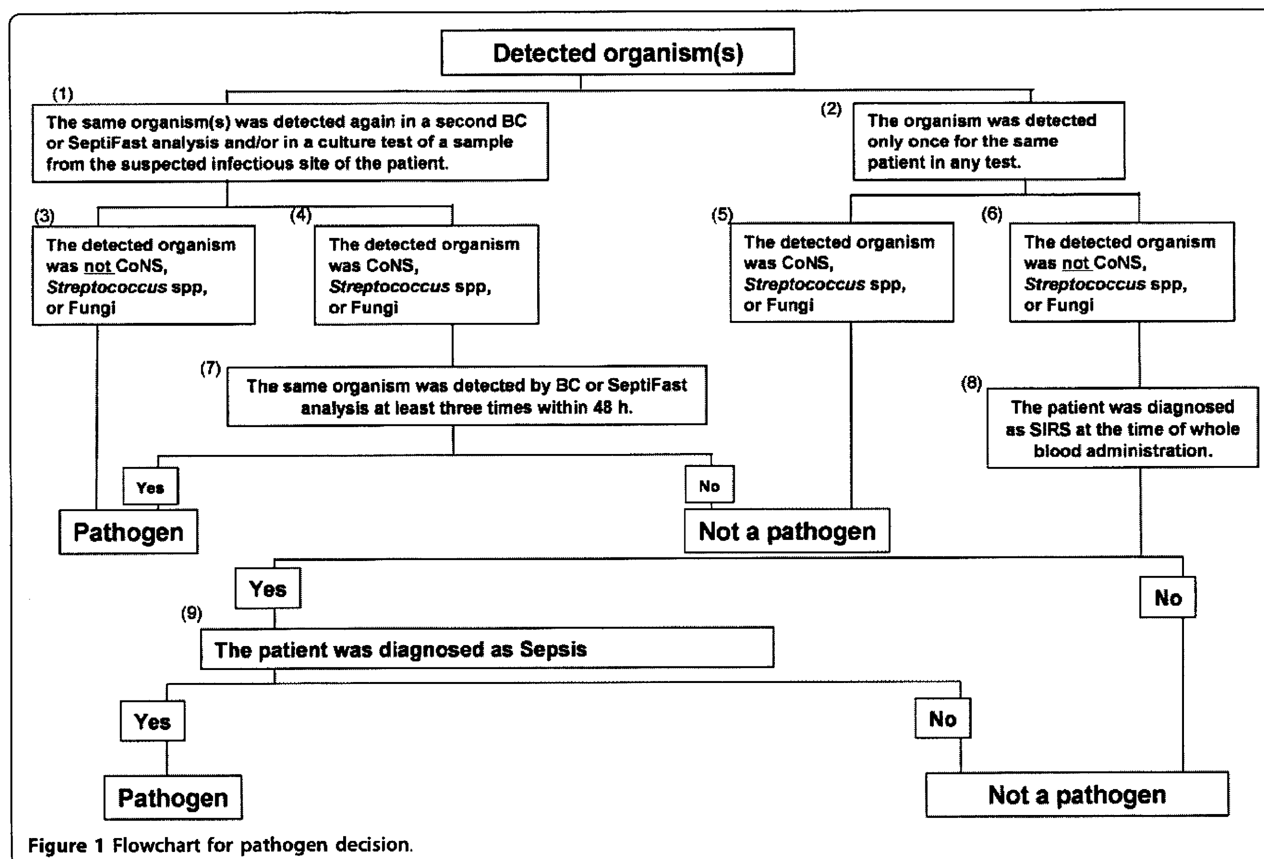

Figure 1 Flowchart for pathogen decision.

Results

Correlation between SeptiFast and blood culture analyses

The patients consisted of 137 males and 75 females. Table 2 demonstrates that Septi*Fast* analysis detected more organisms in patients than blood culture analysis.

Figure 2 shows the correlation between blood culture and Septi*Fast* analyses. No specific pathogen could be identified in seven of the samples (by either method). These samples were therefore eliminated from the study since they did not meet the definition of sepsis, leaving a total of 400 samples that were evaluated. The DNA Detection Kit identified a pathogen in 11.3% (45/400) of the samples, and blood culture analysis identified a pathogen in 8.0% (32/400) of the samples. The difference between positive and negative results for each assay was statistically different, as measured using McNemar's test ($P < 0.04$). Of the 22 samples in which pathogens were detected by both blood culture and DNA Detection Kit analyses, there was one sample in which there was a discrepancy in the pathogen that was detected. In this sample, *E. faecium* was detected by blood culture analysis but *E. coli* was detected by Septi*Fast* analysis. We confirmed *E. coli* and *E. faecium* were detected from the other sample of the same patient. Thus, it was decided that both organisms were pathogens. Table 3 summarizes the number of samples in which each of the listed organisms was identified. The detected pathogen is total 56 because we count both *E. coli* and *E. faecium* as pathogens.

Twenty-three pathogens were detected by Septi*Fast* only. All of the pathogens detected only by DNA Detection Kit were identified as the same organism from the other culture. Pathogens were detected in 10 of the samples only by blood culture analysis. The organisms identified in five of these samples, *Bacteroides spp.*, Gram-positive rod and *Morganella morganii* (in 2, 2 and 1 samples respectively), are not listed as organisms that can be detected by Septi*Fast* analysis. Of the remaining five samples, MRSA was detected in two of the samples and *Pseudomonas aeruginosa, Klebsiella and Enterococcus faecium* were each detected in one of the remaining samples.

Figure 3 shows the change in the number of samples testing positive for a pathogen when the positive results of blood culture and Septi*Fast* were combined. This figure demonstrates that the number of samples testing positive in SIRS samples only, increased from 9.0% (35/387) to 16.0% (62/387) when organisms that were detected by blood culture analysis, and those that were detected by Septi*Fast* analysis, were combined.

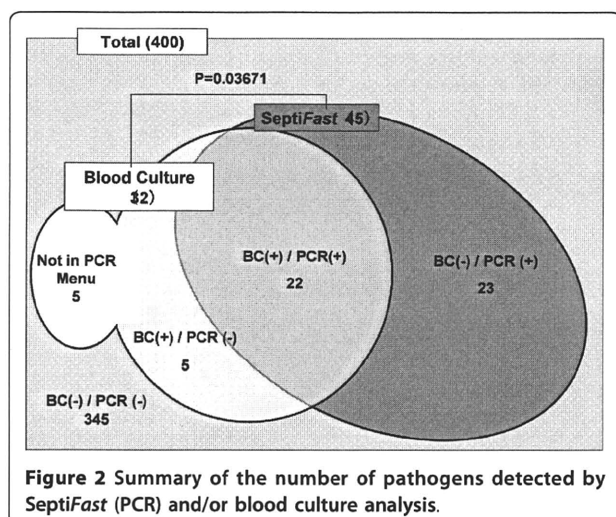

Figure 2 Summary of the number of pathogens detected by Septi*Fast* (PCR) and/or blood culture analysis.

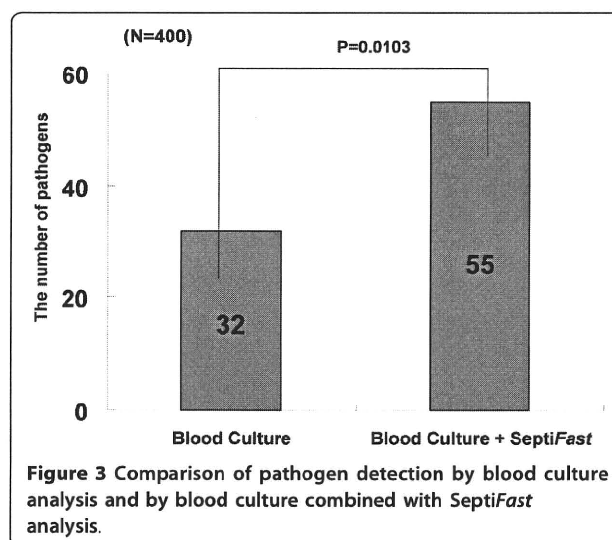

Figure 3 Comparison of pathogen detection by blood culture analysis and by blood culture combined with Septi*Fast* analysis.

A significant difference in the number of positive samples from the combined tests compared to that in the individual tests was observed using a two-sample test for equality of proportions (P = 0.01).

MRSA detection

In this study, 12 samples tested positive for *S. aureus* as a pathogen. Of these 12 samples, 10 were detected by Septi*Fast* analysis and 9 were detected by blood culture analysis. However, while blood culture analysis detected MRSA in six samples, Septi*Fast* analysis only detected MRSA in four samples. Two samples were diagnosed as being infected by MRSA based on the analysis shown in the decision tree (Figure 1).

Table 3 Pathogens detected by Septi*Fast* and blood culture analyses

Pathogen	Strain detected		
	Only by BC	Only by Septi*Fast*	Both methods
S.aureus (MSSA)	0	3	3
S.aureus (MRSA)	2	0	4
S.pneumoniae	0	1	0
Streptococcus spp.	0	2	1
Enterococcus faecalis	0	1	0
Enterococcus faecium	2	0	0
Enterobacter aerogenes/ cloacae	0	3	1
Escherichia coli	0	3	9
Klebsiella pneumoniae/ oxytoca	1	5	1
Pseudomonas aeruginosa	1	4	1
Candida albicans	0	1	0
Candida tropicalis	0	1	1
Sub-total	6	24	21
Not detectable by SeptiFast	5	0	0
Total	11	24	21

The affect of antibiotics administration

As shown in Figure 2, a total of 55 pathogens were detected by Septi*Fast* or blood culture analysis. Of these 55 samples, 40 samples (72.7%) were from patients which had been administered antibiotics and 32 of these 40 samples (80.0%) were from patients that had been administered antibiotics that matched the spectra of the antibiotics. These 32 samples were evaluated for the presence of pathogens by blood culture and DNA Detection Kit. Septi*Fast* analysis detected pathogens in 21 samples, while blood culture analysis detected pathogens in 10 samples, indicating that DNA Detection Kit analysis detected significantly more pathogens than blood culture analysis (P = 0.02) under these conditions. These data further suggest that detection of pathogens by blood culture analysis was affected by antibiotics, since there were 15 samples in which pathogens were detected only by DNA Detection Kit, but not by blood culture analysis. Of the four samples in which pathogens were detected by blood culture analysis but not by Septi*Fast* analysis, one of these samples was identified as containing the pathogen *Bacteroides caccae*, which is an organism that cannot be detected by Septi*Fast*.

Discussion

Sepsis is an infection frequently found in transplant patients, in patients with hematological neoplasms or in patients admitted to an intensive care unit (ICU) following surgery. Rapid pathogen identification and the appropriate chemotherapy are important to improve patient prognoses. Definitive identification of bacterial species with a microarray platform was highly expected [16].

A rapid pathogen detection and diagnosis kit for sepsis called Septi*Fast* has recently been developed [17]. This kit will reduce the turn-around time to detect pathogens. Louie *et al.* surveyed Septi*Fast* pathogen detection times using samples from seven patients and reported that the average pathogen detection time was 6.54 ± 0.27 hours [18].

As shown in Figure 2, we confirmed that Septi*Fast* analysis significantly detected more pathogens than blood culture analysis. However, a discrepancy between the results of Septi*Fast* and blood culture analysis was noted for one sample. In this sample, *E. coli* was detected by Septi*Fast* analysis, but *E. faecium* was detected by blood culture analysis. We rechecked the presence of these organisms in more samples from the patient and found that *E. coli* had been detected by Septi*Fast* and blood culture analysis in samples that were submitted three days before and that *E. faecium* was detected by blood culture analysis two days after. Therefore, it was considered that bacterial translocation had occurred in this patient. In 23 of the samples assayed in this study, pathogens were only identified by DNA Detection Kit. One possible reason why a pathogen was not detected in these samples by blood culture analysis was that blood culture analysis might have been affected by the treatment of the patients with antibiotics. Indeed, 15 of these 23 patients (65.2%) had been administered antibiotics appropriate for the pathogen in question. In 10 samples in this study, pathogens were detected only by blood culture analysis. The reason that Septi*Fast* analysis could not detect these pathogens was considered to be that the concentration of these pathogens was very low and therefore it was outside the limit of detection (LOD) of Septi*Fast* analysis.

Of the 12 samples that tested positive for *S. aureus* in this study, 10 were detected by DNA Detection Kit but only 9 were detected by blood culture analysis. However, as shown in Table 3, blood culture analysis detected MRSA in six samples whereas Septi*Fast* detected MRSA in only four samples. This discrepancy may be caused by the LOD gap mentioned above. Thus, the sensitivity of detection of *S. aureus* and the mecA gene was 30 CFU/mL for the Septi*Fast* assay system, but the LOD is 7.7 CFU/mL for *S. aureus* and 24.2 CFU/mL for mecA genes [19]. Therefore, the reason why MRSA could not be detected by Septi*Fast* analysis, but could be detected by blood culture analysis, may be due to a difference in the detection sensitivity of these two assay systems.

As shown in Table 4, Septi*Fast* analysis detected more pathogens than blood culture analysis when antibiotics had been administered to the patients. Although the antibiotics used prevented the growth of organisms in blood culture analysis, it appeared that DNA Detection Kit could detect pathogens with relatively little interference by antibiotics. Our results are in agreement with the information provided by the Septi*Fast* manufacturer that antibiotics do not interfere with Septi*Fast* detection of pathogens [6]. These data suggest that Septi*Fast* will have clinical utility for analysis of pathogens in patients with a background of unknown pre-treatment of antibiotics due to being referred from other hospitals, and for patients receiving antibiotics before blood collection for testing due to the severity of their condition. Another clinical benefit of Septi*Fast* is that the test result is achieved faster than the result of blood culture analysis, and thus will allow a speedier de-escalation from a broad- to a narrow-spectrum antibiotic. According to the "Surviving Sepsis Campaign Guidelines (SSCG) 2008", antibiotic administration within an hour is recommended in patients suspected of having severe sepsis [20]. Therefore, the use of the DNA Detection Kit, whose pathogen detection ability is not susceptible to the effects of antibiotic administration, should contribute to implementation of these guidelines.

Table 4 Comparison of pathogen detection by Septi*Fast* and blood culture analyses following treatment with the antibiotic appropriate to the pathogen

		Blood Culture		
		Positive	Negative	Total
SeptiFast	Positive	6	15	21
	Negative	4[a]	7	11
Total		10	22	32

[a] One of these four pathogens was a pathogen that is not detectable by Septi*Fast*.

In Japan, blood culture analysis is the gold standard of pathogen analysis when sepsis is suspected. However, it is anticipated that if Septi*Fast* analysis is introduced, it will facilitate the selection of antibiotics based on EBM due to earlier pathogen detection and to the detection of more pathogens. DNA Detection Kit analysis cannot replace blood culture analysis because it cannot detect all sepsis pathogens. However, by combining Septi*Fast* and blood culture analyses, the detection rate of pathogens will significantly increase. A faster detection rate will be especially useful for SIRS patients since more precise sepsis treatment will become feasible. Since the use of the DNA Detection Kit requires skilled clinical laboratory technicians and suitable facilities, the kit should be used in university hospitals where severe sepsis patients are gathered.

The extended duration of surgical antibiotic prophylaxis for up to seven days and multicoverage for empiric therapy of suspected sepsis is performed in Japan. Thus, our results are not easily applicable to other regions since the diagnostic value of conventional blood culture

systems in this study may have been decreased by very frequent previous antibiotic exposure.

Conclusions

Although DNA Detection Kit analysis could not detect all sepsis pathogens, Septi*Fast* analysis did detect potentially important pathogenic DNA that could not be detected by blood culture analysis. Simultaneous testing of samples from patients with demonstrated SIRS using blood culture analysis and DNA Detection Kit showed a high pathogen detection rate. This rapid multiplex pathogen detection system complemented traditional culture-based methods and offered some added diagnostic value for the timely detection of causative pathogens, particularly in antibiotic pre-treated patients. Furthermore, the ability of Septi*Fast* analysis to identify pathogens when the background of antibiotic administration is unknown may allow a change to narrower-spectrum antibiotics. The combined data suggest that Septi*Fast* may ultimately contribute both to the improvement of patient safety and to future medical economic efficiency. Clearly, adequately designed intervention studies are urgently needed to prove its clinical effectiveness in improving appropriate antibiotic selection and patient outcomes.

Key messages

- This rapid multiplex pathogen detection system showed a higher pathogen detection rate in comparison with blood culture analysis.
- This system offered some added diagnostic value for the timely detection of causative pathogens, particularly in antibiotic pre-treated patients.
- However, the well designed intervention studies are urgently needed to prove the clinical effectiveness.

Abbreviations

ALL: acute lymphoma leukemia; AML: acute myelogenous leukemia; Cp: crossing point; EBM: evidence-based medicine; IC: internal control; ICU: intensive care unit; ITS: internal transcribed spacer; LOD: limit of detection; ML: malignant lymphoma; MRSA: methicillin-resistant *Staphylococcus aureus*; PCR: polymerase chain reaction; SIRS: systemic inflammatory response syndrome; SSCG:Surviving Sepsis Campaign Guidelines.

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Authors' contributions

KY, YK, SK, KS, SA, HG and MK carried out the molecular genetic studies, participated in the sequence alignment and drafted the manuscript. MT, KT, SK, MS, HS and TS participated in the sequence alignment. OT, AM, YI, SO, NA and SH participated in the design of the study and performed the statistical analysis. HO, AI, NH, JT, MM, YK and YS conceived of the study, and participated in its design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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ORIGINAL ARTICLE

Air-leak syndrome following allo-SCT in adult patients: report from the Kanto Study Group for Cell Therapy in Japan

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We retrospectively investigated air-leak syndrome (ALS), including pneumothorax and mediastinal/s.c. emphysema, following allogeneic hematopoietic SCT. Eighteen patients (1.2%) developed ALS among 1515 undergoing SCT between 1994 and 2005 at the nine hospitals participating in the Kanto Study Group on Cell Therapy. The median onset of ALS was at 575 days (range: 105–1766) after SCT and 14 patients (77.8%) had experienced late onset noninfectious pulmonary complications (LONIPC) before ALS. Chronic GVHD (cGVHD) was the strongest risk factor for ALS (odds ratio 13.5, $P = 0.013$ by multivariate analysis). Repeat SCT, male sex and age <38 years at the time of transplantation were also significant risk factors for ALS. Patients with ALS had a significantly worse survival rate than those without ALS (61.5 vs 14.9% at 3 years; $P = 0.000$). The main cause of death was respiratory complications in 8 of the 18 patients. In conclusion, ALS is a rare complication of SCT that is more likely to occur in relatively young male patients with cGVHD and/or LONIPC. It is possible that better understanding and treatment of LONIPC may lead to prevention of ALS.

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Introduction

The survival of patients with hematological disorders who receive allo-SCT has shown continuing improvement due to the introduction of various innovative therapeutic approaches. However, organ dysfunction/damage and infection remain problematic for long-term survivors after SCT, especially those with chronic GVHD (cGVHD), and such complications affect both their quality of life and survival. Late onset noninfectious pulmonary complications (LONIPC) are one of the most common manifestations of organ damage during the late phase after allo-SCT,[1–5] and have been reported to be the major cause of death for patients in this phase. Unlike LONIPC, air-leak syndrome (ALS), which includes pneumothorax (PT), mediastinal emphysema (ME) and s.c. emphysema (SE), is a relatively rare complication of SCT.[6–9] Although patients with ALS after SCT had a fatal outcome according to some case reports or small-scale retrospective studies, the clinical features of post transplant ALS remain obscure. The purpose of this study was to clarify the characteristics and risk factors for ALS after SCT, as well as its effect on survival.

Materials and methods

Patients

We retrospectively surveyed 1515 patients aged $\geqslant 15$ years who received allo-SCT between January 1994 and March 2005 at nine hospitals participating in the Kanto Study Group on Cell Therapy (KSGCT) in Japan. Detailed clinical data were collected by reviewing the medical records of each institution, whereas baseline pretransplant and post transplant information on the patients was retrieved from the KSGCT database.

Definitions

Air-leak syndrome was diagnosed from chest X-ray films and/or computed tomography scans as follows: PT was diagnosed by detection of extra-alveolar air in the left and/or right hemithorax, ME was defined as the presence of extra-alveolar air in the mediastinal space and SE was defined as extra-alveolar air in the s.c. tissue. Iatrogenic ALS that occurred after procedures such as trans-bronchial lung biopsy or mechanical ventilation was excluded. cGVHD and LONIPC were diagnosed from previously reported criteria.[10,11]

The case report form for ALS patients included the following information: date of diagnosis of ALS, type of ALS (described above), initial symptoms (cough, dyspnea and chest pain), presence of active cGVHD at the onset of ALS (yes or no), type of cGVHD (limited or extensive), presence of LONIPC (bronchiolitis obliterans (BO), bronchiolitis obliterans with organizing pneumonia (BOOP) and interstitial pneumonia (IP)), time from LONIPC to ALS, immunosuppressive therapy at the onset of ALS (prednisolone (PSL), CYA, tacrolimus (FK) and others), treatment of ALS (drain, initiate or increase the dose of immunosuppressive therapy, decrease the dose of immunosuppressive therapy, pleurodesis, observation and others), response to treatment (improved, stable or worsened), outcome and cause of death.

Statistical analysis

To identify risk factors for ALS, we tested the following variables by univariate and multivariate analyses: recipient age and sex, stem cell source, conditioning regimen (conventional vs reduced intensity), use of TBI, number of SCT procedures (first vs second or more), GVHD prophylaxis (CYA based vs FK based), grade of acute GVHD (0–I vs II–IV) and cGVHD (none vs limited or extensive). Comparison of categorical variables was carried out by the χ^2-test or Fisher's exact test, whereas comparison of continuous variables was performed with Student's t-test. To evaluate the independence of potential risk factors for ALS, we performed multiple logistic regression analysis. In all analyses, $P<0.05$ was considered to indicate statistical significance. Survival curves after the occurrence of ALS were estimated by the Kaplan–Meier method and the survival of patients with ALS was compared to that of those without ALS by the log-rank test, treating the occurrence of ALS as a time-dependent variable. To determine whether ALS was an independent poor prognostic factor for long-term survival after SCT, we performed Cox proportional hazards analysis including the following variables: recipient age and sex, disease risk (standard risk diseases were acute leukemia in the first or second CR, aplastic anemia, refractory anemia, refractory anemia with ringed sideroblasts, CML in the first chronic phase, multiple myeloma in PR or CR and malignant lymphoma in the first or second CR, whereas all other diseases/states were considered to be high risk), stem cell source, type of conditioning regimen and use of TBI. Statistical analyses were performed with SPSS software (SPSS Inc., Chicago, IL, USA).

Results

Clinical features of ALS

Air-leak syndrome was diagnosed in 18 patients (1.2% of all patients) after allo-SCT. Table 1 shows a summary of the baseline characteristics of these patients. Sixteen patients were men and the median age was 29.5 years. Grade II–IV acute GVHD occurred in 11 patients (61%) and all but one patient (94%) had cGVHD. The clinical presentation and outcome of ALS are shown in Table 2. The median time of onset was day 575 (range: days 105–1766) after SCT. ALS was classified as PT in seven patients, ME/SE in six patients and PT combined with ME/SE (mixed ALS) in five patients. At the onset of ALS, 16 patients had active cGVHD. Before ALS occurred, 14 patients had also experienced LONIPC, including 4 with BO, 4 with BOOP, 5 with IP and 1 with IP along with BOOP. At the diagnosis of ALS, LONIPC had resolved in two patients (nos. 5 and 18), but persisted in the remaining 12 patients. The median time from the diagnosis of LONIPC to the onset of ALS among the patients who had persistent LONIPC was 74 days (range: 3–1177 days). Seventeen patients (94%) had been treated with steroids and 13 patients (72%) were on steroids at the time of diagnosis of ALS. Treatment of ALS included drainage in 11 patients and pleurodesis in 3. ALS improved in 12 of 18 patients, remained stable in 2 patients and progressed despite treatment in 4 patients. The three patients (nos. 7, 12 and 13) who underwent pleurodesis all had progressive ALS (Table 2).

Eleven patients died at a median of 222 days after the occurrence of ALS (range: 6–944 days). Respiratory complications were the direct cause of death in eight patients, including ALS in four, BO in two and IP in two. Patient 10 did not have LONIPC before the occurrence of ALS, but BO appeared after improvement of ALS and became progressively worse. Two nonrespiratory deaths were attributable to relapse of the primary disease and one was due to multiple organ failure that was unrelated to ALS. Among the patients with PT or ME/SE, seven out of nine who responded to treatment are still alive, whereas all four patients whose initial therapy

Table 1 Summary of baseline characteristics of the patients with ALS

No. of patients (%)	18 (1.2%)
Sex (male/female)	16/2
Median age (range)	29.5 (16–53)
Primary disease (AML/ALL/CML/ML)	9/5/2/2
Disease status at HSCT (CR or CP/NR or BC)	14/4
Donor source (RBM/UBM/PB/CB)	6/8/4/0
Conditioning (myeloablative/nonmyeloablative)	17/1
TBI or TLI (yes/no)	15/3
No. of SCT (first/second or more)	5/13
GVHD prophylaxis (CYA based/FK based)	8/8
aGVHD (0–I/II–IV)	6/12
cGVHD (no/limited/extensive)	1/2/15

Abbreviations: aGVHD = acute GVHD; BC = blastic crisis; CB = cord blood; cGVHD = chronic GVHD; CP = chronic phase; FK = tacrolimus; ML = malignant lymphoma; NR = non remission; RBM = related BM; RPB = related PBSCs; TLI = total lymphoid irradiation; UBM = unrelated BM.

Table 2 Clinical presentation and outcome of 18 patients with ALS

Patient no.	*Time from SCT to ALS (days)*	*Type of ALS*	*Initial symptoms*			*Active cGVHD at onset of ALS/ type of cGVHD*	*Active LONIPC at onset of ALS*	*Time from LONIPIC to ALS (days)*	*Immuno-suppression at onset of ALS*	*Treatment of ALS*	*Response to therapy*	*Outcome (cause of death)*
			Cough	*Dyspnea*	*Chest pain*							
1	1332	PT (one side)	+	+	–	Yes/Limit	BOOP	264	—	Drainage	Improved	Alive
2	296	ME/SE	+	+	–	Yes/Ext	BO	36	PSL + FK	Observation	Improved	Alive
3	257	ME/SE	–	–	–	Yes/Ext	IP	20	—	Observation	Improved	Died (IP)
4	636	PT (both sides)	–	+	–	No	—		—	Drainage	Improved	Alive
5	600	PT (one side)	–	–	+	Yes/Ext	(BOOP; resolved)[a]		PSL + FK	Drainage	Improved	Alive
6	1766	ME/SE	–	–	–	Yes/Ext	BOOP	170	PSL + CYA	Increase IS	Improved	Alive
7	806	PT (both sides)	+	+	–	Yes/Ext	BOOP	112	PSL + FK	Drainage, increase IS, pleurodesis	Progressed	Died (ALS)
8	155	PT (one side)	+	+	+	Yes/Ext	BO	3	PSL + CYA	Drainage, increase IS	Improved	Died (primary disease)
9	145	Mixed	–	–	–	Yes/Ext	IP	13	PSL + FK	Observation	Improved	Died (primary disease)
10	185	PT (both sides)	–	–	+	Yes/Ext	—		PSL + FK	Drainage	Unchanged	Died (BO)
11	163	Mixed	–	–	+	Yes/Limit	IP	18	PSL + FK	Drainage	Improved	Died (IP)
12	584	Mixed	+	+	+	Yes/Ext	BO	170	PSL + FK	Drainage, increase IS, pleurodesis	Progressed	Died (ALS)
13	1378	Mixed	+	+	+	Yes/Ext	BOOP/IP	1177	PSL + FK	Drainages, increase IS, pleurodesis	Progressed	Died (ALS)
14	105	ME/SE	–	–	–	Yes/Ext	—		PSL + CYA	Observation	Improved	Alive
15	565	Mixed	–	+	–	Yes/Ext	—		PSL + CYA	Drainage	Improved	Died (MOF)
16	841	ME	+	+	–	Yes/Ext	BO	170	—	Start IS	Unchanged	Died (BO)
17	209	ME/SE	–	+	–	Yes/Ext	IP	23	PSL + CYA	Increase PSL	Progressed	Died (ALS)
18	1438	PT (one side)	–	+	+	No	(IP; resolved)[a]		—	Drainage	Improved	Alive

Abbreviations: ALS = air-leak syndrome; BO = bronchiolitis obliterans; BOOP = bronchiolitis obliterans with organizing pneumonia; Ext = extensive type; FK = tacrolimus; IP = interstitial pneumonia; IS = immunosuppressants; Limit = limited type; ME = mediastinal emphysema; mixed = PT with ME/SE; MOF = multiple organ failure; PSL = prednisolone; PT = pneumothorax; SE = s.c. emphysema.
[a]At the diagnosis of ALS, LONIPC had already resolved.

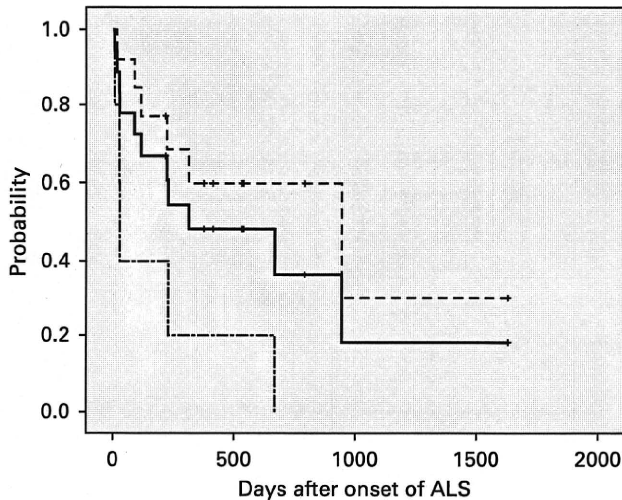

Figure 1 OS after the onset stratified according to the type of ALS. ——— OS of all 18 patients. ---- OS of patients with PT and ME/SE ($n=13$). –·–·– OS of patients with mixed ALS ($n=5$). Patients with PT and ME/SE showed better survival than those with mixed ALS ($P=0.017$).

failed eventually died of LONIPC and/or ALS. In contrast, all five patients with mixed ALS died regardless of their responses to treatment and three of them died of progressive lung disease.

For all 18 patients, OS at 1 year after the onset of ALS was 48.5±12.1% and the 3-year survival rate was 18.2±14.6% (Figure 1). Patients with PT and ME/SE showed better survival than did those with mixed ALS (59.8 vs 20.0% at 1 year and 29.9 vs 0% at 3 years, respectively; $P=0.017$; Figure 1). The patients without active LONIPC at the diagnosis of ALS ($n=6$) had a higher survival rate at 3 years than those with active LONIPC (53.3 vs 0%; $P=0.15$; data not shown).

Risk factors for ALS

Air-leak syndrome was always diagnosed more than 3 months after SCT in this study, so we compared the clinical features of patients with or without ALS who survived for more than 90 days after transplantation (1142 recipients). According to univariate analysis, ALS was significantly more frequent in recipients with cGVHD ($P=0.001$), those who received a second or subsequent SCT ($P=0.043$), younger recipients ($P=0.013$) and male recipients ($P=0.013$; data not shown). We also evaluated the risk factors for ALS by logistic regression analysis in 1047 recipients, after excluding 95 recipients (8.3%) because complete data were not available. The median age of the recipients was 38 years, so we divided them into two groups aged <38 and ⩾38 years. Chronic GVHD was identified as the strongest risk factor for ALS (odds ratio (OR), 13.48; $P=0.013$), whereas second or subsequent SCT (OR, 7.91; $P=0.021$), male sex (OR, 4.95; $P=0.038$), age <38 years (OR, 3.55; $P=0.033$) and FK-based GVHD prophylaxis (OR, 3.3; $P=0.025$) were also identified as independent risk factors (Table 3).

Table 3 Multivariate analysis of factors related to ALS

Variable	*HR*	*95% CI*	P-*value*
Recipient age (⩾38)	3.55	1.11–11.37	0.033
Recipient sex, male	4.95	1.10–22.36	0.038
Donor source, unrelated	0.62	0.30–1.27	0.19
No. of SCT ⩾2	7.91	1.57–39.91	0.021
Reduced-intensity conditioning	0.22	0.02–2.49	0.219
TBI	0.96	0.26–3.54	0.953
GVHD prophylaxis with FK	3.30	1.17–9.36	0.025
Acute GVHD (grade II–IV)	1.51	0.56–4.10	0.193
Chronic GVHD	13.48	1.75–103.89	0.013

Abbreviation: FK, tacrolimus.

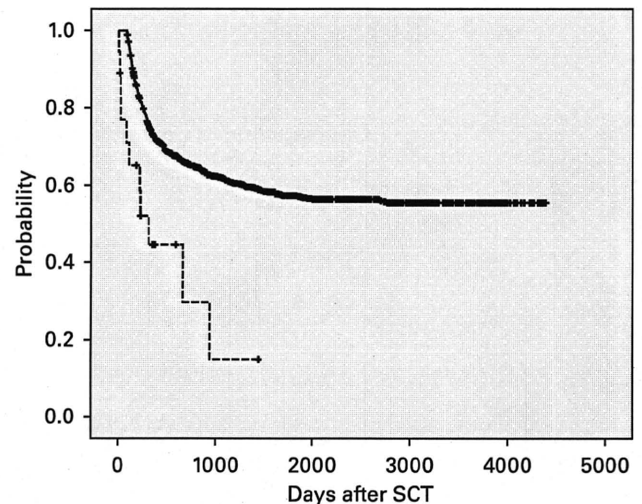

Figure 2 Kaplan–Meier curves for OS after SCT in patients with or without ALS. ——— ALS(−) ($n=1124$). ---- ALS(+) ($n=18$). Recipients with ALS showed significantly worse survival after SCT than those without ALS ($P=0.002$).

Impact of ALS on OS

The patients with ALS had significantly lower survival rates (44.7% at 1 year and 14.9% at 3 years) compared with those without ALS (72.7% at 1 year and 61.5% at 3 years; $P=0.002$; Figure 2). We analyzed factors associated with lower OS among 1079 recipients who lived for more than 90 days after allo-SCT by multivariate Cox regression analysis (63 patients (5.5%) were excluded because of incomplete data). ALS was identified as an independent predictor of worse survival after SCT (OR, 3.468; $P=0.001$), as was a high-risk disease status (OR, 2.851; $P=0.000$) and an age ⩾38 years (OR, 1.267; $P=0.016$; Table 4).

Discussion

All forms of thoracic air leak are defined as ALS according to Franquet *et al.*,[12] including spontaneous pneumomediastinum or pneumopericardium, SE, interstitial emphysema and spontaneous PT. There have been only a few reports about ALS associated with SCT. Recently, Toubai *et al.*[8] performed a single-institution retrospective study and found ALS in 5 out of 213 recipients (2.3%)

Table 4 Multivariate analysis of factors associated with worse OS after SCT

Variable	*HR*	*95% CI*	P-*value*
Recipient age (⩾38)	1.27	1.05–1.54	0.016
Recipient sex, male	0.89	0.73–1.08	0.231
Donor source, unrelated	1.07	0.88–1.30	0.528
Reduced-intensity conditioning	0.75	0.53–1.06	0.105
TBI	0.97	0.75–1.25	0.795
Disease status, high risk	2.85	2.34–3.48	0.000
ALS	3.47	1.63–7.40	0.001

Abbreviation: ALS = air-leak syndrome.

following allo-SCT, whereas Vogel *et al.*[9] reported ALS in 7 out of 300 recipients (2.3%). The incidence of ALS in the present study was slightly lower than in these previous studies. In this retrospective multicenter study, we tried to identify the characteristics, risk factors and prognosis of ALS after SCT, but the following limitations of our study must be considered. Unrecognized biases might have influenced the results of this retrospective study, especially as patients with asymptomatic ALS could not be detected. All cases of ALS were diagnosed more than 100 days after SCT in our series. Other studies and case reports have also shown that this complication occurs more than 100 days after SCT,[6–9] and all authors have agreed that ALS can be classified as a late complication of allo-SCT.

We confirmed that cGVHD, second or subsequent SCT, male sex, age <38 years and FK-based GVHD prophylaxis were independent risk factors for ALS. Several case reports have indicated that ALS following SCT is associated with severe cGVHD or noninfectious pulmonary complications such as BO.[6,9,13,14] In our series, 17 out of 18 recipients with ALS experienced cGVHD, and this is the first report to confirm statistically that the occurrence of ALS is strongly associated with cGVHD. It has also been reported that cGVHD is a significant risk factor for the development of LONIPC.[1–4] In this series, most ALS patients also had LONIPC based on cGVHD. Although the mechanism leading to chronic pulmonary GVHD is unknown, it is thought that host-reactive donor T cells cause injury to the lungs.[15] Continuous inflammation due to cGVHD may lead to fibrotic change of the peripheral airways that decreases lung compliance. Chronic GVHD appears to cause the progression of LONIPC, resulting in the occurrence of ALS. Our recipients who received a second or subsequent SCT would have suffered pulmonary damage by the conditioning regimen, which could have contributed to the development of ALS, although the mechanism of lung injury differs from that of cGVHD. According to a review of the literature,[6,7,13,14,16,17] ALS occurred after SCT in 17 men and 6 women with a median age of 30 years (range: 8–51 years). Our study confirmed by multivariate analysis that younger (<38 years old) men have a high risk of developing ALS after SCT. FK-based GVHD prophylaxis is generally chosen if stem cells are obtained from high-risk donors for GVHD, such as unrelated or HLA-mismatched related donors. This might explain the association between FK-based GVHD prophylaxis and development of ALS in our study.

The survival rate of patients after the occurrence of ALS was significantly impaired ($18.2 \pm 14.6\%$ at 3 years), and the prognosis was related to the type of ALS (mixed ALS vs others). Alveolar rupture can occur because of an elevated intra-alveolar pressure, following damage to the alveolar walls, or for both reasons.[9] The subtypes of ALS reflect the extent of pulmonary/thoracic tissue damage, rather than different pathophysiological processes. This may explain the very poor outcome of mixed ALS. It is well known that the prognosis of secondary PT in patients with COPD is worse compared with idiopathic PT because it usually takes longer to reexpand the lung after a chest tube is inserted and failure of treatment is common.[16] It can be suggested that the onset of ALS in recipients with LONIPC leads to more severe lung tissue damage and a poor prognosis.

In conclusion, we were able to identify a subgroup of SCT recipients with a high risk of developing ALS, namely younger (<38 years) men with cGVHD, second or subsequent SCT and/or FK-based GVHD prophylaxis. As ALS is rare and its etiology is multifactorial, trials of new treatments are not feasible. A more promising strategy may be to improve our understanding and treatment of LONIPC, which should then lead to prevention of ALS.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

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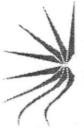

Alloantigen expression on non-hematopoietic cells reduces graft-versus-leukemia effects in mice

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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.

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

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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

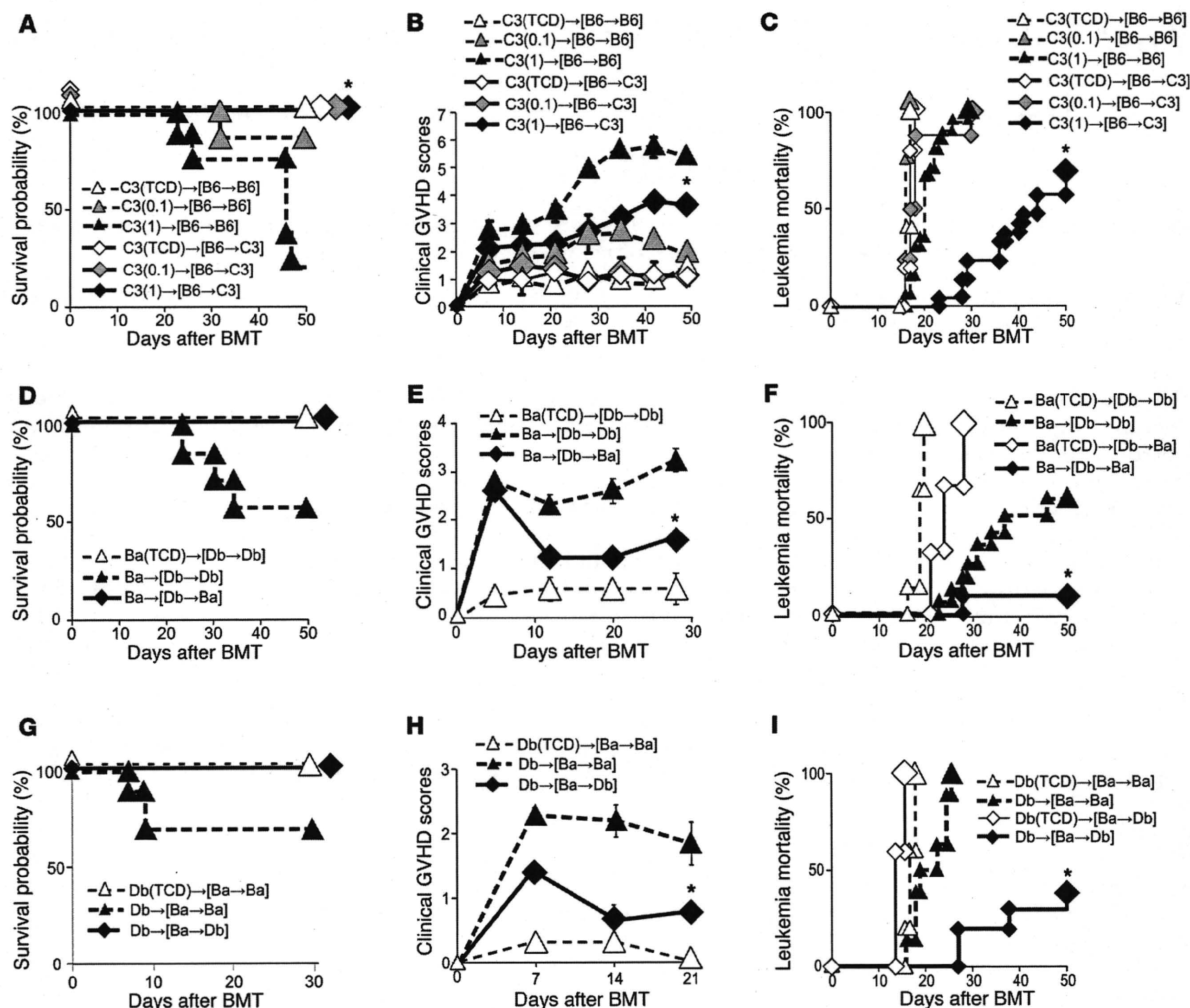

Figure 1

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

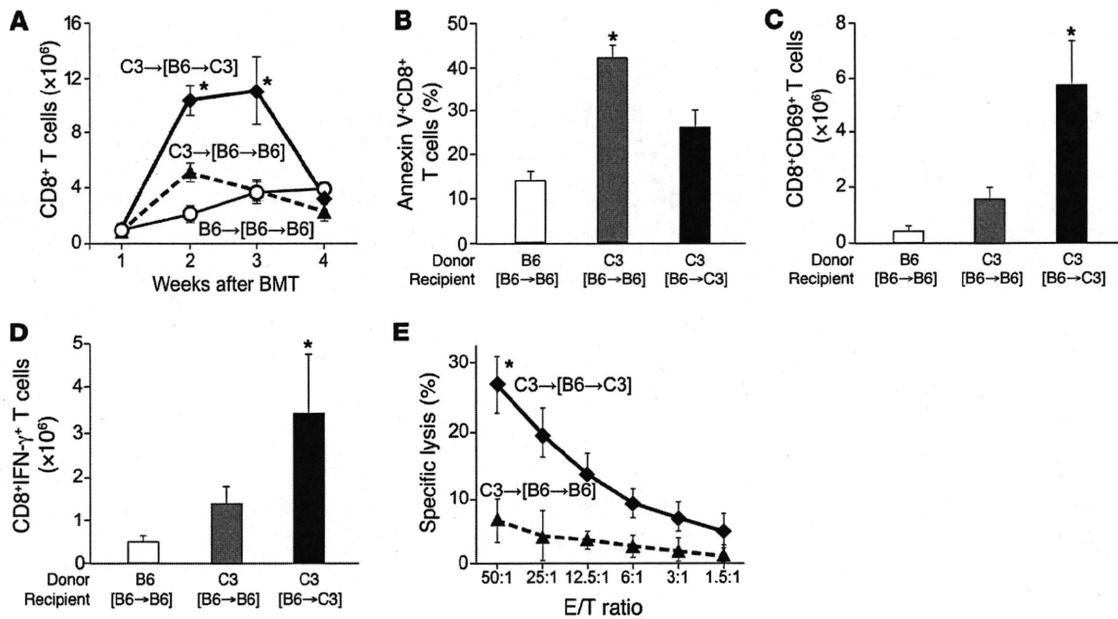

Figure 2

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).

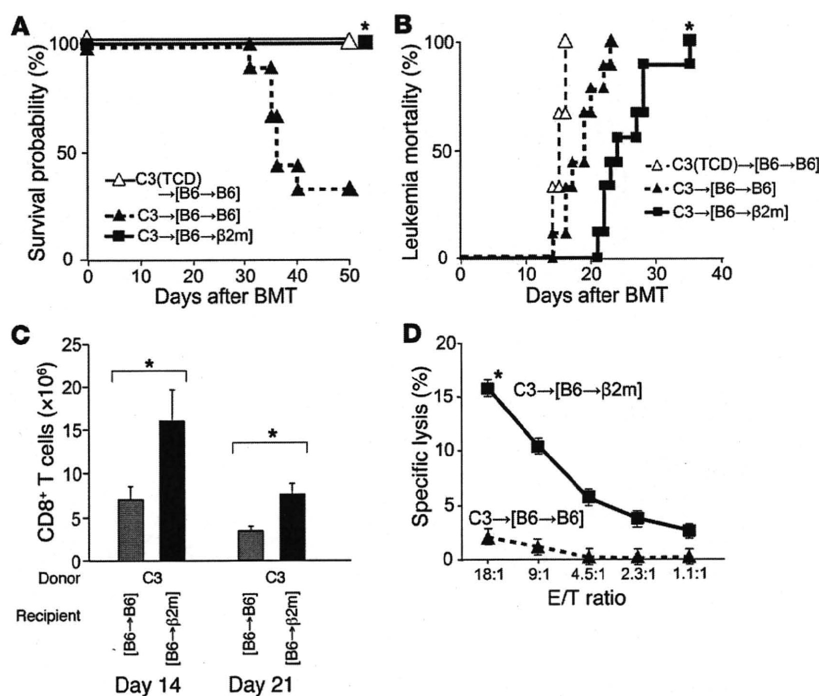

Figure 3

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-

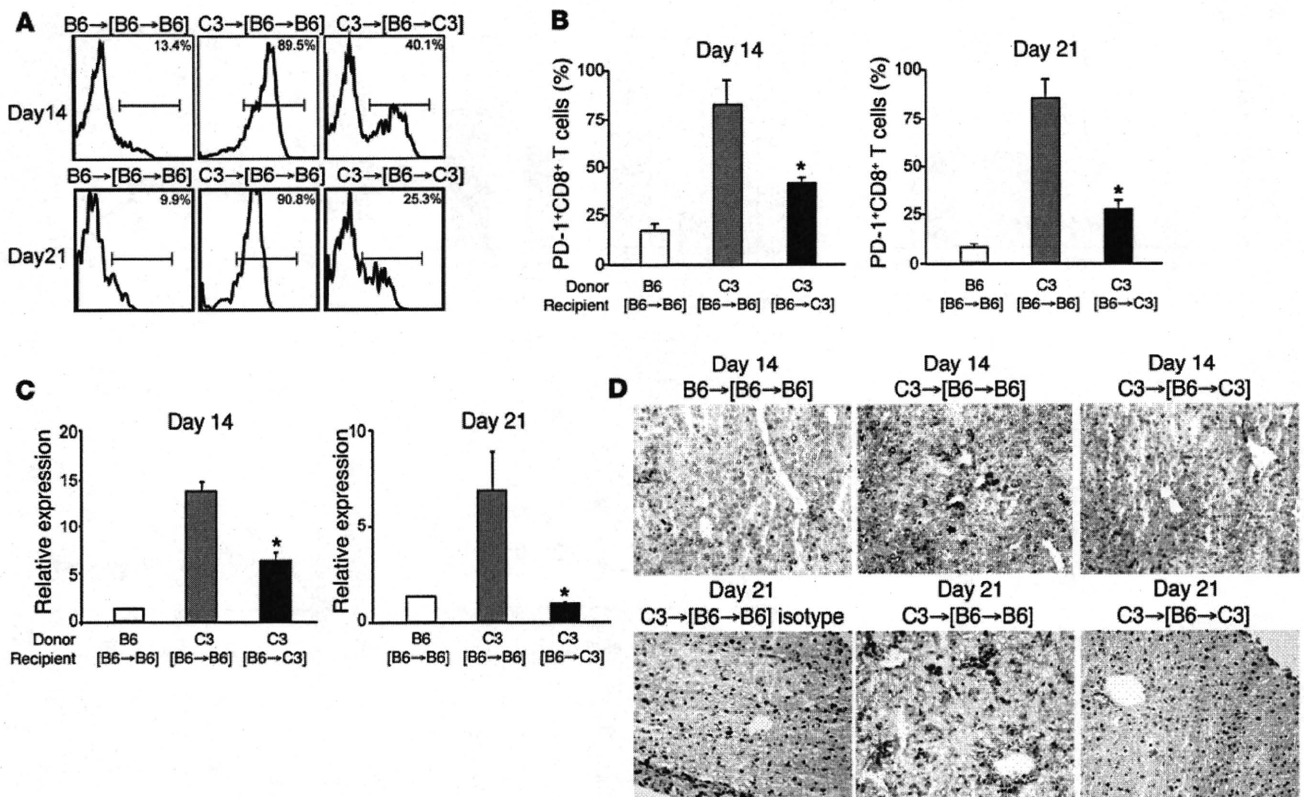

Figure 4

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-

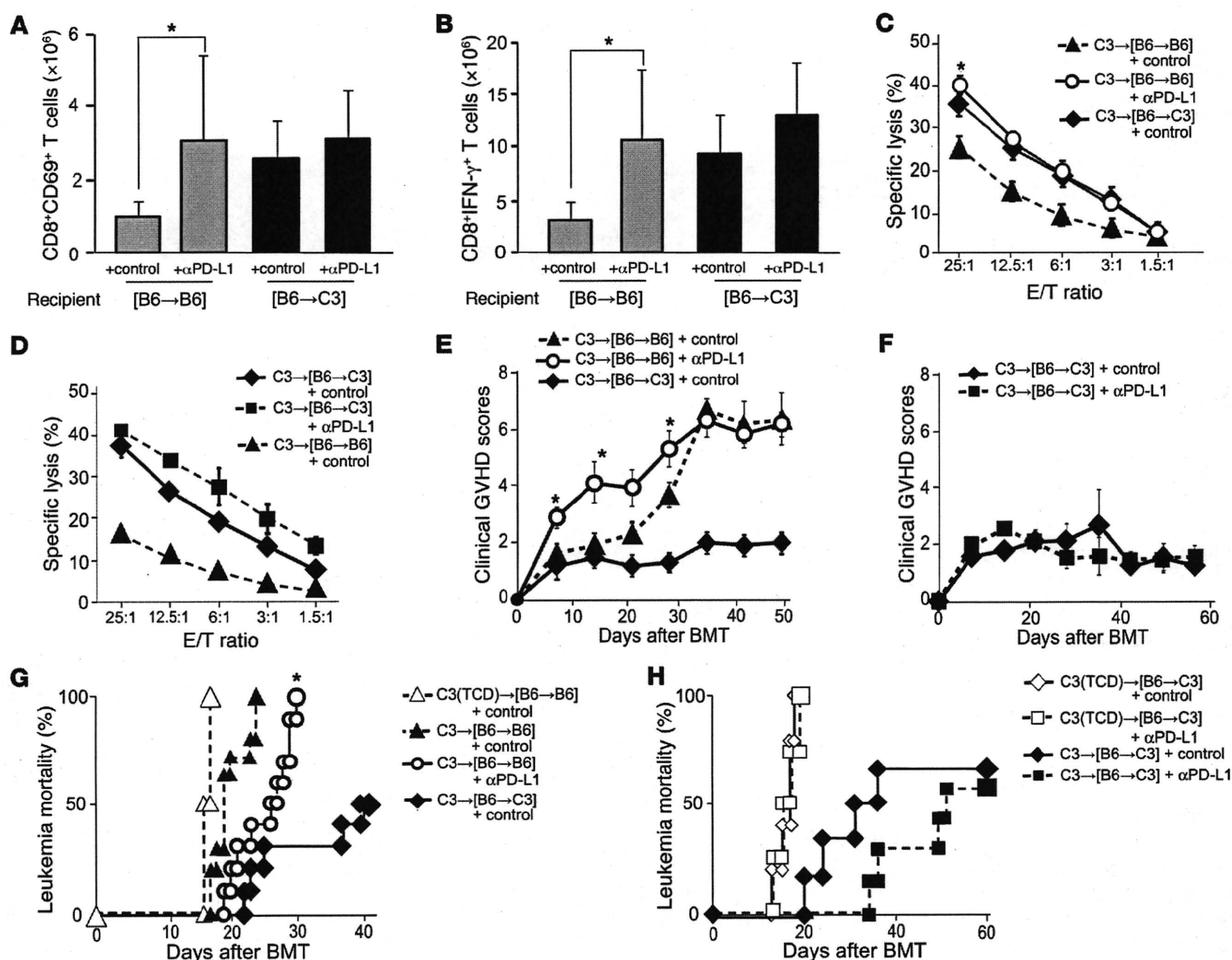

Figure 5

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

and/or spleen, or hind leg paralysis. GVHD death was defined as the absence of leukemia and by the presence of clinical signs of GVHD, assessed by using a clinical scoring system. Animals surviving beyond the observation period of BMT were sacrificed, and the spleen and liver were harvested for histological evaluation to determine leukemia-free survival.

Flow cytometric analysis. The mAbs used were FITC-, PE-, PerCP-, Cy5.5-, or APC-conjugated anti-mouse CD5.1, CD8, CD45.1, CD45.2, CD69, and PD-1 (BD Biosciences). Cells positive for 7-amino-actinomycin D (BD Biosciences) were excluded from the analysis. For the analysis of donor T cell apoptosis, the cells were stained with Annexin V (MBL). For intracellular IFN-γ staining, the splenocytes were incubated for 4 hours with leukocyte activation cocktail and BD GolgiPlug (BD Biosciences) at 37°C. Then, the cells underwent permeabilization with a BD Cytofix/Cytoperm solution (BD Biosciences) and were stained with FITC-conjugated anti-IFN-γ mAbs (BD Biosciences). For intracellular CTLA-4 staining, cells were stained with PE-conjugated anti-CTLA-4 mAbs (eBioscience). At least 5,000 live events were acquired for the analysis using a FACSCalibur flow cytometer (BD Biosciences).

CTL assay. Splenocytes were removed from chimeric recipients 14 days after BMT, and the mononuclear cells were then separated by density gradient centrifugation. The percentage of $CD8^+$ cells in this fraction was determined by flow cytometry, and counts were normalized for $CD8^+$ cell numbers. Tumor targets, 2×10^6 P815 or EL4, were labeled with 100 μCi of ^{51}Cr sodium salt (Amersham Biosciences) for 2 hours. After washing 3 times, the labeled targets were resuspended in 10% FCS in RPMI and plated at 10^4 cells per well in U-bottom plates (Corning-Costar Corp.). Allogeneic splenocyte preparations, as described above, were added to quadruplicate wells at varying effector-to-target ratios and incubated for 4 hours. Maximal and background release were determined by adding 1% SDS and media alone to the targets, respectively. ^{51}Cr activity in the supernatants collected 4 hours later was determined using a Wallac 1470 WIZARD Gamma Counter (Wallac Oy), and lysis was expressed as a percentage of maximum: percentage of specific lysis = 100 (sample count – background count/maximum count – background count).

Quantitative real-time PCR. Total RNA was isolated from the frozen liver using ISOGEN (Nippon Gene). cDNA was synthesized from 150 μg RNA using a QuantiTect Reverse Transcription Kit (QIAGEN). *Pdl1* mRNA levels were quantified by real-time PCR using the 7500 Real-Time PCR System (Applied Biosystems). TaqMan Universal PCR MasterMix, primers, and the fluorescent TaqMan probe specific for murine PD-L1 (Mm00452054-m1) and a house keeping gene, mGAPDH (Mm99999915-g1), were purchased from Applied Biosystems. The standard was obtained using RNA extracted from syngeneic controls.

Immunohistochemistry. For immunohistochemical analysis, isolated livers were frozen in Tissue-Tek (Sakura Finetek), and 5-μm cryostat sections were prepared. Slides were fixed in 100% acetone and air dried. Endogenous peroxidase activity was blocked with peroxidase blocking reagent (Dako). The sections were incubated with purified rat anti-mouse PD-L1 mAb (clone MIH5; eBiosciences). The primary Abs were detected using the Histofine Simple Stain Mouse MAX PO (Rat) kit and DAB solution (Nichirei). The images were captured using an Olympus BH2 microscope with a Nikon DS-5M color digital camera (Nikon), controlled by Nikon ATC-2U software version 1.5. An Olympus ×10/20 ocular lens and a ×20/0.46 NA objective lens were used. Images were cropped using Adobe Photoshop (Adobe Systems) and were composed using Adobe Illustrator.

Statistics. We used the Kaplan-Meier product-limit method to obtain survival probability and the log-rank test to compare survival curves. The Mann-Whitney *U* test was used to analyze the clinical scores. A *P* value less than 0.05 was considered statistically significant.

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