PD-1 deficiency results in the development of fatal myocarditis in MRL mice

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Abstract

The deficiency of programmed cell death 1 (PD-1, Pdcd1), a negative immuno-receptor belonging to the CD28/CTLA-4 family, can support various tissue-specific autoimmune conditions. Here, we analyzed the effect of PD-1 deficiency in MRL mice that is genetically predisposed to systemic autoimmunity. MRL-Pdcd1²/² mice developed a fatal myocarditis, which is reminiscent of CTLA-4-deficient (Ctla4²/²) mice. Massive infiltration of CD4⁺ and CD8⁺ T cells and myeloid cells was found in hearts of MRL-Pdcd1²/² mice concomitant with the production of high-titer auto-antibodies against cardiac myosin. In contrast to Ctla4²/² mice in which most of the CD4⁺ T cells are non-specifically activated and invade various organs, T cells in the heart but not in the spleen and lymph nodes are activated in MRL-Pdcd1²/² mice, suggesting that myocarditis is mediated by antigen-specific autoimmune response. Heart infiltrating myeloid cells strongly suppressed the allogenic response of T cells in vitro, suggesting that these Mac1⁺Gr1⁺ myeloid cells are phenotypically similar to myeloid suppressor cells, which can be found in tumor-bearing hosts. These findings unravel the hidden heart-specific autoimmune predisposition of MRL mice and provide MRL-Pdcd1²/² mice as a useful animal model of lymphocytic myocarditis.

Keywords: animal model, auto-antibody, autoimmune disease, co-stimulation, genetic predisposition

Introduction

Programmed cell death 1 (PD-1, Pdcd1), a member of the CD28/CTLA-4 family, inhibits antigen stimulation upon interacting with either of its two ligands, PD-1 ligand 1 (PD-L1, B7-H1) and PD-1 ligand 2 (PD-L2, B7-DC) (1, 2). Because PD-Ls are expressed on both antigen-presenting cells (APC) and tissue parenchymal cells, PD-1 expressing lymphocytes can be suppressed both at activation and at effector steps. The negative regulation by PD-1 is particularly important for the prevention of autoimmunity as evidenced by the spontaneous development of autoimmune diseases in Pdcd1¹/⁻ mice (1, 2). Interestingly, PD-1 deficiency can support various tissue-specific autoimmune conditions, suggesting that PD-1 deficiency can be a useful tool to unveil autoimmune predisposition of mice with different genetic background (1). Recently, it has become evident that PD-1-dependent immune effects are not limited to autoimmunity. For example, PD-1 induces functional impairment of virus-specific T cells in chronic viral infections, including infections by HIV and hepatitis C virus. PD-1 also suppresses immunity against tumor cells expressing PD-L1, and the level of PD-L1 expression on tumor cells correlates negatively with a good prognosis in various cancers (3–6).

Mouse models are especially useful for the study of autoimmunity and many models have been reported until now. MRL-Fas⁻/⁻ mouse is one of the most widely used animal models of human lupus erythematosus, whose major genetic determinant is unequivocally the loss-of-function mutation in the Fas gene (lpr) (7, 8). Besides the lpr mutation, MRL chromosome seems to harbor additional genetic factors that promote autoimmunity because Fas-intact MRL mice also develop lupus-like phenotypes in later life and the introduction of the lpr mutation into other strains of mice including...
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C3H/HeJ, AKR or C57BL/6 does not induce severe autoimmune myocarditis, although the mechanisms of the autoimmune myocarditis in MRL and MRL-Fas+/− mice have been analyzed in detail (10), the precise feature of the autoimmune predisposition of the MRL background remains elusive due to its high complexity. Because ipr mutation allows the survival of virtually all autoreactive lymphocytes resulting in the multiple reactivity of the autoimmune myocarditis, the autoimmune myocarditis of the strain can be more appropriately analyzed in the presence of intact Fas gene. However, the autoimmune myocarditis phenotypes of MRL mice are very mild and appear very late in their life (7, 11).

Myocarditis is one of the major causes of sudden death in children and young adults and is a frequent precursor of dilated cardiomyopathy (DCM), most common indication for cardiac transplantation. Although cardiotropic viruses are considered to be the most common causative agent, viral genome cannot be isolated from the myocardium in the majority of the cases and the etiology of the disease is therefore unknown (12). To date, most of the studies on myocarditis have been performed using animal models in which autoimmune reaction is induced either by injection of cardiotropic viruses or by immunization with cardiac myosin (CM) in adjuvants (13–15). Therefore, it is important to establish a spontaneous model of myocarditis that represents human myocarditis better. Spontaneous development of myocarditis has been reported in mice deficient in several genes including those for CTLA-4 and transforming growth factor-β1 (16–18). However, virtually all T cells are non-specifically activated in these animals and it is likely that the inflammatory response is not antigen specific. We have previously reported that BALB/c-Pdcd1−/− mice die of heart disease, which is similar to human DCM (19). Interestingly, there is almost no inflammation in the heart of BALB/c-Pdcd1−/− mice and subsequent analyses revealed that auto-antibodies against cardiac troponin I are responsible for the disease (20).

Myeloid suppressor cells (MSCs), which are most commonly defined as Mac1Gr1+ cells, have been reported to accumulate in both tumor-bearing mice and cancer patients (21, 22). MSCs play a critical role in the tumor-induced immune dysfunction because depletion of MSCs improves the efficacy of tumor vaccination (23). MSCs have been reported to inhibit the T-cell proliferative responses induced by alloantigens, CD3 ligation or various mitogens as well as NK cell activity in vitro using nitric oxide, arginase I and reactive oxygen species (21, 22). MSCs also expand in acute and chronic infection suggesting that they might suppress anti-infectious immunity as well (24). Recently, the involvement of MSCs in experimental autoimmune encephalomyelitis has been reported (25).

In the present study, we introduced PD-1 deficiency into MRL mice to exaggerate its genetic predisposition of autoimmunity, in particular tissue-specific autoimmunity. MRL-Pdcd1−/− mice developed fatal myocarditis by 10 weeks of age that is reminiscent of Ctlad4−/− mice, suggesting that hearts are at risk of autoimmunity in MRL mice. Massive infiltration of both CD4+ and CD8+ T cells and myeloid cells was found in hearts of MRL-Pdcd1−/− mice concomitant with the production of high-titer auto-antibodies against CM. In addition to T cells, a large number of Mac1Gr1+ myeloid cells with immunosuppressive function was observed in inflamed hearts of MRL-Pdcd1−/− mice, suggesting that MSCs are involved in the regulation of autoimmune myocarditis.

Materials and methods

Animals

MRL/MpJ−/− (MRL wild-type), MRL/MpJ-Fas+/− (MRL-Fas+/−) and C57BL/6 mice were purchased from Japan SLC (Hamamatsu, Japan). MRL-Pdcd1−/− mice were generated by backcrossing C57BL/6-Pdcd1−/− mice (26, 27) on MRL-Fas+/− mice for 10 generations and subsequently on MRL wild-type mice for two generations. All mouse protocols were approved by the Institute of Laboratory Animals, Graduate School of Medicine, Kyoto University. All animals were maintained under specific pathogen-free conditions.

Histological analysis

MRL-Pdcd1−/− mice were sacrificed when they became moribund. Organs were fixed in 10% buffered formalin, processed and embedded in paraffin. Sections were stained with hematoxylin and eosin using standard techniques.

Reagents

APC- and PE-conjugated streptavidin; APC-conjugated antibodies against CD3, CD8, B220, Mac1 and Gr1; Cy5-conjugated antibodies against CD8 and B220; PE-conjugated antibodies against CD4, B220, CD44, CD62L, CD69, PD-L1, PD-L2, F4/80, Tim3 and Mac1; FITC-conjugated antibodies against CD4, CD8, CD25 and CD3 and biotinylated antibody against Tim3 were purchased from eBioscience (San Diego, CA, USA). Biotinylated antibodies against B7.1, B7.2, CD11c, Vβ2, Vβ3, Vβ4, Vβ5/15.2, Vβ6, Vβ8/1/8.2, Vβ11, Vβ12, Vβ14 and Vβ17 and PE-conjugated antibodies against Vβ8.3 and Vβ10 were purchased from Becton Dickinson (San Diego, CA, USA). Polyclonal antibodies against PD-L1 and PD-L2 were purchased from R&D Systems (Minneapolis, MN, USA). FITC- and Cy5-conjugated polyclonal antibodies against goat IgG- and TxRed-conjugated polyclonal antibody against mouse IgM were purchased from Jackson Immunoresearch (West Grove, PA, USA).

Immunohistochemistry

Heart, stomach and ovary were collected from indicated animals and snap frozen in OCT-compound. Cryosections were fixed with CytoFix (Becton Dickinson) and stained with indicated antibodies. Signals were observed with axio-vision (Bio-Rad, Hercules, CA, USA).

Western blotting

Crude heart extracts were prepared with a Polytron homogenizer in lysis buffer (150 mM NaCl, 20 mM Tris–HCl (pH 7.4), 5 mM EDTA, 1% NP-40 and protease inhibitor cocktail (complete™, Roche, Basel, Switzerland)), separated by SDS–PAGE and transferred onto Hybond-P filter (GE Healthcare UK Ltd, Buckinghamshire, UK). Filters were incubated with serially diluted mouse sera for 2 h at room temperature.
and visualized by HRP-labeled anti-mouse IgG antibody (Kirkegaard and Perry Laboratories, Gaithersburg, MD, USA) with ECL system (GE Healthcare UK Ltd). Reactivity against purified CM (Sigma-Aldrich, St Louis, MO, USA) was examined as above.

Transfer experiment

Twenty million splenocytes from moribund MRL-Pdcd1−/− mice were transferred into sub-letally irradiated (4.5 Gy) MRL wild-type mice through tail vein. Mice were analyzed 3 weeks later.

Isolation of heart infiltrate

Hearts were removed from moribund MRL-Pdcd1−/− mice, minced by scissors and incubated in a digestion buffer [PBS, 2% FCS and 0.5 mg ml−1 collagenase (Wako Chemicals, Tokyo, Japan)] for 15 min at 37°C with continuous agitation. Digested hearts were passed through 24-gauge needle and further incubated for 10 min at 37°C. After centrifugation, cells were re-suspended in 40% buffered Percoll (Sigma-Aldrich) and layered on 75% buffered Percoll. After spinning, heart infiltrates were collected from the surface of the 40% layer and washed twice with PBS. On average, 1 × 10⁶ cells were isolated from one heart.

Real-time PCR using TaqMan Low Density Arrays

Total RNA was isolated from heart using TRIzol (Life Technologies, Carlsbad, CA, USA). Single-strand cDNA was synthesized using Quantitect Reverse Transcription (Qiagen, Hilden, Germany), mixed with Taqman Universal PCR Master Mix (Life Technologies) and loaded into 384 wells of a Taqman Low Density Array Immuno Profiling Plate (Life Technologies). PCRs were performed on an ABI PRISM 7900 HT system according to the manufacturer’s protocol. Gene expression profiling was achieved using the comparative cycle threshold (Ct) method of relative quantification. The GAPDH reference gene was used as an endogenous control, and the calibrator samples were from healthy subjects. Genes with Ct > 35 were eliminated for lack of reproducibility. ΔCt represents the Ct of the target minus that of the endogenous control, and ΔACt represents the ΔCt of each target minus that of the calibrator. Relative quantities were determined using the formula: relative quantity = 2−ΔACt.

Suppression assay

Mac1+ cells were positively selected from heart infiltrates using the MACS system (Miltenyi Biotech, Gladbach, Germany). Thy1+ splenocytes were prepared from MRL-Pdcd1−/− and MRL wild-type mice and Thy1+ splenocytes were prepared from C57BL/6 wild-type mice using the MACS system. Using the manufacturer’s protocol, a purity of 95% Thy1+ and Thy1+ cells was routinely achieved as determined by flow cytometry. Four hundred thousand CFSE-labeled Thy1+ cells from either MRL-Pdcd1−/− or MRL wild-type mice were mixed with an equal number of mitomycin C-treated Thy1+ cells from C57BL/6 mice in 96-well plate for 4 days and analyzed by FACS Calibur (Becton Dickinson). Four thousand (1%), 8000 (2%) or 12 000 (4%) Mac1+ cells were added. Relative proliferation was calculated by dividing the frequency of proliferating cells with that in the absence of MSCs.

Results

Fetal myocarditis in MRL-Pdcd1−/− mice

MRL-Pdcd1−/− mice were generated by backcrossing C57BL/6-Pdcd1−/− mice on MRL mice for 12 generations and housed in the specific pathogen-free condition. As shown in Fig. 1(A), MRL-Pdcd1−/− mice became moribund from 4 weeks of age and ~70% of them died by 10 weeks of age. Whereas, only ~10% of the MRL-Pdcd1−/− and MRL wild-type mice died by 30 weeks of age. Autopsy examination revealed that all diseased MRL-Pdcd1−/− mice exhibited dilatation of the heart with occasional thrombus formation in the atrium, pleural effusion, ascites and congested liver, suggesting congestive heart failure as the cause of premature death (Fig. 1B). Histological examination revealed a massive infiltration of lymphocytes as well as multi-nucleated cells in heart (Fig. 1D and I). In addition, inflammatory foci were found in the liver (Fig. 1E, upper panel, 88% of MRL-Pdcd1−/− mice at 4–8 weeks of age, 22 of 25), lung (Fig. 1F, 96%, 24 of 25), salivary gland (Fig. 1G, 33 and 60%, 12 of 25) with little to no tissue destruction. The remaining 30% of the MRL-Pdcd1−/− mice died by 35 weeks of age. Those which died later than 15 weeks showed severe glomerulonephritis but not myocarditis (data not shown). In the 8- and 20-week-old MRL wild-type mice, inflammatory foci were found in the lung (Fig. 1F, 22 and 60%, respectively), salivary gland (Fig. 1G, 33 and 100%, respectively), stomach (Fig. 1H, 0 and 50%, respectively) and liver (Fig. 1E, 0 and 30%, respectively), but not in heart (Fig. 1D). These findings indicate that the autoimmune response against heart is strictly regulated by PD-1, while other organs are at risk of the autoimmune damage on the MRL background, which can be accelerated by PD-1 deficiency (Table 1). Immunohistological analysis revealed that the mononuclear cells consisted primarily of CD4+ and CD8+ T cells, while B cells were rarely detected (Fig. 1J).

No polyclonal activation of T cells in MRL-Pdcd1−/− mice

We examined the lymphoid organs of moribund MRL-Pdcd1−/− mice and compared with those of age-matched and aged (20-week old) MRL wild-type mice. In MRL-Pdcd1−/− mice, thymus was generally smaller than age-matched MRL wild-type mice and large mediastinal lymph nodes were found next to thymus as reported for MRL-Pdcd1−/− and MRL wild-type mice with myocarditis. Strong expression of PD-L1 upon inflammation to suppress autoreactive T cells that may express PD-1, as suggested for the expression of PD-L1 on pancreatic β cells in non-obese diabetic (NOD) mice (28, 29).
However, the increase in splenocytes is quite modest compared with the difference between 8-week-old and 20-week-old MRL wild-type mice (~80% increase). The absolute numbers of CD4+ and CD8+ T cells and B cells are comparable with age-matched MRL wild-type mice (Fig. 2B).

We next analyzed the expression of CD44, CD62L and CD69 on T cells to examine the activation status. The expression of CD69 on CD4+ T cells and CD44 and CD69 on CD8+ T cells was slightly augmented in MRL-Pdcd1−/− mice compared with that in age-matched MRL wild-type mice (Fig. 2C and D). However, the augmentation was quite modest compared with that in Cnta4−/− or aged MRL wild-type mice (17, 18). Hyperactivation of T cells was not observed in mediastinal lymph nodes as well (data not shown). Therefore, in contrast to the non-specific activation of T cells in Cnta4−/− mice, most of the T cells are rather quiescent in MRL-Pdcd1−/− mice.

### Table 1. Summary of autoimmune phenotypes of MRL-Pdcd1−/− mice

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>Pdcd1−/− (4–8 weeks)</th>
<th>Wild type (8 weeks)</th>
<th>Wild type (20 weeks)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Myocarditis</td>
<td>96% (24/25)</td>
<td>0% (0/9)</td>
<td>0% (0/10)</td>
</tr>
<tr>
<td>Sialadenitis</td>
<td>60% (15/25)</td>
<td>33% (3/9)</td>
<td>100% (10/10)</td>
</tr>
<tr>
<td>Gastritis</td>
<td>48% (12/25)</td>
<td>0% (0/9)</td>
<td>50% (5/10)</td>
</tr>
<tr>
<td>Pneumonitis</td>
<td>96% (24/25)</td>
<td>22% (2/9)</td>
<td>60% (6/10)</td>
</tr>
<tr>
<td>Hepatitis</td>
<td>88% (22/25)</td>
<td>0% (0/9)</td>
<td>30% (3/10)</td>
</tr>
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</table>
Myocarditis in MRL-Pdcd1−/− mice

Enhanced production of auto-antibodies against CM

Autoimmune diseases in Pdcd1−/− mice often accompanies auto-antibody production and MRL mice are known to produce auto-antibodies quite frequently (20, 32). Especially, BALB/c-Pdcd1−/− mice develop DCM that is dependent on auto-antibodies against cardiac troponin I. Therefore, we examined the production of auto-antibodies against heart in MRL-Pdcd1−/− mice. As shown in Fig. 5(A–D), MRL-Pdcd1−/− mice produced auto-antibodies against nucleus as well as various organs, including heart, stomach and ovary. PD-1 deficiency augmented both the frequency and the titer of auto-antibodies against heart and all the MRL-Pdcd1−/− mice examined had high-titer auto-antibodies against heart. In contrast, the frequency and the titer of auto-antibodies against nucleus, ovary and stomach were not affected by PD-1 deficiency so much (Fig. 5E).

We next performed western blotting to examine the antigen specificity of the cardiac auto-antibodies. We extracted protein from normal heart and probed with sera from MRL-Pdcd1−/− mice with myocarditis. All the sera from sick MRL-Pdcd1−/− mice strongly recognized a band ~200 kDa (Fig. 5F). Of note, sera from sick MRL-Pdcd1−/− mice rarely recognized cardiac troponin I, whose molecular weight is ~30 kDa (data not shown). CM was suspected as a candidate antigen based on its molecular weight and tissue specificity. All the sera from sick MRL-Pdcd1−/− mice often accompanied auto-antibodies against CM. Therefore, PD-1 deficiency may simply facilitate the development of myocarditis proclivity in MRL mice.

Infiltration of myeloid cells with suppressive function

As mentioned above, we found a massive infiltration of Mac1+ Gr1+ myeloid cells in heart (Fig. 3A and B). When we analyzed the expression of surface markers by FACS, the cells but not the sensitivity of the heart. We transferred total splenocytes of sick MRL-Pdcd1−/− mice into sub-lethally irradiated MRL wild-type mice and examined their hearts 3 weeks later. All the recipient mice showed severe myocarditis, which is pathologically indistinguishable from that in donor mice (Fig. 4A). Inflammatory foci were also observed in the lung, salivary gland and stomach (Fig. 4B–D). FACS analysis confirmed that types of infiltrated cells in the hearts of recipient mice were similar to those in donor mice with slight increase of CD8+ T cells and modest reduction of Mac1+ Gr1+ myeloid cells (Fig. 4E). We also examined the usage of V segments of the TCRβ chain by FACS. Compared with splenocytes, Vβ6+, Vβ8.1/8.2+ CD4+ T cells and Vβ6+ and Vβ8.1/8.2+ CD8+ T cells are increased, whereas Vβ3+, Vβ10+ and Vβ14+ CD4+ T cells and Vβ3+, Vβ10+ and Vβ14+ CD8+ T cells are reduced in hearts of MRL-Pdcd1−/− mice. The increase of Vβ8.1/8.2+ CD4+ and CD8+ T cells was augmented by transfer, whereas the increase of Vβ2+ and Vβ6+ CD4+ T cells and Vβ6+ CD8+ T cells was not reproduced by transfer (Fig. 4F and G). These results suggest that both CD4+ and CD8+ T cells with high affinity may play critical roles in the pathogenesis of myocarditis in MRL-Pdcd1−/− mice.

Tn1-biased activation of heart infiltrates

We next characterized heart infiltrates by FACS. We digested inflamed heart with collagenase and isolated infiltrating cells as described in methods (Fig. 3A). Consistent with the immunohistological analysis, the major populations of heart infiltrates were CD8+ and CD4+ T cells (35.4±2.7% and 19.7±2.0%, respectively), while B cells were quite few (3.8±0.5%). We also found an infiltration of Mac1+ Gr1+ myeloid cells (23.6±2.7%) (Fig. 3A and B). Both CD8+ and CD4+ T cells are activated in heart as evidenced by the higher expression of CD44, CD69 and CD25 and the reduced expression of the CD62L (Fig. 3C). Heart infiltrating T cells expressed a Tn1-specific marker of Tim3 but not a Tn2-specific marker of Tim2 (Fig. 3C) (31). In accordance with the high Tim3 expression, we detected augmented expression of mRNAs for Tn1 cytokines including IFNγ (3048-fold increase compared with normal heart, Fig. 3D) in inflamed heart. Whereas, the amounts of mRNAs for most of the Tn2 cytokines (IL-4, IL-5, IL-9 and IL-13) and IL-17 were under the detection limit (Fig. 3D). The expression of granzyme B and perforin 1 was also higher in inflamed heart (1153- and 442-fold increase, respectively). Therefore, the myocarditis in MRL-Pdcd1−/− mice is most likely mediated by a Tn1-biased immune response.

Next, we performed a transfer experiment to confirm that PD-1 deficiency affects the aggressiveness of autoactive
expression of PD-L1 and F4/80 but not PD-L2, B7.1, B7.2 and CD11c was augmented in heart infiltrating Mac1^+Gr1^+ cells compared with the same fraction in spleen (Fig. 6A–D). Some of the tumor-bearing mice and cancer patients have been reported to accumulate Mac1^+Gr1^+ cells, which are designated as MSCs for their suppressive function against the host immune system. So, we expected that these Mac1^+Gr1^+ cells in inflamed hearts might have immunosuppressive function. We purified Mac1^+Gr1^+ cells from inflamed heart of MRL-Pdcd1^−/−/− mice and examined their suppressive function in allogenic response. As shown in Fig. 7A–D, addition of heart infiltrating Mac1^+Gr1^+ cells efficiently suppressed the proliferation of both CD4^+ and CD8^+ T cells from MRL wild-type mice stimulated by allogenic thy1^+ cells from C57BL/6 mice. Therefore, heart infiltrating Mac1^+Gr1^+ cells take an acute course and these mice die prematurely ~3 to 5 weeks of age (Fig. 1). Pathologically, both MRL-Pdcd1^−/−/− and Cita4^−/− mice develop lymphocytic myocarditis and massive infiltration of T cells can be observed (Figs 1J and 3). However, there are several different aspects between these mice. First, the tissue specificity of autoimmune response is more strictly regulated and heart is predominantly affected in MRL-Pdcd1^−/−/− mice (Fig. 1). Second, the frequency of myocarditis in MRL-Pdcd1^−/−/− mice
is slightly lower than that of Ctla4+/− mice (Fig. 1). Third, no polyclonal activation of lymphocytes was observed in MRL-Pdcd1+/− mice (Fig. 2), while T cells are non-specifically activated in Ctla4+/− mice. Fourth, MRL-Pdcd1+/− mice produced high-titer auto-antibodies against CM (Fig. 5), which was not reported in Ctla4+/− mice. Lastly, the phenotype of Pdcd1+/− mice is strongly influenced by the genetic background of the mice. The polyclonal activation of T cells in Ctla4+/− mice has been reported to be cell non-autonomous based on the observation that wild-type T cells could suppress the autoaggressiveness of Ctla4+/− T cells (33, 34). In the case of MRL-Pdcd1+/− mice, co-transfer of MRL wild-type and MRL-Pdcd1+/− splenocytes into sub-lethally irradiated MRL mice still induced severe myocarditis (three of three recipients), so the activation of heart reactive T cells by PD-1 deficiency is likely due to the hyperactivation of effector cells but not the developmental and/or functional deficit of regulatory T cells. Taken together, CTLA-4 and PD-1 play similar but yet non-redundant roles in the regulation of autoimmunity. The heart-specific autoimmune damage in MRL-Pdcd1+/− mice is most likely due to the tissue-specific loss of immunological tolerance rather than a manifestation of a systemic autoimmune inflammation.

As mentioned above, Pdcd1+/− mice develop DCM on BALB/c background (19). Although the affected organ is the same between Pdcd1+/− mice on BALB/c and MRL backgrounds, phenotypes are quite different as summarized in Table 3. For example, there is almost no inflammation in hearts of BALB/c-Pdcd1+/− mice, while hearts of MRL-Pdcd1+/− mice are heavily infiltrated by lymphocytes and myeloid cells. Although both BALB/c-Pdcd1+/− and MRL-Pdcd1+/− mice produce cardiac auto-antibodies, their antigens are different [cardiac troponin I (∼30 kDa) and CM (∼200 kDa) in BALB/c-Pdcd1+/− and MRL-Pdcd1+/− mice, respectively]. Further studies are required to unravel why hearts are preferentially attacked in Pdcd1+/− mice both on MRL and on BALB/c backgrounds.

MRL-Faslpr/lpr mice have been widely used as an animal model of lupus erythematosus and several autoimmune susceptible loci have been identified on its chromosome (6). These loci seem to function even in the presence of the intact Fas gene because MRL wild-type mice have also been reported to develop lupus-like syndrome as they age (7, 11). However, the immunological function of these loci especially in organ-specific autoimmunity was not well understood. Current analysis clearly demonstrated that MRL chromosome can support autoimmune myocarditis in conjunction with PD-1 deficiency. Preliminary intercross experiments suggested the presence of several loci that promote or protect autoimmune myocarditis on MRL chromosome. Interestingly, adoptive transfer of splenocytes from sick MRL-Pdcd1+/− mice failed to induce fatal myocarditis in other mouse strains that share the same MHC haplotype with MRL (AKR and C3H, data not shown), suggesting that some of these loci may affect the sensitivity of cardiomyocytes against autoreactive T cells.

Another intriguing finding of the current study is the identification of MSCs in the inflamed hearts. Because MSCs from inflamed heart highly expressed PD-L1, we expected that MSCs suppress T-cell response in a PD-1-dependent manner and that the defect in the MSC function is the main cause of myocarditis in MRL-Pdcd1+/− mice. However, T cells from MRL-Pdcd1+/− mice were apparently suppressed by MSCs albeit to a lesser extent compared with those from MRL wild-type mice in our in vitro experimental system (Fig. 7), suggesting that the reduced sensitivity to MSCs may not be the main
Fig. 5. Augmented production of auto-antibodies against CM by MRL-Pdcd1^{−/−} mice. (A–D) Sera from sick MRL-Pdcd1^{−/−} mice recognized cardiomyocytes (A), gastric parietal cells (B), nucleus (C) and granulosa cells in the ovary (D). Pancreatic section is shown as an example of nuclear staining in (C). (E) Frequencies of mice with auto-antibodies against heart, stomach, ovary and nucleus are shown for indicated genotype. The titer of auto-antibodies are shown by bars: black bar, positive with ×1000 diluted sera (×10 000 for stomach); gray bar, positive with ×300 diluted sera (×1000 for stomach) and white bar, positive with ×100 diluted sera (×300 for stomach); n = 20 (KO), 12 (age-matched WT) and 10 (aged WT). (F) MRL-Pdcd1^{−/−} mice produced auto-antibodies against 200 kDa cardiac antigens. Sera from MRL-Pdcd1^{−/−} (lanes 1–4), age-matched (lanes 5–7) and aged (lanes 8–10) MRL wild-type mice are diluted 1000 times and examined for the reactivity with heart extract. (G) MRL-Pdcd1^{−/−} mice produced auto-antibodies against CM. Sera from MRL-Pdcd1^{−/−} (lanes 1–4), age-matched (lanes 5–7) or aged (lanes 8–10) MRL wild-type mice are diluted ×1000 and examined for the reactivity with purified CM. (H) Sera from age-matched and aged MRL wild-type mice weakly recognized CM. Lanes 1 and 2, ×3000 diluted sera from MRL-Pdcd1^{−/−} mice; lanes 3–8, ×1000, ×300 and ×100 diluted sera from age-matched MRL wild-type mice; lanes 9–14, ×1000, ×300 and ×100 diluted sera from aged MRL wild-type mice.

Fig. 6. Expression of PD-L1 on heart infiltrating Mac1^{+}Gr1^{+} cells. (A and B) Representative FACS profiles of heart infiltrates (A) and splenocytes (B) are shown with dot plot. (C and D) Expression of PD-L1, PD-L2, F4/80, B7.1, B7.2 and CD11c on Mac1^{+}Gr1^{+} (upper panel), Mac1^{+}Gr1^{+} (middle panels) and Mac1^{−}Gr1^{+} (lower panels) cells is shown with histogram plot for heart infiltrates (C) and splenocytes (D).
siveness of splenocytes. The increase of MSCs in *T. cruzi* infection is dependent on IFNγ because the increase of MSCs could not be observed in IFNγ receptor-deficient mice. Collectively, it is likely that MSCs migrated into hearts as the result of strong Th1 response to suppress the autoimmune inflammation in hearts of MRL-Pdcd1−/− mice. However, MSCs could not suppress myocarditis due to its insufficient suppressive function or late arrival. Further studies are required for the understanding of the actual contribution of MSC-dependent suppression of autoimmunity.

While we are preparing this manuscript, Lucas et al. (35) reported that PD-L1-deficient MRL mice (MRL-Pdcd1lg1−/− mice) develop autoimmune myocarditis and pneumonitis. Although the overall phenotypes of MRL-Pdcd1lg1−/− and MRL-Pdcd1−/− mice are similar, phenotypes of MRL-Pdcd1−/− mice seem to be milder. All the MRL-Pdcd1lg1−/− mice die of myocarditis by 8 weeks, while only 70% of MRL-Pdcd1−/− mice die of myocarditis by 8 weeks. Interestingly, not only PD-L1 on target cells but also on bone marrow (BM)-derived cells play important roles in the prevention of myocarditis in MRL-Pdcd1lg1−/− mice because BM cells from MRL-Pdcd1lg1−/− mice can transfer myocarditis to lethally irradiated MRL mice. It is tempting to speculate that PD-L1 on MSCs plays regulatory role in both PD-1-dependent and -independent manner and that the presence of PD-1-independent regulatory function of PD-L1 on MSCs is the reason why phenotypes of Pdcd1−/− mice is milder than those of Pdcd1lg1−/− mice. In addition, myocarditis occurs in a comparable frequency (~80%) in the presence of *lpr* mutation of Fas gene in Pdcd1lg1−/− mice, while *lpr* mutation significantly attenuates myocarditis in Pdcd1−/− mice (supplementary Figure 1 available at *International Immunology* Online) 20 and 75% in the presence and absence of *lpr* mutation, respectively. It is reported that *lpr* mutation prevents autoimmune diabetes in NOD mice by protecting target cells from terminal destruction, preventing physiological β-cell death in the young or directly dampening T-cell reactivity (36–38). Because adoptive transfer of splenocytes from sick MRL-Pdcd1lg1−/− mice induced severe myocarditis in sub-lethally irradiated MRL-Faslpr/lpr mice (data not shown), it is less likely that autoreactive cells damage cardiomyocytes in a Fas-dependent manner. Further studies are needed to clarify the reason why Fas deficiency, which is required for the systemic autoimmunity in MRL-Faslpr/lpr mice, rather prevent tissue-specific autoimmunity in MRL-Pdcd1−/− mice.

**Supplementary data**

Supplementary Figure 1 is available at *International Immunology* Online.

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