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ORIGINAL ARTICLE

# PD-L1 Ameliorates Murine Acute Graft-Versus-Host Disease by Suppressing Effector But Not Regulatory T Cells Function

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## Abstract

There is increasing evidence that interaction between programmed death 1 (PD-1) and its ligands PD-1 (PD-L1) plays a critical role in the pathology of acute graft-versus-host disease (aGVHD). However, the role of PD-L1 in the development of aGVHD has been controversial in recent mouse studies. In this study, we carried out studies in a murine aGVHD model to clarify the role of PD-L1 in aGVHD pathogenesis. We found that systemic overexpression of PD-L1 by hydrodynamic gene transfer (HGT) method in vivo ameliorates aGVHD-induced lethality in mice. Systemic overexpression of PD-L1 inhibits the donor T cells activation, effector memory status, as well as Th1 and Th17 cells responses in vivo. In addition, PD-L1 Ig treatment significantly suppressed T cells' proliferation, promoted T cells' apoptosis, and reduced pro-inflammatory cytokines expression by effector T cells in vitro in the stimulation of anti-CD3/CD28 and allogeneic dendritic cells. However, we found that PD-L1 overexpression did not affect Treg cells' differentiation in vivo and in vitro, depletion of Treg cells in PD-L1 HGT recipients did not aggravate aGVHD mortality. Therefore, our results demonstrated that systemic treatment with PD-L1 protein ameliorates aGVHD by suppressing effector but not regulatory T cell function. Our findings suggest that systemic treatment with PD-L1 may be a potential strategy to prevent or ameliorate aGVHD.

**Keywords** PD-1 · PD-L1 · aGVHD · Treg cells

## Introduction

Allogeneic hematopoietic stem cell transplantation (allo-HSCT) is one of the most effective therapies for the treatment of hematological malignant diseases. However, acute graft-versus-host disease (aGVHD) is one of the major complications that severely disturb the outcome of patients (Ghimire et al. 2017). Currently, systemic corticosteroids remain the recommended first-line therapy of aGVHD. However, these methods can affect the immune reconstruction and increase the patients' risk of infection and weakened the graft-versus-leukemia (GVL) effect (Holtan et al. 2014). Therefore, seeking novel therapies is urgently needed for the prevention and treatment of aGVHD.

Programmed death 1 (PD-1) is one of the most important immune checkpoints corresponding to T-cell dysfunction (Keir et al. 2008). PD-1 interacts with its ligands programmed death ligand 1 (PD-L1) and PD-L2 to deliver inhibitory signals that regulate T-cell activation, tolerance, and immune-mediated tissue damage (Keir et al. 2008; Sharpe and Pauken 2018). Most recently, monoclonal antibodies against PD-1 and PD-L1 have been approved by the U.S. Food and Drug Administration

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for the treatment of melanoma and lung cancer (Ribas and Wolchok 2018). PD-1 is mainly expressed on the surface of activated T cells (Freeman et al. 2000). PD-L1 is constitutively expressed on T and B cells, dendritic cells (DCs), macrophages, mesenchymal stem cells, and bone marrow (BM)-derived mast cells and its expression will be upregulated after activation (Yamazaki et al. 2002).

Currently, there is increasing evidence that interaction between PD-1 and PD-L1 plays a critical role in the pathology of aGVHD. In clinical, PD-1 was found elevated on donor $CD4^+$ and $CD8^+$T cells in patients with aGVHD and associated with mortality after allo-HSCT (Gallez-Hawkins et al. 2009; Hossain et al. 2017; Schade et al. 2016). PD-1 and PD-L1 were also found upregulated on activated T cells and the aGVHD-target tissues in mouse (Asakura et al. 2010; Saha et al. 2013; Tkachev et al. 2015). The previous study has shown that blockade PD-1 on donor cells drives T-cell expansion, increases interferon (IFN)-γ production, and accelerates GVHD onset (Blazar et al. 2003). Donor PD-1 genotype was also found associated with the risk of grades II to IV aGVHD (Santos et al. 2018). Although no studies have defined the critical role of host PD-1 in aGVHD, Fujiwara et al. (2014) reported that PD-1 in host tissues suppressed Th17/Th1-mediated experimental chronic GVHD. In addition, blockade of PD-1/PD-L1 interactions partially restored T-cell effector functions and improved GVL effect (Asakura et al. 2010; Michonneau et al. 2016). In addition to the certain role of PD-1 in regulating GVHD, PD-L1 in host tissues decreases the severity of GVHD in allogeneic recipients (Li et al. 2012; Saha et al. 2013), while PD-L1 expression on donor T cells drives the severity of GVHD by augmenting the expansion and survival of donor $CD4^+$ and $CD8^+$ T cells (Saha et al. 2016). However, it was reported that in the absence of donor $CD4^+$ T cells, block of the interactions of PD-L1 with PD-1 on donor $CD8^+$ T cells causes their tolerance, thereby preventing GVHD (Ni et al. 2017). Additionally, PD-L1 was more critical than PD-L2 in mediating aGVHD (Saha et al. 2013). Therefore, the effect of PD-L1 on the development of GVHD remains to be further investigated.

In the present study, we attempted to clarify the role of PD-L1 in aGVHD pathogenesis. We found that systemic overexpression of PD-L1 by hydrodynamic gene transfer (HGT) method in vivo ameliorates aGVHD in mice. Systemic overexpression of PD-L1 inhibits the effector function of donor T cells, but does not affect regulatory T (Treg) cells function in vivo and in vitro.

## Materials and Methods

### Mice

Six to eight week-old female C57BL/6 ($H\text{-}2^b$) and BALB/c ($H\text{-}2^d$) mice were purchased from Shanghai Laboratory Animal Center (Shanghai, China). C57BL/6 FoxP3-eGFP mice (CD45.2) were provided by Dr. Zhinan Yin (Jinan University, Guangzhou, China). C57BL/6 CD45.1 mice were obtained from Beijing Vital River Laboratory Animal Technology Co. Ltd (Beijing, China). All mice were maintained in a specific pathogen-free room at Animal Facilities of Soochow University. Experiments were carried out and approved according to the guidelines of the animal care and use committee at Soochow University.

### Induction and Assessment of aGVHD

Murine aGVHD was induced as described previously (Cai et al. 2018; Han et al. 2017). Briefly, BALB/c mice were given lethally 650 cGy (X-Ray, 325 cGy per dose with 4 h interval) total-body irradiation, irradiated BALB/c mice were transplanted with $1 \times 10^7$ C57BL/6 BM cells and $5 \times 10^6$ C57BL/6 splenocytes via the tail vein. The recipients were monitored daily for survival and every 2 days for body weight changes and clinical signs of aGVHD. The severity of aGVHD was assessed using an aGVHD scoring system (Cai et al. 2018; Han et al. 2017). For histopathologic analysis, 2 weeks after transplantation, liver, lung, small intestine and skin were obtained from the transplanted recipients and fixed in 10% formalin. Samples were then embedded in paraffin, sectioned and were stained with hematoxylin and eosin. Tissue damage was assessed based on a semiquantitative scoring system as described previously (Cai et al. 2018; Han et al. 2017). The paraffin-embedded section of mouse liver were stained with an anti-PD-L1 antibody (1:500, EPR20665, Abcam, USA), followed by HRP-labeled goat anti-rabbit IgG. The slides were washed and visualized by adding the substrate diaminobenzidine tetrahydrochloride.

### PD-L1 Overexpression Plasmid Construction and HGT

The mature peptide sequence of murine PD-L1 was amplified from cDNA bought from Sino Biological (Beijing, China). The PD-L1 gene fragment was constructed into pcDNA3.1 plasmid. Plasmids were purified and injected in vivo by HGT technique to express in vivo as described previously (Liu et al. 2015). Briefly, BALB/c mice were injected with plasmid through the tail vein at a concentration of 40 μg/ml in a total of 2 ml saline solution within

10 s 24 h before transplantation. All mice survive well after a temporary cardiovascular system failure induced by the sudden fluid overload.

### Cell Co-Culture and Mix Lymphocytes Reaction

$CD4^+CD25^-$ effector T (Teff) cells were sorted from the spleen of C57BL/6 by MACS system (Miltenyi Biotec, USA) and then labeled with CellTrace CFSE (5 μM, Invitrogen, Life Technologies, USA) according to the manufacturer's protocol. Plates were pre-coated with 2 μg/ml anti-CD3 and/or 0.4 μg/ml anti-CD28 antibodies (Biolegend, CA, USA) overnight. $1 \times 10^5$ purified T cells were cultured for 3 days in the presence of PD-L1-Ig (40 μg/ml, cat. 50010-M02H, Sino Biological, China) or control mouse IgG1-Fc (μg/mL, Sino Biological, USA). For mixed lymphocyte reaction (MLR), BM-derived DCs were generated and expanded from BALB/c mice with granulocyte–macrophage colony-stimulating factor (10 ng/ml) and interleukin (IL)-4 (10 ng/ml) for 7 days. DCs ($1 \times 10^4$) treated were irradiated (30 Gy) and cocultured with $1 \times 10^5$ allogeneic CFSE labeled T cells in U-bottom 96-microwell plates in the presence of PD-L1-Ig or control mouse IgG1-Fc. T cells were then analyzed by flow cytometry to determine the cell proliferation, apoptosis, and activation. For Treg cells' suppression assay, $CD4^+CD25^-$ Teff cells were sorted from spleen of CD45.1 mice and labeled with CFSE, stimulated with plate-coated anti-CD3. Treg cells ($CD4^+Foxp3\text{-}eGFP^+$) were sorted from spleen of C57BL/6 FoxP3-eGFP mice (CD45.2). Teff cells were co-cultured with or without Treg cells in the presence of PD-L1 Ig or IgG-Fc for 3 days. CFSE dilute staining were gated on PD-$1^+$ or PD-$1^-$ Teff cells.

### Flow Cytometry

Splenocytes were obtained from mice and analyzed by flow cytometry. The fluorescence antibodies used for FACS staining included anti-mouse CD3, CD4, CD8, CD25, CD44, CD62L, CD69, PD-1, $H2k^b$, $H2k^d$, IFN-γ, tumor necrosis factor (TNF)-α, IL-2, IL-10, IL-17 (all purchased from BioLegend, San Diego, CA, USA). For intracellular cytokine staining, cells were blocked with brefeldin A (10 μg/ml) for 6 h. Intracellular staining for Foxp3 was performed by using a Foxp3 staining kit (eBioscience, San Diego, CA, USA). Flow cytometric analyses were performed using NovoCyte (ACEA Biosciences, USA) and analyzed by FlowJo software.

### ELISA

Serum samples were obtained from recipients 14 days after BM transplantation (BMT) and separated by centrifugation. Culture supernatants were collected at indicated time by centrifugation. The levels of PD-L1 (Boster Biological Technology, Wuhan, China. Catalog #EK1449) and IL-10 (Multi Sciences, Hangzhou, China) were examined by ELISA kit according to the manufacturer's instructions.

### Statistical Analysis

Survival data were analyzed by log-rank test and Kaplan–Meier survival curves were generated using GraphPad Prism (GraphPad 6.0, San Diego, CA, USA). The data were expressed as the mean ± SD. Two-tailed Student's *t* test was used for statistical comparison between two groups. The significance levels are marked $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

## Results

### Systemic Overexpression of PD-L1 in Vivo Ameliorates aGVHD in Mice

Previous studies using genetic models have demonstrated the paradoxical role of donor and host-derived PD-L1 in aGVHD. PD-L1 on host tissues reduced aGVHD, whereas PD-L1 on donor T cells aggravated aGVHD (Saha et al. 2013, 2016). To further address the effect of PD-L1 on aGVHD development in vivo, we systemically overexpressed PD-L1 in mice. Figure 1a shows that the serum levels of PD-L1 reached the peak on the 2nd day and maintained for about 3 weeks after HGT, suggesting that HGT could successfully upregulate the PD-L1 expression in vivo. Immunohistochemistry showed that liver tissues from mice that received PD-L1 HGT showed a higher level of PD-L1 expression compared with those of control mice (Fig. Suppl 1A). In addition, the immune phenotypic analysis indicated that PD-L1 overexpression did not significantly affect the immune responses in normal mice (Fig. Suppl 1B–E). We then assessed the role of PD-L1 in an MHC-mismatched aGVHD model. The data showed that the survival time was significantly prolonged in the PD-L1 HGT group, while in the vector control group all died within 35 days (Fig. 1b). PD-L1 HGT mice showed significantly lower aGVHD scores compared with vector control mice (Fig. 1c). In addition, histologic assessment revealed that the pathological damage in the liver, intestine, lung, and skin of PD-L1 HGT mice was markedly decreased compared with vector control mice (Fig. 1d, e). Accordingly, our data suggested that systemic overexpression of PD-L1 in vivo significantly ameliorates aGVHD as evidenced by prolonged survival and reduced aGVHD scores following allo-BMT.

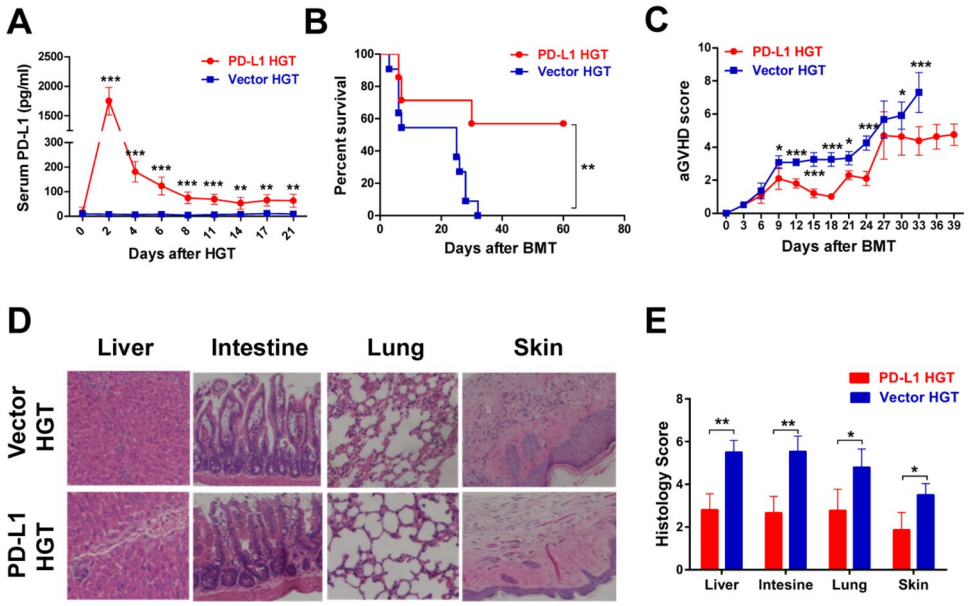



**Fig. 1** Systemic overexpression of PD-L1 in vivo ameliorates aGVHD in mice. **a** PD-L1 overexpressing plasmid or vector control plasmid were injected into mice by hydrodynamic gene transfer (HGT), serum PD-L1 levels were examined by ELISA ($n=3$ per time point). BALB/c mice were injected with PD-L1 overexpressing plasmid or vector control plasmid 24 h before transplantation, and then were given lethally 650 cGy total-body irradiation. Irradiated BALB/c mice (vector group, $n=11$; PD-L1 group, $n=8$) was transplanted with $1\times10^7$ C57BL/6 bone marrow (BM) cells and $5\times10^6$ C57BL/6 splenocytes via the tail vein. PD-L1 HGT significantly prolongs mice survival (log-rank, $P<0.01$) (**b**) and reduced aGVHD severity (**c**) compared with vector control. Histological analysis revealed that there was decreased pathological damage in the liver, lung, intestine and skin of recipients receiving PD-L1 HGT 14 days after BMT (**d** and **e**, $n=3$ per group). Survival data were analyzed by log-rank test and Kaplan–Meier survival curves were generated using GraphPad Prism. Data shown are mean ± SD. $^*P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$

## Systemic Overexpression of PD-L1 Inhibits Effector Function of Donor T Cells in Mice

To explore the mechanisms underlying the reduction of aGVHD by overexpression of PD-L1 in vivo, we examined the effect of PD-L1 on T cell function during the aGVHD. The results showed that the absolute numbers of donor $CD4^+$T, and $CD8^+$T cells (Fig. 2a), as well as the frequency of activated $CD4^+$T ($CD4^+$ $CD69^+$ subset), activated $CD8^+$T cells ($CD8^+CD69^+$ subset) were significantly decreased in PD-L1 HGT aGVHD mice on day 7 after BMT (Fig. 2b, c). In addition, the absolute numbers (Fig. 2d) and the frequency (Fig. 2e, f) of effector $CD4^+$T ($CD4^+$ $CD62^-$ $CD44^+$ subset) and $CD8^+$T cells ($CD8^+$ $CD62^-$ $CD44^+$ subset) were both reduced in PD-L1 HGT group compared with vector control group, suggesting that overexpression of PD-L1 impaired the effector function of T cells during aGVHD. We further evaluated the pro-inflammatory cytokines' production by donor $CD4^+$ and $CD8^+$T cells. The data showed that PD-L1 HGT resulted in a significant decrease in the production of IFN-γ and TNF-α by $CD4^+$T cells (Fig. 2g, h), as well as TNF-α by $CD8^+$ T cells (Fig. 2i, j). Therefore, our data indicated that systemic overexpression of PD-L1 inhibited effector function of donor T cells in aGVHD.

## PD-L1 Inhibits the Effector Function of Teff Cells in Vitro

To further investigate the effect of PD-L1 on Teff cell function, we sorted $CD4^+$ $CD25^-$ Teff cells and treated with recombinant PD-L1 Ig protein in vitro. The results showed that PD-L1 Ig treatment significantly suppressed T cells' proliferation (Fig. 3a, b, e, f), and promoted T cells' apoptosis (Fig. 3c, g), in the stimulation of anti-CD3 alone or plus anti-CD28. In addition, PD-L1 Ig treatment also reduced the expressions of IL-2, IFN-γ, and TNF-α by Teff cells (Fig. 3d, h). Since donor T cells allorecognition of host antigens is a prerequisite for the onset of GVHD, we carried out the MLR assay to mimic the alloreactive responses in vitro. $CD4^+$ $CD25^-$ T cells from C57BL/6 mice were stimulated with the irradiated DCs from BABL/c mice. It was found that PD-L1 Ig could also inhibit the proliferation of alloreactive Teff cells (Fig. 3i, j), promoted cell apoptosis (Fig. 3k), reduced pro-inflammatory cytokines expression (Fig. 3l). Therefore, PD-L1 ameliorates aGVHD maybe through inhibiting the proliferation and effector function of Teff cells.

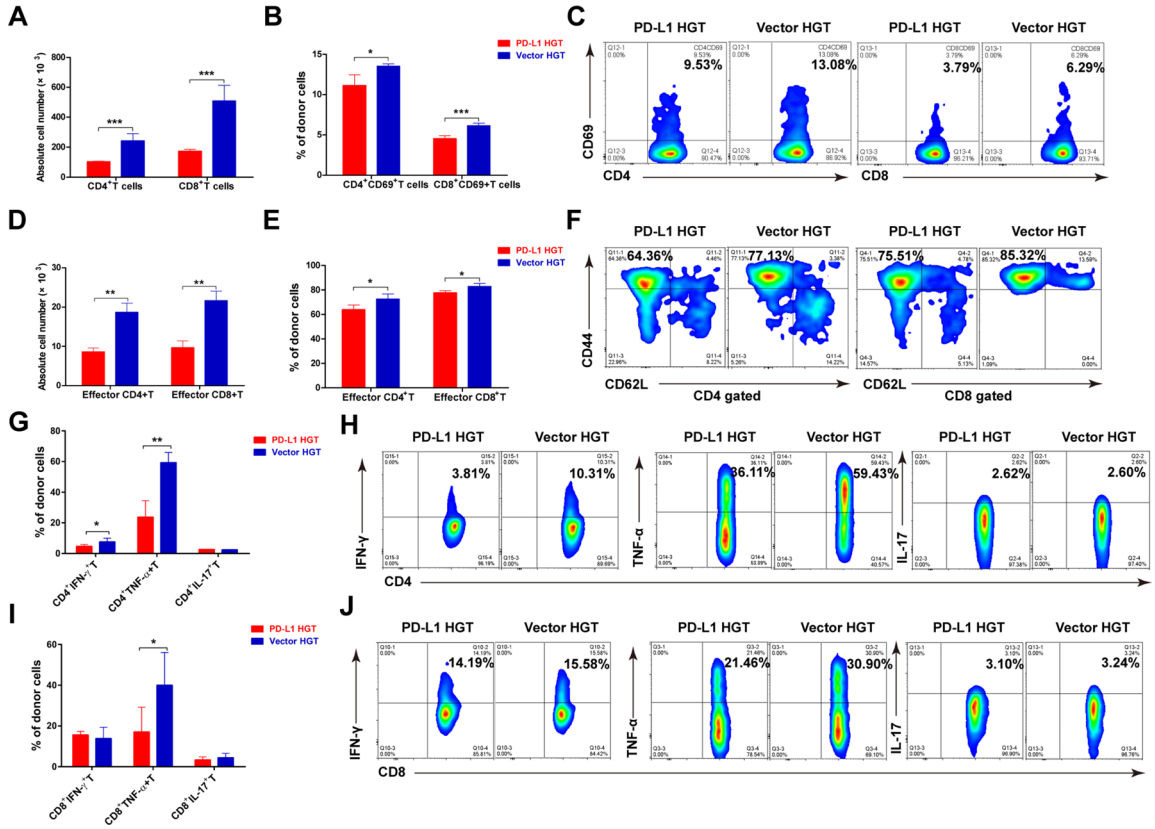



**Fig. 2** PD-L1 inhibits effector function of donor T cells in vivo. 7 days after BMT, splenocytes were obtained from mice ($n=6$ per group) and analyzed by flow cytometry. **a** Donor $CD4^+$T, and $CD8^+$T cells, (**b, c**) activated $CD4^+$T ($CD4^+$ $CD69^+$ subset), activated $CD8^+$T cells ($CD8^+$ $CD69^+$ subset), **d–f** effector $CD4^+$T ($CD4^+$ $CD62^-$ $CD44^+$ subset) and $CD8^+$T cells ($CD8^+$ $CD62^-$ $CD44^+$ subset) were examined by flow cytometry. **g–j** The IFN-γ, TNF-α and L-17 production by $CD4^+$ and $CD8^+$T cells of recipients' mice 7 days after BMT was examined by intracellular staining. Data shown are mean ± SD. $^*P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$

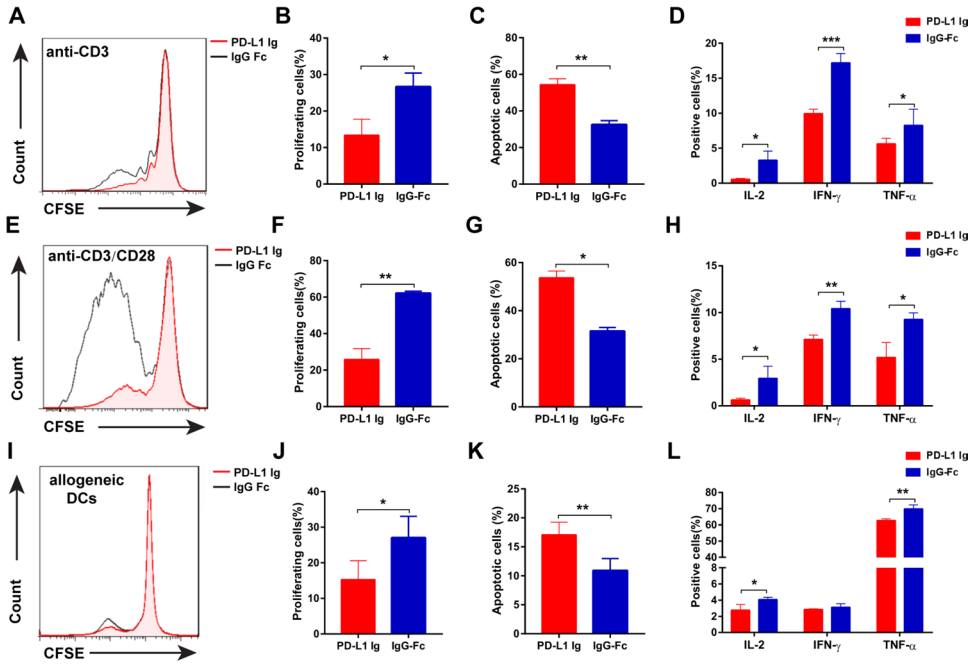
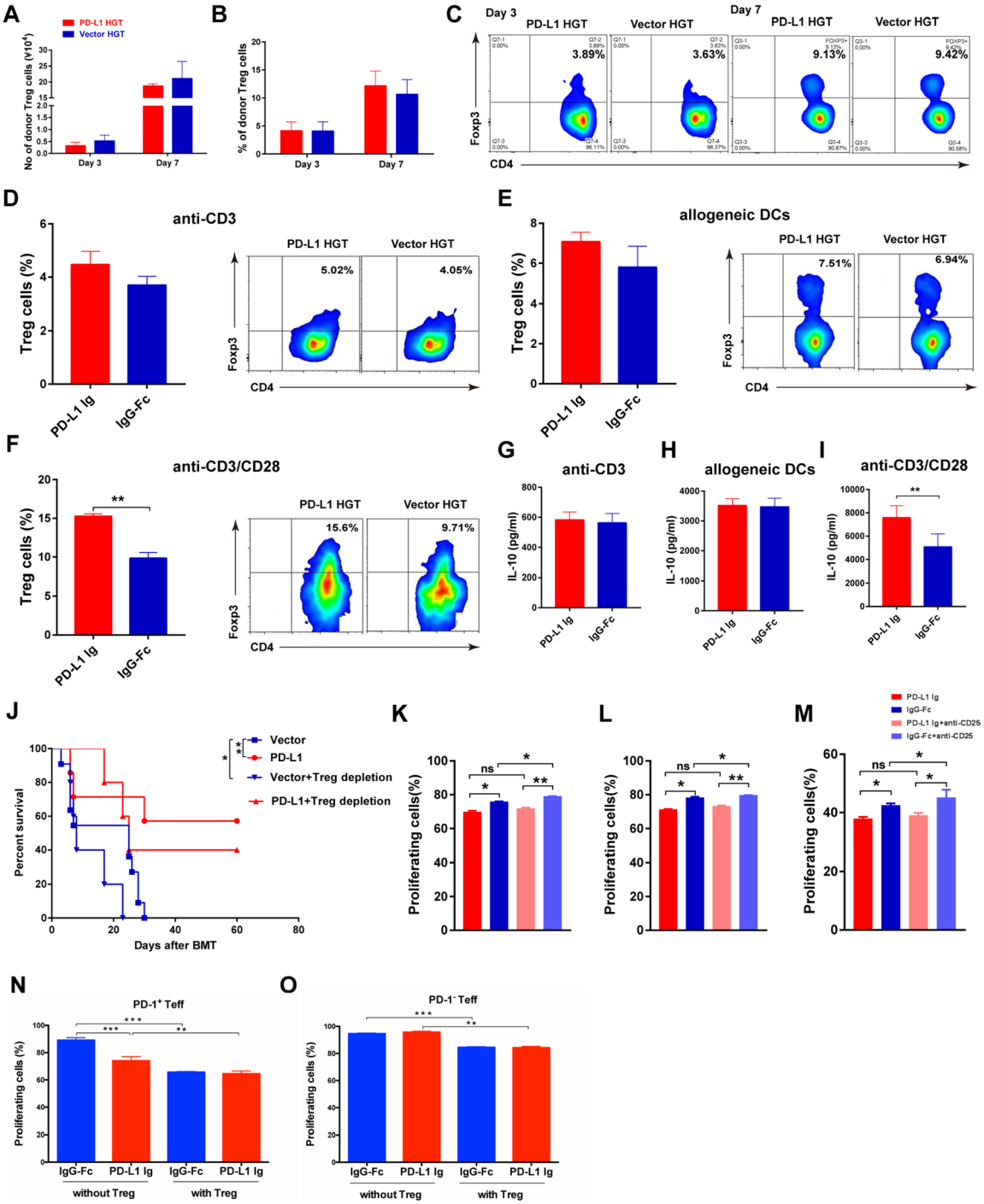



**Fig. 3** PD-L1 inhibits the effector function of Teff cells in vitro. $CD4^+$ $CD25^-$ Teff cells were sorted from spleen of C57BL/6 mice, and stimulated with plate-coated anti-CD3, or anti-CD3 plus anti-CD28, or allogeneic DCs, in the presence of PD-L1-Ig (40 μg/mL) or control mouse IgG1-Fc (40 μg/ml) for 3 days. Cell proliferation (**a** and **b, e** and **f, i** and **j**) was evaluated by CFSE dilute. Cell apoptosis (**c, g** and **k**) was examined by Annexin V/PI staining. The expressions of IL-2, IFN-γ and TNF-α by Teff cells (**d, h** and **l**) were examined by intracellular staining. Data shown are mean ± SD. $^*P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$




## PD-L1 Ameliorates aGVHD is Independent of Donor Treg Cells

Since Tregs are able to modulate the pathogenesis of GVHD, and PD-1/PD-L1 pathway plays an important role in the regulation of the differentiation and function of Treg cells (Keir et al. 2008; Wang et al. 2008), leading us to ask whether GVHD amelioration by PD-L1 overexpression was mediated by donor Treg cells. We found that the frequency and the absolute number of donor Treg cells has no significant changes after PD-L1 HGT treatment at day 3 and day 7 post-BMT (Fig. 4a–c), suggesting that systemic overexpression of PD-L1 in vivo did not affect Treg cells.

To further elucidate the role of PD-L1 in Treg cells, we treated CD25-depleted $CD4^+$T cells with PD-L1 Ig in the conditions of anti-CD3 alone or plus anti-CD28 stimulation, as well as allogeneic DCs conditions, and examined the Treg cells differentiation in vitro. The results showed that

◂**Fig. 4** PD-L1 ameliorates aGVHD is independent of donor Treg cells. **a, c** 3 and 7 days after BMT, splenocytes were obtained from mice ($n=6$ per group), Treg cells were examined by flow cytometry. **d–f** $CD4^+$ $CD25^-$ Teff cells were sorted from spleen of C57BL/6 mice, and stimulated with plate-coated anti-CD3, or anti-CD3 plus anti-CD28, or allogeneic DCs, in the presence of PD-L1-Ig (40 μg/ml) or control mouse IgG1-Fc (40 μg/ml) for 3 days. Treg cells were examined by flow cytometry. **g–i** The levels of IL-10 in the culture supernatant were examined by ELISA. **j** Lethally irradiated BALB/c recipients were transplanted with BM cells plus B6 splenocytes or CD25-depleted splenocytes ($n=6$ per group). Survival data were analyzed by log-rank test and Kaplan–Meier survival curves were generated using GraphPad Prism. **k–m** $CD4^+$ $CD25^-$ Teff cells were sorted from spleen of C57BL/6 mice, and stimulated with plate-coated anti-CD3, or anti-CD3 plus anti-CD28, or allogeneic DCs, in the presence of PD-L1-Ig (40 μg/ml) or control mouse IgG1-Fc (40 μg/ml) for 3 days, anti-CD25 neutralization antibody were added in the culture, Cell proliferation was evaluated by CFSE dilute. **n–o** $CD4^+$ $CD25^-$ Teff cells were sorted from spleen of CD45.1 mice and labeled with CFSE, stimulated with plate-coated anti-CD3. Treg cells ($CD4^+$ $Foxp3\text{-}eGFP^+$) were sorted from spleen of C57BL/6 FoxP3-eGFP mice (CD45.2). Teff cell were co-cultured with or without Treg cells in the presence of PD-L1 Ig or IgG-Fc for 3 days. CFSE dilute staining were gated on $PD\text{-}1^+$ or $PD\text{-}1^-$ Teff cells. Data shown are mean ± SD. $^*P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$

PD-L1 Ig treatment did not impact Treg cells differentiation in the condition of CD3 stimulation and allogeneic antigen-presenting cells stimulation (Fig. 4d, e), The production of IL-10 in the culture supernatant also had no significant changes (Fig. 4g–h). However, we found that PD-L1 Ig could promote Treg cells differentiation only in the stimulation of anti-CD3 and anti-CD28 (Fig. 4f), the levels of IL-10 in the culture supernatant were also upregulated in PD-L1 Ig-treated cells (Fig. 4i). Our data suggest that PD-L1 does not affect Treg cells differentiation in vitro in the allogeneic conditions, which is in accordance with the in vivo findings.

To further confirm the effect of PD-L1 on aGVHD was independent of Treg cells, lethally irradiated BALB/c recipients were transplanted with BM cells plus B6 splenocytes or CD25-depleted splenocytes. The survival curve showed that depletion of Treg cells in PD-L1 HGT recipients did not aggravate aGVHD mortality compared with PD-L1 HGT recipients, while depletion of Treg cells in vector HGT recipients caused more severity of GVHD lethality (Fig. 4j). In addition, we found that the inhibitory effect of PD-L1 Ig on cell proliferation was not rescued by adding anti-CD25 neutralization antibody in the culture system in vitro (Fig. 4k–m). To further confirm the independent effect of PD-L1 on Treg cells function, we performed in vitro Treg suppression assay. $CD4^+CD25^-$ Teff cells sorted from CD45.1 mice, and co-cultured with Treg cells from FoxP3-eGFP mice (CD45.2) in the presence of PD-L1 Ig or IgG-Fc for 3 days. CFSE dilute stainings were gated on $PD\text{-}1^+$ or $PD\text{-}1^-$ Teff cells. The results demonstrated that PD-L1 could not enhance Treg cells' suppressive capacity towards to Teff cells (Fig. 4n, o), regardless $PD\text{-}1^+$ or $PD\text{-}1^-$ Teff cells. Therefore, the in vitro and in vivo data confirm that PD-L1 ameliorates aGVHD is independent of donor Treg cells.

## Discussion

PD-1 and its two ligands PD-L1 and PD-L2 are critical regulators of immune tolerance, and play a critical role in the pathology of aGVHD. However, the role of PD-L1 in the development of aGVHD has been controversial in recent mouse studies. PD-L1 on host tissues reduced aGVHD, whereas PD-L1 on donor T cells aggravated aGVHD (Saha et al. 2013, 2016). In this study, we carried out studies in a murine aGVHD model to clarify the role of PD-L1 in aGVHD pathogenesis. Our results demonstrated for the first time that systemic treatment with PD-L1 protein ameliorates aGVHD by suppressing effector but not regulatory T-cell function.

In this study, we found that systemic overexpression of PD-L1 by HGT method in vivo significantly prolonged the survival time of mice and alleviated aGVHD symptoms. Overexpression of PD-L1 could decrease the frequency of activated $CD4^+$T and $CD8^+$T cells, and reduce effector $CD4^+$T and $CD8^+$T cells, as well as the production of IFN-γ and TNF-α by $CD4^+$T and $CD8^+$T cells, which resulted in the downregulated effector function of donor T cells in aGVHD. PD-1/PD-L1 pathway plays an important role in the regulation of the differentiation and function of Treg cells (Keir et al. 2008; Wang et al. 2008), while Treg cells are key mediators of peripheral tolerance that actively suppress Teff cells and inhibit immune-mediated tissue damage, such as aGVHD (Di Ianni et al. 2011; Hoffmann et al. 2002). In the current research, however, we found that systemic overexpression of PD-L1 in vivo does not affect Treg cells. This is inconsistent with the previous report that PD-L1 can improve the expression of Treg cells in GVHD (Deng et al. 2015; Ni et al. 2017). To further validate our findings, we set up aGVHD model with transfusion spleen cells that removed Treg cells, the result showed that the depletion of Treg cells in PD-L1 HGT recipients did not aggravate aGVHD mortality. Meanwhile, we found that the inhibitory effect of PD-L1 Ig on cell proliferation was not rescued by adding anti-CD25 neutralization antibody in the culture system in vitro. Classical in vitro Treg suppression assay also showed that PD-L1 could not enhance Treg cells' suppressive capacity towards Teff cells, regardless $PD\text{-}1^+$ or $PD\text{-}1^-$ Teff cells. Combined with the results of in vivo and in vitro, it was proved that PD-L1-alleviated aGVHD is independent of donor Treg cells.

PD-L1-Ig augmented human $CD4^+$ T expansion and increased IL-2, IFN-γ, and IL-10 production under stimulation with a suboptimal concentration of anti-CD3 in vitro (Dong et al. 1999). In contrast, another report showed that

PD-L1-Ig reduced $CD4^+$ T cell expansion and reduced the production of cytokines under stimulation with anti-CD3 and anti-CD28 in vitro (Francisco et al. 2009). In the current study, we sorted $CD4^+$ $CD25^-$ Teff cells and treated with recombinant PD-L1 Ig protein in vitro under three conditions. The results showed that PD-L1 protein inhibited the proliferation, activation, and the expression of cytokines of Teff cells in the stimulation of anti-CD3 alone or plus anti-CD28, as well as allogeneic DCs. This is consistent with previous literature reports that activated PD-1/PD-L1 pathways can inhibit T-cell function (Patsoukis et al. 2012). Therefore, PD-L1 ameliorates aGVHD maybe by inhibiting the proliferation and effector function of Teff cells.

It has been reported that PD-L1 can promote differentiation and maintain the function of induced Treg cells (Francisco et al. 2009). In our study, we found that PD-L1 Ig could promote Treg cells differentiation only in the stimulation of anti-CD3 and anti-CD28, indicating that anti-CD28 plays a key role in Treg cells differentiation. There were two pieces of literatures reporting that activation of CD28/B7 signaling pathway plays an important role in inhibiting PD-L1 signaling. Hui et al. (2017) found that PD-1 suppresses T cell function primarily by inactivating CD28 signaling. Kamphorst et al. (2017) demonstrated that the recovery and proliferation of T cells remained dependent on activation of the CD28/B7 signaling pathway, even after the targeted therapy that blocked the PD-L1 signal. Further studies are needed to explore the molecular mechanism of PD-L1 in regulating Tregs development and function.

Nowadays, much success has been realized with treatment of cancer with anti-PD-1/PD-L1 monoclonal antibodies. However, for GVHD, PD-L1 checkpoint enforcement, but not blockade, can prevent GVHD after HCT (Cassady et al. 2018). Since the role of PD-L1 in the development of aGVHD has been controversial, more detail about PD-1/PD-L1 interaction in GVHD needs to be clarified. Our data suggested that systemic treatment with PD-L1 fusion protein might ameliorate aGVHD, clinical translation of PD-L1 needs more convinced data in mouse model and human studies in the future.

In conclusion, our results demonstrated for the first time that systemic treatment with PD-L1 ameliorates aGVHD by suppressing effector but not regulatory T-cell function. PD-L1 inhibits T-cell activation, proliferation and secretion of cytokines and promotes T-cell apoptosis directly, and the effects are not subject to the influence of presence or absence of Treg cells. Our study clarified the role and the mechanism of systemic PD-L1 in the experimental aGVHD model. Our findings suggest that systemic treatment with PD-L1 protein may provide a new therapeutic strategy for treatment of aGVHD.

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**Author contributions** AS, DW and SM designed the study; LT and SM performed the experiments; HG, JW and YX contributed to the experiments; LT and SM analyzed the data and wrote the manuscript. All authors have discussed and revised the manuscript.

## Compliance with Ethical Standards

**Conflict of interest** The authors have no financial conflicts of interest.

**Ethics statement** All animal experiments were carried out and approved according to the guidelines of the animal care and use committee at Soochow University.

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