version of the Ag molecule after CAR-T cell therapy (2). Although CAR-T cells recognize very low levels of the target Ag, they cannot recognize completely Ag-negative cells. The strategy of administering CAR-T therapy as a first-line treatment, or in earlier phases with the aim of earlier eradication of target cells, may prevent immunological escape by negative conversion of the Ag.

One limitation of the current study is that we assessed the threshold using only CD20 and CD20CAR systems. Because the threshold may be influenced by many other factors, such as affinity (42), structure (43), epitope localization of individual CAR-Ag pairs (44, 45), and the expression of a coreceptor on target cells (46), the threshold may vary among mAbs and target Ags. We also could not investigate the relationship between the expression of CD20 and the ADCC activity of mAbs because NK cell activity is predominant in the CD20-CEM system, and a clear threshold has not been observed (28). Although the potential relationship between target Ag density and ADCC activity has been investigated in other experimental systems, $>10^4$ Ag molecules per cell are needed to demonstrate significant ADCC (47). In the current study, the minimum threshold of CAR recognition was 3-log units lower than that of mAbs to trigger CDC. CAR can also directly mobilize T cells to target cells, whereas mAb therapy mainly depends on indirect cytotoxicity such as CDC or ADCC (3–5, 19, 28). Thus, the lytic and activating thresholds of CAR are considered significantly lower than those of mAbs.

We concluded that CAR-T cells can recognize and lyse cells expressing considerably low levels of the target Ag and were activated and expanded upon such stimulation. CD20CAR–T cell therapy may also be applicable for the treatment of CD20-positive lymphoid malignancies.

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ORIGINAL ARTICLE

Soluble interleukin-2 receptor level on day 7 as a predictor of graft-versus-host disease after HLA-haploidentical stem cell transplantation using reduced-intensity conditioning

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Abstract In the present study, we analyzed the kinetics of serum soluble interleukin-2 receptor (sIL-2R) using data from 77 patients undergoing HLA-haploidentical transplantation using reduced-intensity conditioning (RIC), who were at an advanced stage or at high risk for relapse, to clarify the usefulness of sIL-2R as a biomarker of acute graft-versus-host disease (GVHD). Anti-T-lymphocyte globulin and methylprednisolone were used as GVHD prophylaxis. While the median sIL-2R in 38 patients not developing GVHD was suppressed at levels <740 U/ml, sIL-2R in 25 patients developing severe GVHD peaked on day 11 (1,663 U/ml), and thereafter decreased to <1,000 U/ml after day 30. The occurrence of GVHD was not limited to times of high sIL-2R level, but occurred at any time point on the sIL-2R curve. Most patients developing GVHD, however, experienced a higher sIL-2R level early in their transplant course. The combination of RIC and glucocorticoids sufficiently suppressed sIL-2R levels after HLA-haploidentical transplantation. In a multivariate analysis to identify factors associated with GVHD, day 7 sIL-2R >810 U/ml was the only factor significantly associated with the occurrence of severe GVHD ($p = 0.0101$).

Keywords Allogeneic stem cell transplantation · Graft-versus-host disease · Soluble interleukin-2 receptor · Alloreactive response · HLA-haploidentical transplantation

Introduction

Bone marrow transplantation (BMT) from siblings genotypically matched for human leukocyte antigen (HLA) improves long-term survival in patients with hematologic malignancies [1]. However, more than 70 % of patients who could benefit from allogeneic BMT do not have a matched sibling donor. On the other hand, there is a >90 % chance of promptly identifying an HLA-haploidentical donor within the family; therefore, the number of patients receiving HLA-haploidentical stem cell transplantation (SCT) is gradually increasing [2–6]. The major drawback of HLA-haploidentical SCT is graft-versus-host disease (GVHD). To overcome GVHD after HLA-haploidentical SCT, several breakthroughs in transplant methodology, including drastic ex vivo T cell purging coupled with the use of megadose of stem cells [2], and in vivo T cell purging through the use of anti-T-lymphocyte globulin (ATG) [4, 5, 7], or the use of cyclophosphamide at post-transplant, have been done [6]. We and others have been studying HLA-haploidentical SCT using in vivo T cell purging method using ATG [4, 5, 7]. In this transplant setting, although the severity of GVHD is within a permissible range, GVHD still continues to be the problem, but an appropriate monitoring method of GVHD has not been established yet.

Basically, GVHD is induced by the immunological response of donor T cells. In general, once activated, T cells express the interleukin-2 receptor (IL-2R), consisting of at least three subunits (α, β and γc) on their membrane

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[8, 9]. The soluble form of IL-2R is produced by proteolytic cleavage of IL-2Rα, and the release of soluble interleukin-2 receptor (sIL-2R) into the circulation has been found to be proportional to its membrane bound expression [10, 11]. Thus, serum sIL-2R levels reflect the magnitude of the T cell immunological response and are associated with the incidence and severity of GVHD in allogeneic BMT settings. In fact, sIL-2R is reported to be the most reliable biomarker among several useful biomarkers [12].

The role of sIL-2R as a GVHD biomarker has been studied mainly in the transplant settings of HLA-matched myeloablative SCT for patients mostly in complete remission (CR) [12–17]. Reduced-intensity conditioning (RIC), which has been used also in HLA-haploidentical transplant settings, may contribute to the reduction of the incidence and severity of GVHD [18–20]. We and others reported that HLA-haploidentical reduced-intensity conditioning stem cell transplantation (RIST) was useful for patients who did not have a suitable HLA-matched donor [5, 7]; however, there are no reports analyzing whether sIL-2R is still a useful biomarker of GVHD in this transplant setting.

Despite the usefulness of sIL-2R as a GVHD biomarker, transplant-related complications, including severe infection, graft rejection, and hepatic veno-occlusive disease, are known to increase sIL-2R levels [13, 15, 21]. Furthermore, leukemia- or lymphoma-associated elevation of serum sIL-2R levels has been reported [22–25]. The coexistence of these conditions could reduce the value of sIL-2R as a biomarker of GVHD.

Therefore, in the present study, after excluding data of patients with conditions that increase sIL-2R levels other than GVHD, we retrospectively studied the usefulness of sIL-2R as a GVHD biomarker using data from 77 patients, with poor prognosis or in an advanced stage of disease, who underwent HLA-haploidentical RIST.

Patients and methods

Patients

To retrospectively evaluate the role of the sIL-2R level as a biomarker of acute GVHD, we analyzed data from patients who underwent HLA-haploidentical RIST at the Hospital of Hyogo College of Medicine between January 2009 and June 2012. All patients had hematologic malignancies and were at an advanced stage or had a poor prognosis at the time of transplantation.

The inclusion criteria were as follows: donor-type engraftment, survival for at least 30 days after transplantation, the absence of hepatic veno-occlusive disease, and severe infections (CRP >10), including sepsis [13, 15, 21]. Furthermore, to avoid the effect of tumor-associated sIL-2R [22–25], data from patients who showed a tumor-associated increase in sIL-2R >2,000 U/ml before conditioning, which did not decrease to <1,000 U/ml on day 0, were excluded. Consequently, data from 20 % of the total transplant patients were excluded based on the exclusion criteria described above, and we analyzed data from 77 patients who underwent transplantation using a graft from an HLA-haploidentical donor (2–3 antigen-mismatches in GVH direction). The patients' characteristics are shown in Table 1.

Table 1 Patients' characteristics

	GVHD Grade 0	Grade I	Grade II–III
Number of patients	38	14	25
Sex			
Male/female	24/14	5/9	10/15
Age (years)			
Median (range)	42.5 (17–63)	46.5 (20–61)	55 (14–65)
Disease			
AML/MDS	17	10	12
ALL	11	0	3
Lymphoma	6	2	6
Others	4	2	4
Disease status			
Good (CR/RA/CP)	2	0	2
Intermediate (PR/RAEB/AP)	4	1	1
Poor	32	13	22
HLA disparity in GVH direction			
2 antigen	18	9	13
3 antigen	20	5	12
Number of times of transplant			
First	17	7	20
Second or later	21	7	5
Conditioning combination chemotherapy			
Busulfan-containing	8	3	9
Melphalan-containing	26	8	15
Others	4	3	1
TBI			
Containing	25	8	15
Non-containing	13	6	10

Institutional review board approval was obtained for the treatment protocol, and written informed consent was obtained from the patients and their families.

Preparative regimen for transplantation

Sixty-nine patients received a regimen consisting of fludarabine (30 mg/m^2/day on days −9 to −4), cytarabine (2 g/m^2 on days −9 to −6), ATG (thymoglobulin: total

2.5 mg/kg divided on days -3 to -1), and busulfan (4.0 mg/kg/day on days −6 and −5) or melphalan (70 mg/m^2 on days −3 and −2) with or without TBI 3–4 Gy on day 0. The remaining 8 patients received other agents instead of busulfan or melphalan because of chemoresistance. GVHD prophylaxis consisted of tacrolimus and methylprednisolone (mPSL) 1 mg/kg [5]. All patients except 2 received peripheral blood stem cells. T cell depletion was not performed.

Diagnosis of graft-versus-host disease and supportive care

Acute GVHD was graded according to standard criteria [26]. GVHD was treated as previously described [5]. Each patient was isolated in a laminar air-flow room or protective environment room, and standard decontamination procedures were followed. Oral antibiotics (ciprofloxacin, vancomycin, itraconazole or voriconazole) were administered to sterilize the bowel. Patients who were negative for cytomegalovirus (CMV) IgG received blood products from CMV-seronegative donors. Intravenous immunoglobulin was administered at a minimum dose of 100 mg/kg every 2 weeks until day 100. Cotrimoxazole was given for at least 1 year for prophylaxis of *Pneumocystis carinii* infections. Acyclovir was administered at a dose of 1,000 mg/day for 5 weeks after transplantation to prevent herpes simplex virus or varicella-zoster virus infection, and then the agent was continued for at least 2 years at a dose of 200 mg/day. Ganciclovir 7.5 mg/kg divided into 3 doses per day was administered from day −10 to day −3 as prophylaxis for CMV infection.

Measurement of serum sIL-2R

The serum sIL-2R level was monitored from the start of conditioning three times a week until hospital discharge. The serum sIL-2R concentration was evaluated using a commercially available sandwich enzyme-linked immunosorbent assay (ELISA) with two murine anti-human sIL-2R antibodies (CELLFREE Human sIL-2R ELISA Kit; Thermo Fisher Scientific Inc., Rockford, IL, USA). The normal sIL-2R level is <534 U/ml.

Statistics

The background levels of serum sIL-2R were decided using data from 38 patients who did not develop GVHD. The difference in sIL-2R levels on day 7 between patients who developed grade 0–I and grade II–III GVHD was analyzed using the Mann–Whitney *U* test. In addition, we determined the appropriate cutoff value of the sIL-2R level on day 7 to discriminate patients with and without severe GVHD through receiver operating characteristic (ROC) analysis, in which sensitivity and specificity were calculated as a function of the cutoff value, (1-specificity) was plotted against the sensitivity, and the area under the ROC curve (AUC) was calculated. Cumulative incidence of GVHD for patients with sIL-2R on day 7 of >810 or <810 U/ml was estimated using the Kaplan–Meier method, treating death and relapse as competing risks. Gray's test was used for comparison of cumulative incidence in the 2 groups. To identify factors associated with GVHD, using variables including the donor source, age, disease status before transplantation, sex, number of times of transplantation, HLA disparity in GVH direction, disease, and day 7 sIL-2R level, univariate and multivariate analyses were performed using the Cox proportional hazards model. Results were considered significant when $p \leq 0.05$. Statistical analyses were performed with EZR [27, 28].

Results

Background level of sIL-2R based on the data from patients who did not develop GVHD

Serum sIL-2R levels were monitored 3 times a week to analyze the relationship between sIL-2R levels and the development of GVHD in detail. We first identified the serum background level of sIL-2R based on data from 38 patients who did not develop acute GVHD. As shown in Fig. 1, sIL-2R was slightly high, but mostly <1,200 U/ml during 2 weeks after transplantation, and thereafter slightly decreased to <1,000 U/ml. The median value of sIL-2R

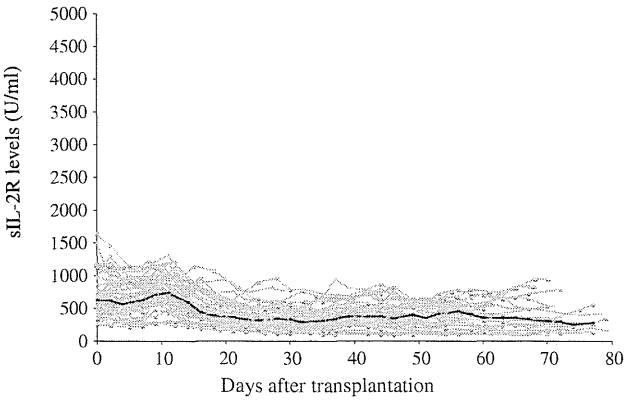

Fig. 1 The kinetics of sIL-2R in patients not developing acute GVHD. To identify the background levels of sIL-2R, changes of serum sIL-2R of 38 patients who did not develop GVHD were plotted. The normal sIL-2R level is <534 U/ml. sIL-2R was slightly high, but mostly <1,200 U/ml during 2 weeks after transplantation, and thereafter slightly decreased to <1,000 U/ml. The *bold line* shows a median sIL-2R level

was slightly increased after transplantation, peaked on day 11 (the peak level was 740 U/ml), and thereafter decreased to levels as low as between 290 and 450 U/ml.

The kinetics of sIL-2R in patients who developed severe GVHD

Next, we analyzed the kinetics of sIL-2R in 25 patients who developed severe (grade II–III) GVHD. Four patients developed skin-only GVHD, 16 gut-only GVHD, and 5 skin/gut GVHD. Those patients developed grade II–III GVHD at a median 28 days (range 3–67 days). The sIL-2R curves were found to vary patient-to-patient in the peak level or in the timing of the peak. The median sIL-2R levels increased after transplantation, reach on day 11 (the peak value of 1,663 U/ml), and thereafter decreased to low levels of <1,000 U/ml after day 30 (Fig. 2).

Regarding the relationship between the kinetics of sIL-2R and the onset of GVHD, 4 patterns were observed. Eight (32 %) patients, in whom sIL-2R increased rapidly after transplantation, developed GVHD at an increasing phase or at the peak level of sIL-2R curve by day 30 (Fig. 3a). These patients developed GVHD at a median of 9 days (range 5–26 days), with the median value of sIL-2R of 1,795 U/ml (range 1,134–4,341 U/ml) at the onset of GVHD. Four (16 %) patients developed GVHD at a decreasing phase of sIL-2R over the peak of sIL-2R (Fig. 3b). These patients developed GVHD at a median of 18.5 days (range 14–21 days), with the median value of sIL-2R of 1,465.5 U/ml (range 618–2,004 U/ml) at the onset of GVHD. Ten (40 %) patients, in whom sIL-2R increased to a high level after transplantation, with the median peak level of 1,711 U/ml (range 1,200–2,977 U/ml) at a median 5.5 days (range 0–16 days), developed GVHD after sIL-2R levels decreased to almost the normal range of sIL-2R (Fig. 3c). These patients developed GVHD at a median of 38 days (range 28–67 days), with the median value of sIL-2R of 642.5 U/ml (range 336–950 U/ml) at the onset of GVHD. Three (12 %) patients, in whom sIL-2R did not increase over the background levels of sIL-2R after transplantation, developed GVHD after day 30, when sIL-2R was slightly increasing over the background level of sIL-2R (Fig. 3d). GVHD in this group of patients occurred latest at a median of 44 days (range 37–59 days), with the median value of sIL-2R of 984 U/ml (range 935–1,054 U/ml) at the onset of GVHD. These results show that GVHD can occur on any point of the sIL-2R curve of patients with GVHD.

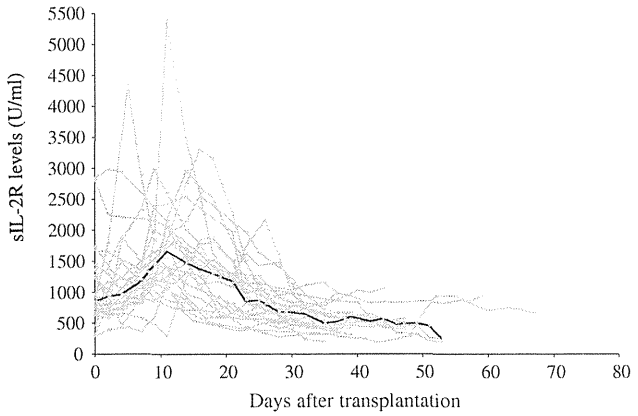

Fig. 2 The kinetics of sIL-2R in 25 patients who developed acute GVHD (grade II–III). Changes of serum sIL-2R in 25 patients who developed severe GVHD were plotted. The sIL-2R curves were found to vary patient-to-patient in the peak level or in the timing of the peak. The *bold line* shows a median sIL-2R level

Prediction of severe acute GVHD by serum sIL-2R levels on day 7

The relationship between sIL-2R change and the onset of GVHD (Fig. 3a–d) shows that the occurrence of GVHD is not limited at the time of high level of sIL-2R or at an increasing phase of sIL-2R; however, the majority of patients who developed severe GVHD showed a high level of sIL-2R early in their transplant course. Therefore, we considered that sIL-2R in the early phase of transplantation may be associated with the development of severe GVHD.

We compared sIL-2R levels on day 7 in patients who developed grade II–III GVHD or grade 0–I GVHD. Consequently, patients with grade II–III GVHD showed significantly higher sIL-2R on day 7 than those with grade 0–I GVHD ($p < 0.0001$). To determine the appropriate cutoff value of sIL-2R on day 7 to discriminate patients with and without severe GVHD, ROC analysis (Fig. 4a) was performed, and the optimal grade II–III GVHD cutoff point was found to be 810 U/ml. The area under the ROC curve (AUC) was 0.790 (CI 0.675–0.904). The relationship between the incidence of severe GVHD and day 7 sIL-2R levels was analyzed using competing risk analysis, treating death and relapse as competing risks. As shown in Fig. 4b, the cumulative incidence of severe GVHD was 43.2 % (CI 28.2–57.3 %) and 6.5 % (CI 1.1–18.9 %) for patients with day 7 sIL-2R >810 U/ml and those with day 7 sIL-2R <810 U/ml, respectively. Patients with day 7 sIL-2R >810 U/ml had a significantly higher risk of GVHD than those with day 7 sIL-2R <810 U/ml ($p = 0.00076$, log-rank test).

Next, using variables including the donor source (offspring vs others), HLA disparity (2 antigen vs 3 antigen) in the GVH direction, older age (>47 years), disease status before transplantation (CR vs non-CR), sex, number of times of transplantation, disease (ALL vs others), and day 7 sIL-2R, factors that were significantly associated with the development of severe GVHD were analyzed using the

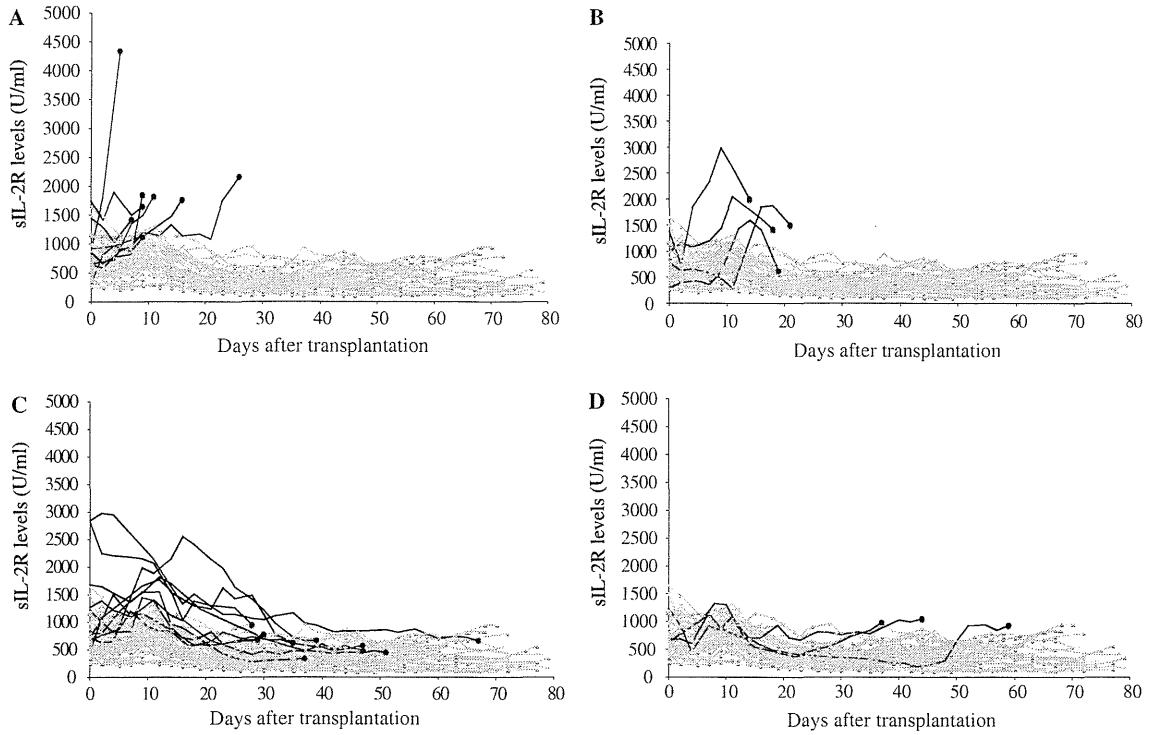

Fig. 3 The relationship between the kinetics of sIL-2R and onset of GVHD in patients who developed severe acute GVHD. *Bold lines* show changes of sIL-2R in patients who developed severe GVHD. *Closed circles*, which are at the end of the *bold lines*, show the onset of GVHD. *Gray lines* show changes of sIL-2R in patients not developing GVHD. **a** GVHD occurred at an increasing phase of sIL-2R or at the peak level by day 30. **b** GVHD occurred at a decreasing phase of sIL-2R (still at a high level) over the peak of sIL-2R. **c** GVHD occurred after returning to the background level of sIL-2R, which passed through the high levels after transplantation. **d** GVHD occurred at a time point slightly increased from the background level of sIL-2R after day 30

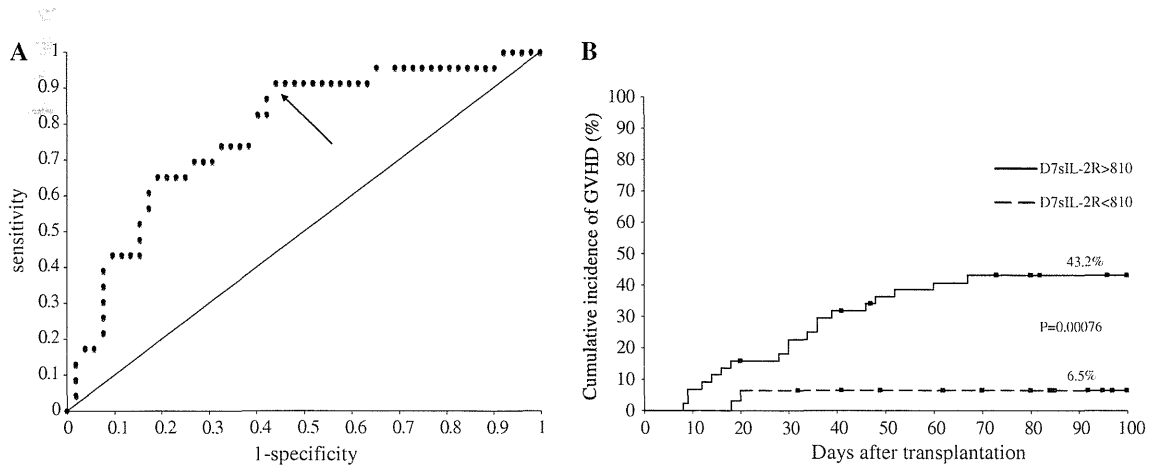

Fig. 4 **a** ROC curve of sIL-2R level on day 7 for the prediction of severe GVHD. To determine the appropriate cutoff value of sIL-2R levels on day 7 to discriminate patients with and without severe GVHD, ROC analysis was performed, and the optimal grade II–III GVHD cutoff point was found to be 810 U/ml, shown by the *arrowhead*. The area under the ROC curve (AUC) was 0.790. **b** Cumulative incidence of severe GVHD for patients with sIL-2R on day 7 of >810 and <810 U/ml. Cumulative incidence of acute GVHD for patients with sIL-2R on day 7 of >810 U/ml and <810 U/ml was estimated using the Kaplan–Meier method, treating death and relapse as competing risks. Gray's test was used for comparison of cumulative incidence in the 2 groups. Patients with sIL-2R on day 7 of >810 U/ml had a significantly higher risk of severe acute GVHD than those with day 7 sIL-2R of <810 U/ml ($p = 0.00076$, log-rank test)

Cox proportional hazards model (Table 2). In a univariate analysis, day 7 sIL-2R >810 U/ml, offspring, age >47 years, and first transplantation were significantly associated with the occurrence of severe GVHD. In a multivariate analysis, day 7 sIL-2R >810 U/ml was only a factor significantly associated with the occurrence of

Table 2 Analysis of factors related to the development of severe GVHD

	Univariate analysis		Multivariate analysis	
	p	CI	*p*	CI
sIL-2R on day 7 (>802 vs <802)	0.0024	2.214–40.431	0.0101	1.597–31.999
Donor source (offspring vs others)	0.0125	1.287–8.102	0.6601	0.238–9.617
Age (>47 vs <47 years)	0.0089	1.389–9.905	0.8337	0.144–11.068
Disease status before transplantation (CR vs non-CR)	0.7203	0.307–5.527	0.1965	0.576–14.591
Sex (female vs male)	0.1877	0.769–3.820	0.526	0.525–3.523
Number of times of transplant (retransplantation vs first transplantation)	0.0155	0.112–0.795	0.2003	0.100–1.621
HLA disparity in GVH direction (2 antigen vs 3 antigen)	0.8831	0.430–2.067	0.5993	0.322–1.922
Disease (ALL vs others)	0.2713	0.152–1.698	0.490	0.181–2.265

CI confidence interval, *CR* complete remission, *non-CR* non-complete remission

severe GVHD ($p = 0.0101$, CI 1.597–31.999). Offspring, age >47 years, and first transplantation had no significant impact on the occurrence of severe GVHD.

Discussion

In the present study, using data from 77 patients who underwent HLA-haploidentical RIST, we investigated the thorough kinetics of serum sIL-2R after transplantation to elucidate the usefulness of sIL-2R as a GVHD biomarker, and found that sIL-2R on day 7 was useful as a predictor of severe GVHD.

In the present study, data from other pathological situations that increase serum sIL-2R were excluded from the analysis. Serum sIL-2R levels reflect the magnitude of the activation and proliferation of T cells, but are not specific to the GVH reaction. This is an inevitable drawback in the diagnosis of GVHD using sIL-2R, as a non-specific T cell reaction of donor or recipient origin produces sIL-2R in some particular transplant complications, such as infection. To avoid the effect of these complications on sIL-2R analysis, other researchers also excluded data from patients with these complications, who represent 15 % of allogeneic recipients [12]. In the present study, a tumor-associated increase in sIL-2R was observed in a slightly high incidence because the majority of patients treated in our institute were in non-CR at the time of transplantation; therefore, data from a slightly higher proportion (20 %) of patients were excluded.

In the absence of GVHD, the median serum sIL-2R was slightly increased after transplantation, peaked on day 11 (the peak level was 740 U/ml), and thereafter decreased to as low as between 290 and 450 U/ml (Fig. 1). On the other hand, in the presence of GVHD, the median serum sIL-2R increased after transplantation, peaked in a median of 11 days (the peak level was 1,663 U/ml), and thereafter decreased to low levels of <600 U/ml (Fig. 2). Compared with the previous studies [14, 16], in which sIL-2R levels peaked 2–3 weeks after transplantation with the peak level of 3,000–5,000 U/ml, sIL-2R in the present study reached the peak level slightly earlier, but the peak levels were lower. The use of ATG-containing RIC regimen and the incorporation of glucocorticoids into the GVHD prophylaxis are considered to contribute to the decrease in the peak level of sIL-2R, which is probably the main reason for a low incidence of severe GVHD observed in our regimen for HLA-haploidentical RIST [5]. Miyamoto et al. [14], in the study of allogeneic SCT using myeloablative conditioning, reported that sIL-2R in patients with GVHD started to increase on day 3, and that the elevation of sIL-2R on day 3 predicted the occurrence of acute GVHD. In the present study, sIL-2R in patients with GVHD started to increase on day 7, as shown in Fig. 2. This discrepancy may be explained by the use of RIC in this study, which induces mixed chimerism status between donor and recipient in the early transplant period, retarding the start of GVH reaction.

The previous studies of sIL-2R only showed that sIL-2R peaked on weeks 2 and 3, or that the peak level of sIL-2R was associated with the severity of GVHD [14, 16]. From the analysis of the onset of GVHD, GVHD was found to occur in 4 different phases of sIL-2R curve: GVHD occurred (1) at an increasing phase or at the peak level of sIL-2R after transplantation (Fig. 3a), (2) at a decreasing phase of sIL-2R (still at a high level) over the peak of sIL-2R (Fig. 3b), (3) after returning to the background level of sIL-2R, which passed through the high levels after transplantation (Fig. 3c), and (4) at a time point slightly increased from the background level of sIL-2R after day 30 (Fig. 3d). Although the prophylactic use of glucocorticoids is considered to contribute to the reduction in sIL-2R levels, as described above, there was no difference among 4 patterns of patients with GVHD in the administration schedule of steroids until the onset of GVHD. The occurrence of GVHD in 4 different phases of sIL-2R curve of

GVHD is not limited to HLA-haploidentical RIST, but observed also in other types of allogeneic SCT, including related HLA- matched, unrelated bone marrow, and umbilical cord blood SCT (data not shown). These results show that GVHD occurs at any time point on the sIL-2R curve, indicating that sIL-2R is not a suitable marker for real-time monitoring of the development of GVHD. Host organ-associated factors [29–31], other than donor T cell activation, must be also associated with the ultimate development of GVHD.

In fact, while sIL-2R peaked at a median of 11 days in patients developing GVHD, GVHD occurred at a median of 28 days. This time lag between the peak level of sIL-2R and the onset of GVHD may be explained as follows. According to the pathophysiology of GVHD that Ferrara et al. [32] proposed, donor T cell activation precedes a series of the subsequent various immunological reactions leading to the development of GVHD. In addition, GVHD may become clinically evident as the dose of immunosuppressive agents is tapered.

On the other hand, the fact that the majority of patients developing GVHD showed a high level of sIL-2R early in their transplant course indicates that sIL-2R levels in the early transplant phase could be a predictor of severe GVHD. In a univariate analysis, day 7 sIL-2R >810 U/ml, offspring, age >47 years, and first transplantation were significantly associated with the occurrence of severe GVHD; however, in a multivariate analysis, day 7 sIL-2R >810 U/ml was only a factor significantly associated with the occurrence of severe GVHD (Table 2). Thus, for the first time, we showed that sIL-2R in the early transplant phase was useful as a GVHD predictor.

The occurrence of events, such as VOD or sepses, until day 7 may result in non-specific increase in sIL-2R on day 7, which make it unable to predict GVHD using day 7 sIL-2R; however, the predictability of GVHD by sIL-2R on day 7 is not basically affected by such pathological events that occur after day 7. Regarding non-specific increase in sIL-2R on day 7, whether we can detect or diagnose such events (inducing increase in sIL-2R) on day 7 is practically important because the GVHD predictor should not be applied if a given patient has such events and shows sIL-2R >810 U/ml. The diagnosis of VOD or severe infections is relatively easy, whereas accurate quantification of residual tumor burden that may lead to tumor-associated increase in sIL-2R may be sometimes difficult. In general, in case of tumor-associated increase, sIL-2R levels are usually high since before transplantation or during conditioning, and tend to gradually or rapidly decrease after transplantation in this early transplant period, whereas, in GVHD-associated increase, sIL-2R levels are increasing at day 7 in most cases, as shown in Fig. 2. Therefore, when applied to patients undergoing allogeneic SCT in CR status, sIL-2R will be more useful as a GVHD predictor. In addition, even if such a non-specific increase blunts the usefulness of sIL-2R as GVHD predictor, when sIL-2R levels are <810 U/ml on day 7, the incidence of GVHD is only 6.5 %, as shown in Fig. 4b, indicating that patients with such low sIL-2R levels have an extremely low risk of developing severe GVHD even in HLA-haploidentical SCT.

In conclusion, in this HLA-haploidentical SCT using the combination of ATG-containing RIC regimen and the incorporation of glucocorticoid into GVHD prophylaxis, sIL-2R levels were mostly suppressed after transplantation compared with other studies on sIL-2R, which possibly lead to a low incidence of severe GVHD. sIL-2R on day 7 was useful as a predictor of GVHD in this transplant setting.

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Conflict of interest The authors declare no conflict of interest.

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Bone Marrow Graft-versus-Host Disease: Evaluation of Its Clinical Impact on Disrupted Hematopoiesis after Allogeneic Hematopoietic Stem Cell Transplantation

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Idiopathic cytopenias are frequently observed in patients after allogeneic hematopoietic stem cell transplantation (allo-HSCT). We have previously reported the effect of graft-versus-host disease (GVHD) on bone marrow (BM) in murine models, indicating that the osteoblast injury mediated by donor T cells was associated with bone marrow suppression and delayed immune reconstitution. In this study, we prospectively evaluated the relevance of these findings in 51 patients. Patients with chronic GVHD manifested the loss of osteoblasts, contributing to cytopenic symptoms ($P = .0427$ compared with patients without cytopenic symptoms). The loss of osteoblasts was significantly associated with the extensive type of chronic GVHD ($P = .012$), and flow cytometric analyses revealed lower numbers of $CD19^+$ B cells and a significantly increased CD4 to CD8 ratio ($P = .0002$) in these patients. Our data, for the first time to our knowledge, summarize the detailed analyses of the effect of GVHD on BM in the clinical allo-HSCT patients.

INTRODUCTION

Allogeneic stem cell transplantation (allo-HSCT) is currently established as a curative therapy for hematologic malignancies. However, graft-versus-host disease (GVHD) still remains a major complication after allo-HSCT and, therefore, developing better strategies for the prophylaxis and treatment of GVHD is essential to improve outcomes of allo-HSCT. The principal target organs of acute GVHD are the skin, liver, and gastrointestinal tract [1]. However, cytopenias and bone marrow (BM) suppression are often observed in association with GVHD in patients undergoing allo-HSCT, suggesting that BM is a potential target of GVHD. Clinical and experimental data have shown that immunologic reconstitution is impaired by GVHD [2-5], and GVHD-associated myelosuppression and lymphoid hypoplasia have been reported [6-8]. Recently, we demonstrated the destruction of BM hematopoietic niches, especially osteoblasts, by donor T cells in murine models of GVHD, resulting in BM suppression, including B lymphopoiesis. We identified this phenomenon as *BM GVHD* [9]. Here, we report clinical research investigating BM GVHD in patients after allo-HSCT. We analyzed 51 patients undergoing allo-HSCT who were evaluable with BM biopsy samples both before and after allo-HSCT.

METHODS

Study Design and Patients

For our prospective analyses of BM GVHD, we enrolled 57 patients who underwent allo-HSCT from February 2010 to June 2012 in Hokkaido University Hospital. A total of 51 patients were assessed for BM biopsy specimens before and after allo-HSCT (6 patients who did not have BM biopsies at all after allo-HSCT were excluded). The study protocol was approved by the review board of Hokkaido University Graduate School of Medicine on January 29, 2010. Patients provided written informed consent before being enrolled in the protocol. Characteristics of patients, as well as of the transplantation procedures, are summarized in Table 1.

Evaluation of GVHD

Diagnosis and clinical grading of acute and chronic GVHD were performed according to established criteria [10-12].

Bone Marrow Samples

We performed BM biopsies and aspirations for patients before and after allo-HSCT. BM aspirates were analyzed for B and T cell profiles by flow cytometry. Biopsied specimens were stained with hematoxylin and eosin, as well as with CD56 for immunohistochemical assessments of cellularity, morphology, and presence or absence of osteoblasts [13]. We categorized the loss of osteoblasts into 3 groups: (1) not affected, if the osteoblasts were intact or the decrease was moderate, up to 30%; (2) partial loss, if the

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Table 1
Characteristics of Patients

Characteristics	Value
No. of transplantations	51
Age, median (range), yr	41 (19-66)
Patient sex	
Male	27 (53%)
Female	24 (47%)
Disease	
AML	16 (31%)
MDS	5 (10%)
ALL/LBL	12 (24%)
ML	9 (18%)
ATL	3 (6%)
AA	3 (6%)
Others	3 (6%)
Donor sources	
U-BMT	28 (55%)
R-PBSCT	8 (16%)
R-BMT	5 (10%)
CBT	10 (20%)
Preparative regimen	
CST	20 (39%)
RIST	31 (61%)
Immuno suppression	
CyA based	13 (25%)
FK based	38 (75%)

AML indicates acute myelogenous leukemia; MDS, myelodysplastic syndrome; ALL/LBL, acute lymphocytic/lymphoblastic lymphoma; ML, malignant lymphoma; ATL, acute T cell leukemia; AA, aplastic anemia; U-BMT, unrelated bone marrow transplantation; R-PBSCT, related peripheral blood stem cell transplantation; R-BMT, related bone marrow transplantation; CBT, cord blood transplantation; CST, conventional stem cell transplantation; RIST, reduced-intensity stem cell transplantation; CyA, cyclosporin A; FK, tacrolimus.
Data presented are n (%) unless otherwise indicated.

osteoblasts were partially lost, in between 30% to 90% of the bone trabeculae in the pathological sections; and (3) complete loss, if more than 90% of osteoblasts were lost.

Assessment of Cytopenias

We identified the cytopenic condition as *idiopathic cytopenias* after excluding the following conditions. We excluded bacterial, fungal, and viral infections by routine screening tests (serological as well as culture tests). Additionally, thrombo microangiopathy and hemophagocytic syndrome that also cause cytopenias in patients were excluded. Insufficient hematopoiesis after engraftment was also excluded when the patient showed recovery (confirmed retrospectively) in hematopoiesis without any specific treatment for the cytopenias.

Statistical Analysis

Median values and ranges were used for continuous variables and percentages were used for categorical variables (Table 1). Gray's test was used for group comparisons of cumulative incidences of acute and chronic GVHD. Statistical analyses were performed using chi-square test and *t*-test, as appropriate. JMP software version 8.0.2 (SAS Institute, Cary, NC) was used for most of the statistical analyses. Analysis of cumulative incidences was carried out with EZR (Saitama Medical Center, Jichi Medical University, http://www.jichi.ac.jp/saitama-sct/SaitamaHP.files/statmedEN.html), which is a graphical user interface for R (The R Foundation for Statistical Computing, version 2.13.0) [14]. All *P* values were 2 sided and a value of $P = .05$ was used as a cut off for statistical significance.

RESULTS

Patients' Characteristics, Sample Collections, and Acute and Chronic GVHD after Allo-HSCT

The patients' characteristics are shown in Table 1. The median age at allo-HSCT for the 27 males and 24 females was 41 years (range, 19 to 66). Of 51 patients analyzed, 32 developed acute GVHD (Figure 1A) and 29 developed chronic GVHD (Figure 1B). BM biopsies were performed before (median, day −22; range, day −174 to day −8) and after (median, day 63; range, day 18 to day 527) allo-HSCT together with BM aspirations. The average number of the BM biopsies performed after allo-HSCT was 1.7 per patient (range, 1 to 6). We found a significant decrease of BM cellularity in samples collected from patients suffering from cytopenias in the peripheral blood (Supplemental Table 1). The characteristics of GVHD, in terms of the duration from its onset to BM biopsy, as well as the percentage of donor chimerism in the samples with cytopenic symptoms are shown in Supplemental Table 2.

BM GVHD and BM Suppression during Acute GVHD

We analyzed a total of 56 BM samples biopsied from day 0 to day 100 after allo-HSCT (Figure 2). Of these 56 samples, 15 were harvested when the patients had acute GVHD. Eight of these 15 samples were harvested from patients suffering from cytopenias and 2 of them displayed partial loss of osteoblasts, identifying 1 sample as an idiopathic cytopenia. We also identified 3 samples presenting the partial loss of osteoblasts; however, none of these 3 samples were collected when patients showed clinical manifestation of acute GVHD symptoms. The causes of cytopenias for these 3 samples included disease relapse and delayed engraftment. Taken together, during the early period after allo-HSCT, we did not observe a strong correlation between loss of osteoblasts and idiopathic cytopenias.

BM GVHD and BM Suppression during Chronic GVHD

We analyzed a total of 33 samples biopsied beyond day 100 (Figure 3A). Of 14 samples harvested from patients when they exhibited symptoms of chronic GVHD and concurrent idiopathic cytopenias, 4 samples displayed partial loss of

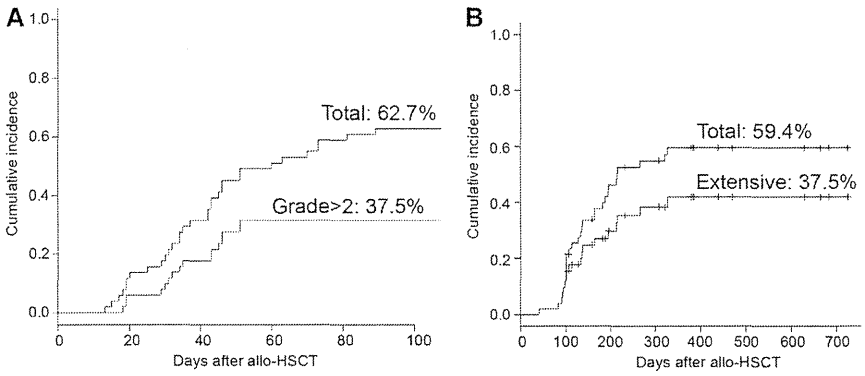

Figure 1. Cumulative incidences of acute (A) and chronic (B) GVHD after allogeneic hematopoietic stem cell transplantation.

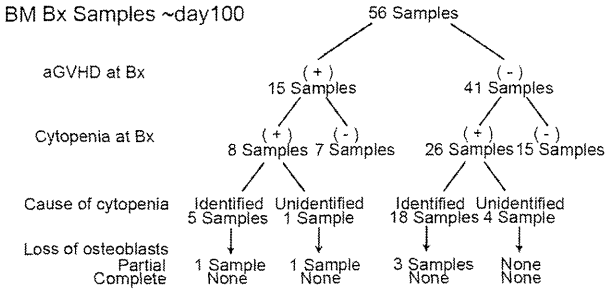

Figure 2. Assessments of bone marrow biopsy samples from patients up to day 100 after allogeneic hematopoietic stem cell transplantation. aGVHD indicates acute graft-versus-host disease; Bx, biopsy.

osteoblasts and another 6 samples displayed complete loss of osteoblasts. We further detailed the types of chronic GVHD affecting these idiopathic cytopenias and found 10 of 19 samples from patients suffering from idiopathic cytopenias displayed extensive chronic GVHD (Figure 3B). The loss of osteoblasts was significantly correlated with the extensive type of chronic GVHD (Table 2, $P = .012$) and also with idiopathic cytopenias in patients with chronic GVHD (Table 3, $P = .0427$). Among samples collected when patients had no cytopenic symptoms, no loss of osteoblasts were observed. We observed a significantly higher frequency of GVHD treatment with steroids in patients with osteoblast loss during chronic GVHD (Table 4). Characteristic pathological analyses of these cases, as well as a control BM sample, are summarized below.

Case 1: A patient with no GVHD and no cytopenias

Figure 4A indicates a pathological sample from a 47-year-old female who had no episodes of GVHD symptoms and no cytopenias when her BM sample was harvested on day 41. In hematoxylin and eosin staining, osteoblasts lining bone trabeculae are well observed before allo-HSCT and on day 41 (arrowheads). The lower panels show CD56 staining from the

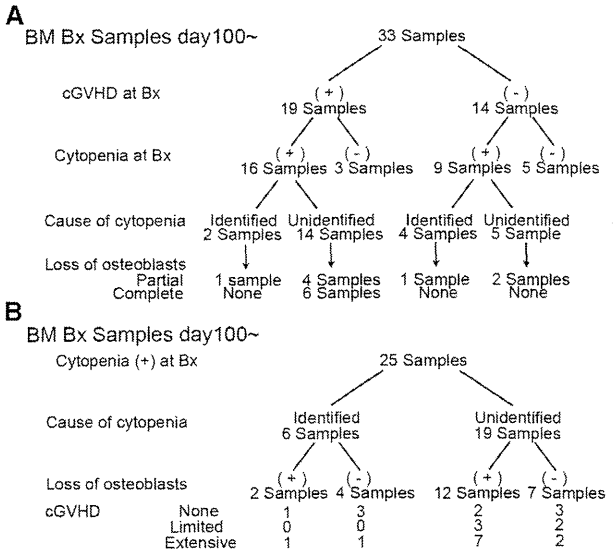

Figure 3. Assessments of bone marrow biopsy samples from patients after day 100 after allogeneic hematopoietic stem cell transplantation. (A) Relations between loss of osteoblasts and idiopathic cytopenias. (B) Grades of chronic GVHD and their relations to idiopathic cytopenias.

Table 2
Loss of Osteoblasts and Its Correlation with Chronic GVHD in BM Samples after Day 100

Chronic GVHD	Loss of Osteoblasts (+) (n = 14 Samples)	Loss of Osteoblasts (−) (n = 19 Samples)	*P* Value
Any chronic GVHD	11	8	**.0324**
Limited chronic GVHD	3	5	.7451
Extensive chronic GVHD	8	3	**.0120**

GVHD indicates graft-versus-host disease; BM, bone marrow.
Bold values indicate 2-tailed chi-square test.

same patient. Previous reports indicate neural cell adhesion molecule (NCAM, CD56) is strongly expressed by human osteoblasts [13]; therefore, we used CD56 staining for our samples to specifically identify osteoblasts.

Case 2: A patient with chronic liver and skin GVHD with cytopenias

A 57-year-old male patient underwent allo-HSCT from an HLA identical sibling. His underlining disease (anaplastic large cell lymphoma) relapsed on day 134 and tacrolimus was tapered off afterwards. Chronic extensive GVHD of the liver manifested on day 168, followed by deterioration of cutaneous and oral chronic GVHD and cytopenias, including platelets and red blood cells (grade 4 in platelets and grade 2 in hemoglobin by Common Terminology Criteria for Adverse Events version 4). BM biopsy on day 176 shows the complete loss of osteoblasts (Figure 4B). Chronic GVHD and cytopenias were then improved by the resumption of low-dose tacrolimus, and BM biopsy on day 521 showed recovery of osteoblasts.

Case 3: A patient with sustained cytopenias with skin GVHD

Figure 4C shows BM from a 37-year-old male patient receiving allo-HSCT from an HLA mismatched unrelated donor. Gradual cytopenias was observed from day 90 after allo-HSCT with stage 1 cutaneous GVHD. BM biopsy on day 127 displays complete loss of osteoblasts. When he recovered from these symptoms, osteoblasts reappeared on the sample taken on day 260.

Case 4: A patient with gradual loss of osteoblasts with worsening GVHD

A 27-year-old male patient underwent allo-HSCT from an unrelated donor. He achieved neutrophil engraftment on day 20; however, he remained dependent on platelet and red blood cell transfusions. He developed stage 1 skin GVHD on day 34. Red blood cell and platelet engraftment were

Table 3
Loss of Osteoblasts and its Correlation with Idiopathic Cytopenias in BM Samples after Day 100

Loss of Osteoblasts*	cGVHD (+), Idiopathic Cytopenia (+) (n = 14 Samples)	cGVHD (+), Idiopathic Cytopenia (−) (n = 5 Samples)†	*P* Value
Any osteoblast loss	10	1	**.0427**
Partial loss	4	1	.7032
Complete loss	6	0	**.0324**

BM indicates bone marrow; cGVHD, chronic graft-versus-host disease.
Bold values indicate 2-tailed chi-square test.
* Loss of osteoblasts is defined as *partial* if loss of osteoblasts is observed in 30% to 90% of the bone trabeculae in the pathological sections and *complete* if more than 90% of the osteoblast are lost.
† These samples include n = 3 samples without cytopenias and n = 2 with cytopenias with identified causes.

Table 4
Steroid Administration and Loss of Osteoblasts in Chronic GVHD BM Samples after Day 100

Steroid Therapy at BM Biopsy	Loss of Osteoblasts (+) cGVHD (+) (n = 11 Samples)	Loss of Osteoblasts (−) cGVHD (+) (n = 8 Samples)	*P* Value
(+)	8	2	.0360
(−)	3	6	

GVHD indicates graft-versus-host disease; BM, bone marrow; cGVHD; chronic graft-versus-host disease.

achieved on day 106 (reticulocytes >1%) and day 30 (>20,000/μL), respectively. Osteoblasts were partially lost on BM samples taken on day 36 (Figure 4D). Cytopenias continued, and he then developed chronic lung GVHD exacerbating from day 97, which was successfully treated with steroid therapy. However, the cytopenias persisted and the complete loss of osteoblasts was observed in BM samples taken on day 147. These results demonstrate the potential correlation between systemic (and supposedly affecting BM) GVHD and loss of BM osteoblasts leading to cytopenias. The patient did not develop bronchiolitis obliterans syndrome.

Suppression of CD19$^+$ B Cells and Increased CD4 to CD8 Ratio in BM Samples with the Loss of Osteoblasts

We next examined the BM aspirates samples taken at the same time points of the BM biopsies. We analyzed samples collected after day 100 by categorizing them into 3 subgroups based on the status of osteoblasts: not affected, partial loss, and complete loss. We observed decreased numbers of CD19$^+$ B cells and CD3$^+$ T cells in parallel with the loss of osteoblasts (Figure 5). Also, we found the ratio of CD4 and CD8 T cells was significantly increased with the loss of osteoblasts (P = .0002, not affected versus complete loss). These data indicate the effects of BM GVHD, resulting in disrupted hematopoiesis after allo-HSCT and are consistent with our mouse model data in the setting of BM GVHD [9].

DISCUSSION

In the settings of clinical allo-HSCT, patients frequently suffer from sustained cytopenias that parallel systemic GVHD. Some patients in the outpatient clinics after day 100 develop cytopenias without any signs of infection or GVHD. The causes for these cytopenias include relapse of original disease, viral (or bacterial) infections, and/or side effects of drugs, and it is very important to identify the cause as it directly affects the treatment decision for these patients. By

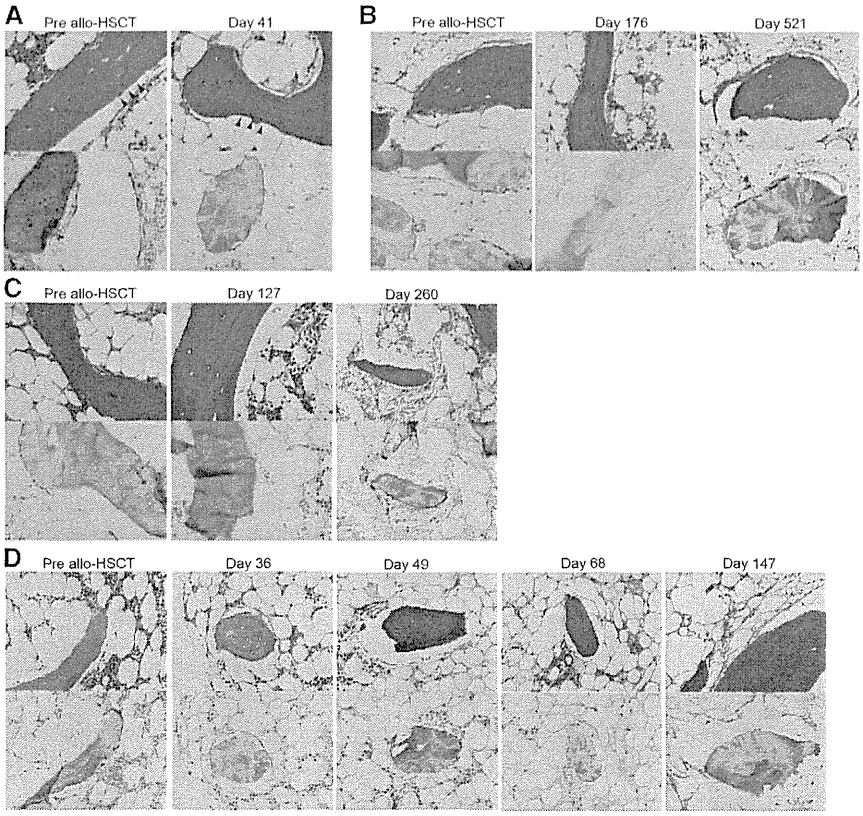

Figure 4. Loss of osteoblasts during GVHD and cytopenias. Biopsied bone marrow (BM) samples were stained with hematoxylin and eosin (upper panels for all pictures), as well as with CD56 (lower panels for all pictures) for assessments of cellularity, morphology, and presence or absence of osteoblasts. Magnification for the images is ×400. (A) BM samples from a 47-year-old patient after unrelated bone marrow transplantation who had no episode of GVHD and cytopenias when BM biopsies were performed. Osteoblasts are well preserved. Arrowheads indicate osteoblasts. (B) A 57-year-old patient who underwent related peripheral blood stem cell transplantation and relapsed on day 134 developed symptoms of chronic GVHD and cytopenias after day 160. Complete loss of osteoblasts is shown on day 176, and when he recovered from those symptoms osteoblasts are back on day 521. (C) A 37-year-old patient after related peripheral blood stem cell transplantation gradually developed cytopenias after day 90 with stage 1 skin GVHD. The BM samples on day 127 show clear loss of osteoblasts. On day 260, when his symptoms subsided, osteoblasts recovered to a normal level. (D) A 27-year-old patient who underwent unrelated bone marrow transplantation attained engraftment of white blood cells on day 20; however, cytopenias had persisted and he developed stage 1 skin GVHD on day 34. Partial loss of osteoblasts was observed from day 36 to day 68 and he had been on high demand of red blood cell and platelet transfusions. The BM samples on day 147 indicate complete loss of osteoblasts.

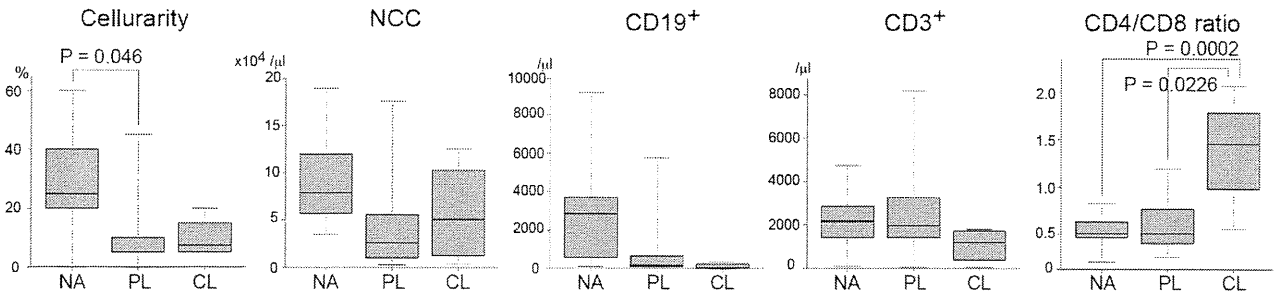

Figure 5. Flow cytometric analyses of BM aspirates in patients after day 100. BM aspiration was performed at the same time of BM biopsy in these patients. Cellularity, nucleated cell count (NCC), $CD19^+$ cells, $CD3^+$ cells, and the ratio of $CD4^+$ to $CD8^+$ cells are shown.

evaluating BM biopsy samples with hematoxylin and eosin, as well as CD56 immunohistochemical staining, we analyzed BM osteoblasts and compared these results with the respective clinical courses. As a result, we confirmed the disappearance of osteoblasts in samples from patients with idiopathic cytopenias and chronic GVHD during the late stage after allo-HSCT, suggesting the correlation between chronic GVHD and BM GVHD, resulting in BM suppression. Bone damage after allo-HSCT [15], especially suppression of B lymphopoiesis during GVHD, has been reported in both clinical and experimental studies [2,6,16-19], and our previous study in murine models unveiled new details of the mechanisms involved in this phenomenon, focusing on the destruction of hematopoietic niches by donor T cells in the course of GVHD [9]. In this article, we also reported the analyses of various clinical factors in BM, including cellularities and B cell analyses, which indicated the correlation with BM GVHD in human chronic GVHD cases. Consistent with our findings in murine GVHD models, we observed a decreased number of $CD19^+$ B cells and an increased CD4 to CD8 ratio in patients with osteoblast destruction; however, these findings were not observed during the early period after allo-HSCT, when in the murine models, donor $CD4^+$ T cells mediated strong BM GVHD. It is possible that in the clinical settings, patients are treated with immunosuppressive therapy and this could have prevented acute BM GVHD [20,21]. In cases of patients treated with steroids, it is quite difficult to separate the effects of chronic GVHD on osteoblasts from those of treatment with steroids, as steroids also decrease osteoblastic proliferation and activity [22]. We observed a higher frequency of GVHD treatment with steroids in patients who had idiopathic cytopenias with osteoblast loss (Table 4), indicating more severe systemic GVHD with BM GVHD that required steroid therapy.

In conclusion, we have shown for the first time, to our knowledge, the direct proof of BM GVHD and the loss of osteoblasts in chronic GVHD patients. Further studies with a large number of patients are warranted; however, our findings explain the cause of idiopathic cytopenias after allo-HSCT and give valuable insights for clinicians to use in treating patients suffering from BM suppression after allo-HSCT.

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SUPPLEMENTARY DATA

Supplementary data related to this article can be found online at http://dx.doi.org/10.1016/j.bbmt.2013.12.568.

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Programmed Death-1 Pathway in Host Tissues Ameliorates Th17/Th1-Mediated Experimental Chronic Graft-versus-Host Disease

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Chronic graft-versus-host disease (GVHD) is a major cause of late death and morbidity after allogeneic hematopoietic cell transplantation, but its pathogenesis remains unclear. We investigated the role of the programmed death-1 (PD-1) pathway in chronic GVHD using a well-defined mouse model of B10.D2 (H-2^d) donor to BALB/c (H-2^d) recipients. PD-1 expression on allogeneic donor T cells was upregulated continuously in chronic GVHD development, whereas PD-L1 expression in host tissues was transiently upregulated and declined to basal levels in the late posttransplant period. Blockade of the PD-1 pathway by anti–PD-1, anti–PD-L1, or anti–PD-L2 mAbs exacerbated clinical and pathologic chronic GVHD. Chimeric mice revealed that PD-L1 expression in host tissues suppressed expansion of IL-17$^+$IFN-γ^+ T cells, and that PD-L1 expression on hematopoietic cells plays a role in the development of regulatory T cells only during the early transplantation period but does not affect the severity of chronic GVHD. Administration of the synthetic retinoid Am80 overcame the IL-17$^+$IFN-γ^+ T cell expansion caused by PD-L1 deficiency, resulting in reduced chronic GVHD damage in PD-L1$^{-/-}$ recipients. Stimulation of the PD-1 pathway also alleviated chronic GVHD. These results suggest that the PD-1 pathway contributes to the suppression of Th17/Th1-mediated chronic GVHD and may represent a new target for the prevention or treatment of chronic GVHD. *The Journal of Immunology*, **2014, 193: 2565–2573.**

Alloreactive T cell activation, expansion, cytokine secretion, and effector function require two signals: 1) interaction between the TCR and antigenic peptide–MHC complex on APCs, and 2) Ag-independent costimulatory molecules expressed on APCs (1–3). However, some of these costimulatory molecules deliver negative signals that could regulate T cell tolerance. The programmed death-1 (PD-1) receptor is involved in the B7:CD28 family and is associated with regulatory function with its ligands, PD-L1 (B7-H1) and PD-L2 (B7-DC) (4–9). PD-1 is expressed by activated CD4$^+$ and CD8$^+$ T cells, B cells, and myeloid cells (4, 10). Expression of PD-L1 is upregulated on dendritic cells (DCs), monocytes, and B cells, as well as in nonlymphoid organs, such as vascular endothelium, pancreatic islets, and keratinocytes (6, 7, 11, 12). PD-L1 is also upregulated on APCs and nonlymphoid organs by a major proinflammatory cytokine, IFN-γ (11, 13).

Previous studies have reported a role for PD-1/PD-L in acute graft-versus-host disease (GVHD), which is mainly Th1 biased and organ damage is CD8 T cell mediated, involving cytotoxic and inflammatory mediators, such as IFN-γ, TNF-α, and IL-1. Blazar et al. (14, 15) revealed that systemic IFN-γ levels were augmented by PD-1/PD-L blockade and that this accelerated acute GVHD lethality. Li et al. (16) showed that the absence of PD-L1 expression allowed donor CD8$^+$ T cell expansion and exacerbated GVHD lethality, using an acute GVHD model. In contrast, chronic GVHD is primarily dependent on CD4$^+$ T cells, and the pathophysiology of chronic GVHD differs from that of acute GVHD. Although chronic GVHD is a major cause of late death and morbidity after allogeneic hematopoietic cell transplantation, the role of the PD-1 pathway in chronic GVHD is not fully defined. In this study, we investigated the role of the PD-1 pathway in the development of Th subsets in a well-defined chronic GVHD model (B10.D2 into BALB/c). Furthermore, many reports have shown that PD-L1 plays an important role in the expansion of regulatory T cells (Tregs), and Yi et al. reported that PD-L1 deficiency on host APCs, not tissues, caused impaired Treg expansion in the mouse GVHD model (17, 18). However, it remains unknown whether PD-L1 deficiency on host APCs is associated with chronic GVHD. In this study, we used different chimeric recipients with PD-L1 expression only on hematopoietic cells or host tissues and clarified that PD-L1 deficiency in host tissues, not hematopoietic cells, is associated with exacerbated chronic GVHD.

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H.F. conducted the experiments, analyzed the data, and wrote the manuscript; Y.M. designed the experiments, supervised the research, and wrote the manuscript; K.K. and H.N. performed the research; T.T. performed histopathologic analyses of the organs; L.C., M.A., and H.Y. provided vital mice and mAbs for the study; and K.M., N.F., E.K., and M.T. supervised the research.

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The online version of this article contains supplemental material.

Abbreviations used in this article: BM, bone marrow; BMT, BM transplant; DC, dendritic cell; GVHD, graft-versus-host disease; pLN, peripheral lymph node; PD-1, programmed death-1; TCD-BM, T cell–depleted BM; Treg, regulatory T cell; WT, wild-type.

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Materials and Methods

Mice

Female B10.D2 (H-2^d) donor mice were purchased from Japan SLC (Hamamatsu, Japan). Female BALB/c (H-2^d) recipient mice were purchased from Charles River Japan (Yokohama, Japan). PD-1–deficient (PD-$1^{-/-}$) mice on a BALB/c background were generated previously and provided by T. Honjo (Kyoto University, Kyoto, Japan) via RIKEN BRC (Tsukuba-shi, Japan) (19–21). PD-L1–deficient (PD-$L1^{-/-}$) mice on a BALB/c background were generated previously and kindly provided by Dr. M. Azuma (Tokyo Medical and Dental University, Tokyo, Japan) with permission of Dr. L. Chen (Yale University, New Haven, CT) (22). PD-$1^{-/-}$ mice and PD-$L1^{-/-}$ mice on a B10.D2 background were generated by backcrossing for 10 generations. All mice were maintained under specific pathogen-free conditions and used at 8–12 wk of age. All animal experiments were performed according to the regulations of the Institutional Animal Care and Research Advisory Committee, Okayama University Advanced Science Research Center, Okayama, Japan.

Bone marrow transplantation

Mice received transplants according to standard protocols described previously (23). In brief, recipient BALB/c mice received a single dose of 5.8 Gy X-ray total body irradiation and were injected with 2×10^6 spleen T cells and 8×10^6 T cell–depleted bone marrow (TCD-BM) cells from BALB/c or B10.D2 donors. Chimeric mice were generated with transplants according to protocols described previously (24). Bone marrow (BM) was isolated from PD-$L1^{-/-}$ or wild-type (WT) BALB/c donor mice. PD-$L1^{-/-}$ or WT BALB/c recipient mice received a single dose of 5.8-Gy total body X-ray irradiation and immediately after irradiation were injected with 5×10^6 cells. Full donor chimerism was confirmed by evaluating PD-L1 expression on splenic $CD11c^+$ DCs after at least 12 wk after BM transplant (BMT) (24). After BMT, animals were weighed twice/week and scored for skin manifestations of GVHD (23).

Tissue histopathology

Shaved skin from the interscapular region (~2 cm^2) and liver specimens of recipients were fixed in 10% formalin, embedded in paraffin wax, sectioned, mounted on slides, and stained with H&E. Masson trichrome staining was used for fibrosis. Slides were scored by a pathologist (T.T.) blinded to the experimental group. Skin was scored on the basis of dermal fibrosis, fat loss, inflammation, epidermal interface changes, and follicular dropout (0–2 for each category; the maximum score was 10) (23). Liver slides were also scored according to bile duct injury and inflammation (0–4 for each category; the maximum score was 8) (25). Salivary gland slides were scored based on atrophy and inflammation (0–3 for each category) and the maximum score was 6 (26).

Immunofluorescence analysis

PE-conjugated anti-CD25 (PC61.5), anti–PD-1 (CD279, RMP1-30), anti–PD-L1 (CD274, MIH5), anti–PD-L2 (TY25), FITC-conjugated anti-CD4 (RM4-5), anti-CD8 (53-6.7), anti-CD11c (N418), anti-Foxp3 (FJK-16s), PerCP-Cy5.5–conjugated anti-CD4 (RM4-5), allophycocyanin-conjugated anti-CD8 (53-6.7), 7-AAD, and control Abs were purchased from eBioscience (Affymetrix Japan K.K., Tokyo, Japan). Cells were analyzed using a FACSCalibur flow cytometer and CellQuest software or the MACSQuant flow cytometer with the FlowJo software.

Immunohistochemistry

Back skin tissues from syngeneic and allogeneic recipients were removed surgically, embedded in Tissue-Tek (Sakura, Tokyo, Japan), frozen, and stored at −80°C until use. Cryostat sections (5 μm thick) were fixed in

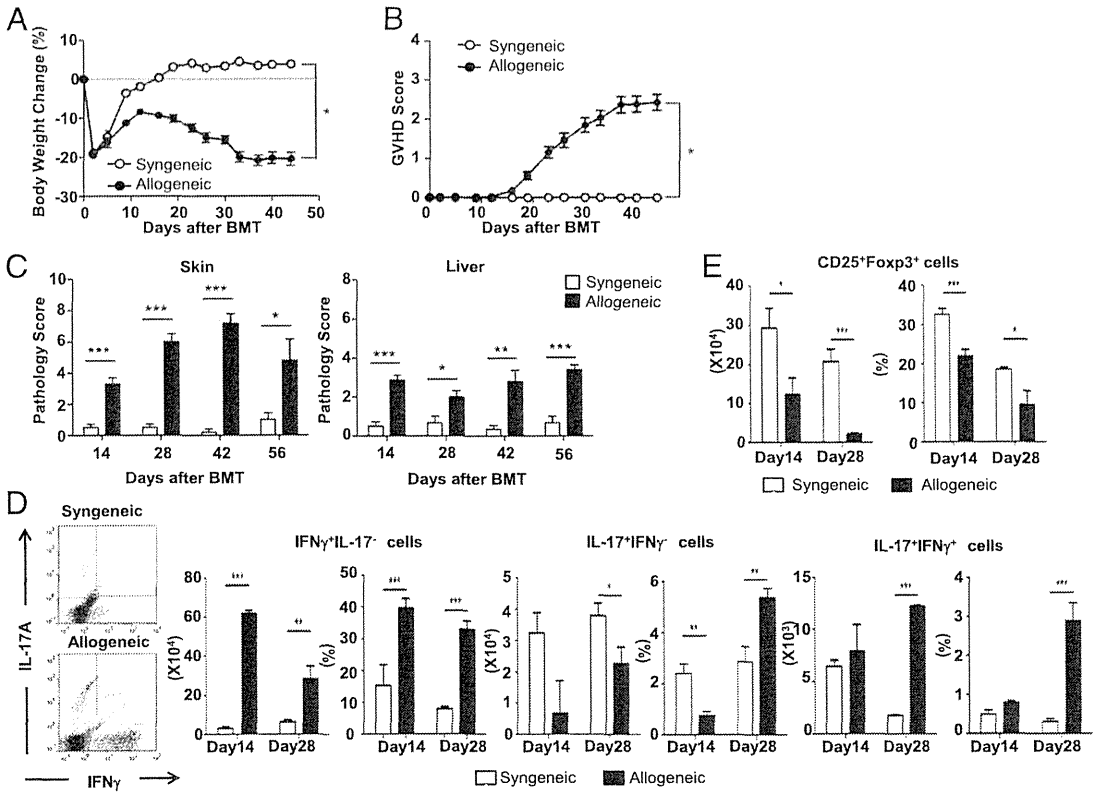

FIGURE 1. Th17 and Th1 cells are increased during chronic GVHD. (**A–E**) Sublethally irradiated (5.8 Gy) BALB/c mice were transplanted with 2×10^6 spleen T cells and 8×10^6 TCD-BM cells from B10.D2 mice (allogeneic group). The syngeneic group received a transplant of the same dose of splenocytes and TCD-BM from BALB/c mice. Body weight change (A) and clinical GVHD skin scores (B) are shown; data shown are from 1 representative of ≥3 independent experiments (n = 24 in each group). Pathology scores of skin and liver (C) from days 14 to 56 of BMT are shown. Data shown are from 1 representative of ≥3 independent experiments (n = 8 in each group). (D) Representative staining for intracellular IL-17 and IFN-γ on $CD4^+$ cells on day 28 for syngeneic and allogeneic mice. The numbers and percentages of donor-derived $CD4^+$ T cells expressing IFN-γ^+IL-17^-, IL-17^+IFN-γ^-, and IL-17^+IFN-γ^+ cells from pLNs of syngeneic and allogeneic mice on days 14 and 28 are shown. (E) The numbers and percentages of donor-derived $CD4^+$ T cells expressing $CD25^+$ $Foxp3^+$ cells from pLNs of syngeneic and allogeneic mice on days 14 and 28 are shown. The means (± SE) of each group are shown. Data shown are from 1 representative of ≥3 independent experiments (n = 8 in each group). *$p < 0.05$, **$p < 0.01$, ***$p < 0.005$.

absolute acetone and subjected to enzymatic immunohistochemistry. After blocking, sections were incubated with the primary mAb against PD-L1 (MIH5; eBioscience) overnight at 4°C. The primary Abs were detected using the Histofine Simple Strain Mouse MAX PO kit and diaminobenzidine solution (Nichirei Biosciences, Tokyo, Japan). Sections were counterstained with hematoxylin. The images were captured using an Olympus BH2 microscope with a Nikon DS-5M color digital camera (Nikon, Tokyo, Japan), controlled by the ATC-2U software (version 1.5; Nikon). An Olympus ×10/20 ocular lens and a ×20/0.46 NA objective lens were used.

Real-time PCR

Total RNA from snap-frozen skin tissues of syngeneic and allogeneic recipients was extracted using the TRIzol reagent (Life Technologies, Tokyo, Japan) according to the manufacturer's protocol. cDNA was synthesized using oligo(dT) primers and Superscript II reverse transcriptase (Invitrogen). Target cDNA levels were quantified using real-time quantitative PCR with an ABI Prism 5300 system (Applied Biosystems, Tokyo, Japan). TaqMan Universal PCR Master mix, primers, and the fluorescent TaqMan probe specific for murine PD-L1 (Mm00452054-m1) and a housekeeping gene, mGAPDH (Mm99999915-g19), were purchased from Applied Biosystems. The mRNA levels of individual genes were normalized relative to GAPDH, using the equation Δ threshold cycle = $Ct_{target} - Ct_{GAPDH}$.

Intracellular cytokine staining and cytokine analysis

Cells were stimulated in vitro with 50 ng/ml PMA (Sigma-Aldrich) and 100 ng/ml ionomycin (Sigma-Aldrich) at 37°C for 3 h. Cells were then incubated with GolgiPlug (BD Biosciences) for an additional 2 h. mAbs to PE-conjugated anti–IL-17A (eBio17B7) and FITC-conjugated anti–IFN-γ (XMG1.2) were used to assess the cell populations (eBioscience). Total cells were adjusted to 1×10^6/ml in cultures.

Administration of Abs and Am80

Neutralizing mAbs against mouse PD-1 (RPMI-14), PD-L1 (MIH6), and PD-L2 (TY25) for in vivo experiments were prepared as described previously (10, 14, 27). Neutralizing mAbs against mouse PD-1 (RMPI-14) and PD-L2 (TY25) were kindly provided by Dr. H. Yagita, and a neutralizing mAb against mouse PD-L1 (MIH6) was kindly provided by Dr. M. Azuma. Anti–PD-1, –PD-L1, and –PD-L2 mAbs or control rat IgG (Sigma-Aldrich) 250 μg were administered i.p. on days 14, 16, 19, 21, 24, and 26 after BMT. Anti-mouse PD-1 agonistic mAb (PIM2) for in vivo experiments was prepared as described previously, and 200 μg was administered i.v. on days 14, 17, 20, 23, and 26 after BMT (28). Recipients were administered Am80 (1.0 mg/kg body weight; Nippon Shinyaku) or vehicle solution orally daily from day 0.

Statistical analyses

Group comparisons of skin chronic GVHD scores and pathology scores were performed using the Mann–Whitney *U* test or Kruskal–Wallis test. Cell populations, mean weights, and gene expression data were analyzed by unpaired Student *t* tests. Survival was evaluated using the log-rank test. All data were analyzed using the GraphPad Prism software (version 5.0). The *p* values <0.05 were taken to indicate statistical significance.

Results

Upregulated PD-L1 expression in targeted cells declined in the late posttransplant period

To evaluate the role of the PD-1 pathway in the development of chronic GVHD, we used a common chronic GVHD model, the MHC-compatible, murine minor histocompatibility Ag-incompatible allogeneic BMT model (B10.D2 into BALB/c). Sublethally irradiated (5.8 Gy) BALB/c mice were transplanted with 2×10^6 spleen T cells and 8×10^6 TCD-BM cells from B10.D2 mice. We used Ly9.1 as a marker to distinguish B10.D2 donor cells from BALB/c recipients and confirmed full donor chimerism (>95% donor cells) of spleens and peripheral lymph nodes (pLNs) on days 14 and 28 (29). Allogeneic recipients showed severe weight loss, increased clinical chronic GVHD, and obvious histopathologic damage to the skin and liver (Fig. 1A–C). Cells isolated from pLNs were harvested and analyzed for cytokine expression as reported previously (29). On day 28 after BMT, IL-17$^+$IFN-γ$^-$ and IL-17$^+$IFN-γ$^+$ CD4$^+$ T cells from pLNs of allogeneic recipients increased and were detected more frequently (Fig. 1D). Because of GVHD-induced lymphopenia, the absolute number of IFN-γ$^+$IL-17$^-$ CD4$^+$ T cells from pLNs of allogeneic recipients decreased on day 28, whereas

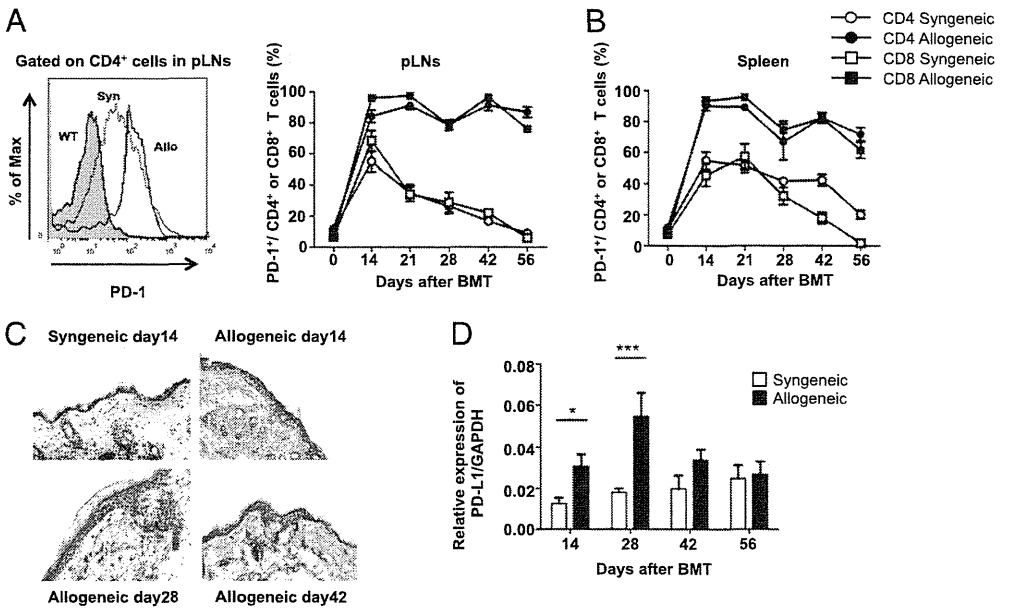

FIGURE 2. PD-1 expression on donor cells and PD-L1 expression in host tissues during chronic GVHD development. Sublethally irradiated BALB/c mice were transplanted from allogeneic B10.D2 or syngeneic BALB/c donors. (**A**) Representative histograms of PD-1 expression on CD4$^+$ T cells from pLNs of syngeneic and allogeneic groups on day 14 of BMT are shown (*left panel*). Percentages of PD-1$^+$ among CD4$^+$ T cells from pLNs and spleen of syngeneic and allogeneic groups from day 0 to day 56 after BMT are shown. (**B**) Percentages of PD-1$^+$ among CD8$^+$ T cells from pLNs and spleen of syngeneic and allogeneic groups from day 0 to day 56 after BMT are shown. (**C**) Representative images of PD-L1 expression of skin stained with anti–PD-L1 mAb from day 14 to day 42 after BMT are shown (original magnification ×100–200). (**D**) PD-L1 mRNA from skins of syngeneic and allogeneic recipients on days 14, 28, 42, and 56 after BMT are shown. The means (± SE) of each group are shown. Data shown are from 1 representative of ≥3 independent experiments (n = 6–8 in each group). *$p < 0.05$, ***$p < 0.005$.

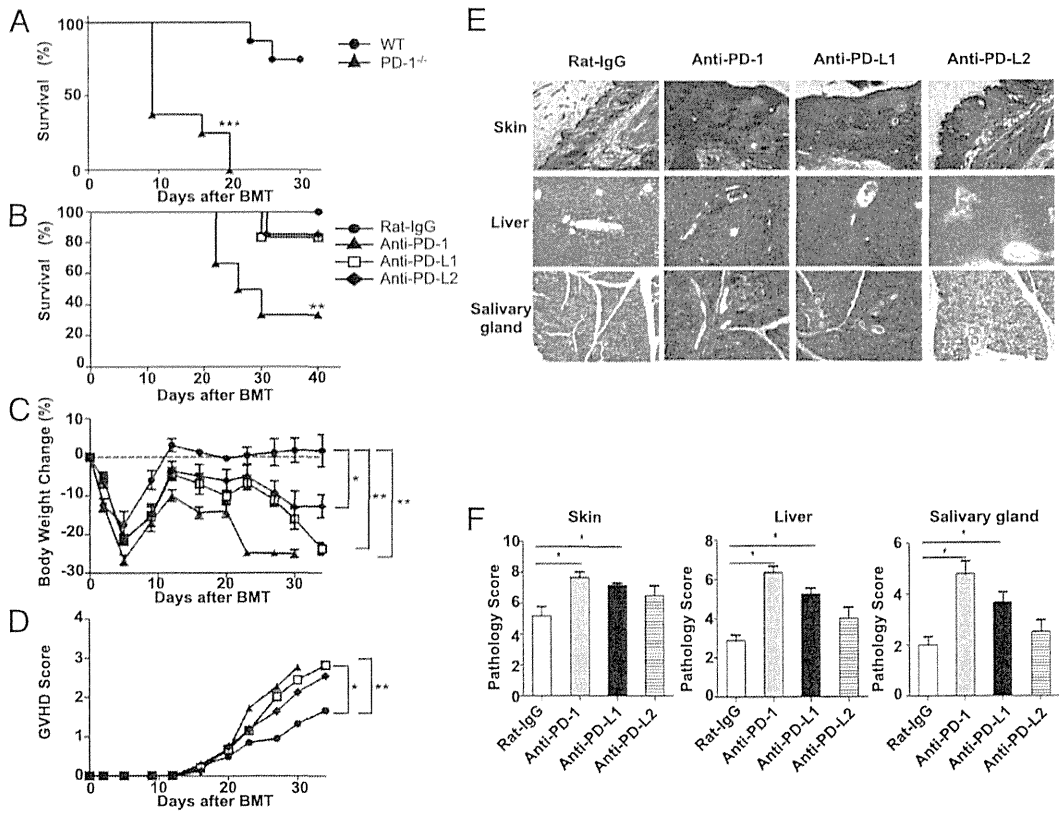

FIGURE 3. PD-1/PD-L blockade exacerbates chronic GVHD. (**A**) Sublethally irradiated BALB/c recipients were transplanted with 2×10^6 spleen T cells and 8×10^6 TCD-BM cells from WT or PD-$1^{-/-}$ B10.D2 donors. Survival data are shown; data shown are from 1 representative of ≥ 3 independent experiments ($n = 8$ in each group). (**B–F**) Sublethally irradiated BALB/c recipients were transplanted from WT B10.D2 donors. Recipients were injected with anti–PD-1, –PD-L1, –PD-L2 mAbs or control rat IgG (250 μg/mouse) on days 14, 16, 19, 21, 24, and 26 after BMT. Survival (B) and body weight change (C) and clinical GVHD skin score (D) are shown; data shown are from 1 representative of ≥3 independent experiments ($n = 8$ in each group). (E and F) Skin, liver, and salivary gland from recipients were taken on day 36 after BMT. (E) Representative images are shown (original magnification ×100). (F) Pathology scores of skin, liver, and salivary gland on day 36 after BMT are shown. The means (± SE) of each group are shown. Data shown are from 1 representative of ≥3 independent experiments ($n = 4$–6 in each group). $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.005$.

the rate of those was consistently high on days 14 and 28. Different from Th17/Th1 cells, $CD4^+$ $CD25^+$ $Foxp3^+$ Tregs were consistently detected at lower percentages in allogeneic recipients on days 14 and 28 (Fig. 1E).

We next assessed PD-1 expression on donor T cells in pLNs and spleen on days 14, 21, 28, 42, and 56 after transplantation. Before transplantation, donor cells had low expression levels (<20% of total cells) of PD-1. On day 14 after transplantation, donor $CD4^+$ and $CD8^+$ T cells in the pLNs showed increasing levels of PD-1 in both syngeneic and allogeneic recipients (Fig. 2A). From day 21 onward, PD-1 expressions on donor $CD4^+$ T and $CD8^+$ cells from syngeneic recipients showed a time-dependent decrease, whereas $CD4^+$ T and $CD8^+$ T cells from allogeneic recipients maintained significantly higher expression levels of PD-1 in the pLNs. PD-1 expressions on $CD4^+$ T and $CD8^+$ cells in the spleen showed a similar pattern to those in the pLNs (Fig. 2B).

Previous studies revealed that parenchymal cell expression of PD-L1 was induced by IFN-γ derived from infiltrating T cells, and IFN-γ^+IL-17^- T cells were detected more frequently in pLNs of allogeneic recipients on both days 14 and 28 (Fig. 1D) (13, 30, 31). As a next step, we evaluated donor cell inhibitory signal ligands and PD-L1 expression in host tissues after BMT. PD-L1 expressions on donor T cells from allogeneic recipients showed slightly higher than those in syngeneic recipients but sequentially reduced (Supplemental Fig. 1A, 1B). PD-L1 expression on $CD11c^+$ DCs from the allogeneic group was higher, whereas PD-L2 expression on $CD11c^+$ DCs was almost identical between syngeneic and allogeneic groups (Supplemental Fig. 1C, 1D). Immunohistochemical analyses of skin from allogeneic recipients showed higher PD-L1 expression than in syngeneic recipients from days 14 to 28, whereas it decreased to baseline on day 42 (Fig. 2C). mRNA levels showed similar results; from days 14 to 28, the skin from allogeneic recipients revealed significantly increased levels of PD-L1 compared with skin from syngeneic recipients, and a decrease was observed after day 42 (Fig. 2D). These results indicate that although expression of PD-1 on donor T cells from allogeneic recipients was continuously upregulated, PD-L1 expression in host tissues was transiently upregulated and declined to basal levels in the late posttransplant period when allogeneic recipients showed significant signs of chronic GVHD.

PD-1/PD-L1 blockade exacerbated chronic GVHD

To analyze the influence of the PD-1 pathway, we used PD-$1^{-/-}$ mice on a B10.D2 background as a donor and evaluated the contribution of PD-1 on donor cells to chronic GVHD. PD-$1^{-/-}$ donor induced severe weight loss, and more than half died within 1 wk (Fig. 3A). To avoid early death and to examine the roles of the PD-1 pathway in chronic GVHD, we used neutralizing mAb against PD-1, PD-L1, and PD-L2 in allogeneic recipients from day 14 after BMT, immediately before the development of chronic GVHD. The anti–PD-1 mAb treatment group showed exacerbated chronic GVHD and poorer survival compared with the control group ($p < 0.01$; Fig. 3B). The anti–PD-L1–treated group also showed severe weight loss and worse clinical GVHD scores than