# Prognostic significance of pleural or pericardial effusion and the implication of optimal treatment in primary mediastinal large B-cell lymphoma: a multicenter retrospective study in Japan

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## ABSTRACT

The prognosis of patients with primary mediastinal large B-cell lymphoma has improved over recent years. However, the optimal treatment strategy including the role of radiotherapy remains unknown. We retrospectively analyzed the clinical outcomes of 345 patients with newly diagnosed primary mediastinal large B-cell lymphoma in Japan. With a median follow up of 48 months, the overall survival at four years for patients treated with R-CHOP (n=187), CHOP (n=44), DA-EPOCH-R (n=9), 2nd- or 3rd-generation regimens, and chemotherapy followed by autologous stem cell transplantation were 90%, 67%, 100%, 91% and 92%, respectively. Focusing on patients treated with R-CHOP, a higher International Prognostic Index score and the presence of pleural or pericardial effusion were identified as adverse prognostic factors for overall survival in patients treated with R-CHOP without consolidative radiotherapy (IPI: hazard ratio 4.23, 95% confidence interval 1.48-12.13, $P$=0.007; effusion: hazard ratio 4.93, 95% confidence interval 1.37-17.69, $P$=0.015). Combined with the International Prognostic Index score and the presence of pleural or pericardial effusion for the stratification of patients treated with R-CHOP without radiotherapy, patients with lower International Prognostic Index score and the absence of effusion comprised approximately one-half of these patients and could be identified as curable patients (95% overall survival at 4 years). The DA-EPOCH-R regimen might overcome the effect of these adverse prognostic factors. Our simple indicators of International Prognostic Index score and the presence of pleural or pericardial effusion could stratify patients with primary mediastinal large B-cell lymphoma and help guide selection of treatment.

## Introduction

Primary mediastinal large B-cell lymphoma (PMBL) is characterized by distinct clinical, pathological and genetic features and comprises a subtype of diffuse large B-cell lymphoma (DLBCL) according to the current World Health Organization (WHO) classification.[1] The disease is more common in younger females and often presents with bulky mediastinal mass without extrathoracic spread and pleural or pericardial effusion.[2-5]

Prior to the introduction of rituximab, the outcomes of patients treated with anthracycline-containing chemotherapies, including cyclophosphamide, doxorubicin, vincristine and prednisolone (CHOP), had a suboptimal progression-free survival (PFS) of 38%-52%.[5,6] Several retrospective analyses revealed that the outcomes of the 2nd- and 3rd-generation chemotherapeutic regimens, such as methotrexate, leucovorin, doxorubicin, cyclophosphamide, vincristine, bleomycin and prednisolone (MACOP-B), might be superior to those of CHOP regimens.[5,7-10] High-dose chemotherapy followed by autologous stem cell transplantation (HDT/ASCT) was also associated with encouraging results (PFS >75% for newly diagnosed PMBL patients).[3,11,12] These reports indicate that intensive regimens might be beneficial in a certain proportion of PMBL patients.

In the rituximab era, the combination of rituximab and

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chemotherapy has improved outcomes in various subtypes of B-cell lymphoma.[13-22] In the literature, more than 80% of patients with PMBL receiving immunochemotherapy with or without radiotherapy (RT) also achieved long-term overall survival (OS).[17-22] Despite the outstanding advances with R-CHOP, 20%-30% of patients still experience progression or relapse and have poor outcomes. Moreover, approximately 80% of long-term survivors treated with R-CHOP required consolidative RT for residual mediastinal disease.[20-23] Considering late adverse events induced by the mediastinal RT, namely the increased risk of secondary breast cancer and cardiac toxicity, the risk of RT should be minimized, especially for younger patients.[24-26]

Recently, Dunleavy *et al.* reported excellent outcomes for dose-adjusted etoposide, cyclophosphamide, doxorubicin, vincristine, prednisolone and rituximab (DA-EPOCH-R) when restricting candidates for RT according to the results of positron-emission tomography/computed tomography (PET/CT).[27] Although outcomes were reported from a phase II trial, the regimen might be a promising treatment strategy to reduce the risk of RT. Meanwhile, the DA-EPOCH-R regimen is somewhat complicated and expensive, requiring continuous infusion for 96 h in each cycle and frequent evaluation of complete blood counts. Considering R-CHOP-based regimens without RT could provide curative potential for a significant proportion of PMBL patients without hospitalization,[19,21] it would, therefore, be beneficial to identify the subset of patients that could be cured with this treatment strategy.

The goal of the present multicenter co-operative retrospective study in Japan was to investigate the optimal treatment strategy for PMBL patients by evaluating the clinical outcomes in response to various treatments and to assess a risk-stratified treatment strategy to minimize the risk of late adverse events in PMBL patients.

## Methods

### Patients

A total of 363 patients with PMBL newly diagnosed between May 1986 and September 2012 at one of any of the 65 participating hospitals in Japan were retrospectively analyzed. We registered consecutive patients who were diagnosed with PMBL at each institution in accordance with the WHO classification.[1] The time period during which we could collect the clinical data from each institution varied due to the differences in the length of time medical records are kept there. Medical record data since the 1980s were collected from three institutions, while data since the 1990s and 2000s were available from 10 and 65 institutions, respectively. In this study, PMBL was defined as patients with a dominant mass within the anterior mediastinum, irrespective of the tumor size. In addition, a central pathological review was performed by a hematopathologist (SN) for 196 patients for whom histological paraffin-embedded tissue materials could be provided. Eighteen of the 363 patients were excluded from analysis due to disease other than PMBL (n=10) by central pathological review or due to the absence of important clinical information (n=8). For the remaining patients who were not available for the central review, the histological diagnosis of PMBL was re-confirmed by a pathologist at each institution, according to the current WHO classification. Therefore, 345 patients were finally analyzed for the present study. Patients were treated according to each institution's treatment standards. The study protocol was approved by the institutional review boards of Nagoya Daini Red Cross Hospital where this study was organized and of each participating hospital. The study complied with all the provisions of the Declaration of Helsinki.

### Immunohistochemistry

Immunohistochemistry was performed using formalin-fixed, paraffin-embedded tissue sections using the avidin-biotin peroxidase complex method. Monoclonal antibodies targeting the following proteins were used: CD20, CD30, CD3, CD10, BCL6, MUM1 and CD15 (Dako). In addition, programmed cell death ligand-1 (PDL1) was evaluated, as previously described.[28] To evaluate PDL1, we used a polyclonal rabbit antibody for CD274 (ab82059; Abcam) according to the manufacturer's instructions. The cut-off values for these markers were 20% for CD30, and 30% for Bcl-6, MUM1 and PDL1.[29-31]

### Treatment

Initial treatments were performed based on the physicians' decisions at each institution, as there had been no uniform treatment guidelines for PMBL in Japan. Patients who received CHOP or a CHOP-like regimen, with or without rituximab, were categorized and analyzed as the R-CHOP or CHOP group, respectively. Patients who received 2nd-/3rd-generation treatments were categorized and analyzed as the 2nd-/3rd-generation regimen group, irrespective of the use of rituximab. Patients who received the DA-EPOCH-R regimen[27] were analyzed as the DA-EPOCH-R group. Patients who underwent consolidative HDT/ASCT after initial therapy were analyzed as the HDT/ASCT group, irrespective of the use of rituximab. CHOP- or R-CHOP-based regimens were mainly selected in 46 institutions. Physicians at six institutions selected 2nd-/3rd-generation chemotherapeutic regimens other than CHOP- or R-CHOP-based regimens as the first-line treatment. HDT/ASCT as the first-line treatment was performed at 13 institutions. Consolidative RT was performed according to the treatment strategy used at each institution.

### Response assessment

Clinical data were collected from case report forms. In principle, an effusion was evaluated by CT and/or echocardiography, as per the usual pre-treatment evaluation. Responses were evaluated by each investigator in accordance with the 1999 International Workshop Criteria.[32]

### Statistical analysis

Overall survival was defined as the period from diagnosis to death or last follow up. Progression-free survival (PFS) was defined as the period from diagnosis to disease progression, relapse, death from any cause, or last date of follow up. Patients who did not achieve a complete remission (CR) or partial response (PR) were considered to have primary refractory disease. Early relapse was defined as relapse occurring less than 12 months after diagnosis. PFS and OS were analyzed using Kaplan-Meier methods and results were compared using the log rank test. Univariate and multivariate Cox regression analyses were performed to assess the effects of prognostic factors. Multivariate analysis was built with a forward/backward, step-wise method using threshold values for removal from and addition to the model of $P$=0.20 and $P$=0.05, respectively. The individual factors of IPI were entered into the model in multivariate analysis. All probability values were two-sided and had an overall significance level of 0.05. Statistical analyses were performed with Stata SE 12 software (StataCorp LP, College Station, TX, USA).

## Results

### Patients' characteristics

Patients' characteristics are summarized in Table 1. Median age was 32 years (range 17-83 years) and females were predominant (58%). The median diameter of mediastinal mass was 10 cm (range 3-32 cm). Stage I/II disease, low-risk disease according to the International Prognostic Index (IPI), and performance status (PS) 0/1 were also predominant (67%, 52% and 75%, respectively). The presence of pleural or pericardial effusion, elevated lactate dehydrogenase (LDH) level and more than one extranodal lesion were observed in 46%, 80% and 9% of patients, respectively. For the patients who had extra-nodal involvement, major extra-nodal sites were lung (n=44), effusion (n=49) and cardiac (n=28). Pathological features are listed in Table 1. Lymphoma cells in all patients expressed CD20. Further, CD30, BCL6, and MUM1 expression was detected in 71%, 61%, and 96%, respectively. PDL1 was expressed in 62% of 110 evaluable patients.

Table 1. Patients' characteristics.

| Characteristic | All | | CHOP | | R-CHOP | | DA-EPOCH-R | | 2nd/3rd generation | | HDT/ASCT | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | N. | % | N. | % | N. | % | N. | % | N. | % | N. | % |
| Median follow up (months) | 48 | | 118 | | 36 | | 19 | | 48 | | 101 | |
| Patient number | 345 | | 44 | | 187 | | 9 | | 45 | | 57 | |
| Age at diagnosis (years) | | | | | | | | | | | | |
| Median | 32 | | 31.5 | | 33.5 | | 30 | | 31 | | 27 | |
| Range | 17-83 | | 17-77 | | 17-83 | | 24-64 | | 18-76 | | 17-63 | |
| >60 years | 47 | 14 | 10 | 23 | 30 | 16 | 1 | 11 | 3 | 7 | 3 | 5 |
| Gender, male | 146 | 42 | 18 | 41 | 85 | 45 | 4 | 44 | 12 | 27 | 27 | 47 |
| PS, ≥2 | 84/338 | 25 | 12/42 | 29 | 40/182 | 22 | 3 | 33 | 8 | 18 | 20 | 3 |
| Extranodal sites, >1 | 64/334 | 19 | 7/40 | 17 | 31/181 | 18 | 0 | 0 | 11 | 24 | 15/56 | 27 |
| Stage, I/II | 230/342 | 67 | 27 | 61 | 133/184 | 72 | 7 | 78 | 31 | 69 | 31 | 54 |
| LDH at diagnosis, ≥ULN | 270/337 | 80 | 35/41 | 85 | 134/183 | 73 | 8 | 89 | 37 | 82 | 54/56 | 96 |
| B symptoms, present | 90/337 | 27 | 15/42 | 36 | 40/183 | 22 | 2 | 22 | 11 | 24 | 22/55 | 40 |
| IPI | | | | | | | | | | | | |
| Low | 175/334 | 52 | 19/40 | 48 | 103/181 | 57 | 5 | 56 | 26 | 58 | 21/56 | 38 |
| Low-intermediate | 84/334 | 25 | 11/40 | 28 | 44/181 | 24 | 3 | 33 | 9 | 20 | 16/56 | 29 |
| High-intermediate | 43/334 | 13 | 4/ 40 | 10 | 21/181 | 12 | 0 | 0 | 5 | 11 | 12/56 | 21 |
| High | 32/334 | 10 | 6/40 | 15 | 13/181 | 7 | 1 | 11 | 5 | 11 | 7/56 | 13 |
| Bulky tumor size | | | | | | | | | | | | |
| Median | 10 | | 10 | | 9.2 | | 12.6 | | 10.5 | | 10 | |
| ≥10 cm | 166/324 | 51 | 20/36 | 56 | 84/180 | 47 | 6 | 67 | 26 | 59 | 30/56 | 58 |
| s-IL2R after first-line therapy, ≥1000 U/mL | 141/305 | 46 | 20/30 | 67 | 91/175 | 52 | 2/8 | 25 | 17/40 | 43 | 33/49 | 67 |
| Presence of pleural or pericardial effusion | 159/343 | 46 | 15/43 | 35 | 83/186 | 43 | 5 | 56 | 23 | 51 | 31 | 54 |
| WBC, >10×10⁹/L | 23/339 | 7 | 2/42 | 5 | 12/184 | 7 | 0 | 0 | 5 | 11 | 3/56 | 5 |
| Hemoglobin, ≤12 g/dL | 119/329 | 36 | 16/39 | 41 | 57/81 | 31 | 3 | 33 | 21 | 47 | 19/52 | 37 |
| Platelet count, <150×10⁹/L | 20/331 | 6 | 2/40 | 5 | 16/182 | 9 | 0 | 0 | 0 | 0 | 2/52 | 4 |
| ALC at diagnosis, <0.5×10⁹/L | 62/321 | 19 | 2/33 | 6 | 29/180 | 16 | 5 | 56 | 12 | 27 | 13/52 | 25 |
| IHC staining, positive | | | | | | | | | | | | |
| CD20 | 152/152 | 100 | 15/15 | 100 | 99/99 | 100 | 5/5 | 100 | 8/8 | 100 | 25/25 | 100 |
| CD10 | 4/129 | 3 | 1/11 | 9 | 2/85 | 2 | 0/5 | 0 | 0/7 | 0 | 1/21 | 5 |
| CD30 | 100/140 | 71 | 9/13 | 69 | 62/85 | 70 | 5/5 | 100 | 5/8 | 63 | 18/25 | 72 |
| BCL6 | 72/116 | 61 | 8/11 | 73 | 46/75 | 61 | 2/5 | 40 | 4/6 | 67 | 12/19 | 63 |
| MUM1 | 105/109 | 96 | 10/11 | 91 | 67/68 | 99 | 4/5 | 80 | 6/6 | 100 | 18/19 | 95 |
| PDL-1 | 68/110 | 62 | 7/11 | 64 | 44/68 | 65 | 2/5 | 40 | 1/5 | 20 | 14/21 | 67 |
| Treatment | | | | | | | | | | | | |
| Administration of rituximab | 267 | 77 | 0 | 0 | 187 | 100 | 9 | 100 | 28 | 62 | 43 | 75 |
| Consolidation RT | 145 | 42 | 21 | 48 | 64 | 34 | 4 | 44 | 30 | 67 | 24 | 42 |
| Late adverse event | | | | | | | | | | | | |
| Secondary cancer | 7 | 2 | 1 | 2 | 4 | 2 | 0 | 0 | 0 | 0 | 2 | 4 |
| Cardiac toxicity | 10 | 3 | 0 | 0 | 9 | 5 | 0 | 0 | 0 | 0 | 1 | 2 |

*CHOP: cyclophosphamide, adriamycin, vincristine and prednisone; R: rituximab; DA-EPOCH-R: dose-adjusted etoposide, cyclophosphosphamide, doxorubicin, vincristine, prednisolone and rituximab; HDT/ASCT: high-dose chemotherapy followed by autologous stem cell transplantation; PS: performance status; LDH: lactate dehydrogenase; ULN: upper limit of normal; IPI: international prognostic index; s-IL2R: soluble interleukin-2 receptor; WBC: white blood cell count; ALC: absolute lymphocyte count; IHC: immunohistochemical staining; RT: radiation therapy.*

### Treatment regimen

In all, 267 patients received rituximab-containing chemotherapy. CHOP and R-CHOP chemotherapy groups consisted of 44 and 187 patients, respectively. DA-EPOCH-R chemotherapy was administered to 9 patients. In the 2nd-/3rd-generation regimen group (n=45), 28 patients received MACOP-B with (n=18) or without (n=10) rituximab, 15 patients received cyclophosphamide, vincristine, bleomycin, etoposide, doxorubicin and prednisolone (CyclOBEAP)[33] with (n=12) or without (n=3) rituximab, and 2 patients received vincristine, cyclophosphamide, doxorubicin, ranimustine, vindesine, etoposide carboplatin and prednisone (JCOG-LSG15 study regimen).[34] In the HDT/ASCT group (n=57), 43 patients received rituximab-containing chemotherapy as the initial chemotherapy. Consolidative RT was given to 42% of all patients. After approval of the use of rituximab for DLBCL in Japan in 2003, the use of rituximab-containing regimens rapidly increased, as shown in *Online Supplementary Table S1*. There was a moderate decrease in the use of HDT/ASCT and radiation therapy after initial treatment. The DA-EPOCH-R regimen was selected in the latest period.

### Clinical outcomes

With a median follow up of 48 months in surviving patients, the OS and PFS at four years were 87% and 70%, respectively (Figure 1A and B). The OS and PFS of patients treated with rituximab-containing chemotherapy were superior to those of patients receiving chemotherapy without rituximab (4-year OS: 91% *vs.* 77%, $P<0.001$; 4-year PFS: 75% *vs.* 54%, respectively, $P<0.001$). There was no difference in the risk of central nervous system (CNS) relapse between patients treated with and patients treated without rituximab as first-line treatment (3.8% *vs.* 1.3%; $P=0.251$). The OS at four years for patients treated with CHOP, R-CHOP, DA-EPOCH-R, the 2nd-/3rd-generation regimens, and HDT/ASCT was 67%, 90%, 100%, 91% and 92%, respectively, with median follow-up durations of 118 months, 36 months, 19 months, 48 months and 101 months, respectively ($P<0.001$) (Figure 1C); PFS at four years was 40%, 71%, 100%, 83% and 76%, respectively ($P<0.001$) (Figure 1D).

Secondary malignancies and cardiac toxicity developed after treatment in 7 and 10 patients, respectively. The median age of these 17 patients was 62 years. Seven of 17

Table 2. **Risk factors for overall survival, progression-free survival and early relapse for patients treated with R-CHOP without consolidative radium therapy.**

| | OS | | | | PFS | | | | Early relapse | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | univariate analysis | | | | univariate analysis | | | | multivariate analysis | | | | univariate analysis | | | | multivariate analysis | | | |
| Variables | HR | 95% CI | | P | HR | 95% CI | | P | HR | 95% CI | | P | HR | 95% CI | | P | HR | 95% CI | | P |
| Effusion present | 4.93 | 1.37-17.69 | | 0.015 | 4.67 | 2.28-9.57 | | <0.001 | 3.53 | 1.69-7.40 | | 0.001 | 6.45 | 2.45-16.98 | | <0.001 | 6.11 | 2.30-16.24 | | <0.001 |
| Age > 60 years | 2.23 | 0.75-6.68 | | 0.150 | 0.62 | 0.26-1.48 | | 0.282 | – | – | | – | 0.14 | 0.019-1.05 | | 0.056 | – | – | | – |
| Sex Male | 1.35 | 0.47-3.89 | | 0.584 | 0.93 | 0.50-1.73 | | 0.821 | – | – | | – | 0.67 | 0.31-1.43 | | 0.299 | – | – | | – |
| PS | > 1 | 4.50 | 1.56-12.97 | 0.005 | 2.85 | 1.49-5.47 | | 0.002 | – | – | | – | – | 2.68 | 1.25-5.73 | 0.011 | – | – | | – |
| Extranodal sites > 1 | 2.47 | 0.83-7.37 | | 0.106 | 2.28 | 1.16-4.51 | | 0.017 | – | – | | – | 2.38 | 1.08-5.27 | | 0.032 | 1.75 | 0.79-3.91 | | 0.169 |
| Stage III/IV | 1.75 | 0.61-5.06 | | 0.300 | 2.76 | 1.47-5.18 | | 0.002 | 2.16 | 1.14-4.11 | | 0.018 | 2.89 | 1.37-6.09 | | 0.005 | – | – | | – |
| LDH > ULN | 1.80 | 0.50-6.46 | | 0.369 | 3.72 | 1.45-9.53 | | 0.006 | 2.28 | 0.86-6.00 | | 0.096 | 3.02 | 1.05-8.71 | | 0.041 | – | – | | – |
| B symptoms present | 0.74 | 0.17-3.32 | | 0.697 | 1.08 | 0.49-2.35 | | 0.853 | – | – | | – | 1.55 | 0.68-3.53 | | 0.292 | – | – | | – |
| IPI ≥ 3 | 4.23 | 1.48-12.13 | | 0.007 | 2.94 | 1.55-5.57 | | 0.001 | – | – | | – | 2.95 | 1.40-6.25 | | 0.005 | – | – | | – |
| Tumor diameter ≥ 10 cm | 1.31 | 0.44-3.90 | | 0.150 | 2.40 | 1.26-4.60 | | 0.088 | – | – | | – | 3.69 | 1.61-8.43 | | 0.002 | – | – | | – |
| s-IL2R > 1000 U/L | 1.88 | 0.57-6.25 | | 0.302 | 2.40 | 1.18-4.90 | | 0.016 | – | – | | – | 1.93 | 0.85-4.37 | | 0.115 | – | – | | – |
| Serum albumin < 3.5 g/dL | 1.82 | 0.56-5.89 | | 0.322 | 1.46 | 0.69-3.10 | | 0.321 | – | – | | – | 1.80 | 0.79-4.10 | | 0.159 | – | – | | – |
| ALC < 0.5×10⁹/L | 1.15 | 0.26-5.15 | | 0.855 | 1.17 | 0.49-2.79 | | 0.728 | – | – | | – | 1.33 | 0.50-3.50 | | 0.566 | – | – | | – |
| Hemoglobin <12 g/dL | 1.86 | 0.64-5.37 | | 0.253 | 1.17 | 0.60-2.29 | | 0.643 | – | – | | – | 1.20 | 0.55-2.59 | | 0.651 | – | – | | – |
| Platelet count <150×10⁹/L | 2.15 | 0.48-9.65 | | 0.316 | 1.82 | 0.71-4.67 | | 0.316 | – | – | | – | 0.93 | 0.22-3.91 | | 0.919 | – | – | | – |

*OS: overall survival; PFS: progression-free survival; R-CHOP: rituximab, cyclophosphamide, adriamycin, vincristine and prednisone; RT: radiation therapy; HR: hazard ratio; CI: confidence interval; Effusion: pleural or pericardial effusion; PS: performance status; LDH: lactate dehydrogenase; ULN: upper limit of normal; IPI: international prognostic index; s-IL2R: soluble interleukin-2 receptor; ALC: absolute lymphocyte count.*

patients received RT or ASCT as first-line treatment. In addition, 3 of 7 patients who developed secondary malignancies received RT during the initial series of treatment. Among the secondary malignancies, myelodysplastic syndrome (MDS) or acute myeloblastic leukemia (AML) was reported in 2 patients. The patient who developed MDS received HDT/ASCT as a first-line treatment. The patient who developed AML received CHOP as a first-line treatment and ICE as a salvage treatment. Among the 187 patients treated with R-CHOP, 9 experienced cardiac toxicity, and 4 developed a secondary cancer. The median time to development of a secondary malignancy was 40.5 months (range 9-200 months).

### *Patients' characteristics and clinical outcomes in the R-CHOP group*

Detailed characteristics of patients in the R-CHOP group are shown in *Online Supplementary Table S2*. We divided this group into four subgroups according to the disease status after R-CHOP or R-CHOP-like regimen and the presence or absence of consolidative RT: namely, R-CHOP+RT with residual mass, R-CHOP+RT in CR, R-CHOP with residual mass and R-CHOP in CR. Among the 187 patients in the R-CHOP group, 64 patients received consolidative RT after R-CHOP (*Online Supplementary Table S3*). Elderly age and higher IPI score were less common in those who received consolidative RT. Thirty-three of 64 patients received consolidative RT with residual mass after R-CHOP, while 31 of 64 patients received RT in CR after R-CHOP. Among the remaining 123 patients without consolidative RT, 34 patients did not achieve CR after R-CHOP, and 89 patients were in CR after R-CHOP, respectively. Among 34 patients with residual mass who were treated with R-CHOP, 16 patients developed progressive disease (PD), and 4 patients received follow up without RT based on the negative findings on PET/CT after the initial series of treatment. Of 89 patients who achieved a CR after R-CHOP but did not receive RT, 14 patients experienced relapse. Among these 14 patients, 9 developed the relapsed disease in their mediastinum, while the remaining 5 relapsed in other sites. The OS and PFS at four years of patients receiving consolidative RT were 100% and 85%, respectively, in the group with residual mass, and 96% and 90%, respectively, in the

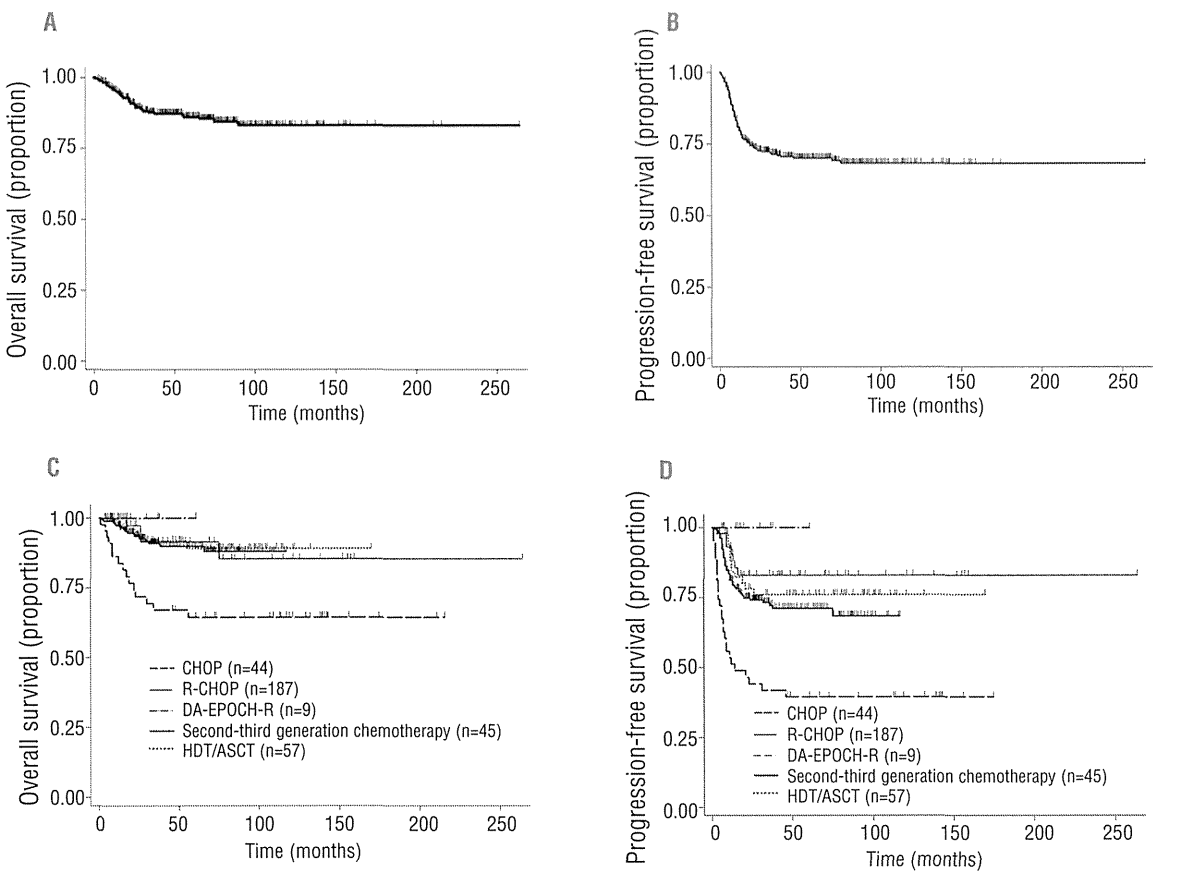


Figure 1. Survival of patients with primary mediastinal large B-cell lymphoma. (A) Overall survival (OS) of all patients with primary mediastinal large B-cell lymphoma (PMBL). (B) Progression-free survival (PFS) of all patients with PMBL. (C) OS of patients with PMBL treated with CHOP (n=44), R-CHOP (n=188), DA-EPOCH-R (n=9), 2nd- or 3rd-generation regimens (n=45), and HDT/ASCT (n=57). (D) PFS of patients with PMBL treated with CHOP (n=44), R-CHOP (n=188), DA-EPOCH-R (n=9), 2nd- or 3rd-generation regimens (n=45), and HDT/ASCT (n=57). CHOP: cyclophosphamide, adriamycin, vincristine and prednisone; R: rituximab; DA-EPOCH-R: dose-adjusted etoposide, cyclophosphosphamide, doxorubicin, vincristine, prednisolone and rituximab; HDT/ASCT: high-dose chemotherapy followed by autologous stem cell transplantation.

group in CR (OS: $P$=0.15; PFS: $P$=0.80) (*Online Supplementary Figures S1 and S2*). Meanwhile, the OS and PFS at four years of patients who did not receive consolidative RT were 64% and 35%, respectively, in the group with residual mass without disease progression, and 95% and 77%, respectively, in the group in CR (OS: $P$<0.001; PFS: $P$<0.001). Taken together, these data indicate that a significant proportion of patients achieving CR after R-CHOP can be cured without consolidative RT.

### Prognostic factors and survival for patients treated with R-CHOP and without consolidative radiotherapy

One hundred and twenty-three patients receiving R-CHOP without consolidative RT were analyzed. The analysis of potential prognostic factors is shown in Table 2. On univariate analysis, the presence of pleural or pericardial effusion, performance status (PS) over 1 and higher IPI were adverse prognostic factors for OS, and the presence of pleural or pericardial effusion, advanced stage, extranodal involvement, PS, LDH, soluble interleukin-2 receptor (sIL-2R), and higher IPI were adverse prognostic factors for PFS. On multivariate analysis, we could not identify significant prognostic factors for OS. The presence of pleural or pericardial effusion [hazard ratio (HR), 3.53; 95% confidence interval (CI), 1.69-7.40; $P$=0.001] and advanced stage (stage III/IV; HR, 2.16; 95%CI: 1.14-4.11; $P$=0.018) were identified as adverse prognostic factors for PFS. As almost all the patients with progression after R-CHOP developed disease within 12 months after diagnosis, we performed Cox regression analyses to determine the predictive factors for primary refractory or early relapse within 12 months after diagnosis. On multivariate analysis, only the presence of pleural or pericardial effusion was predictive of primary refractory or early relapse within 12 months (HR, 6.11; 95%CI: 2.30-16.24; $P$<0.001). In this cohort, only 5 (8%) of 65 patients without pleural or pericardial effusion experienced primary refractory or early relapse within 12 months; meanwhile, 25 (43%) of 58 patients with pleural or pericardial effusion ($P$<0.001) had refractory or early relapsed disease.

As IPI and the presence of pleural or pericardial effusion were prognostic factors for OS on univariate analysis, and these were not related (correlation coefficient = 0.39), the OS and PFS in patients receiving R-CHOP without RT were analyzed according to these prognostic factors. The OS and PFS in patients receiving R-CHOP without RT were analyzed according to the presence of pleural or pericardial effusion and IPI. As expected (Figure 2A and B), the best OS and PFS were observed in patients with low IPI and without pleural or pericardial effusion. The OS and

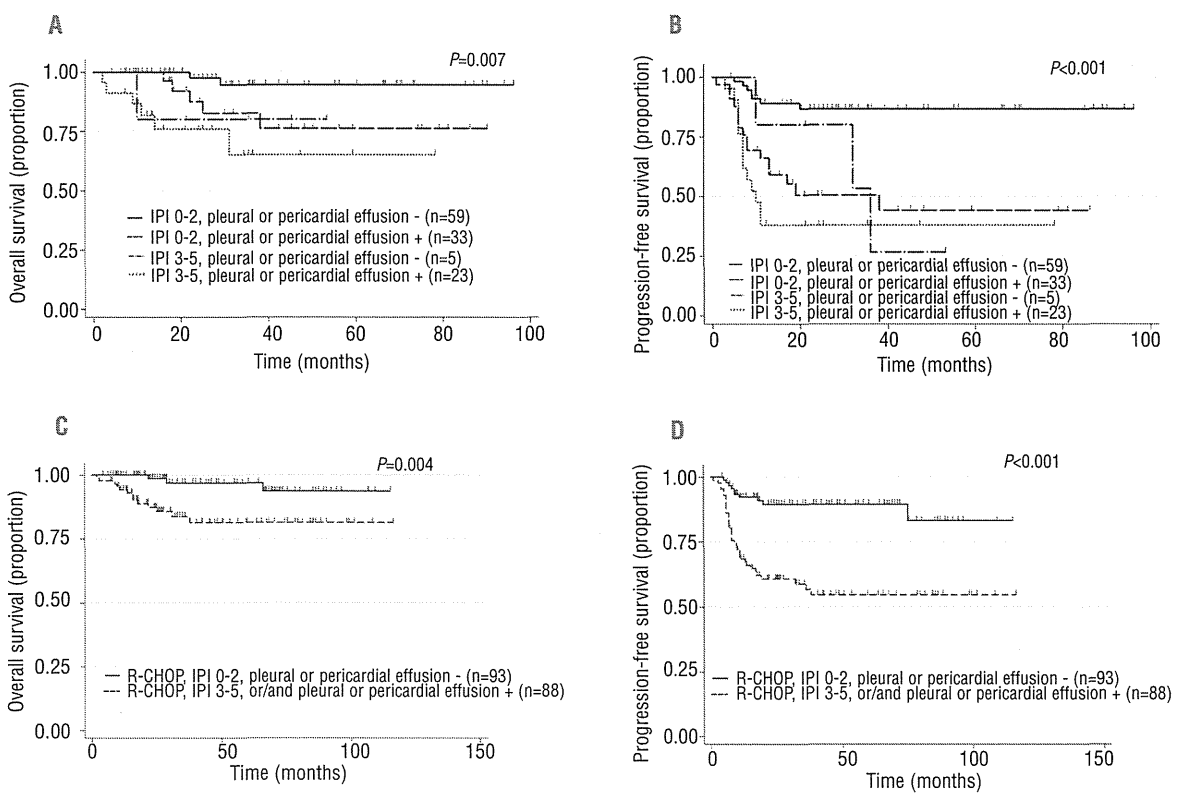


Figure 2. Survival of patients with primary mediastinal large B-cell lymphoma according to the International Prognostic Index and the presence pleural or pericardial effusion. (A) Overall survival (OS) of patients with primary mediastinal large B-cell lymphoma (PMBL) treated with R-CHOP without radiation therapy (RT) according to the international prognostic index (IPI) and the presence pleural or pericardial effusion. (B) Progression-free survival (PFS) of patients with PMBL treated with R-CHOP without RT according to the IPI and the presence pleural or pericardial effusion. (C) OS of patients with PMBL treated with R-CHOP according to the IPI and the presence pleural or pericardial effusion. (D) PFS of patients with PMBL treated with R-CHOP according to the IPI and the presence pleural or pericardial effusion. R-CHOP: rituximab, cyclophosphamide, adriamycin, vincristine and prednisone; RT: radiation therapy.

PFS at four years of these 58 patients were 95% and 87%, respectively. Meanwhile, based on individual factors of LDH, B symptom, and pleural or pericardial effusion identified on multivariate analysis for PFS, the OS and PFS were also analyzed (*Online Supplementary Figures S3 and S4*). Although the OS and PFS could be well stratified, the number of patients categorized into the well stratified low-risk category was lower than that of patients under the stratification using IPI and effusion. Taken together, these data indicate that a significant proportion of patients with low IPI and without pleural or pericardial effusion at the time of diagnosis can be potentially cured by the R-CHOP regimen without consolidative RT.

Meanwhile, the treatment should be considered for patients with higher IPI and the presence of pleural or pericardial effusion. As shown in Figure 2C and D, the outcomes of R-CHOP were not satisfactory in patients with higher IPI and/or the presence of pleural or pericardial effusion (4-year OS: 97% *vs.* 81%, $P$=0.004; 4-year PFS: 89% *vs.* 54%, $P$<0.001, respectively).

## Discussion

It is important to establish a more effective and less toxic standard treatment for PMBL, as affected patients tended to be young and can be cured when properly treated. The present study investigated a larger cohort than other studies and indicated that almost all PMBL patients with lower IPI and the absence of the pleural or pericardial effusion could be cured by the R-CHOP regimen without consolidative RT. Considering the excellent outcomes of the recent promising regimen DA-EPOCH-R, reported by Dunleavy *et al.*,[27] the initial treatment regimen for PMBL could be stratified according to our simple indicators of IPI score and the presence of pleural or pericardial effusion; DA-EPOCH-R or R-CHOP could be selected for high- or low-risk PMBL patients, respectively.

Consistent with other studies, patients who received rituximab-containing chemotherapies showed better outcomes.[17-22,27,35] HDT/ASCT and 2$^{nd}$-/3$^{rd}$-generation regimens that were more intensive and that have been historically used as first-line treatment for PMBL resulted in better outcomes than those seen in response to CHOP chemotherapy.[11,17,18,36] In the present study, similar OS and PFS was observed among patients treated with a 2$^{nd}$-/3$^{rd}$-generation regimen, HDT/ASCT, and R-CHOP. This suggests that R-CHOP regimen might have curative potential in a significant proportion of PMBL patients without utilizing 2$^{nd}$-/3$^{rd}$-generation regimen or HDT/ASCT and thereby avoiding their associated toxicities.

Late toxicities are another important issue to consider when weighing the benefits of different curative regimens. In the current study, 17 patients had late adverse events (secondary cancer, n=7; cardiac toxicity, n=10). Previous reports indicated that RT to the mediastinum significantly increased the risk of breast cancer and cardiac toxicity.[24-26,37] Although longer follow up is required to evaluate for late toxicities, we investigated whether we could omit the consolidative RT from the current treatment strategies. We analyzed the outcomes of patients treated with R-CHOP without consolidative RT, and identified higher IPI and the presence of pleural or pericardial effusion as adverse risk factors for OS. Moreover, the presence of the effusion was identified as an adverse risk factor for early relapse. Considering that previous studies had reported that the presence of pleural effusion was associated with poor outcomes in patients with PMBL and Hodgkin lymphoma,[21,38] our results might be universal. Our simple indicators could identify patients who could be cured in response to R-CHOP without consolidative RT; however, patients with these factors comprised only approximately one-half of patients receiving R-CHOP. This means the remaining patients should be treated with an alternative regimen. The fact that excellent outcomes were seen in patients with higher IPI and the presence of the effusion receiving DA-EPOCH-R regimen in this study, as well as in another recent report,[27] suggests that it may be reasonable to use this approach in high-risk PMBL patients. A prospective trial of this strategy is warranted.

Another approach to stratify PMBL patients is currently being investigated in Europe. The prospective IELSG-37 trial is investigating whether consolidative RT could be omitted according to the presence or absence of FDG-PET or PET/CT findings after the initial series of treatments. In clinical practice, we frequently encounter patients in whom it is difficult to judge FDG-PET positivity.[39,40] Unfortunately, we could not evaluate the role of PET/CT in this study because of retrospective settings. Meanwhile, the very recent report from the IELSG-26 study clarified the role of PET/CT after treatment in PMBL patients.[41] Considering the difficulty of re-biopsy of the suspected mediastinal mass after treatment, using the optimal cut-off value on PET/CT after treatment reported by IELSG could be an important tool to assess the risk of treatment failure.

This study has several limitations. First, its retrospective nature might have unrecognized biases and the results should be interpreted with care. Regarding evaluation of response, evaluation of the residual mass might have been heterogeneous at each institution because of the retrospective setting. Therefore, the CR rate in our study could be over-estimated. Second, patients received various treatment regimens and consolidative RT according to each institution's preferred strategy; thus, treatment outcomes might have been over-estimated or under-estimated. In particular, patients who did not receive consolidative RT might have had clinical indicators that physicians considered favorable, resulting in an overestimation of the clinical outcomes in response to R-CHOP without consolidative RT. However, in the present analysis, the proportion of patients with higher IPI and with the presence of effusion was not low in patients who did not receive consolidative RT compared with that in patients who did receive RT. This suggests that the base-line characteristics and outcomes of patients without consolidative RT were not necessarily favorable and that they might not have been over-estimated. Finally, we carried out a central pathological review for only 196 patients. We tried to collect as much pathological histological paraffin-embedded tissue materials as possible. However, in some cases, sufficient materials were not available because they were too old. In addition, the period during which data could be submitted differed because clinical data were kept for different lengths of time at the different institutions. Therefore, the number of institutions who could submit clinical data in the 1980s and 1990s was smaller than in the 2000s: 10 and 65 institutions before and after the year 2000, respectively. Furthermore, although gene expression or methylation profiling can help to diagnose PMBL correctly, for the moment we cannot use these tools in routine clinical prac-

tice. Further study of the utility of these biological tools is necessary to improve diagnosis and management of this disease.

In conclusion, the present study demonstrated that IPI and the presence of pleural or pericardial effusion were adverse prognostic factors for risk stratification of PMBL patients treated with R-CHOP. R-CHOP without consolidative RT can achieve a high rate of cure for approximately one-half of PMBL patients, while alternative regimens, including DA-EPOCH-R, should be offered to the remaining patients. Prospective studies to validate these prognostic factors and a risk-adopted treatment strategy are warranted.

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### *Authorship and Disclosures*

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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-$\gamma^+$ 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-$\gamma^+$ 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-$\gamma$ (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-$\gamma$, TNF-$\alpha$, and IL-1. Blazar et al. (14, 15) revealed that systemic IFN-$\gamma$ 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 $2 \times 10^6$ spleen T cells and $8 \times 10^6$ 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 $5 \times 10^6$ 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



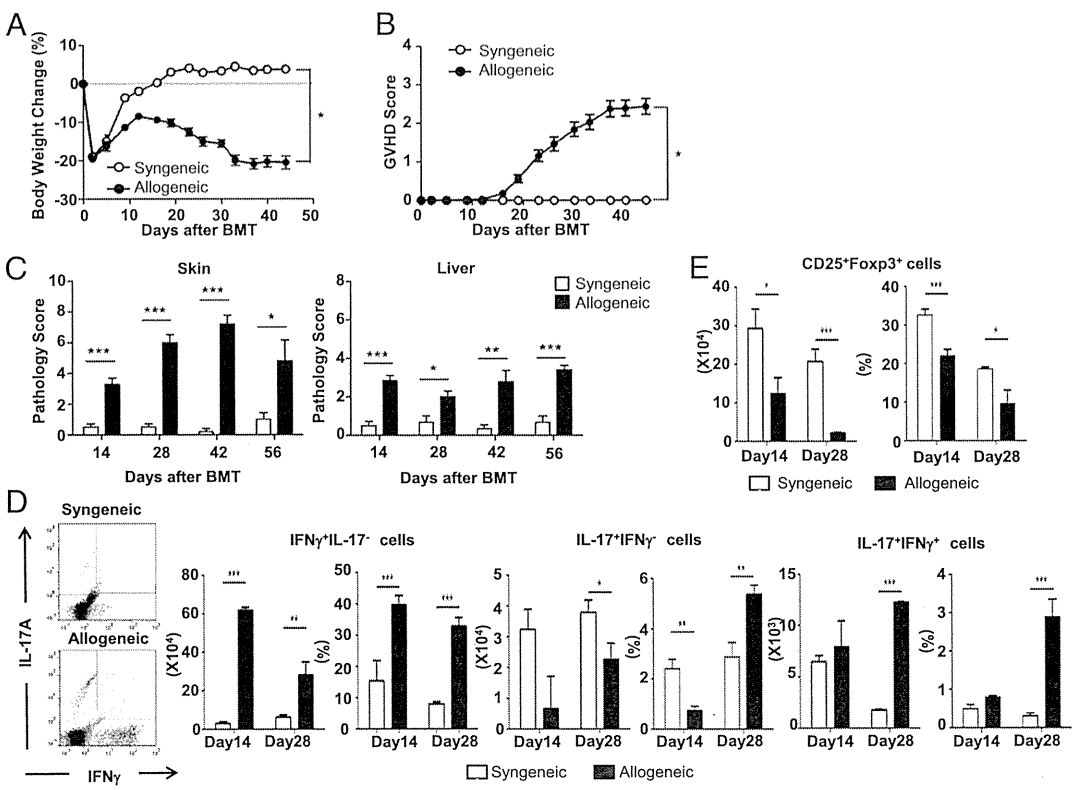


**FIGURE 1.** Th17 and Th1 cells are increased during chronic GVHD. (**A–E**) Sublethally irradiated (5.8 Gy) BALB/c mice were transplanted with $2 \times 10^6$ spleen T cells and $8 \times 10^6$ TCD-BM cells from B10.D2 mice (allogeneic group). The syngeneic group received a transplant of the same dose 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-$\gamma^+$IL-$17^-$, IL-$17^+$IFN-$\gamma^-$, and IL-$17^+$IFN-$\gamma^+$ 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 $1 \times 10^6$/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 $2 \times 10^6$ spleen T cells and $8 \times 10^6$ 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

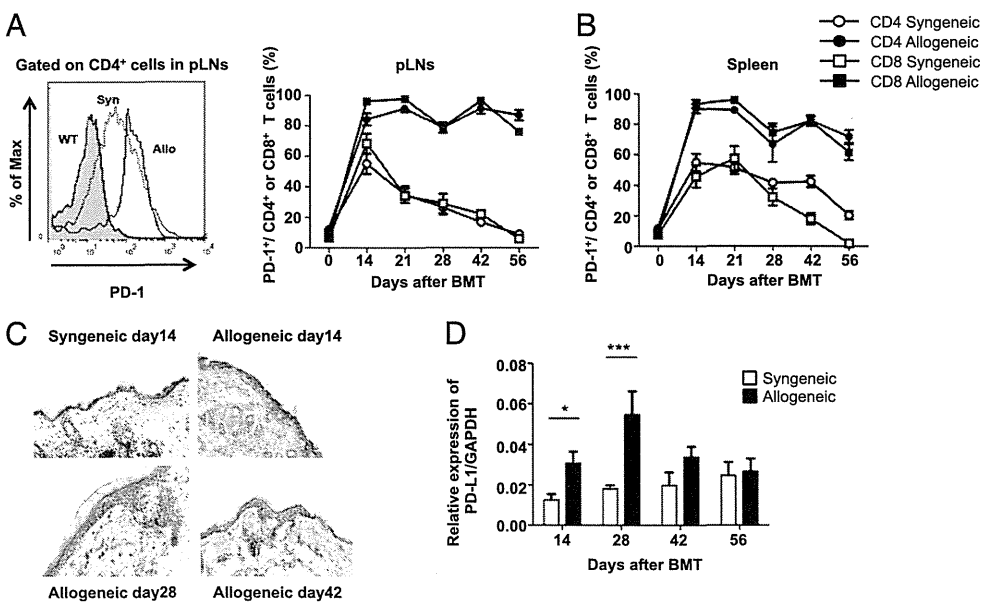


**FIGURE 2.** PD-1 expression on donor cells and PD-L1 expression in host tissues during chronic GVHD development. Sublethally irradiated BALB/c mice were transplanted from allogeneic B10.D2 or syngeneic BALB/c donors. (**A**) Representative histograms of PD-1 expression on CD4$^+$ T cells from pLNs 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). *$p < 0.05$, ***$p < 0.005$.

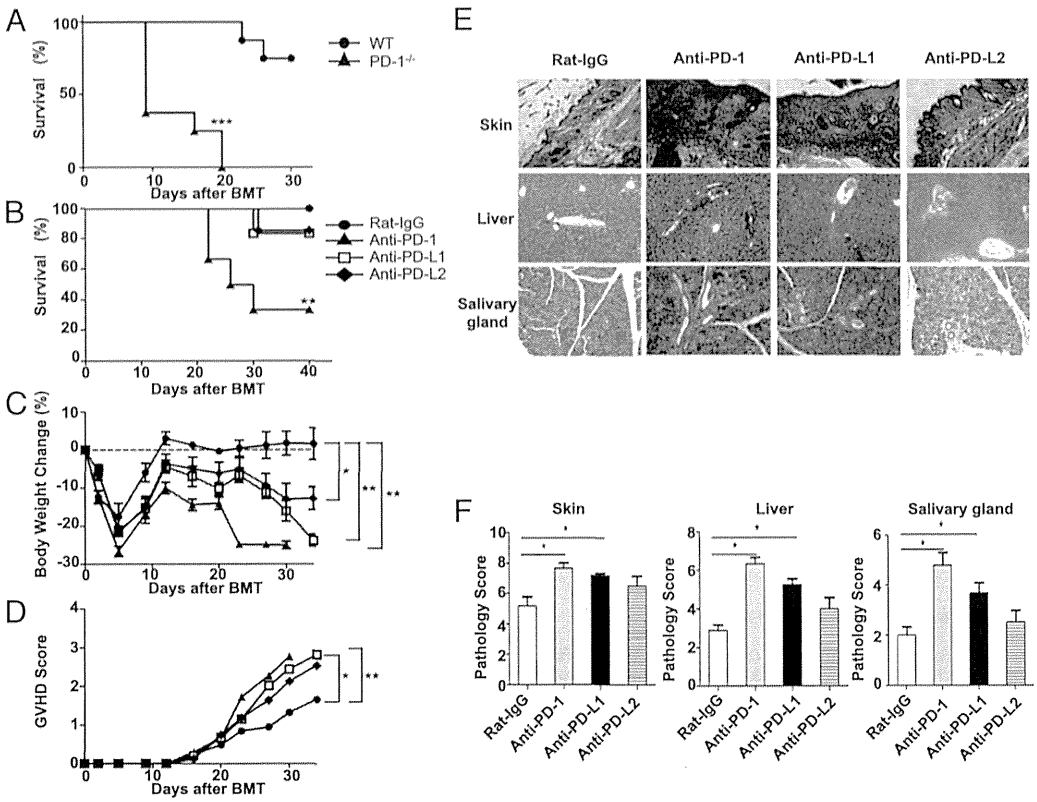


**FIGURE 3.** PD-1/PD-L blockade exacerbates chronic GVHD. (**A**) Sublethally irradiated BALB/c recipients were transplanted with $2 \times 10^6$ spleen T cells and $8 \times 10^6$ TCD-BM cells from WT or PD-$1^{-/-}$ B10.D2 donors. Survival data are shown; data shown are from 1 representative of $\geq 3$ 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 $\geq 3$ 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 $\geq 3$ 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-$\gamma^+$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

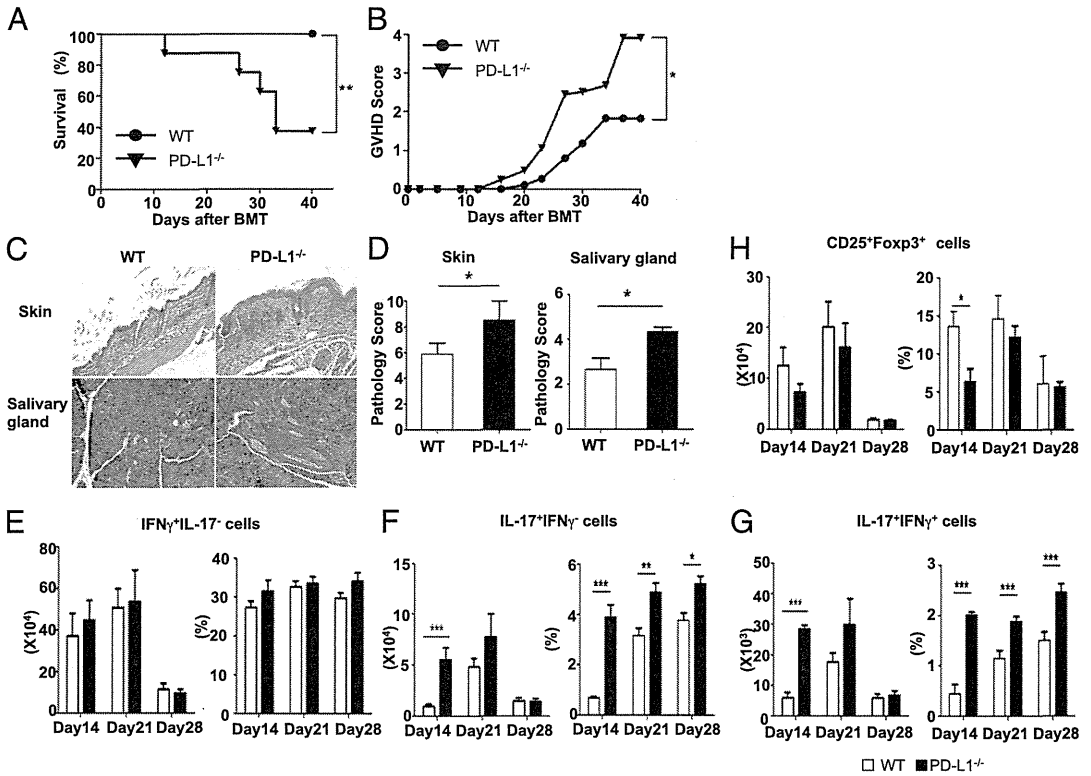


**FIGURE 4.** PD-L1 deficiency in recipients exacerbates chronic GVHD with Th17/Th1 cell expansion. (**A–D**) Sublethally irradiated WT or PD-L1$^{-/-}$ BALB/c recipients were transplanted from WT B10.D2 donors. Survival (A) and clinical GVHD skin score (B) are shown; data shown are from 1 representative of ≥3 independent experiments ($n$ = 8 in each group). (C and D) Skin and salivary gland from indicated recipients were taken on day 36 after BMT. (C) Representative images with Masson trichrome staining are shown (original magnification ×100). (D) Pathology score of skin and salivary gland on day 36 after BMT is shown. The numbers and percentages of donor-derived CD4$^+$ T cells expressing IFN-γ$^+$IL-17$^-$ (**E**), IL-17$^+$IFN-γ$^-$ (**F**), and IL-17$^+$ IFN-γ$^+$ cells (**G**), and CD25$^+$ Foxp3$^+$ cells (**H**) from pLNs on days 14, 21, and 28 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). *$p < 0.05$, **$p < 0.01$, ***$p < 0.005$.

those of the control group ($p < 0.01$, Fig. 3C; $p < 0.05$, Fig. 3D). Pathologic scores of skin and liver were significantly higher in anti–PD-L1–treated mice than in the controls (skin: 7.17 ± 0.17 versus 5.20 ± 0.58, $p < 0.05$; liver: 5.25 ± 0.31 versus 2.75 ± 0.25, $p < 0.05$; salivary gland: 3.67 ± 0.42 versus 2.00 ± 0.32, $p < 0.05$; Fig. 3E, 3F). Clinical and pathogenic scores tended to be worse in anti–PD-L2–treated mice, as compared with those treated with control, although it was not statistically significant (Fig. 3E, 3F). These findings suggest that the PD-1 pathway, especially the PD-1/PD-L1 pathway, plays a critical role in suppressing lethal chronic GVHD.

### *Lack of PD-L1 expression exacerbated chronic GVHD with IL-17$^+$IFN-γ$^+$ T cell expansion*

The anti–PD-L1 mAb neutralized PD-L1 on host cells, as well as on donor cells. To evaluate the contribution of host PD-L1 to chronic GVHD, we used PD-L1$^{-/-}$ mice on BALB/c background as recipients. On transferring WT donor T cells into PD-L1$^{-/-}$ recipients, survival was shortened significantly ($p < 0.01$; Fig. 4A) and skin chronic GVHD scores were enhanced in comparison with WT recipients ($p < 0.05$; Fig. 4B). Histopathologic examination of skin and salivary gland showed that exacerbated GVHD in PD-L1$^{-/-}$ recipients was not simply shifted toward acute GVHD, but rather significantly exacerbated chronic GVHD pathology with decreased fat, dermal fibrosis, epidermal interface changes, diffuse hair loss, and inflammatory cell invasion of skin, fibrosis, and atrophy of salivary gland (skin: 5.88 ± 0.85 versus 8.38 ± 0.38, $p < 0.05$; salivary gland: 2.67 ± 0.49 versus 4.33 ± 0.21, $p < 0.05$; Fig. 4C, 4D).

Our previous study and the current results (Fig. 1D, 1E) showed that Th17/Th1 cell expansion was detected during chronic GVHD and contributed to chronic GVHD progression (27). We next assessed Th subsets from pLNs of WT and PD-L1$^{-/-}$ recipients. Absolute numbers of IFN-γ$^+$IL-17$^-$, IL-17$^+$IFN-γ$^-$, and IL-17$^+$ IFN-γ$^+$ CD4$^+$ T cells from pLNs of PD-L1$^{-/-}$ recipients were modestly increased from days 14 to 21 and declined to the same levels between WT PD-L1$^{-/-}$ recipients because of lymphocytopenia of chronic GVHD (Fig. 4E–G). Intracellular staining showed that no differences were observed in frequency of IFN-γ$^+$ IL-17$^-$ T cells between PD-L1$^{-/-}$ and WT recipients; however, IL-17$^+$IFN-γ$^+$ T cells were detected significantly more frequently in PD-L1$^{-/-}$ recipients from days 14 to 28 ($p < 0.005$; Fig. 4G). In contrast, CD4$^+$ CD25$^+$ Foxp3$^+$ Tregs from PD-L1$^{-/-}$ recipients were detected less frequently on day 14 than in WT recipients ($p < 0.05$), but levels were similar on days 21 and 28 (Fig. 4H). These results suggest that host PD-L1 deficiency exacerbated chronic GVHD in conjunction with IL-17$^+$IFN-γ$^+$ T cell expansion.

### *PD-L1 expression on host tissues contributes to chronic GVHD augmentation*

To separate the role of PD-L1 on host APCs from host tissues, we generated chimeric recipients expressing PD-L1 on only hematopoietic cells or host tissues. Three types of chimeras were prepared: (WT→WT), (WT→PD-L1$^{-/-}$), and (PD-L1$^{-/-}$→WT). The three types of chimera mice were sublethally irradiated and then transplanted with 2 × 10$^6$ spleen T cells and 8 × 10$^6$ TCD-BM cells from B10.D2 mice. (PD-L1$^{-/-}$→WT) recipients showed

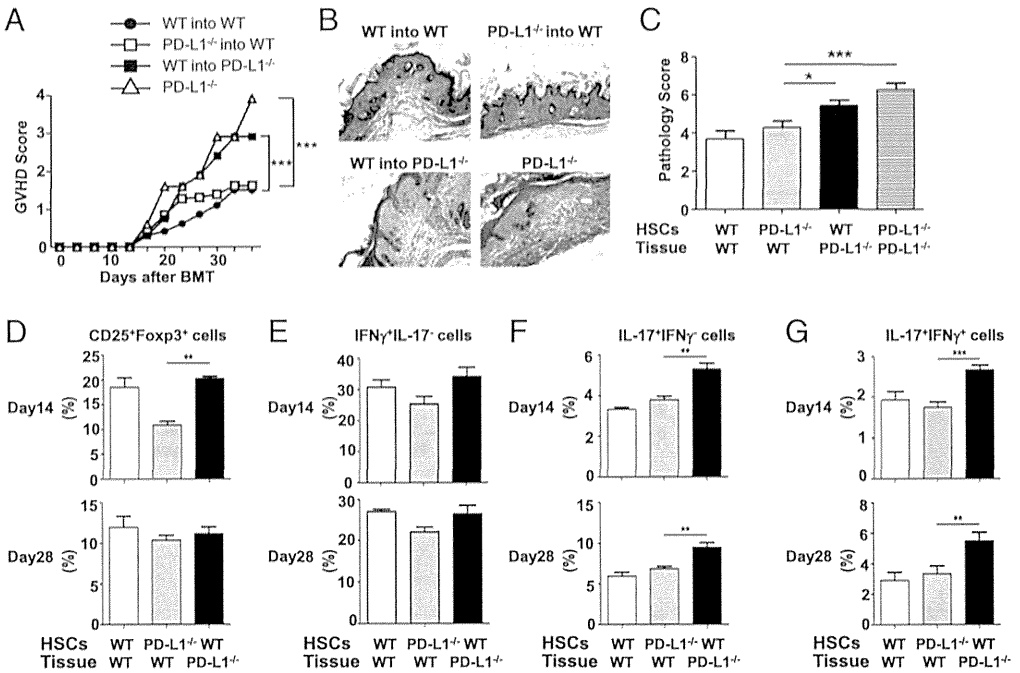


**FIGURE 5.** PD-L1 expression on host tissues contributes to chronic GVHD augmentation. (**A–G**) Sublethally irradiated (WT into WT), (PD-L1$^{-/-}$ into WT), (WT into PD-L1$^{-/-}$) chimera BALB/c recipients and PD-L1$^{-/-}$ BALB/c recipients were transplanted from WT B10.D2 donors. Clinical GVHD skin scores (A) are shown; data shown are from 1 representative of ≥3 independent experiments ($n$ = 8 in each group). Skin tissues from the recipients were taken on day 36 after BMT. (B) Representative images are shown (original magnification ×100). (C) Pathology scores of skin on day 36 after BMT are shown. The percentages of donor-derived CD4$^+$ T cells expressing CD25$^+$ Foxp3$^+$ (D), IFN-γ$^+$IL-17$^-$ (E), IL-17$^+$IFN-γ$^-$ (F), and IL-17$^+$IFN-γ$^+$ cells (G) from pLNs of (WT into WT), (PD-L1$^{-/-}$ into WT), and (WT into PD-L1$^{-/-}$) recipients 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 ($n$ = 6–8 in each group). *$p < 0.05$, **$p < 0.01$, ***$p < 0.005$.

similar clinical chronic GVHD to (WT→WT) recipients. In contrast, clinical chronic GVHD scores were exacerbated significantly in (WT→PD-L1$^{-/-}$) recipients compared with (WT→WT) recipients (Fig. 5A). Histopathologic examination also showed significantly exacerbated chronic GVHD pathology in (WT→PD-L1$^{-/-}$) recipients compared with (WT→WT) recipients (5.43 ± 0.30 versus 3.67 ± 0.42; $p < 0.05$; Fig. 5B, 5C).

We assessed CD4$^+$ CD25$^+$ Foxp3$^+$ Tregs and Th17/Th1 expansion in pLNs of chimera recipients. CD4$^+$ CD25$^+$ Foxp3$^+$ Tregs from (PD-L1$^{-/-}$→WT) recipients were detected less frequently on day 14 than in (WT→WT) and (WT→PD-L1$^{-/-}$) recipients ($p < 0.01$), but at similar levels on day 28 ($p = 0.36$; Fig. 5D). Intracellular staining also showed that IFN-γ$^+$IL-17$^-$ CD4$^+$ T cells from (WT→PD-L1$^{-/-}$) recipients were almost identical to (PD-L1$^{-/-}$→WT) and (WT→WT) recipients on days 14 and 28 after BMT (Fig. 5E). IL-17$^+$IFN-γ$^-$ and IL-17$^+$IFN-γ$^+$ CD4$^+$ T cells from (WT→PD-L1$^{-/-}$) recipients were increased and detected significantly more frequently than in (PD-L1$^{-/-}$→WT) recipients on days 14 and 28 after BMT (IL-17$^+$IFN-γ$^-$; day 14: $p < 0.01$; day 28: $p < 0.01$; Fig. 5E, IL-17$^+$IFN-γ$^+$; day 14: $p < 0.005$; day 28: $p < 0.01$; Fig. 5F). Collectively, these findings indicated that PD-L1 expression in host tissues was involved in suppressing the expansion of IL-17$^+$IFN-γ$^+$ T cells, attenuating chronic GVHD, and that PD-L1 expression on hematopoietic cells plays a role in the development of Tregs only during the early transplantation period but does not affect chronic GVHD severity.

### *Administration of Am80 overcomes the IL-17$^+$IFN-γ$^+$ T cell expansion caused by PD-L1 deficiency*

Next, we examined whether the synthetic retinoid Am80 could alleviate chronic GVHD in PD-L1$^{-/-}$ recipients, because in a previous study we showed that Am80 suppressed Th17/Th1 cells (29). Recipients were administered Am80 orally (1.0 mg/kg) from day 0 after BMT. Am80 significantly ameliorated the clinical score not only in WT recipients, but also in PD-L1$^{-/-}$ recipients compared with the control group ($p < 0.005$; Fig. 6A). Histopathologic examination showed significantly reduced chronic GVHD skin damage in Am80-treated animals (WT vehicle: 5.50 ± 0.29 versus WT Am80: 2.83 ± 0.40, $p < 0.01$; PD-L1$^{-/-}$ vehicle: 8.13 ± 0.52 versus PD-L1$^{-/-}$ Am80: 3.29 ± 0.47, $p < 0.005$; Fig. 6B, 6C). CD4$^+$ CD25$^+$ Foxp3$^+$ Tregs from the Am80-treated groups of WT and PD-L1$^{-/-}$ recipients were at similarly low frequencies only on day 14 but at similar levels on day 28 in each group (Fig. 6D). In contrast, the Am80-treated groups of both WT and PD-L1$^{-/-}$ recipients showed decreased IFN-γ$^+$IL-17$^-$, IL-17$^+$ IFN-γ$^-$ (day 28, WT vehicle versus WT Am80, $p < 0.05$; PD-L1$^{-/-}$ vehicle versus PD-L1$^{-/-}$ Am80, $p < 0.005$; Fig. 6D) and IL-17$^+$IFN-γ$^+$ cells (day 28, WT vehicle versus WT Am80, $p < 0.005$; PD-L1$^{-/-}$ vehicle versus PD-L1$^{-/-}$ Am80, $p < 0.005$; Fig. 6D) on days 14 and 28. These findings suggest that Am80 administration overcame the IL-17$^+$ IFN-γ$^+$ cell expansion caused by PD-L1 deficiency, resulting in reduced chronic GVHD damage in PD-L1$^{-/-}$ recipients.

### *Administration of anti–PD-1 agonistic Ab alleviates chronic GVHD*

Donor CD4$^+$ and CD8$^+$ T cells in pLNs and spleen from both vehicle- and Am80-treated WT recipients showed similar expression levels of PD-1 (Supplemental Fig. 2A). Immunohistochemical analysis and mRNA quantitation of skin from Am80-treated recipients showed reduced PD-L1 expression compared with that from vehicle-treated recipients (Supplemental Fig. 2B, 2C). Thus, Am80 administration reduced chronic GVHD damage via suppressing IL-17$^+$IFN-γ$^+$ T cell expansion caused by impaired PD-L1 expression and did not directly affect the PD-1 pathway. Finally, to directly assess the role of therapeutic modu-

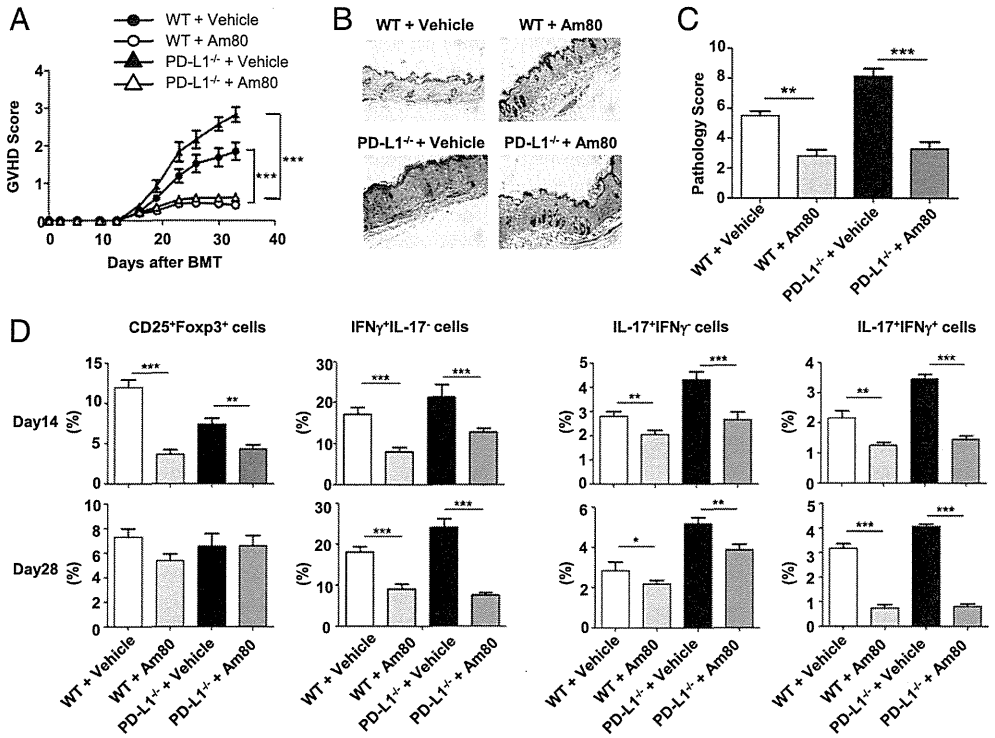


**FIGURE 6.** Administration of Am80 alleviates chronic GVHD enhanced by PD-L1 deficiency. (**A–D**) Sublethally irradiated WT and PD-L1$^{-/-}$ BALB/c recipients were transplanted from WT B10.D2 donors. These recipients received daily administration of Am80 (1.0 mg/kg body weight) or vehicle solution orally after BMT and were assessed for clinical signs of chronic GVHD every 3 d. Clinical GVHD skin scores (A) are shown; data shown are from 1 representative of ≥3 independent experiments ($n$ = 8 in each group). Skin tissues from the recipients were taken on day 35 after BMT. (B) Representative images are shown (original magnification ×100). (C) Pathology scores of skin on day 35 after BMT are shown. (D) The percentages of donor-derived CD4$^+$ T cells expressing CD25$^+$ Foxp3$^+$, IFN-γ$^+$IL-17$^-$, IL-17$^+$IFN-γ$^-$, and IL-17$^+$IFN-γ$^+$ cells from pLNs of WT and PD-L1$^{-/-}$ BALB/c recipients with vehicle or Am80 treatment 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 ($n$ = 6–8 in each group). *$p < 0.05$, **$p < 0.01$, ***$p < 0.005$.

lation of PD-1 in chronic GVHD, we used an anti–PD-1 agonistic mAb in allogeneic recipients from day 14 after BMT. Stimulation of the PD-1 pathway ameliorated clinical chronic GVHD scores compared with the control group (anti–PD-1 agonistic Ab: 1.00 ± 0.24 versus rat IgG: 2.84 ± 0.42; $p < 0.05$; Fig. 7A), and pathologic scores of skin were improved (anti–PD-1 agonistic Ab: 3.50 ± 0.29 versus rat IgG: 6.20 ± 0.20; $p < 0.05$; Fig. 7B, 7C). These results suggest that the PD-1 pathway contributes to the development of chronic GVHD, and that stimulation of the PD-1 pathway alleviates clinical and pathologic chronic GVHD.

## Discussion

The results of this study show that the PD-1 pathway is important in the alleviation of chronic GVHD. Blockade of the PD-1 pathway using anti–PD-1, anti–PD-L1, or anti–PD-L2 mAbs exacerbated chronic GVHD, and chimeric mice showed the importance of PD-L1 expression in host tissues in attenuating chronic GVHD. BMT into PD-L1–deficient recipients revealed IL-17$^+$IFN-γ$^+$ T cell expansion and Am80 administration of 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 with an agonistic anti–PD-1 mAb alleviated chronic GVHD, suggesting a new target for the prevention or treatment of chronic GVHD.

T cell activation via the TCR and costimulatory molecules has been well characterized, whereas coinhibitory pathways, which regulate T cell tolerance, are also known (32). The PD-1R and its ligands were identified and their inhibitory roles have become better understood (5–7, 9, 20, 21, 33). Previous studies have reported a role for PD-1/PD-L in acute GVHD, which is primarily

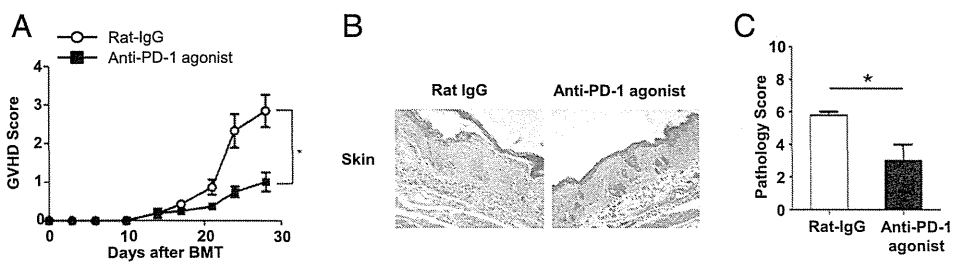


**FIGURE 7.** Administration of anti–PD-1 agonistic Ab alleviates chronic GVHD enhanced by PD-L1 deficiency. Sublethally irradiated BALB/c recipients were transplanted allogeneic B10.D2 donors. Recipients were injected with an anti–PD-1 agonist mAb or control rat IgG (200 μg/mouse) on days 14, 17, 20, 23, and 26 after BMT. (**A**) Clinical GVHD skin scores, (**B**) representative images (original magnification ×100), and (**C**) pathology scores of skin on day 30 after BMT are shown; data shown are from 1 representative of ≥2 independent experiments ($n$ = 5 in each group). *$p < 0.05$.

Th1 biased and CD8 T cell mediated. PD-1/PD-L blockade accelerated donor $CD8^+$ T cell expansion and exacerbated acute GVHD (14–16). In our model, we found that IFN-$\gamma^+$ $CD8^+$ T cells were increased in PD-$L1^{-/-}$ recipients only during the early phase after BMT, but no difference was found between WT and PD-$L1^{-/-}$ recipients thereafter (Supplemental Fig. 3). In contrast, chronic GVHD is dependent primarily on $CD4^+$ T cells; the pathophysiology of chronic GVHD differs from that of acute GVHD. In this study, we investigated the PD-1 pathway in a well-defined chronic GVHD model. PD-$1^{-/-}$ mice on B10.D2 background were backcrossed for 10 generations and used as the donor. Lack of constitutive PD-1 signaling in donor T cells exacerbated GVHD and more than half died within 1 wk. Next, we used mAbs to inhibit the PD-1 pathway immediately before the development of chronic GVHD. Blockade of the PD-1 pathway using anti–PD-1, anti–PD-L1, or anti–PD-L2 mAbs exacerbated chronic GVHD and was confirmed by histopathologic examinations.

Donor tissue expression of PD-L1 provides protection against host T cell responses in cardiac and kidney allografts (34–36). More recently, Saha et al. (15) reported that PD-L1 expression in host tissues played an important role in the suppression of acute GVHD. In contrast, Yi et al. (18) reported that PD-L1 on host APCs, not tissues, was critical for Treg expansion in an autoimmune-like GVHD model. Host APCs, but not parenchymal cells, are replaced by donor cells, and we showed that even upregulated PD-L1 expression in host tissues in early phase was not enough to control or prevent chronic GVHD development and declined to basal levels in the late posttransplant period. In this study, to clarify the role of PD-L1 expression in host tissues during chronic GVHD, we used BM chimeric recipients. Transplantation of WT BM cells into PD-L1–deficient mice (WT→PD-$L1^{-/-}$ chimera) showed chronic GVHD exacerbation. In contrast, transplantation of PD-L1–deficient BM cells into WT mice (PD-$L1^{-/-}$→WT chimera) showed no exacerbation of chronic GVHD. This is consistent with previous observations that expression of PD-L1 on parenchymal cells inhibits self-reactive $CD4^+$ T cell–mediated autoimmune disease and $CD8^+$ T cell–mediated damage in chronic viral infection (24, 37). Taken together, our results indicated that PD-L1 expression in host tissues plays a critical role in alleviating chronic GVHD.

To clarify the mechanism of chronic GVHD exacerbation in PD-$L1^{-/-}$ recipients, we analyzed Treg reconstitution because PD-L1 regulates the development of induced Tregs (17). We found that Tregs were decreased significantly in PD-$L1^{-/-}$ recipients only during the early phase after BMT, and no difference was found between WT and PD-$L1^{-/-}$ recipients thereafter. We next identified the population of donor-derived Th1 and Th17 cells, because it has been shown that Th17 cells play a role in the pathogenesis of experimental autoimmune encephalomyelitis and chronic GVHD by our group and others (29, 38–40). IL-$17^+$ IFN-$\gamma^-$ and IL-$17^+$IFN-$\gamma^+$ T cells were detected significantly more frequently in PD-$L1^{-/-}$ recipients than WT recipients. Furthermore, we showed the importance of PD-L1 expression on host tissues for expansion of IL-$17^+$IFN-$\gamma^+$ T cells. Treatment with Am80 overcame the IL-$17^+$IFN-$\gamma^+$ T cell expansion caused by PD-L1 deficiency and resulted in reduced chronic GVHD in PD-$L1^{-/-}$ recipients. D'Addio et al. (41) showed that PD-L1 blockade was associated with a switch in the Th1 balance toward Th17, leading to breakdown of fetomaternal tolerance. Recent clinical data reported augmentation of Th1 and Th17 responses in patients treated with anti–PD-1 therapy (42). Also, mesenchymal stem cells suppress Th17 proliferation via PD-L1 expression, and IL-27–primed $CD4^+$ T cells inhibit Th17 cell differentiation via PD-L1 (43). Therefore, PD-L1 deficiency plays an important role in Th17 expansion, and the PD-L1/Th17 axis may be a good therapeutic target for chronic GVHD.

In the acute GVHD model, PD-1/PD-L1 blockade accelerated acute GVHD via Th1 skewing; whereas during development of chronic GVHD, PD-L1 deficiency exacerbated histopathologically confirmed chronic GVHD via IL-$17^+$IFN-$\gamma^+$ T cell expansion, but not simply Th1 skewing. The pathophysiology of chronic GVHD includes defects in thymic function/negative selection (44), Tregs (45), clonal deletion (46–48), and clonal anergy (49, 50). In this study, we showed that the PD-1 pathway contributed to the development of chronic GVHD. Modulation of tissue expression of PD-L1 and/or stimulation of the PD-1 pathway of donor T cells may represent a new strategy for the prevention or treatment of chronic GVHD.

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## Disclosures

The authors have no financial conflicts of interest.

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# Mammalian Target of Rapamycin Inhibitors Permit Regulatory T Cell Reconstitution and Inhibit Experimental Chronic Graft-versus-Host Disease

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A B S T R A C T

Chronic graft-versus-host disease (GVHD) remains a major late complication of allogeneic bone marrow transplantation (BMT). In a previous study, impaired thymic negative selection of the recipients permitted the emergence of pathogenic T cells that cause chronic GVHD using MHC class II-deficient (H2-Ab1 KO) B6 into C3H model and $CD4^+$ T cells isolated from chronic GVHD mice caused chronic GVHD when administered into the secondary recipients. In this study, we evaluated the kinetics of regulatory T cell (Treg) reconstitution in wild type B6 into C3H model. After myeloablative conditioning, host Tregs disappeared rapidly, followed by expansion of Tregs derived from the donor splenic T cell inoculum. However, the donor splenic T cell–derived Treg pool contracted gradually and was almost completely replaced by newly generated donor bone marrow (BM)-derived Tregs in the late post-transplantation period. Next, we compared the effects of cyclosporine (CSA) and mammalian target of rapamycin (mTOR) inhibitors on Treg reconstitution. Administration of CSA significantly impaired Treg reconstitution in the spleen and thymus. In contrast, BM-derived Treg reconstitution was not impaired in mTOR inhibitor-treated mice. Histopathological examination indicated that mice treated with CSA, but not mTOR inhibitors, showed pathogenic features of chronic GVHD on day 120. Mice treated with CSA until day 60, but not mTOR inhibitors, developed severe chronic GVHD followed by adoptive transfer of the pathogenic $CD4^+$ T cells isolated from H2-Ab1 KO into C3H model. These findings indicated that long-term use of CSA impairs reconstitution of BM-derived Tregs and increases the liability to chronic GVHD. The choice of immunosuppression, such as calcineurin inhibitor-free GVHD prophylaxis with mTOR inhibitor, may have important implications for the control of chronic GVHD after BMT.



## INTRODUCTION

Chronic graft-versus-host disease (GVHD) is the most serious late complication after allogeneic hematopoietic stem cell transplantation, but the pathophysiology and treatment strategy of chronic GVHD remain poorly defined [1-3]. GVHD prophylaxis using calcineurin inhibitors, such as cyclosporine (CSA) and tacrolimus, reduces the expansion of effector T cells by blocking interleukin (IL)-2 and prevents acute GVHD, but fails to reduce chronic GVHD [4,5]. Administration of CSA for up to 24 months, longer than the standard 6 months of CSA, also did not decrease the risk of chronic GVHD [6]. Several studies have indicated that the efficacy and safety of mammalian target of rapamycin (mTOR) inhibitor, rapamycin (RAPA), in refractory chronic GVHD patients [7-10]. However, a recent randomized trial showed that the combination of RAPA and tacrolimus as GVHD prophylaxis failed to reduce chronic GVHD compared with tacrolimus and methotrexate [11].

$CD4^+CD25^+Foxp3^+$ regulatory T cells (Tregs) have been shown to play an important role in the establishment of tolerance between recipient tissues and donor-derived immunity. A series of animal studies indicated that Tregs in the inoculum can prevent acute GVHD when injected together with donor T cells [12-14]. Based on the role of Tregs in the prevention of GVHD and on their dependence on IL-2, there is considerable concern regarding the impact of blocking IL-2 signaling or IL-2 production by the immunosuppressive agents used for prophylaxis of GVHD. Zeiser et al. reported that Tregs showed relative resistance to RAPA as a result of reduced usage of the mTOR pathway and functional phosphatase and tensin homolog, a negative regulator of the phosphatidyl-inositol 3-kinase/Akt/mTOR pathway in Tregs

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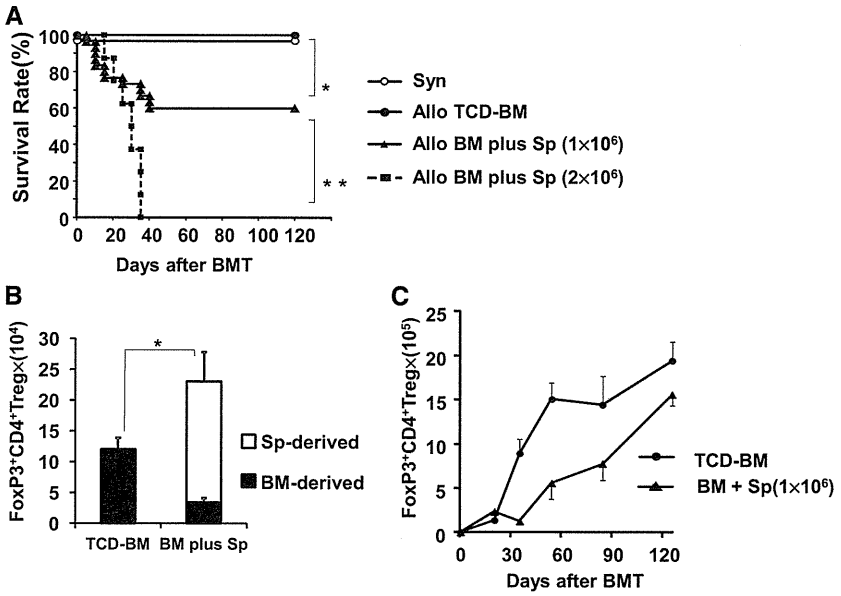


**Figure 1.** Regulatory T cell reconstitution after allogeneic BMT. Lethally irradiated C3H (H-$2^k$) recipient mice received $10 \times 10^6$ T cell–depleted bone marrow (TCD-BM) cells from B6.Ly-5a (H-$2^b$,CD45.1) mice with/without 1 to $2 \times 10^6$ spleen cells from B6 (H-$2^b$,CD45.2) mice. The syngeneic group received transplantation from C3H mice. (A) Survival: the recipients of allogeneic BM plus $1 \times 10^6$ spleen cells (BM plus Sp cells) showed a survival rate of 60% by day 120. Open circle, syngeneic; closed circle, TCD-BM cells only; triangle, -with $1 \times 10^6$ spleen cells; square, -with $2 \times 10^6$ spleen cells. (B) Origin of $CD4^+Foxp3^+$ Treg in the spleen on day 21 post transplantation: $CD45.2^+$ splenic T cell–derived (white bars) and $CD45.2^-$ BM–derived (black bars) are shown. (C) The absolute numbers of Treg in the recipients of BM plus Sp cells (triangles) and TCD-BM (closed circles) are shown. Each group consisted of 7 to 25 mice. The means (±SE) of each group are shown. Data are from a representative of at least 3 independent experiments. $^*P < .05$; $^{**}P < .01$.

compared with conventional T cells [15]. In contrast to CSA, RAPA allowed expansion of adoptively transferred Treg cells and led to reduction of alloreactive T cell expansion when animals received Treg treatment in combination with RAPA. They also showed that a combination of RAPA plus IL-2 increased both expansion of donor natural Tregs and conversion of induced Tregs from donor conventional T cells, and suppressed acute GVHD [16]. These animal data suggest that RAPA and CSA have differential effects on peripheral Tregs after bone marrow transplantation (BMT).

IL-2 signaling is pivotal for Treg homeostasis in the periphery and is also essential for naturally occurring Treg development in the thymus [17-19]. T cell repopulation after BMT is composed of 2 subsets: T cells derived from the donor splenic T cell inoculum and newly arising T cells from bone marrow (BM) inoculum. It has been shown that Tregs from the former pathway play an important role in acute GVHD, whereas, no previous study evaluated whether use of CSA for an extended period affects donor BM-derived Treg generation. We hypothesized that BM-derived Tregs comprise the long-term peripheral Treg pool and that CSA, but not mTOR inhibitors, causes impaired BM-derived Treg reconstitution, which has a negative effect on chronic GVHD. In the present study, we therefore evaluated effects of different immunosuppressants on 2 distinct Treg expansion reconstitution pathways and on the development of chronic GVHD.

## MATERIALS AND METHODS

### *Mice*

Female C57BL/6 (B6: H-$2^b$, $CD45.2^+$) and C3H/HeN (C3H: H-$2^k$) mice were purchased from Charles River Japan (Yokohama, Japan) or from the Okayama University mouse colony (Okayama, Japan). B6-Ly5a (H-$2^b$, $CD45.1^+$) and C3.SW (H-$2^b$, $CD45.2^+$) mice were purchased from Jackson Laboratory (Bar Harbor, ME). B6-background MHC class II-deficient H2-$Ab1^{-/-}$ mice (B6.129-*H2-Ab1*$^{tm1Gru}$ N12) were from Taconic Farms (Germantown, NY) [20]. Mice between 8 and 18 weeks of age were maintained under specific pathogen-free conditions and received normal chow and hyperchlorinated drinking water after transplantation. All experiments involving animals were approved by the Institutional Animal Care and Research Advisory Committee, Okayama University Advanced Science Research Center.

### *BMT*

Mice underwent transplantation according to the standard protocol described previously [21,22]. Briefly, recipient mice received 2 split doses of either 500 cGy (allogeneic C3H and C3.SW recipients) or 650 cGy (syngeneic B6 recipients) total-body irradiation (TBI) 3 to 4 hours apart. Recipients were injected with $10 \times 10^6$ T cell–depleted bone marrow (TCD-BM) cells plus 1 or $2 \times 10^6$ whole spleen cells from B6 donors. [*H2-Ab1*$^{-/-}$→C3H] chimeras were produced by reconstituting lethally irradiated C3H mice with $5 \times 10^6$ TCD-BM cells from *H2-Ab1*$^{-/-}$ mice, as described previously [23]. T cell depletion was performed using anti-CD90–microbeads and an AutoMACS system (Miltenyi Biotec, Auburn, CA) according to the manufacturer's instructions. Donor cells were injected intravenously into the recipients on day 0.

### *Immunosuppressive Treatment*

RAPA was purchased from Toronto Research Chemicals Inc. (North York, ON, Canada). Everolimus (RAD) and CSA were synthesized and provided by Novartis Pharma AG (Basel, Switzerland). Everolimus emulsion was dissolved in distilled water at a concentration of 625 μg/mL and administered to recipients by oral gavage at a dose of 5 mg/kg. RAPA and CSA were given as suspensions in carboxymethylcellulose sodium salt: CMC (C5013; Sigma-Aldrich, St. Louis, MO) at a final concentration of .2% CMC. RAPA and CSA were administered to recipients by peritoneal injection at doses of .5 and 20 mg/kg, respectively [15,24]. Immunosuppressive treatments were performed once daily, starting on day 0 and continuing until death or end of the observation period (day 110 to 125).

### *Adoptive Transfer*

Splenocytes were isolated from [*H2-Ab1*$^{-/-}$→C3H] chimeras 6 to 11 weeks after TCD-BMT. $CD4^+$ T cells were negatively selected from splenocytes by depletion of $CD8^+$, $DX5^+$, $CD11b^+$, Ter-$119^+$, and $B220^+$ cells using the AutoMACS system, as described previously [23]. A total of $2 \times 10^7$ $CD4^+$ T cells per mouse were injected intravenously into recipients after immunosuppressive therapy for 70 days after BMT.

### *Assessment of GVHD*

After BMT, survival was monitored daily, and weight changes were assessed twice per week. The degree of clinically acute GVHD was assessed twice per week using a scoring system that sums changes in 5 clinical