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

Hideaki Fujiwara,* Yoshinobu Maeda,* Koichiro Kobayashi,* Hisakazu Nishimori,* Ken-ichi Matsuoka,* Nobuharu Fuji,* Eisei Kondo,* Takehiro Tanaka,‡ Lieping Chen,‡ Miyuki Azuma,§ Hideo Yagita,¶ and Mitsune Tanimoto*

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-2d) donor to BALB/c (H-2b) 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.

A llloreactive 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).

*Department of Hematology and Oncology, Okayama University Graduate School of Medicine, Dentistry, and Pharmaceutical Sciences, Okayama, Japan 700-8558; ‡Department of Pathology, Okayama University Graduate School of Medicine, Dentistry, and Pharmaceutical Sciences, Okayama, Japan 700-8558; §Department of Immunobiology and Yale Comprehensive Cancer Center, Yale University, New Haven, CT 06519; ¶Department of Molecular Immunology, Graduate School of Medical and Dental Sciences, Tokyo Medical and Dental University, Tokyo, Japan 113-8549; and ©Department of Immunology, Juntendo University School of Medicine, Tokyo, Japan 113-8421

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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.

Address correspondence and reprint requests to Dr. Yoshinobu Maeda, Department of Hematology and Oncology, Okayama University Graduate School of Medicine, Dentistry, and Pharmaceutical Sciences, Shikata-cho 2-5-1, Kitaku, Okayama-city, 700-8558 Japan. E-mail address: yosmaeda@md.okayama-u.ac.jp

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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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 CDB 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.
Materials and Methods

**Mice**

Female B10.D2 (H-2b) donor mice were purchased from Japan SLC (Hamamatsu, Japan). Female BALB/c (H-2b) 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-1^{−/−} mice and PD-L1^{−/−} mice on a B10.D2 background were generated by backcrossing to BALB/c or B10.D2 donors. Chimeric mice were generated with transplants from 106 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 T cell–depleted bone marrow (TCD-BM) cells from B10.D2 mice (allogeneic group). The syngeneic group received a transplant of the same dose of splenocytes or WT BALB/c donor mice. PD-L1^{−/−} mice and TCD-BM from BALB/c mice were surgically, embedded in Tissue-Tek (Sakura, Tokyo, Japan), frozen, and stored at −80°C until use. Cryostat sections (5 μm thick) were fixed in 80˚C until use. Cryostat sections (5 μm thick) were fixed in 3 independent experiments (n = 8 in each group). Data shown are from 1 representative of n ≥ 3 independent experiments (n = 4 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 n ≥ 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, 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 n ≥ 3 independent experiments (n = 8 in each group). *p < 0.05, **p < 0.01, ***p < 0.005.

**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 cm²) 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), allopurinol-conjugated anti–CD8 (53-6.7), 7-AAD, and control Abs were purchased from eBio-science (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 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 n ≥ 3 independent experiments (n = 8 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 n ≥ 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, 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 n ≥ 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 ×10/0.26 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 quantitated 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 $\Delta\Delta^Ct$ threshold cycle = $Ct_{\text{target}} - C_{\text{GAPDH}}$. $Ct$

**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 (eBiol7B17) and FITC-conjugated anti-IFN-γ (XM1G1.2) were used to assess the cell populations (eBioscience). Total cells were adjusted to 1 × 10⁶/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 (RPMI-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.

**Group comparisons of skin chronic GVHD scores and pathology scores**

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 × 10⁶ spleen T cells and 8 × 10⁷ 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
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 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...
IL-17+IFN-γ Lack of PD-L1 expression exacerbated chronic GVHD with especially the PD-1/PD-L1 pathway, plays a critical role in suppressing lethal chronic GVHD.

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.

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 (±SD) 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-L1−/−–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).

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
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 similar 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-
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 on day 35 after BMT are shown. (D) The percentages of donor-derived CD4\(^+\) T cells expressing CD25\(^+\) Foxp3\(^+\), IFN-\(\gamma\)-IL-17\(^-\), IL-17\(^+\)IFN-\(\gamma\)-, and IL-17\(^+\)IFN-\(\gamma\)- 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). \(* p < 0.05, ** p < 0.01, ***p < 0.005.\)

**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-\(\gamma\) T cell expansion and Am80 administration of 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 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 \(\mu\)g/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-L1 blockade accelerated donor CD8+ T cell expansion and exacerbated acute GVHD (14–16). In our model, we found that IFN-γ+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 up-regulated 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−/− recipient 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-γ and IL-17+IFN-γ+γ+ 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-γ+ T cells. Treatment with Am80 overcame the IL-17+IFN-γ+ 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 fetal maternal 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-γ+γ+ 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 clonalergy (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

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References


