transplantation-free survival (TFS) in 40 Japanese children with AA. Survival was investigated using Kaplan-Meier methods. OS for all patients with AA after rATG and cyclosporine as first-line therapy included patients who later received HSCT for nonresponse to rATG. In the analysis of TFS for all patients treated with rATG and CSA, transplantation was considered an event.

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To the editor:

Peripheral blood stem cells versus bone marrow in pediatric unrelated donor stem cell transplantation

The relative benefits and risks of peripheral blood stem cells (PBSCs) versus bone marrow (BM) for allogeneic hematopoietic stem cell transplantation (SCT) are still a matter of highly controversial debates.[1-3] The first randomized study comparing the 2 stem cell sources in unrelated donor SCT recently documented comparable overall and event-free survival, but indicated a higher risk for chronic graft-versus-host disease (GVHD) with PBSCs.[4] Only a few pediatric patients were included in this study even though the long-term sequelae of chronic GVHD are of particular concern in this patient group.

We retrospectively compared the long-term outcome of contemporaneous unrelated donor SCT in 220 children transplanted with BM (n = 102) or PBSCs (n = 118) for hematologic malignancies and reported to the German/Austrian pediatric registry for SCT. All patients had received myeloablative conditioning followed by unmanipulated SCT from HLA-matched unrelated donors. The PBSC and BM groups were comparable with regard to patient and donor age, sex, cytomegalovirus (CMV) serostatus, disease status at transplantation, GVHD prophylaxis, growth factor use, and degree of HLA matching. The groups differed with regard to disease category with slightly more myelodysplastic syndrome patients ($P = .02$) and a higher CD34-cell dose ($P = .001$) in the PBSC group.

Neutrophil and platelet engraftment were achieved significantly faster after PBSC than BM transplantation (Figure 1A-B). In this entirely pediatric cohort, the incidence of clinically relevant grade II-IV acute GVHD (Figure 1C) did not differ. Most importantly, the incidence of chronic GVHD (PBSCs vs BM: 35% vs 33%, respectively; $P = .9$) and extensive chronic GVHD (Figure 1D) proved low and was virtually identical in the 2 groups. With a median follow-up time of 3 years, overall survival (PBSCs vs BM: 50% ± 5% vs 46% ± 6%, respectively; $P = .63$) and event-free survival (PBSCs vs BM: 45% ± 5% vs 44% ± 6%, respectively; $P = .59$) were comparable (Figure 1E-F). In multivariable analysis, taking into account all parameters with $P < .2$ in univariate analysis, the only significant independent risk factor for treatment failure was advanced disease status at the time of transplantation (relative risk = 2.4, 95% confidence interval, 1.5-3.8; $P = .001$). In contrast, stem cell source (PBSCs vs BM) had no effect (relative risk = 1.1, 95% confidence interval, 0.7-1.6; $P = .8$).

Our registry-based analysis provides evidence that in pediatric recipients of HLA-matched unrelated-donor transplantation with consistent antithymocyte globulin (ATG) use during conditioning, transplantation with PBSCs and BM results in comparable clinical outcomes without detectable differences in the risk of acute or, more importantly, chronic GVHD. Consistent with a recent study underscoring the role of ATG for the prevention of acute and chronic GVHD,[5] the use of ATG in 96% of our transplantation procedures compared with only 27% in the above-mentioned randomized study by Anasetti et al[4] might be one of the key factors responsible for the overall low and comparable incidence of

Research article

Alloantigen expression on non-hematopoietic cells reduces graft-versus-leukemia effects in mice

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Allogeneic hematopoietic stem cell transplantation (HSCT) is used effectively to treat a number of hematological malignancies. Its beneficial effects rely on donor-derived T cell–targeted leukemic cells, the so-called graft-versus-leukemia (GVL) effect. Induction of GVL is usually associated with concomitant development of graft-versus-host disease (GVHD), a major complication of allogeneic HSCT. The T cells that mediate GVL and GVHD are activated by alloantigen presented on host antigen-presenting cells of hematopoietic origin, and it is not well understood how alloantigen expression on non-hematopoietic cells affects GVL activity. Here we show, in mouse models of MHC-matched, minor histocompatibility antigen–mismatched bone marrow transplantation, that alloantigen expression on host epithelium drives donor T cells into apoptosis and dysfunction during GVHD, resulting in a loss of GVL activity. During GVHD, programmed death–1 (PD-1) and PD ligand–1 (PD-L1), molecules implicated in inducing T cell exhaustion, were upregulated on activated T cells and the target tissue, respectively, suggesting that the T cell defects driven by host epithelial alloantigen expression might be mediated by the PD-1/PD-L1 pathway. Consistent with this, blockade of PD-1/PD-L1 interactions partially restored T cell effector functions and improved GVL. These results elucidate a previously unrecognized significance of alloantigen expression on non-hematopoietic cells in GVL and suggest that separation of GVL from GVHD for more effective HSCT may be possible in human patients.

Introduction

Donor immunity in allogeneic hematopoietic stem cell transplantation (HSCT) harnesses beneficial graft-versus-leukemia (GVL) effects; therefore, allogeneic HSCT represents a very potent form of immunotherapy for hematological malignancies (1, 2). Induction of GVL is usually associated with the development of graft-versus-host disease (GVHD), which is a major complication after allogeneic HSCT. T cell depletion of the donor inocula prevents GVHD and leads to a loss of the GVL effect (3–5). Both GVL and GVHD are mediated by donor T cells, which recognize alloantigens presented on host APCs (6, 7). Donor CTLs and inflammatory cytokines are major effectors of GVHD, whereas CTLs are primarily responsible for GVL (8, 9). In patients with advanced-stage leukemia and lymphoma, relapse is still a major cause of mortality after allogeneic HSCT even after the development of severe GVHD. Thus, improvements in our understanding of the pathophysiology of GVHD and GVL are urgently needed to develop more effective therapies for malignant diseases.

Alloantigens are expressed on the three major components in HSCT recipients in the context of GVHD and GVL: hematopoietically derived APCs, GVHD target epithelium, and leukemia cells. Several studies have shown that host APCs are crucial for the induction of both GVHD and GVL (6, 7, 9–11). Alloantigen expression on epithelium is also critical for the induction of GVHD in MHC-matched, minor histocompatibility antigen–mismatched (mHA-mismatched) models of bone marrow transplantation (BMT) (10), but GVHD can occur in the absence of alloantigen expression on epithelium in MHC-mismatched models of BMT (9). However, the effect of alloantigen expression on non-hematopoietic cells such as the epithelium in GVL is not well defined. In this study, we addressed this important issue in mHA-mismatched models of BMT.

Results

Alloantigen expression on host non-hematopoietic cells augments acute GVHD but reduces GVL effects. We generated BM chimeric mice that express alloantigens on APCs, which are essential for the induction of both GVHD and GVL (6, 7, 12). BM chimeras were created by reconstituting lethally irradiated C3H.Sw (C3: H-2^b) mice with 5×10^6 T cell–depleted (TCD) BM cells isolated from C57BL/6 (B6, H-2^b) mice that differ from C3 mice at multiple mHAs ([B6→C3] chimeras). Control chimeras, [B6→B6], were identically created. Four months later, donor repopulation of hematopoiesis was confirmed by flow cytometry as shown previously (6, 9, 12). Thus, [B6→C3] chimeric mice expressed B6-derived mHAs on hematopoietically derived APCs but not on non-hematopoietic target cells. In contrast, [B6→B6] mice expressed B6-derived mHAs on both APCs and target epithelium. These chimeras were used as BMT recipients; they were reirradiated and injected with 5×10^6 TCD BM cells alone or with various doses of $CD8^+$ T cells from C3 donors. After BMT, GVHD mortality was higher in [B6→B6] mice than in [B6→C3] mice (Figure 1A). Clinical GVHD scores (13) in surviving animals were also higher in [B6→B6] mice than in [B6→C3] mice (Figure 1B). Mortality and morbidity from GVHD in [B6→C3] mice were almost equivalent to those in [B6→B6] mice given a 1-log lower T cell dose. This finding confirmed the previous observation of a lack of alloantigen expression on host epithelium significantly reducing GVHD across mHA disparity (10). We

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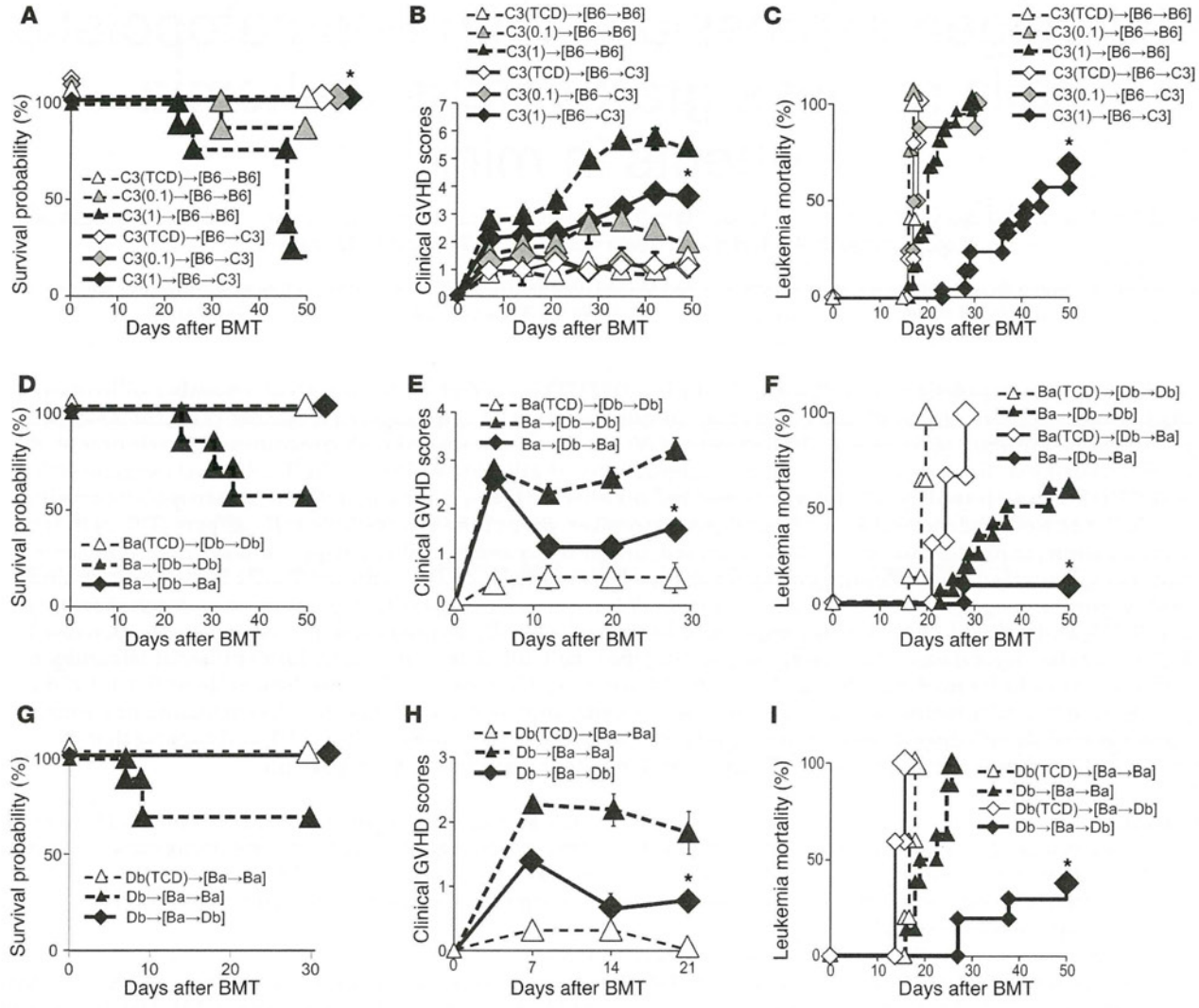

Figure 1

Alloantigen expression on host non-hematopoietic cells augments acute GVHD but reduces GVL effects. (**A**–**C**) [B6→C3] (diamonds) and [B6→B6] chimeras (triangles) were created by reconstituting lethally irradiated C3 and B6 mice with 5 × 10^6 TCD BM cells from B6 mice. Four months later, the chimeras were reirradiated and injected with 5 × 10^6 TCD BM cells alone (open symbols) or with 1 × 10^6 (black symbols) or 0.1 × 10^6 (gray symbols) $CD8^+$ T cells from C3 donors (as indicated in parentheses × 10^6). Survival (**A**) and clinical GVHD scores (**B**) after BMT (n = 3–8/group). (**C**) Leukemia mortality after BMT in chimeras injected with EL4 cells (n = 5–21/group). Data from 3 similar experiments were combined. (**D**–**F**) [Db→Ba] (diamonds) and [Db→Db] (triangles) chimeras were reirradiated and injected with TCD BM alone (open symbols) or with 2 × 10^6 T cells from Ba donors (filled symbols). Survival (**D**) and clinical GVHD scores (**E**) after BMT from a representative experiment of 2 similar experiments (n = 4–7/group). (**F**) Leukemia mortality after BMT in mice injected with P815 cells. Data from 2 similar experiments were combined (n = 6–18/group). (**G**–**I**) [Ba→Db] (diamonds) and [Ba→Ba] (triangles) chimeras were similarly transplanted with 5 × 10^6 TCD BM cells alone (open symbols) or with 2 × 10^6 T cells from Db donors (filled symbols). Survival (**G**) and clinical scores (**H**) after BMT (n = 3–10/group). (**I**) Leukemia mortality after BMT in chimeras injected with A20 cells (n = 5–10/group). Data from 2 similar experiments were combined. Clinical scores are shown as the mean ± SEM. *P < 0.05 compared with allogeneic controls.

then tested the effect of alloantigen expression on GVHD target epithelium on GVL effects. These chimeric mice were transplanted as described above together with 2,500 B6-derived EL4 cells as a model of residual leukemia after BMT. As expected, 100% of both types of chimeric mice that received TCD BM cells died from leukemia by day +20 after BMT (Figure 1C), whereas leukemia-free survival was significantly prolonged in mice that received donor T cells, demonstrating a significant GVL effect. However, this GVL effect was not potent in [B6→B6] mice, and all mice subsequently died from leukemia. Surprisingly, leukemia mortality was significantly lower in [B6→C3] mice that did not express alloantigens on their non-hematopoietic cells (62% vs. 100%; P < 0.05). GVL effects in [B6→B6] mice appeared to be almost equivalent to those in [B6→C3] mice given a 1-log lower T cell dose.

We further confirmed these observations in a different strain combination: BALB/c (Ba, H-2^d) and DBA/2 (Db, H-2^d) mice that

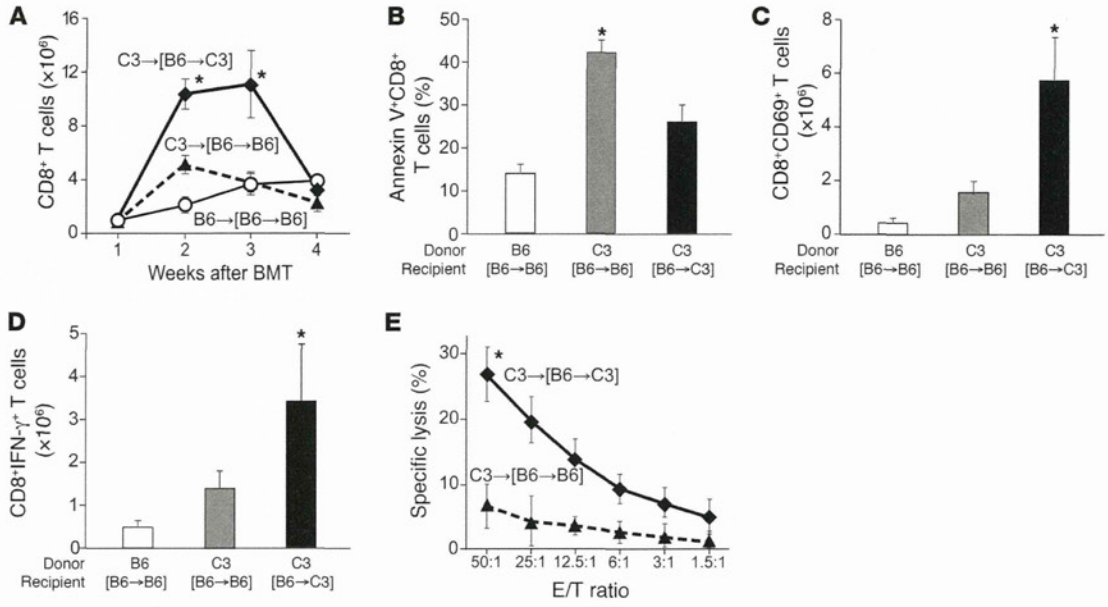

Figure 2

Alloantigen expression on host non-hematopoietic cells enhances the apoptosis and dysfunction of alloreactive T cells. [B6→C3] (diamonds and black bars) and [B6→B6] (triangles and gray bars) chimeras were transplanted as indicated in the legend for Figure 1. Syngeneic controls were [B6→B6] recipients of B6.Ly5.1 (CD45.1$^+$) cells (open circles and white bars). (**A**) Numbers of donor CD8$^+$ T cells in spleens. (**B**) Frequencies of annexin V$^+$ donor CD8$^+$ T cells. (**C**) Numbers of annexin V$^-$ donor CD69$^+$CD8$^+$ T cells. (**D**) Numbers of annexin V$^-$ IFN-γ–producing donor CD8$^+$ T cells. (**E**) CTL activity against EL4. (**B**–**E**) Analysis was performed 14 days after BMT (n = 3–8/group). Representative data from 1 of the experiments are shown as the mean ± SD. *P < 0.05 compared with allogeneic controls.

differed at multiple mHAs from each other. [Db→Ba] and control [Db→Db] chimeras were lethally reirradiated and injected with 5 × 10^6 TCD BM cells alone or with 2 × 10^6 Ba T cells. Mortality (Figure 1D, P = 0.08) and morbidity from GVHD (Figure 1E, P < 0.05) were higher in [Db→Db] mice than in [Db→Ba] mice. When cells were transplanted together with 2,000 Db-derived P815 cells, leukemia mortality was significantly lower in [Db→Ba] mice than in [Db→Db] mice (10% vs. 60%; P < 0.05) (Figure 1F).

Similar results were obtained when [Ba→Db] and control [Ba→Ba] chimeras were transplanted with 5 × 10^6 TCD BM cells with or without 2 × 10^6 Db T cells. In [Ba→Db] recipients, in which non-hematopoietic cells do not express alloantigens, mortality (Figure 1G, P = 0.08) and morbidity of GVHD (Figure 1H, P < 0.05) were lower, but GVL effects against Ba-derived A20 lymphoma cells were significantly more potent as compared with [Ba→Ba] controls (leukemia mortality: 30% vs. 100%; P < 0.05) (Figure 1I). Taken together, these results demonstrate that GVHD is decreased but GVL activity is enhanced in the absence of alloantigen expression on non-hematopoietic cells.

Alloantigen expression on non-hematopoietic cells enhances apoptosis and dysfunction of alloreactive T cells. GVHD and GVL in the C3 and B6 strain combination is dependent on donor CD8$^+$ T cells (12, 14). To elucidate the mechanisms responsible for the enhancement of the GVL effect in [B6→C3] chimeric mice, which lack alloantigen expression on non-hematopoietic cells, the kinetics of donor CD8$^+$ T cell expansion and activation were evaluated after BMT. Expansion of donor CD8$^+$ T cells identified as CD5.1$^+$CD8$^+$ cells peaked on day +14 in the spleens of allogeneic [B6→B6] recipients and decreased thereafter (Figure 2A), as previously shown in this model (15). CD8 expansion was significantly greater in [B6→C3] mice than in [B6→B6] mice on days +14 and +21. We next assessed donor T cell apoptosis as a determinant of the kinetics of T cell expansion. Frequencies of annexin V$^+$ apoptotic donor CD8$^+$ T cells were significantly greater in the spleen of [B6→B6] mice as compared with that of [B6→C3] mice on day +14 (Figure 2B). Notably, surviving donor CD8$^+$ T cells were significantly less activated in [B6→B6] mice than in [B6→C3] mice when evaluated based on the expression of CD69 (Figure 2C) and intracellular IFN-γ (Figure 2D) on annexin V$^-$ donor CD8$^+$ T cells. We next evaluated CTL activity in donor T cells isolated from the spleen on day +14 after BMT. CTL activity against EL4 targets was significantly reduced in the splenocytes of [B6→B6] mice as compared with [B6→C3] mice (Figure 2E). These results suggest that alloantigen expression on non-hematopoietic cells induces apoptosis and dysfunction of alloreactive T cells.

Absence of alloantigen expression on host non-hematopoietic cells restores GVL effects. Self-recognition in the periphery facilitates the reactivity of mature T cells to foreign antigens (16). Therefore, it is possible that the expression of syngeneic MHC molecules and not the absence of alloantigens on non-hematopoietic cells may be responsible for the enhancement of the GVL effect in [B6→C3] chimeras. This possibility was tested in B6-background $\beta_2m^{-/-}$ mice. [B6→$\beta_2m^{-/-}$] chimeras lacking functional MHC class I molecules on non-hematopoietic cells did not develop GVHD after transplantation with CD8$^+$ T cells from C3 donors, as shown previously (17) (Figure 3A). In these mice, however, leukemia mortality was significantly delayed even in the absence of GVHD as compared with [B6→B6] recipients (Figure 3B, P < 0.05). The expansion and CTL activity of donor CD8$^+$ T cells was significantly greater in [B6→$\beta_2m^{-/-}$] recipients than in [B6→B6] recipients (Figure 3, C and D).

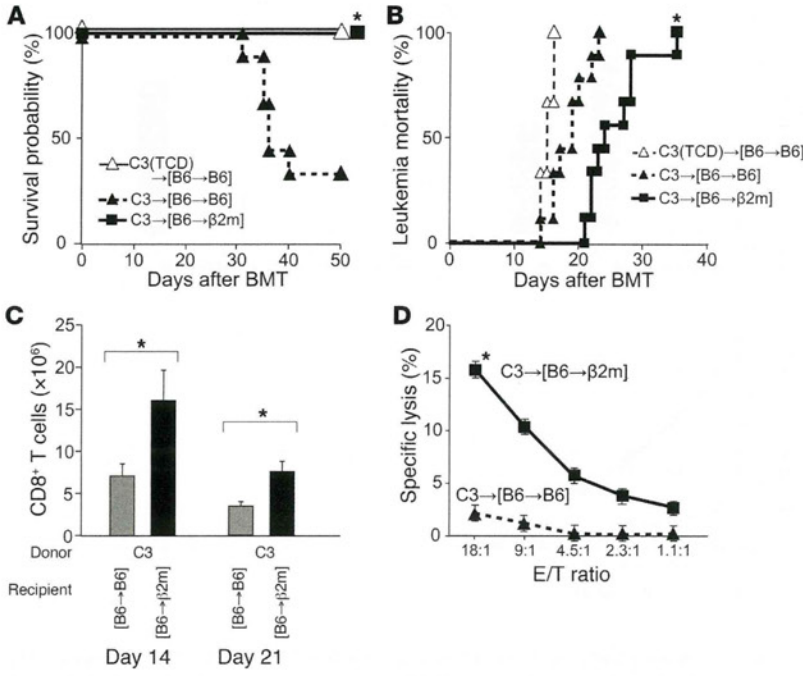

Figure 3
Absence of alloantigen expression on host non-hematopoietic cells restores GVL effects. [B6→B6] (triangles) and [B6→β2m$^{-/-}$] (squares) mice were reirradiated and injected with 5 × 10^6 TCD BM cells alone (open symbols) or with 1 × 10^6 CD8$^+$ T cells from C3 donors (filled symbols). (**A**) Survival after BMT. (**B**) Leukemia mortality in chimeras injected with EL4 cells (n = 6–9/group). Data from a representative experiment of 2 similar experiments are shown. Mean ± SEM numbers of donor CD8$^+$ T cells in spleens (n = 3–6/group) (**C**) and CTL activity against EL4 (**D**). *P < 0.05 compared with allogeneic controls.

These results confirm that alloantigen expression on host epithelium induces apoptosis and dysfunction of alloreactive T cells, which results in impaired GVL effects.

Alloantigen expression on host non-hematopoietic cells stimulates programmed death-1 and its ligand pathway. Programmed death–1 (PD-1) is a negative regulator of activated T cells and regulates T cell exhaustion during chronic infections (18–20). PD-1 interacts with at least 2 ligands: PD ligand–1 (PD-L1) and PD-L2 (21). In particular, the PD-1/PD-L1 pathway has been proposed as one of the most important mechanisms of T cell exhaustion and tolerance induction against infectious agents and tumors (19, 22–25). We therefore hypothesized that the PD-1/PD-L1 pathway plays a role in the loss of GVL effects in [B6→B6] mice. To test this hypothesis, we examined PD-1 expression on donor CD8$^+$ T cells in lymph nodes on day +14 and +21 after BMT. It was significantly upregulated in allogeneic [B6→B6] recipients as compared with syngeneic controls but was low in [B6→C3] mice (Figure 4, A and B). We also investigated the expression of another inhibitory receptor, CTLA-4, on donor CD8$^+$ T cells. Although the expression of cytoplasmic CTLA-4 was slightly upregulated in allogeneic animals as compared with syngeneic animals, its level did not differ between [B6→B6] and [B6→C3] mice (5.5% ± 1.0% vs. 4.5% ± 0.2%, respectively; P = 0.50).

We next examined PD-L1 expression in the liver by real-time PCR after BMT. PD-L1 expression was markedly upregulated in the liver of allogeneic controls as compared with syngeneic controls (Figure 4C). In allogeneic [B6→C3] mice, it was slightly upregulated on day +14 but not on day +21. Immunohistochemical analysis confirmed upregulated expression of PD-L1 in the liver of [B6→B6] mice, as previously reported (Figure 4D) (21, 26). These results showed that alloantigen expression on GVHD target epithelium is associated with upregulation of the PD-1/PD-L1 interactions between donor T cells and GVHD target tissue.

Blockade of the interaction between PD-1 and PD-L1 enhances GVL activity. We next examined whether blocking the PD-1/PD-L1 pathway could enhance GVL activity. [B6→C3] and [B6→B6] chimeras were reirradiated and injected with TCD BM cells and CD8$^+$ T cells from C3 donors. Mice were i.p. injected with 500 μg of anti–PD-L1 mAb on day 0 and then with 200 μg on days +3, +6, +9, +12, +15, and +18 after BMT. In [B6→B6] recipients, injection of anti–PD-L1 mAbs significantly restored T cell functions on day +14, as assessed by CD69 expression (Figure 5A), IFN-γ production (Figure 5B), and CTL activity (Figure 5C). In [B6→C3] mice, it marginally upregulated CD69 expression, IFN-γ production, and CTL activity, although differences were not statistically significant (Figure 5, A, B, and D). As a consequence, anti–PD-L1 mAb administration significantly increased the severity of GVHD in [B6→B6] mice (Figure 5E) but not in [B6→C3] mice (Figure 5F). PD-L1 blockade also significantly augmented GVL activity in [B6→B6] recipients injected with EL4 cells on day 0 (Figure 5G, P < 0.05). It also delayed leukemia death in [B6→C3] mice, although the difference was not statistically significant (Figure 5H, P = 0.38). In controls, PD-L1 blockade did not affect leukemia mortality in TCD-BMT recipients (Figure 5H) or [B6→B6] recipients of syngeneic B6 CD8$^+$ T cells (data not shown).

Discussion

Alloantigens are expressed in three major sites in HSCT recipients: APCs, GVHD target epithelium, and leukemia cells. Alloantigen expression on APCs is essential for the induction of GVHD (6), and an optimal GVL response occurs when alloantigens are expressed on both host APCs and tumor cells (7). Alloantigen expression on the epithelium is also critical for the induction of GVHD across mHA disparities (10), but GVHD can occur in the absence of alloantigen expression on epithelium in MHC-mismatched BMT (9). In this study, we addressed the effect of alloantigen expression on target epithelium in GVL using chimeric mouse models of GVHD and GVL across mHA disparities. Our models mimic clinical BMT in patients not in remission, since most of the mice relapsed after allogeneic BMT. This high tumor burden enabled us to compare the magnitude of GVL activity in our models, and we made sur-

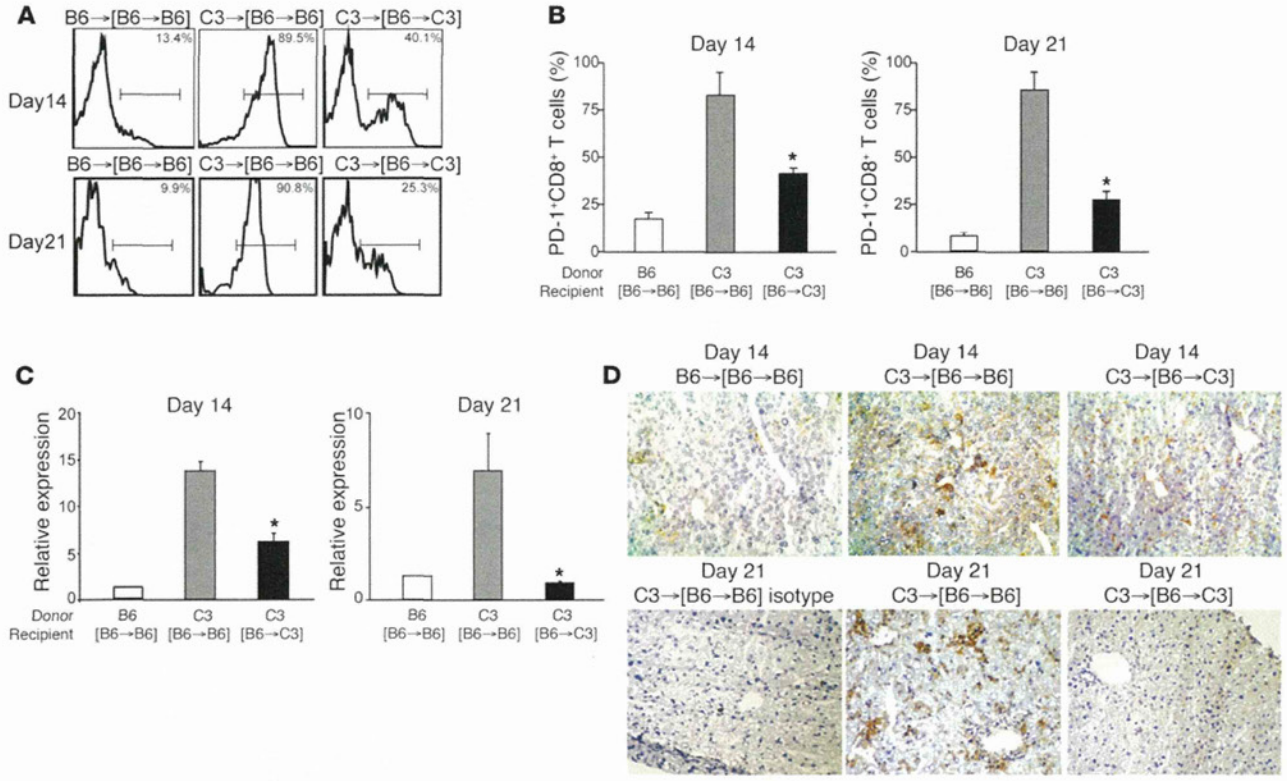

Figure 4

Alloantigen expression on host non-hematopoietic cells stimulates PD-1 and its ligand pathway. [B6→B6] and [B6→C3] chimeras were transplanted as indicated in the legend for Figure 1 (n = 4–8). (**A**) Representative histogram of PD-1 expression among donor CD8$^+$ T cells on day +14 and +21 in syngeneic (left), allogeneic [B6→B6] (middle), and [B6→C3] (right) recipients. (**B**) Frequencies of PD-1$^+$ CD8$^+$ T cells (mean ± SD). (**C**) Relative expressions of *Pdl1* mRNA on day +14 and +21 in the livers of allogeneic [B6→B6] (gray bars) and allogeneic [B6→C3] mice (black bars). Data represent the mean (± SD) of n-fold difference in the amount of *Pdl1* gene expression relative to that in syngeneic mice. (**D**) PD-L1 expression in the liver on day +14 (top row) and +21 (bottom row) from syngeneic (upper left) and allogeneic [B6→B6] (middle) and [B6→C3] (right) recipients. Isotype control of allogeneic [B6→B6] (lower left) is shown. Original magnification, ×200. *P < 0.05 compared with allogeneic controls.

prising observations that alloantigen expression on non-hematopoietic cells inhibited GVL effects but enhanced GVHD. This observation challenges the current paradigm that GVL activity is strongly correlated with the severity of GVHD (1, 2, 27).

We found that alloantigen expression on non-hematopoietic cells induced donor T cell apoptosis and led to a contraction in the size of an alloreactive donor CD8$^+$ T cell pool early after BMT. The remainder of the donor T cells were alive, but their ability to produce cytokines and cytotoxicity were impaired. This defect is similar to T cell exhaustion, which is a principal reason for the inability of the host to eliminate the persisting pathogen in chronic infections (18, 28). CD8$^+$ T cell proliferation and differentiation into cytolytic effectors on an encounter with antigens are variable and change as a consequence of the antigen load (29). As the magnitude of the viral load increases, virus-specific T cells become more functionally impaired. During persistent infection, a high antigen load drives a significant number of virus-specific T cells into activation-induced apoptosis, and the remaining virus-specific T cells remain alive but in a dysfunctional state of cytotoxicity (18, 30–33). In tumor models, antigen quantity determines the behavior of the CD8$^+$ effector cells, including their effector function and sensitivity to apoptosis (34–36). In patients with a larger tumor burden, CD8$^+$ T cells were found to undergo apoptosis (37). Thus, a higher alloantigen load in allogeneic controls as compared with chimeras, in which alloantigen expression is limited to hematopoietic cells and tumor cells, may induce apoptosis and the dysfunction of alloreactive T cells, which leads to the inability of the host to eliminate leukemia.

Our results are consistent with seminal observations by Meunier, Fontaine, and colleagues, who showed that the adoptive transfer of immunodominant mHA (B6^{dom1})–specific T cells eradicates B6^{dom1}-expressing leukemia more efficiently in mice lacking B6^{dom1} expression than in mice expressing B6^{dom1} (38). This was because the widespread expression of B6^{dom1} caused activation-induced apoptosis and dysfunction of donor T cells in mice expressing B6^{dom1} (38, 39). These findings along with our results indicate that allogeneic cellular therapy targeting mHAs exclusively expressed on APCs and tumor cells can induce a potent GVL effect while inducing less-severe GVHD than immunotherapy via targeting of ubiquitously expressed mHAs (40).

The PD-1/PD-L1 pathway is critically involved in T cell exhaustion and tolerance induction in infection and tumor immunology (18–20, 23–25, 41). It is also required for protection against chronic rejection of cardiac allograft, and induction of peripheral dele-