accelerated tapering of immunosuppressant or DLI in an attempt to prevent hematological relapse.[15]

Definitions

The Southwestern Oncology Group/Eastern Cooperative Oncology Group (SWOG/ECOG) classification of cytogenetic risk[16] was used to classify patients by cytogenetics. Favorable risk was defined as follows: inv(16)/t(16;16)/del(16q) with or without other chromosome anomalies and t(8;21) without either del(9q) or part of a complex karyotype (defined as three or more anomalies). Intermediate risk was defined as follows: +8, −Y, +6, del(12p) and normal karyotype. Unfavorable risk was defined as follows: −5/del(5q), −7/del(7q), inv(3q)/t(3,3), abnormal 11q, 20q or 21q, del(9q), t(6;9), t(9;22), abnormal 17p and complex karyotype. Unknown risk was defined as follows: all other clonal abnormalities with less than three anomalies. Diagnosis of acute and chronic GVHD was based on the standard clinical criteria,[17,18] with histopathological confirmation where possible.

Statistical analysis

Ages and characteristics were compared between patients who developed BM relapse and those who developed EM relapse with Mann–Whitney's *U*-test and Fisher's exact test, respectively. Cumulative incidence was used to calculate the probability of relapse, treating non-relapse mortality as a competing risk. The time from SCT to relapse was compared using Mann–Whitney's *U*-test. Patients who never achieved CR after SCT were excluded from the analyses of cumulative incidence of relapse and time to relapse. Post-relapse survival and overall survival after transplantation were calculated using the Kaplan–Meier method and compared using the log-rank test.

Results

Occurrence of BM and EM relapse

Among a total of 57 patients who received haplo-SCT as the first or second SCT, 20 patients had BM relapse and 9 had EM relapse. None of the patients had concomitant BM and EM relapse at the time of the diagnosis of relapse. Two patients (one with BM relapse and the other with EM relapse) who received haplo-SCT as their second SCT were excluded from the analyses described below, because they never achieved CR after the second SCT. The cumulative incidences of BM relapse and EM relapse at 3 years after SCT were 37.7 and 17.1%, respectively (Figure 1a).

Among 38 patients who received haplo-SCT as the first SCT, 15 patients had BM relapse and 4 had EM relapse. The cumulative incidences of BM relapse and EM relapse at 3 years after SCT were 40.5 and 10.9%, respectively (Figure 1b). The time from SCT to BM relapse (median 296 days) was earlier than EM relapse (median 348 days), but this difference was not statistically significant ($P=0.27$).

Among 19 patients who received haplo-SCT as their second SCT, 5 patients had BM relapse and 5 had EM relapse. The cumulative incidences of BM relapse and EM relapse were 30.9 and 31.9%, respectively (Figure 1c).

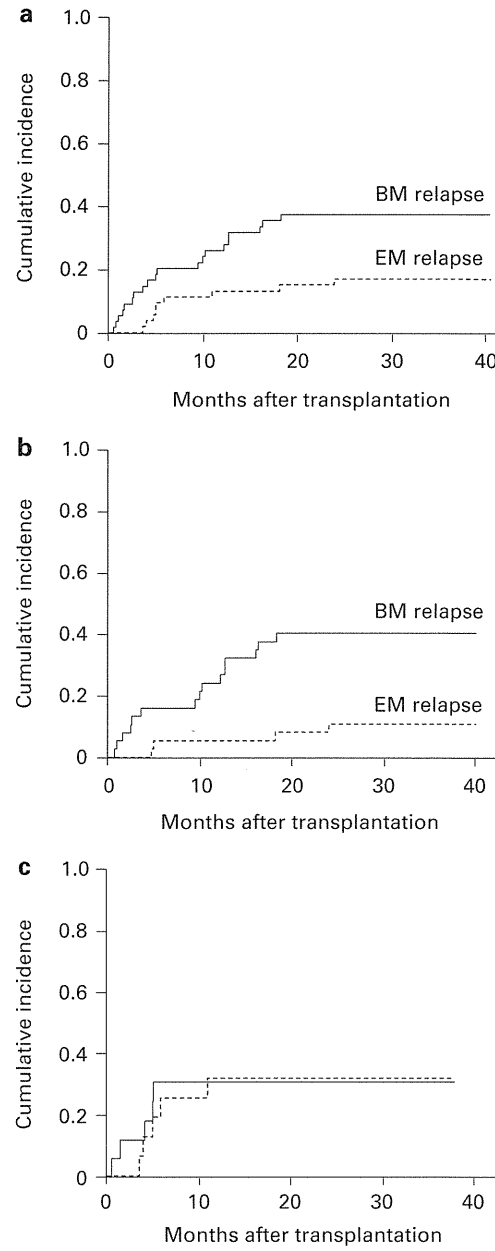

Figure 1 Cumulative incidences of BM and EM relapse after haplo-SCT. The cumulative incidences of BM relapse (solid line) and EM relapse (dotted line) in entire cohort (**a**), in patients who received haplo-SCT performed as first SCT (**b**) and second SCT (**c**). EM relapse occurs less frequently than BM relapse after first SCT (10.9 vs 40.5%), but both patterns of relapse are similar in frequency after second SCT (31.9 vs 30.9%).

The time from SCT to BM relapse (median 121 days) was earlier than EM relapse (median 150 days), but this difference was also not statistically significant ($P=0.25$).

The median time to total relapse (BM relapse + EM relapse) after a second SCT (median 135 days) was earlier than that after a first SCT (median 296 days), but this difference was not statistically significant ($P=0.12$).

The characteristics of the patients who developed either BM or EM relapse, particularly regarding the reported risk

Table 2 Patient characteristics regarding the reported risk factors of EM involvement

	1st SCT (n=38)		*2nd SCT (n=19)*	
	BM relapse (n=15)	*EM relapse (n=4)*	*BM relapse (n=5)*	*EM relapse (n=5)*
Median age, years (range)	49 (26–62)	22 (20–42)	46 (32–56)	33 (19–50)
Subtype				
M4/M5	4	0	1	1
MDS or MDS-AML	6	1	2	1
Others	5	3	2	3
Disease status at transplant				
Any CR	1	0	0	1
Not in remission	14	4	5	4
Cytogenetics				
t(8;21), inv (16)	0	0	0	0
MLL rearrangement	2	0	1	1
Intermediate risk	4	1	2	2
Unfavorable risk (excluding MLL rearrangement)	7	1	2	0
Unknown risk	2	2	0	2
CD56 expression				
Yes	3	2	0	0
No	12	2	4	5
Not tested	0	0	1	0
EM disease before SCT				
Yes	2	1	1	2
No	13	3	4	3
TBI dose				
TBI ⩾ 8 Gy	3	3	0	0
TBI < 8 Gy	0	1	0	0
None	12	0	5	5
Use of ara-C in the conditioning				
Yes	7	3	4	2
No	8	1	1	3
Acute GVHD				
Grade 0–1	14	4	4	4
Grade 2–4	1	0	1	1
Chronic GVHD				
None	6	4	3	3
Limited	1	0	0	1
Extensive	3	0	0	0
Not evaluable	5	0	2	1

Abbreviations: EM = extramedullary; MDS = myelodysplastic syndrome; MLL = mixed-lineage leukemia.

factor of EM involvement, are detailed in Table 2. Patients with EM relapse were significantly younger than those with BM relapse among the patients who underwent haplo-SCT as their first SCT ($P=0.01$); this difference was NS among patients undergoing a second SCT. The proportions of patients with FAB M4/M5 subtype, unfavorable cytogenetics, CD56 expression, history of EM involvement before SCT, use of TBI or use of Ara-C in the preparative regimen, occurrence of acute GVHD and occurrence of chronic GVHD were not significantly different between patients with BM relapse and those with EM relapse.

Clinical course of patients with EM relapse

The characteristics and clinical courses of the patients who developed EM relapses are detailed in Tables 3 and 4 (no. F1 to F4 are patients who developed EM relapse after haplo-SCT performed as first SCT and no. S1 to S5 are those who developed EM relapse after haplo-SCT as second SCT). Sites of involvement included various organs in the body. Two patients (S3 and S4) developed subsequent BM relapse. Local radiotherapy was used in six patients, with or without other treatment modalities. All four patients who developed EM relapse after haplo-SCT performed as first SCT, as well as two of the five patients who developed EM relapse after haplo-SCT as second SCT, underwent a second or third haplo-SCT from other donors. One patient (F2) died from sepsis early after a second SCT and therefore was not evaluable for the response. Among the remaining 5 patients, 4 achieved CR after SCT. However, three of those patients had EM relapse at other sites, and only one patient remains in CR.

Survival after relapse and overall survival after transplantation

Among the patients who underwent haplo-SCT as their first SCT, the probabilities of survival after BM relapse and EM relapse were nearly the same (Figure 2a, 33.3% for BM relapse and 25.0% for EM relapse at 1 year). Among the patients who underwent haplo-SCT as the second SCT, the probability of overall survival after EM relapse appears to be better than that after BM relapse, although the difference was not statistically significant ($P=0.06$, Figure 2b, 0% for BM relapse and 40.0% for EM relapse at 1 year).

Among the patients who underwent haplo-SCT as their first SCT, overall survival at 3 years after SCT was 35.7% in patients who received myeloablative conditioning and 18.8% in patients who received reduced-intensity conditioning (Figure 3a). Among the patients who underwent haplo-SCT as their second SCT, overall survival at 3 years was 15.8% (Figure 3b).

Discussion

The present study had several significant findings. We showed that EM relapse occurred in 10.9% of patients who underwent haplo-SCT performed as the first SCT, which accounted for significant proportion (21%) of total relapses. Although it is difficult to compare the absolute incidence of relapse with other studies due to the exceptionally poor background of the patients in the present study, the proportion of EM relapse among the total number of relapses was higher than or comparable to those found in previous studies.[1,3,19,20] In addition, we found that the incidence of EM relapse after haplo-SCT performed as a second SCT was remarkably high (31.6%), and accounted for half of the total relapses. These findings strongly suggest that a potent GVL effect elicited by HLA disparity occurs preferentially in BM.

Although the precise mechanism is yet to be clarified, previous studies have suggested several possible explanations

Table 3 Characteristics of patients who developed extramedullary relapse after haplo-SCT

No.	*UPN*	*Prior allo-SCT*	*Age (y)/ sex*	*Disease characteristics*					*Donor*		*Conditioning regimen*		*Stem cell source*
				AML subtype	*Stage at SCT*	*Cytogenetics*	*CD56 expression*	*Prior EM involvement*	*Relation*	*No. of HLA mismatch*[a]	*Intensity*	*TBI (dose, Gy)*	
F1	461	No	22/M	M0[b]	Induction failure	Inv (3), −7	Positive	No	Mother	1	MAC	Yes (12)	BM
F2	503	No	21/M	M0	Relapse (BM)	Add (7), del (13)	Positive	Yes	Sibling	2	MAC	Yes (8)	BM
F3	519	No	42/M	MDS-AML	Induction failure	+8, inv (9)	Negative	No	Sibling	2	RIC	Yes (4)	PB
F4	525	No	21/M	M1	Relapse	Normal karyotype	Negative	No	Sibling	3	MAC	Yes (8)	BM
S1	346	Yes (haplo)	19/F	M5	Relapse	Normal karyotype	Negative	No	Mother	2	RIC	No	PB
S2	421	Yes (haplo)	28/F	M1	Relapse (BM)	t (16;21)[c]	Negative	Yes	Mother	2	RIC	No	PB
S3	435	Yes (CBT)	33/M	M1	Relapse (BM)	Normal karyotype	Negative	Yes	Sibling	3	RIC	No	PB
S4	480	Yes (CBT)	50/M	MDS-AML	CR3[d]	t (1;17) (p36;q21)	Negative	No	Sibling	3	RIC	No	PB
S5	481	Yes (CBT)	39/M	M2	Relapse	t (11;19)[e]	Negative	No	Cousin	2	RIC	No	PB

Abbreviations: M = male; F = female; MDS-AML = acute myeloid leukemia evolved from myelodysplastic syndrome; RIC = reduced-intensity conditioning; MAC = myeloablative conditioning.
MDS-AML, acute myeloid leukemia evolved from myelodysplastic syndrome.
[a]Number of serological mismatches in A, B or DR loci in the GVH vector.
[b]Myeloid/NK cell precursor acute leukemia.
[c]Resulting in *TLS/FUS–ERG* fusion gene.
[d]Achieved CR with chemotherapy post-CBT relapse.
[e]Resulting in *MLL–ELL* fusion gene.

Table 4 Clinical course of patients who developed EM relapse

No.	*GVHD*		*Pre-emptive DLI (day)*	*Extramedullary relapse*			*Treatment*	*Response*	*Outcome*		
	Acute	*Chronic*		*Time to relapse*	*Sites of involvement*	*Subsequent BM relapse (d)*			*Status*	*Post-relapse survival (d)*	*Cause of death*
F1	I	None	No	150	Muscle	No	RT, 2nd transplant	PR → multiple EM relapse	Dead	340	Relapse
F2	I	None	No	139	Muscle	No	Chemotherapy, 2nd transplant	NE	Dead	110	Sepsis
F3	0	None	Yes (343)	718	Intraperitoneal	No	Chemotherapy, 2nd transplant, RT	CR → EM relapse at other sites	Dead	261	Relapse
F4	0	None	No	545	Mediastinum	No	RT, 2nd transplant	CR	Alive	532+	
S1	II	None	No	150	Mammary glands	No	RT, DLI, 3rd transplant	CR → EM relapse at other sites and BM relapse	Dead	668	Relapse
S2	0	None	No	119	CNS	No	RT, 3rd transplant	CR → EM relapse at other sites	Dead	403	Relapse
S3	0	None	Yes (39)	175	Skin, nasal sinus	Yes (250)	Chemotherapy	NE	Dead	131	Relapse
S4	0	None	No	108	Adrenal gland, muscle and bone	Yes (120)	Chemotherapy	NE	Dead	38	Relapse
S5	0	Limited	No	328	CNS	No	IT, RT	PR	Alive	654+	

Abbreviations: CNS = central nervous system; IT = intrathecal chemotherapy; NE = not evaluable; RT = radiotherapy.

for the difference in the GVL effect between BM and EM tissues. Effector cells for the GVL response—that is CD8-positive T cells and natural killer cells—are present in much higher numbers in BM than in EM tissues.[21] In addition, the recruitment of accessory cells necessary to achieve efficient local anti-leukemic activity may be deficient at the sites of EM relapse.[22] In other words, the mechanism may be at least partly the same as the one that separates the GVL effect from GVHD after haplo-SCT. In HLA-mismatched SCT, alloreactive T-cell responses are thought to be directed against epitopes on HLA molecules or against peptide-HLA complexes expressed on normal tissues or leukemia cells. This means that the targets of T cells are largely the same in GVL effect and GVHD, and thus, GVL effect could be regarded as a 'lymphohematopoietic GVH response.' Chakraverty *et al.*[23] showed that the presence of inflammation within tissues targeted in GVHD controls the level of trafficking of activated T cells to the affected sites. If what separates GVL from GVHD is the trafficking of T cells, EM sites should be inherently less susceptible to the GVL effect. In the present study, only a few patients had acute or chronic GVHD before the

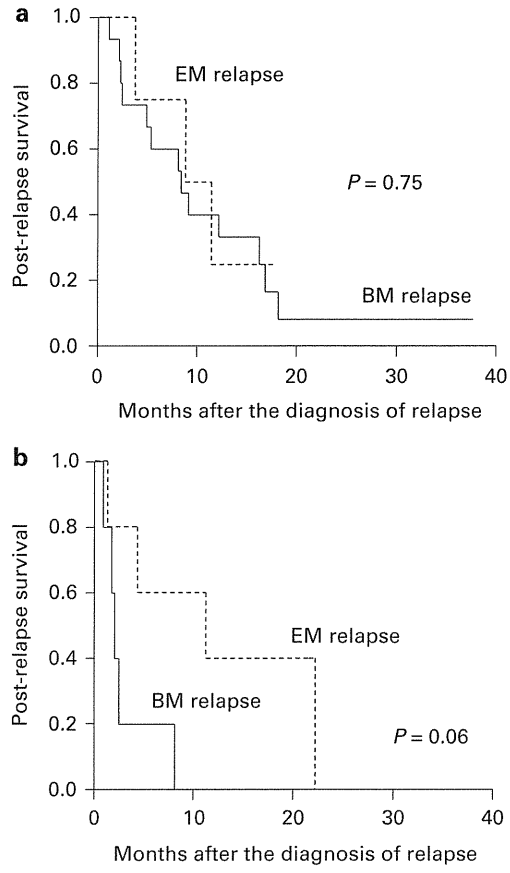

Figure 2 Survival after BM and EM relapse. Probability of survival after BM relapse (solid line) and EM relapse (dotted line) following haplo-SCT performed as first SCT (**a**) and second SCT (**b**). Post-relapse survival was not significantly different between BM and EM relapse patients.

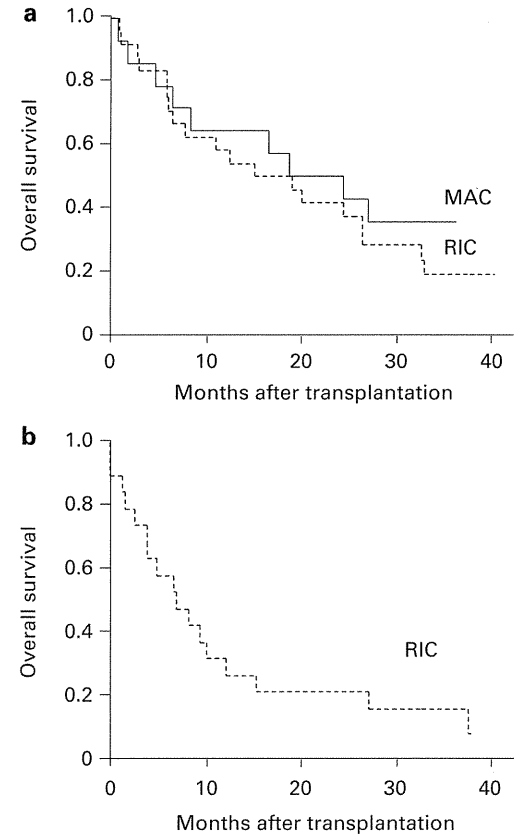

Figure 3 Overall survival after transplantation. Probability of overall survival after haplo-SCT for advanced AML/myelodysplastic syndrome, performed as first SCT (**a**) and second SCT (**b**). The solid line shows overall survival of the patients who received myeloablative haplo-SCT and the dotted line shows overall survival of the patients who received reduced-intensity haplo-SCT. All the patients undergoing second SCT received reduced-intensity conditioning.

occurrence of EM relapse. We speculate that our protocol successfully promoted the GVL effect in BM in the absence of GVHD, but this led to a relatively high incidence of EM relapse.

In addition to these immunological aspects, previous studies have also shown a number of possible intrinsic characteristics of leukemic cells that may predispose them to EM involvement. Such characteristics reported in non-transplant settings include t(8;21),[24] inv(16),[25] and mixed lineage leukemic gene rearrangement,[26] as well as CD56 expression.[27] Such characteristics reported in transplant settings include adverse cytogenetics,[4] the M4 and M5 AML subtypes, EM involvement prior to SCT, younger age and relapse/refractory disease at the time of SCT.[1] However, in the present study, only the age at SCT was shown to be significantly different between patients with BM relapse and EM relapse. Thus, we speculate that the intrinsic characteristics of leukemic cells did not have a major influence on the high incidence of EM relapse in the present study.

We also showed that the prognosis of patients with EM relapse was poor, although several previous studies have reported a better prognosis for patients with EM relapse than for those with BM relapse.[2,3] Notably, four of five evaluable patients who underwent a second or third haplo-SCT for EM relapse from other donors achieved CR after SCT, but eventually developed EM relapse at other sites. Although the role of second SCT for BM relapse is growing,[28] our findings suggest the limitations of this approach for EM relapse, probably due to the preferential occurrence of the GVL effect, as discussed above. In fact, an optimal treatment strategy for EM relapse is yet to be established. Although several reports have shown that local radiotherapy can offer some patients long-term survival,[2,29] most patients develop systemic relapse.[30] Thus, systemic chemotherapy has been combined with radiotherapy in practice, but the chemotherapy may also abrogate the effector cells of the GVL effect. In this regard, it is noteworthy that gemtuzumab ozogamicin has been reported to be effective for EM relapse after SCT.[31,32] Because gemtuzumab ozogamicin does not affect the effector cells of the GVL response and is systemically effective, it could be an attractive option in the treatment of EM relapse. On the other hand, earlier diagnosis of EM relapse may improve the clinical outcome of the patients. Although there have been no established strategies for surveillance

of EM relapse after SCT, recent reports have suggested a usefulness of FDG-PET/CT in the detection of EM relapse of AML.[33–36]

The present study had several limitations. It was a retrospective study including a relatively small number of patients, and the patient characteristics were highly heterogeneous. Moreover, selection bias was unavoidable in patients who underwent a second SCT, which may affect the result. Nevertheless, our findings provide new insights into the mechanism of the GVL effect after haplo-SCT, as well as valuable information regarding the treatment options for EM relapse.

In conclusion, we demonstrated the frequent occurrence of EM relapse after haplo-SCT, particularly when performed as a second SCT. Our findings emphasize the necessity of establishing a future treatment strategy for EM relapse based on the recognition that EM relapse is a different disease entity from BM relapse with regard to its lower susceptibility to the GVL effect.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

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Progressive hearing loss following acquired cytomegalovirus infection in an immunocompromised child

Ken Kato, MD[a,*], Hironao Otake, MD, PhD[a], Mitsuhiko Tagaya, MD, PhD[a], Yoshiyuki Takahashi, MD, PhD[b], Yoshinori Ito, MD, PhD[b], Asahito Hama, MD, PhD[b], Hideki Muramatsu, MD, PhD[b], Seiji Kojima, MD, PhD[b], Shinji Naganawa, MD, PhD[c], Tsutomu Nakashima, MD, PhD[a]

[a]*Department of Otorhinolaryngology, Nagoya University Graduate School of Medicine, 65, Tsurumai-cho, Showa-ku, Nagoya, Japan*
[b]*Department of Pediatrics, Nagoya University Graduate School of Medicine, Nagoya, Japan*
[c]*Department of Radiology, Nagoya University Graduate School of Medicine, Nagoya, Japan*

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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

* Corresponding author. Department of Otorhinolaryngology, Nagoya University Graduate School of Medicine, 65, Tsurumai-cho, Showa-ku, Nagoya 466-8550, Japan. Tel.: +82 52 744 2323; fax: +82 52 744 2325.
E-mail address: katoken@med.nagoya-u.ac.jp (K. Kato).

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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.

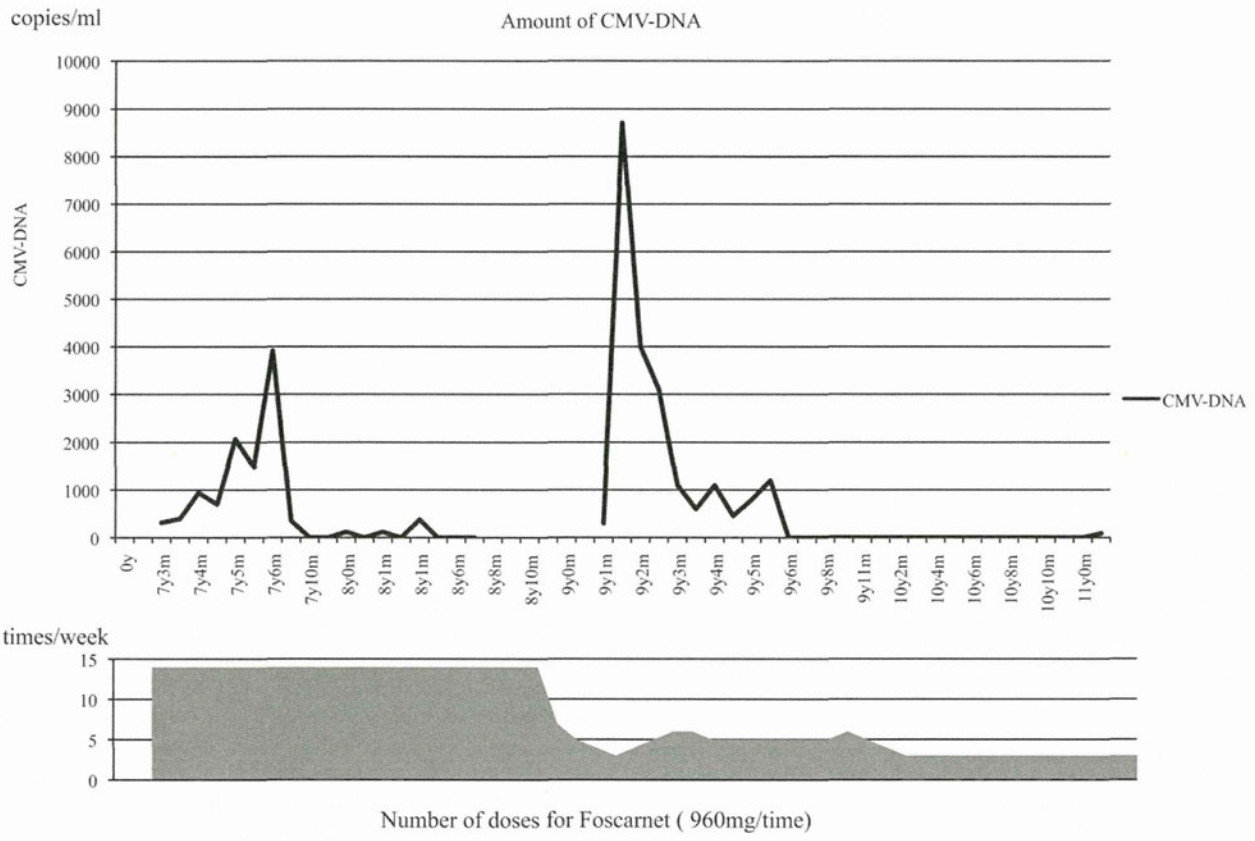

Fig. 1. The upper panel shows the changes in CMV DNA numbers from when she was 7 years old through when she was 11 years old. The lower panel shows the frequency of foscarnet administration during this period.

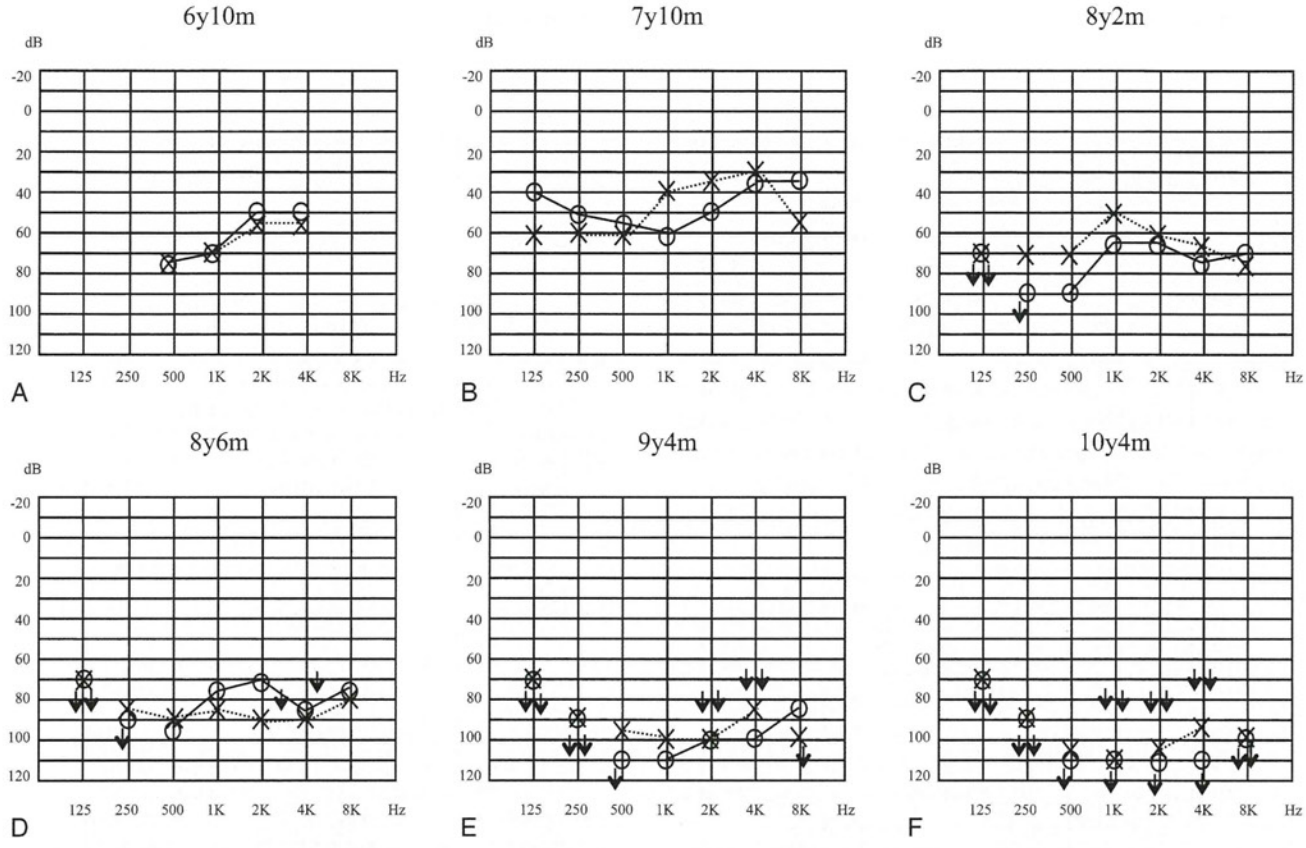

Fig. 2. From January 2007 to the present, the patient's hearing level has deteriorated. In particular, her bony conductive hearing level became worse. This showed that her sensorineural hearing loss was progressing.

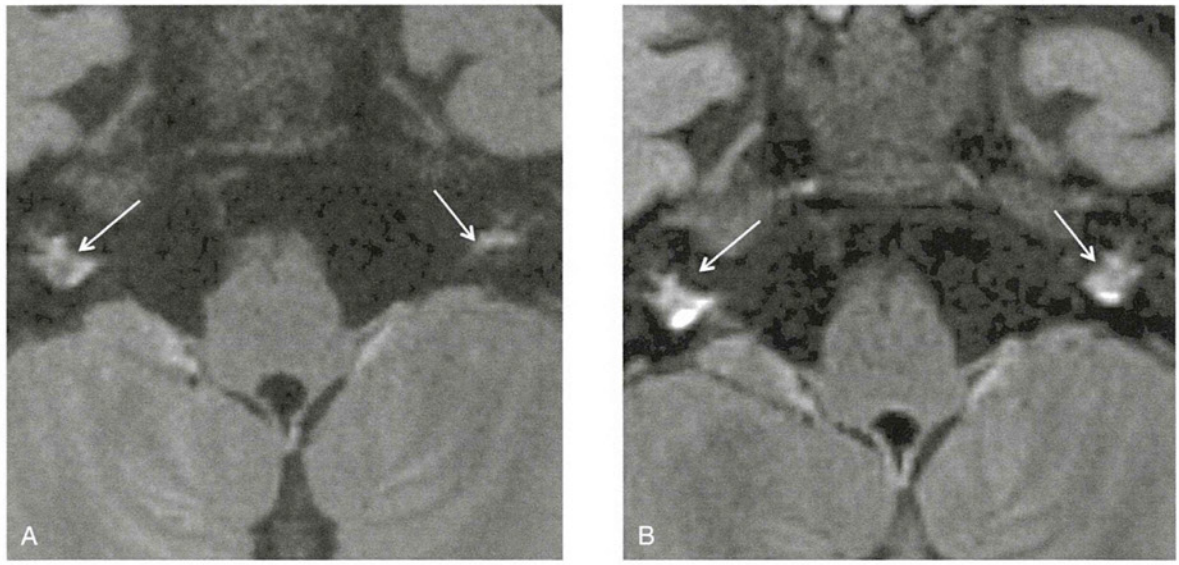

Fig. 3. 3D-FLAIR MRI after intervenous gadolinium administration. A was taken when she was 8 years old, and B was when she was 11 years old. The gadolinium enhancement of the internal auditory canal indicated by arrows was stronger in B than in A.

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

Yoshiyuki Takahashi, Hideki Muramatsu, Naoki Sakata, Nobuyuki Hyakuna, Kazuko Hamamoto, Ryoji Kobayashi, Etsuro Ito, Hiroshi Yagasaki, Akira Ohara, Akira Kikuchi, Akira Morimoto, Hiromasa Yabe, Kazuko Kudo, Ken-ichiro Watanabe, Shouichi Ohga and Seiji Kojima

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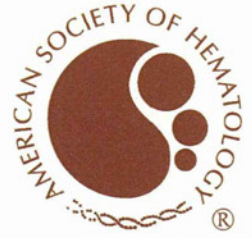

To the editor:

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.

Yoshiyuki Takahashi
Department of Pediatrics, Nagoya Graduate School of Medicine, Nagoya, Japan

Hideki Muramatsu
Department of Pediatrics, Nagoya Graduate School of Medicine, Nagoya, Japan

Naoki Sakata
Department of Pediatrics, Kinki University School of Medicine, Osaka, Japan

Nobuyuki Hyakuna
Center of Bone Marrow Transplantation, Ryukyu University Hospital, Okinawa, Japan

Kazuko Hamamoto
Department of Pediatrics, Hiroshima Red Cross Hospital, Hiroshima, Japan

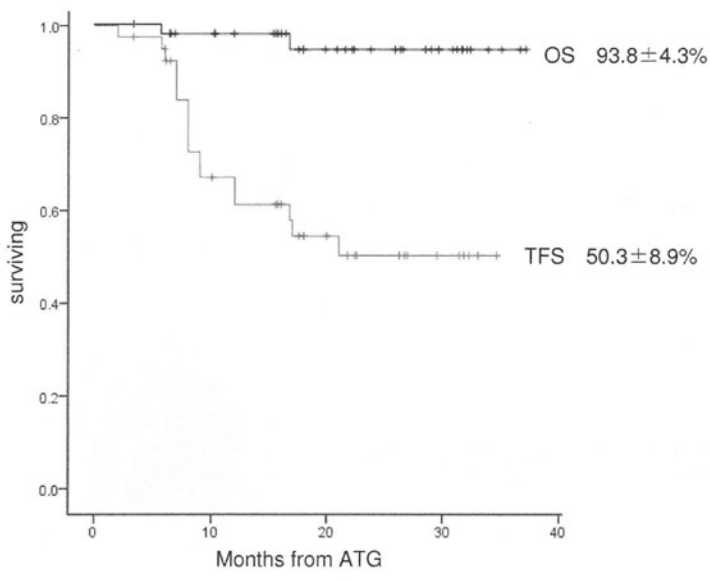

Figure 1. Kaplan-Meier estimates of overall survival (OS) and transplantation-free survival (TFS) in 40 Japanese children with AA. Survival was investigated using Kaplan-Meier methods. OS for all patients with AA after rATG and cyclosporine as first-line therapy included patients who later received HSCT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

Ryoji Kobayashi
Department of Pediatrics, Sapporo Hokuyu Hospital, Sapporo, Japan

Etsuro Ito
Department of Pediatrics, Hirosaki University School of Medicine, Hirosaki, Japan

Hiroshi Yagasaki
Department of Pediatrics, School of Medicine, Nihon University, Tokyo, Japan

Akira Ohara
Division of Blood Transfusion, Toho University Omori Hospital, Tokyo, Japan

Akira Kikuchi
Department of Pediatrics, Teikyo University School of Medicine, Tokyo, Japan

Akira Morimoto
Department of Pediatrics, Jichi Medical University School of Medicine, Tochigi, Japan

Hiromasa Yabe
Department of Cell Transplantation and Regenerative Medicine, Tokai University School of Medicine, Isehara, Japan

Kazuko Kudo
Division of Hematology and Oncology, Shizuoka Children's Hospital, Shizuoka, Japan

Ken-ichiro Watanabe
Department of Pediatrics, Graduate School of Medicine, Kyoto University, Kyoto, Japan

Shouichi Ohga
Department of Perinatal and Pediatric Medicine, Graduate School of Medical Sciences, Kyushu University, Fukuoka, Japan

Seiji Kojima
Department of Pediatrics, Nagoya Graduate School of Medicine, Nagoya, Japan

on behalf of the Japan Childhood Aplastic Anemia Study Group

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Correspondence: Dr Seiji Kojima, Nagoya Graduate School of Medicine, Tsurumai-cho 65, Showa-ku, Nagoya, Ai, Japan 466-8550; e-mail: kojimas@med.nagoya-u.ac.jp.

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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

Shoji Asakura,[1] Daigo Hashimoto,[1,2] Shuichiro Takashima,[3] Haruko Sugiyama,[1] Yoshinobu Maeda,[1] Koichi Akashi,[2,3] Mitsune Tanimoto,[1] and Takanori Teshima[2]

[1]Biopathological Science, Okayama University Graduate School of Medicine and Dentistry, Okayama, Japan. [2]Center for Cellular and Molecular Medicine and [3]Department of Medicine and Biosystemic Science, Kyushu University Graduate School of Science, Fukuoka, Japan.

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

Conflict of interest: The authors have declared that no conflict of interest.

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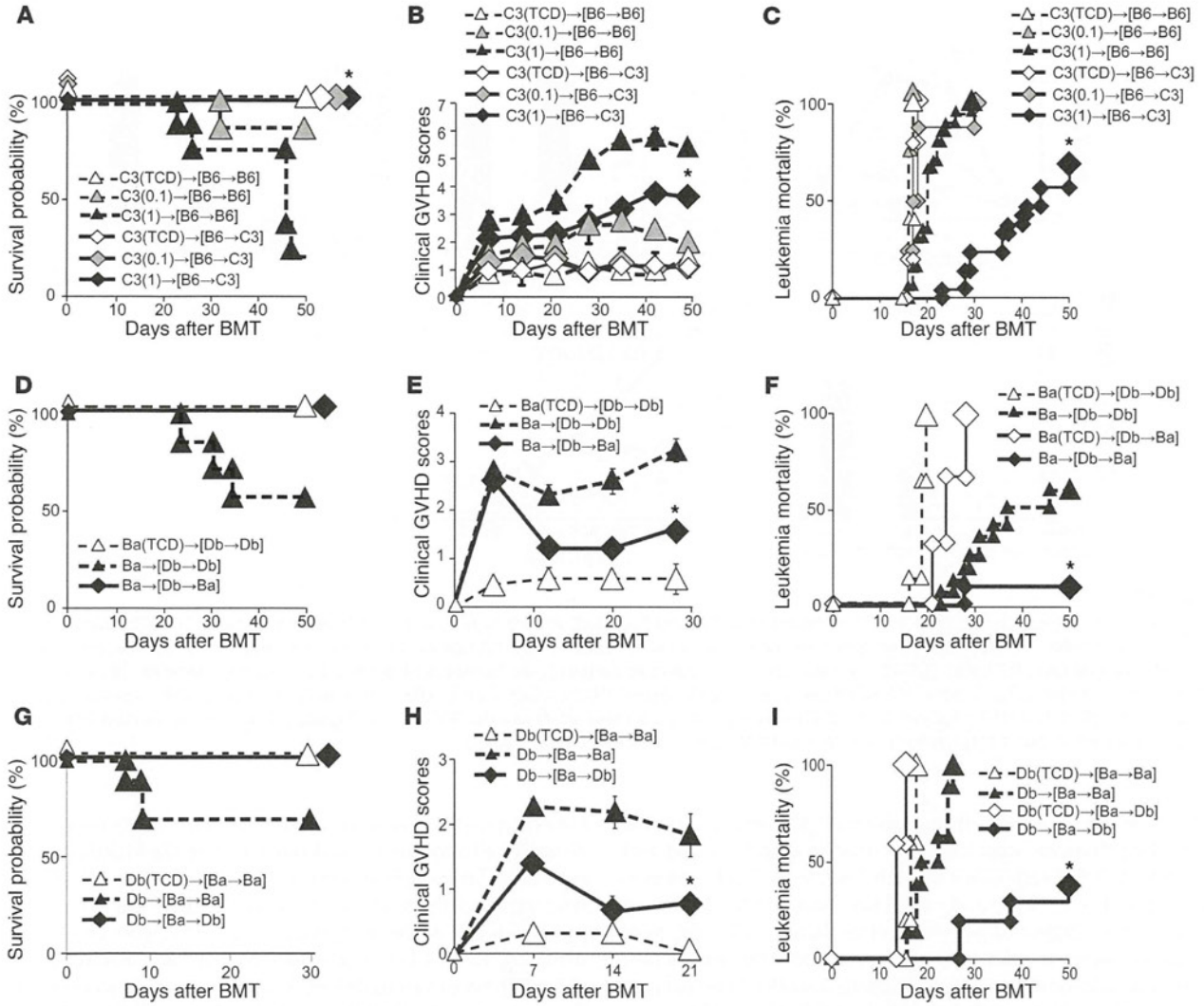

Figure 1

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

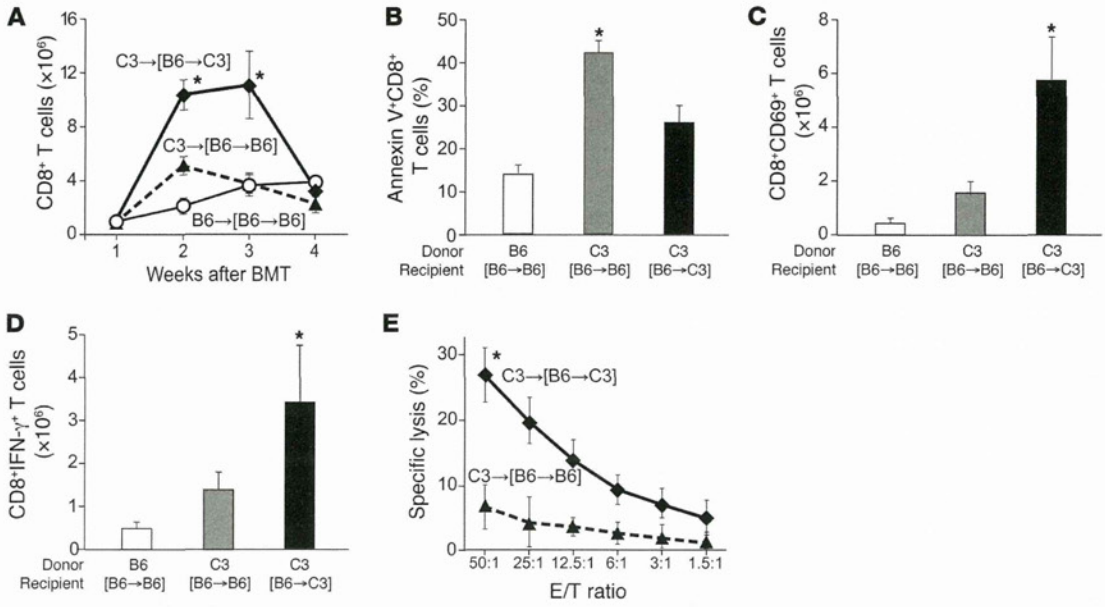

Figure 2

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).

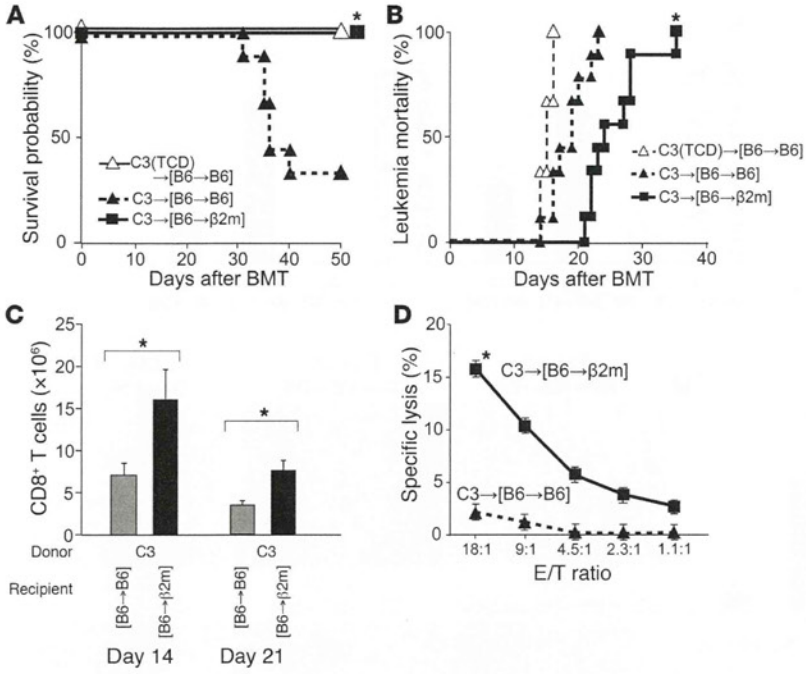

Figure 3
Absence of alloantigen expression on host nonhematopoietic cells restores GVL effects. [B6→B6] (triangles) and [B6→β2m$^{-/-}$] (squares) mice were reirradiated and injected with 5 × 10^6 TCD BM cells alone (open symbols) or with 1 × 10^6 CD8$^+$ T cells from C3 donors (filled symbols). (**A**) 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-

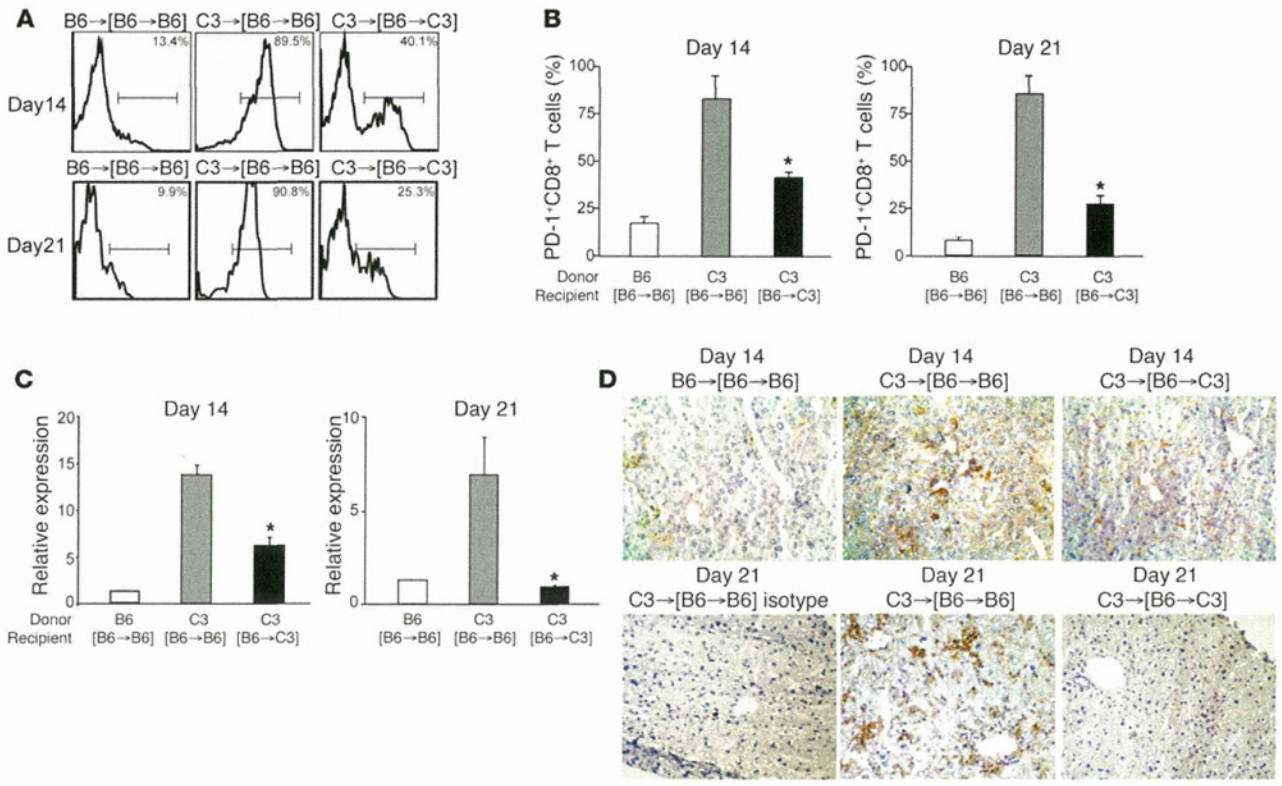

Figure 4

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-

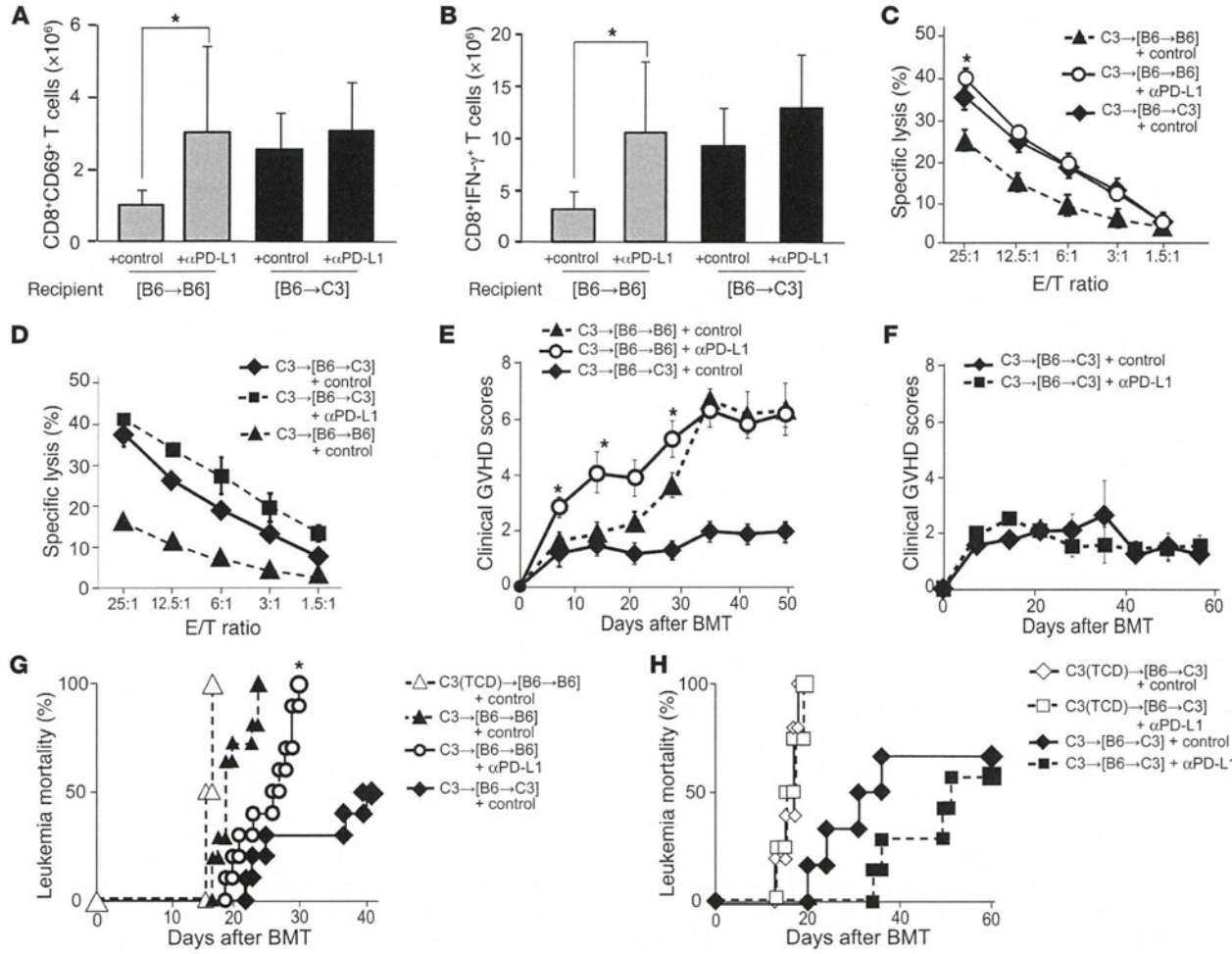

Figure 5

Blockade of the interaction between PD-1 and PD-L1 enhances GVL activity. [B6→C3] and [B6→B6] chimeras were reirradiated and injected with 5 × 10^6 TCD BM cells alone or with 1 × 10^6 $CD8^+$ T cells from C3 donors. Mice were i.p. injected with 500 μg of anti PD-L1 mAbs or controls on day 0 and then 200 μg thereafter on days +3, +6, +9, +12, +15, and +18. Splenocytes were harvested on day +14 to determine the number of $CD8^+CD69^+$ T cells (**A**) and IFN-γ–producing $CD8^+$ T cells (**B**) and CTL activity against EL4 targets (**C** and **D**). Results from a representative experiment of 2 similar experiments (means ± SD, n = 7–8/group). Mean clinical GVHD scores (±SEM) (**E** and **F**) after BMT are shown (n = 5–7/group). (**G** and **H**) Leukemia mortality after BMT in [B6→B6] and [B6→C3] chimeras injected with EL4 cells on day 0 (n = 4–11/group). Data from two similar experiments were combined. αPD-L1, anti–PD-L1 mAbs. *$P < 0.05$ compared with the corresponding controls.

tional tolerance of alloreactive, anti-donor $CD8^+$ T cells to achieve successful engraftment in BMT (42, 43). In this study, we found that PD-1 expression was upregulated in donor T cells and PD-L1 expression was upregulated in GVHD target organs. The expression of PD-1/PD-L1 was markedly reduced in chimeras lacking alloantigen expression on non-hematopoietic cells. PD-1 and PD-L1 expression is induced upon cell activation and inflammation in GVHD (44); therefore, the absence of alloantigen expression on GVHD target epithelium reduced GVHD in chimeric mice, which resulted in insufficient stimulation of the PD-1/PD-L1 interaction. Target tissue expression of PD-L1 is also critical for the induction of T cell exhaustion or tolerance in chronic viral infection, autoimmune diabetes, and cardiac allografting (19, 42, 45).

Both PD-1 and PD-L1 were markedly upregulated in [B6→B6] mice, but they were also modestly upregulated in [B6→C3] mice. Blockade of PD-1/PD-L1 interactions significantly restored T cell effector functions in [B6→B6] mice but modestly restored them in [B6→C3] mice as well. The relevance of these observations is shown by the PD-1/PD-L1 blockade studies. These data showed that the PD-1/PD-L1 pathway is particularly germane to [B6→B6] mice with widespread expression of alloantigens but also applies, at least in part, to [B6→C3] mice, wherein alloantigen expression is only on APCs. While there is likely to be a role for this pathway in the absence of epithelial alloantigen expression, the full negative impact of this pathway on GVL is only seen when alloantigen expression is present on non-hematopoietic tissues.

Of note, the improvement in GVL by the PD-1/PD-L1 blockade was partial, as has been shown in chronic viral infection (46–48). This may be due to the presence of multiple negative regulatory pathways that contribute to T cell exhaustion, including CTLA-4, IL-10, LAG-3, CD160, and 2B4 (20, 47, 49). In addition, the population of exhausted T cells is heterogeneous, and this interven-

tion is effective only for PD-1^{lo} and not PD-1^{hi}, which are subsets of exhausted T cells (50). Many of these inhibitory receptors are either coexpressed by the same exhausted T cells or differentially expressed on different subsets of exhausted cells. As the severity of the infection increases, the number of different inhibitors expressed per cell increases (47). A second inhibitory receptor, CTLA-4, can be overexpressed by exhausted CD4$^+$ T cells in chronic viral infection, but it appears to have a minimal role on exhausted CD8$^+$ T cells (19, 51). Although CTLA-4 was only slightly upregulated on CD8$^+$ T cells in contrast to the marked upregulation of PD-1 in our CD8-dependent model of MHC-matched BMT, the precise inhibitory receptors of therapeutic interest may differ between CD4$^+$-dependent and CD8$^+$-dependent GVHD/GVL. Another key negative regulatory pathway is mediated by Foxp3$^+$ Tregs. However, enhancement of GVL is not due to effects of the PD-1/PD-L1 blockade on Tregs, because blockade of PD-1/PD-L1 interactions enhances the expansion and function of Tregs (52). The hierarchy of these pathways in regulating GVL will need to be studied in the future based on better understanding of the delineation of T cell subsets and models (53). However, our results suggest the detrimental effect of GVHD-induced immunosuppression on GVL responses, regardless of which inhibitory pathway might be dominant clinically.

In addition, the administration of anti–PD-L1 mAb also exacerbated acute GVHD, as has been shown in a previous study (54). Therefore, the beneficial effects of the PD-1/PD-L1 blockade may be offset by the exacerbation of GVHD. Effects of the inhibitory receptor blockade might depend on the magnitude or stage of donor T cell activation and the severity of GVHD; therefore, the timing and duration of the targeting may be important.

In clinical HSCT, alloantigens continue to be presented on MHC class I in non-hematopoietic cells throughout the lifetime of the transplant recipients. However, a substantial number of patients eventually develop tolerance after resolution of GVHD and often experience leukemia relapse. Although activation-induced apoptosis of alloreactive T cells has been proposed as an explanation of this paradox (55), studies monitoring GVHD-specific T cell clones indicate that host-reactive T cells are continuously present after allogeneic HSCT (56–58). Our results provide a logical explanation for this paradox. However, the process of exhaustion is unlikely to occur in patients not developing GVHD, because induction of T cell exhaustion requires antigen-specific activation of T cells and subsequent differentiation into effector T cells. In these patients, tolerance could be induced by other mechanisms, such as functional central and peripheral tolerance mechanisms. It is well known that GVL is not apparent in patients with high leukemia burden. Although leukemia cells used in the current study do not express PD-L1 (22, 59), leukemia cells expressing PD-L1 may also directly limit the GVL response in patients with high leukemia burden (22, 24, 25). However, such insights from animal models must be extrapolated with caution to clinical studies involving humans.

It has been assumed that T cell exhaustion is antigen specific in chronic viral infection. Bystander lysis of T cells has also been reported in the course of viral infections (60), but is of minimal significance because of its limited magnitude and because normal thymic function can replenish the peripheral T cell pool. In contrast, in GVHD, T cell exhaustion occurs after initial T cell activation and the subsequent development of GVHD. GVHD induces bystander apoptosis of non-host-reactive T cells. In addition, GVHD-mediated injury of the thymus and the secondary lymphoid organs inhibits full replenishment of the peripheral T cell pool (55). Thus, establishment of full immune competence probably requires the additional process of T cell reconstitution following T cell exhaustion.

In conclusion, our results indicated the significance of alloantigen expression on non-hematopoietic cells in GVL. Alloantigen expression on non-hematopoietic cells induces the apoptosis of donor T cells and the dysfunction of cytotoxic effector function, which leads to a reduction in GVL activity. T cell dysfunction was partially restored by blocking PD-1/PD-L1 interactions, which suggests that the therapeutic "tuning" of T cell responses via modulation of negative regulatory pathways represents a novel strategy for enhancing GVL. Our results in combination with those of previous studies (6, 7, 9, 10, 38, 39) provide a complete picture of the effect of alloantigen expression on host APCs, GVHD target epithelium, and tumor cells in allogeneic HSCT; alloantigen expression on host non-hematopoietic cells augments GVHD but suppresses GVL effects. This concept may provide an important framework for understanding the pathophysiology of GVHD and allow for the separation of GVHD and GVL.

Methods

Mice. Female C57BL/6 (B6, H-2^b, CD45.2$^+$), BALB/c (Ba, H-2^d), and DBA/2 (Db, H-2^d) mice were purchased from Charles River Japan. B6.Ly5.1 (H-2^b, CD45.1$^+$) and C3H.Sw (C3, H-2^b) mice were purchased from The Jackson Laboratory. B6-background β_2m-deficient mice ($\beta_2m^{-/-}$: B6.129-β_2-m^{tm1Jae}N12) were purchased from Taconic. The age of mice used ranged from 8 to 12 weeks. Mice were maintained in specific pathogen–free conditions and received normal chaw and hyperchlorinated drinking water for the first 3 weeks after BMT. All experiments involving animals were performed according to a protocol approved by the Institutional Animal Care and Research Advisory Committee of Okayama University and Kyushu University.

Generation of bone marrow chimera and induction of GVHD and GVL. Total body irradiation (TBI: X-ray) was split into 2 doses separated by 4 hours to minimize gastrointestinal toxicity. B6 and C3 mice received 10 Gy TBI, whereas Ba and Db mice received 8.5 Gy TBI. To create BM chimeras, lethally irradiated mice were intravenously injected with 5×10^6 TCD BM cells from donors. TCD was performed using anti-CD90 microbeads and AutoMACS (Miltenyi Biotec). Four months later, the chimeric mice were reirradiated and injected with 5×10^6 TCD BM cells plus various doses of CD8$^+$ T cells or 2×10^6 T cells. T cells and CD8$^+$ T cells were negatively isolated from splenocytes by using a T cell isolation kit and a CD8$^+$ T cell isolation kit (Miltenyi Biotec), respectively, and the AutoMACS. In the GVL experiments, EL4 (H-2^b) derived from a B6 mouse, P815 (H-2^d) derived from a Db mouse, and A20 (H-2^d) derived from a Ba mouse were intravenously injected into BMT recipients on day 0 of BMT. Anti–PD-L1 mAbs were purified from the hybridoma supernatant of clone MIH5 (61), which was a gift from Miyuki Azuma of Tokyo Medical and Dental University, Tokyo, Japan, and i.p. injected at a dose of 500 μg/mouse on day 0, followed by 200 μg/mouse on days +3, +6, +9, +12, +15, and +18 after BMT.

Assessment of GVHD and GVL effects. Survival after BMT was monitored daily, and the degree of clinical GVHD was assessed weekly by using a scoring system that sums changes in 5 clinical parameters: weight loss, posture, activity, fur texture, and skin integrity (maximum index, 10) as described previously (13). The cause of each death after BMT was determined by post mortem examination, and was either GVHD or tumor. The most striking leukemia-specific abnormality induced by EL4, P815, and A20 was macroscopic tumor nodules, marked hepatosplenomegaly, and lower limb paralysis (62). Leukemia death induced by EL4, P815, and A20 was therefore defined by the occurrence of hepatosplenomegaly, macroscopic tumor nodules in the liver