Roles of programmed death-1 (PD-1)/PD-1 ligands pathway in the development of murine acute myocarditis caused by coxsackievirus B3

Yoshinori Seko,⁎, Hideo Yagita, Ko Okumura, Miyuki Azuma, Ryozo Nagai

Department of Cardiovascular Medicine, Graduate School of Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, Japan
Department of Immunology, School of Medicine, Juntendo University, Tokyo, Japan
Department of Molecular Immunology, Graduate School, Tokyo Medical and Dental University, Tokyo, Japan

Received 16 September 2006; received in revised form 8 February 2007; accepted 12 March 2007

Abstract

Objective: This study was designed to investigate the roles of programmed death-1 (PD-1) and PD-1 ligands (PD-L) in the development of murine acute myocarditis caused by Coxsackievirus B3. PD-1/PD-L belong to the CD28/B7 superfamily, and the PD-1/PD-L pathway is known to transduce a negative immunoregulatory signal that antagonizes the T-cell receptor-CD28 signal and inhibits T-cell activation.

Methods: We first analyzed the expression of PD-L1/PD-L2 on cardiac myocytes in vivo and in vitro. Second, we examined the effects of in vivo treatment with an anti-PD-1, PD-L1, or PD-L2 monoclonal antibodies on the development of myocardial inflammation in C3H/He mice infected with Coxsackievirus B3. Third, to investigate the effects of anti-PD-1 monoclonal antibody treatment on the activation of the infiltrating cells, we examined the expression of interleukin (IL)-2, interferon (IFN)-γ, CD40 ligand (CD40L), Fas ligand (FasL), and perforin as activation markers in mouse hearts by a semiquantitative PCR method.

Results: PD-L1 was markedly induced on cardiac myocytes with acute myocarditis. In vivo anti-PD-1 or -PD-L1 blocking monoclonal antibody treatment increased the myocardial inflammation whereas anti-PD-1 stimulating monoclonal antibody treatment decreased the myocardial inflammation, and anti-PD-L2 monoclonal antibody treatment had no effect. Anti-PD-1 monoclonal antibody treatment significantly increased the expression of IFN-γ, FasL, CD40L, perforin, and Coxsackievirus B3 genomes in myocardial tissue.

Conclusion: Our findings strongly suggest that the PD-1/PD-L1 pathway played a pivotal role in suppressing myocardial inflammation and raise the possibility of immunotherapy by stimulating the PD-1/PD-L1 pathway to prevent myocardial damage in viral myocarditis.

© 2007 European Society of Cardiology. Published by Elsevier B.V. All rights reserved.

Keywords: Immunology; Infection/inflammation; Myocarditis; Viral diseases

1. Introduction

We formerly reported that costimulatory molecules belonging to the immunoglobulin (Ig) superfamily such as intercellular adhesion molecule-1, vascular cell adhesion molecule-1, and B7-1 as well as to the tumor necrosis factor (TNF) receptor/ligand superfamilies such as CD40/CD40 ligand (CD40L, CD154), 4-1BB (CD137)/4-1BBL, and Fas (CD95)/FasL play an important role in the development of myocardial injury involved in murine viral myocarditis [1–3,5–7]. In contrast to these costimulatory molecules, which deliver positive signals for T-cell activation, cytotoxic T lymphocyte antigen (CTLA)-4, a second B7 receptor expressed on T-cells, transduces a negative signal for T-cell activation competing with CD28. Other known costimulatory molecules which mediate negative signaling for T-cell activation include programmed death-1 (PD-1) and PD-1 ligands, PD-L1 and PD-L2. PD-1/PD-L1 ligands belong to the CD28/B7 family of Ig superfamily. PD-1, an inhibitory receptor isolated by subtractive hybridization technique in 1992 [8], can be induced on not only T-cells but also B-cells...
and myeloid cells, while most of other regulatory co-receptors are induced only on the T-cells [9]. PD-1 delivers a negative costimulatory signal by engagement with its ligands, PD-L1 or PD-L2 [10,11]. The expression of PD-L1 and PD-L2 has been detected in a variety of lymphohematopoietic cell types and nonlymphoid tissues including heart, and their expression was shown to be up-regulated on T-cells and antigen presenting cells by stimulation [11–14]. Studies on PD-1-deficient mice strongly suggested that PD-1/PD-1 ligands pathway may play an important role in peripheral tolerance and prevention of autoimmune diseases [15,16].

The purpose of the present study was to investigate the role of PD-1/PD-1 ligands pathway in the development of myocardial damage in murine acute myocarditis induced by Coxsackievirus B3 (CVB3).

2. Methods

2.1. Animals

The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institute of Health (NIH Publication No. 85-23, revised 1996). Five-week-old male C3H/He mice and pregnant female C3H/He mice were purchased from Shizuoka Laboratory Animal Center (Shizuoka, Japan). This study was carried out in accordance with the guide for the care and use of laboratory animals of University of Tokyo.

2.2. Virus

The preparation of CVB3 (Nancy strain) was as published elsewhere [3]. Five-week-old male C3H/He mice were inoculated intraperitoneally with $1 \times 10^6$ plaque-forming unit of CVB3 in 0.2 mL phosphate-buffered saline.

2.3. Monoclonal antibodies (mAbs)

An anti-mouse PD-1 mAb (hybridoma RMP1-14, rat IgG2a), which was generated against mouse PD-1 transfectant, blocked the binding of both PD-L1-Ig and PD-L2-Ig to PD-1 transfectants just like J43 [17,18]. An anti-mouse PD-L1 mAb (MIH5, rat IgG2a) [19] and an anti-mouse PD-L2 mAb (TY25, 

Table 1

<table>
<thead>
<tr>
<th>Days after infection</th>
<th>Cardiac myocytes</th>
<th>Vascular endothelial cells</th>
<th>Interstitial cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>−</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>3</td>
<td>±</td>
<td>±</td>
<td>±</td>
</tr>
<tr>
<td>5–7</td>
<td>++ and +</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>14</td>
<td>+</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>21</td>
<td>±</td>
<td>++</td>
<td>±</td>
</tr>
<tr>
<td>28</td>
<td>±</td>
<td>++</td>
<td>± and −</td>
</tr>
<tr>
<td>42</td>
<td>± and −</td>
<td>++</td>
<td>±</td>
</tr>
</tbody>
</table>

*The intensity of staining was shown as follows: −, negative; ±, weakly positive; +, moderately positive; ++, strongly positive.
rat IgG2a) [14] were described elsewhere and were used for immunohistochemical study and in vivo mAb treatment study. The preparation of mouse anti-cardiac myosin mAb (CMA19) was as published elsewhere [20]. Another anti-mouse PD-1 mAb (hybridoma PIM-2, rat IgG2a) was recently developed. The procedures for the staining of PD-1 cDNA transfected BHK cells and parental BHK cells with this mAb were as published elsewhere [17].

2.4. In vivo treatment of mice with anti-PD-1/PD-L1/PD-L2 mAbs (Experiment 1)

Five-week-old C3H/He mice were divided into 5 groups, designated as A, B, C, D, and E (8 mice were used for each group). Mice in Group B received the anti-PD-1 blocking mAb (5 mg/kg, intraperitoneally), on the day of virus inoculation (day 0) and on day 3. Mice in Group C received the anti-PD-L1...
mAb (5 mg/kg), mice in Group D received the anti-PD-L2 mAbs (5 mg/kg), mice in Group E received the anti-PD-L1 plus anti-PD-L2 mAbs (5 mg/kg each), and mice in Group A received rat IgG (5 mg/kg) as controls, in the same way.

Mice were euthanized on day 7, and the hearts were laterally sectioned approximately midway between the apex and the atria, which resulted in cross-sections of both ventricles. Half of each heart was fixed in 10% buffered formalin and used for histological study. The other half of each heart was frozen in liquid nitrogen and used for polymerase chain reaction (PCR) and terminal deoxynucleotidyl transferase nick-end labeling (TUNEL) staining. Another lot of 5-week-old C3H/He mice received the anti-PD-1 blocking mAb (5 mg/kg, intraperitoneally) and were euthanized on days 28 and 56 (3 mice were used for each group), and the heart was used for histological study.

2.5. In vivo treatment of mice with anti-PD-1 stimulatory mAb (Experiment 2)

Another lot of 5-week-old C3H/He mice were divided into 2 groups, designated as A and B (8 mice were used for each group). Mice in Group B received the anti-PD-1 stimulatory mAb (PIM-2; 5 mg/kg, intraperitoneally), on the day of virus inoculation (day 0) and on day 3, and mice in Group A received rat IgG (5 mg/kg) as controls, in the same way. The subsequent procedures for histological study were the same as in Experiment 1.

2.6. Histology

The cross-sections of formalin-fixed heart tissue were stained with hematoxylin and eosin, and the percent area of the myocardium undergoing inflammation was determined as published elsewhere [3].

2.7. Preparation of Cultured Cardiac Myocytes

Cultured ventricular cardiac myocytes were prepared from 14- to 16-day-old fetal C3H/He mice as published elsewhere [3]. The isolated cardiac myocytes were treated with recombinant murine interferon (IFN)-γ (10^5 U/L or 3 × 10^5 U/L) (Shionogi & Co., Ltd) for 48 h, then subjected to immunofluorescence study or Northern blot analysis, respectively, as published elsewhere [1,3].

2.8. Immunofluorescence

Mice were killed at each time point after virus inoculation. Cryostat sections (6 μm thick) of heart ventricles were stained with rat anti-mouse PD-1, PD-L1 or PD-L2 mAb by Tyramide Signal Amplification (TSA) technology for fluorescence (TSA™-Direct [Green], NEN Life Science Products) as published elsewhere [3].

For in vitro study, to distinguish cardiac myocytes from non-muscle cells (mainly consisted of fibroblasts), we performed double staining for cardiac myosin heavy chain and PD-L1 or PD-L2 as published elsewhere [3]. To examine the effect of anti-PD-1 blocking mAb treatment on the phenotypes of the infiltrating cells, we also did immunostaining with anti-asialo GM1 for natural killer (NK) cells and anti-Thy 1 for T-cells as described elsewhere [4].

2.9. Preparation of labeled cDNA probes

A full-length mouse PD-L1 cDNA was generated by PCR from mouse spleen cell mRNA with primers 5’-CCGCTCGAGCCCAAAACATGAGGATATTG-3’ and 5’-ATA AAG AAT GC GGC CGC TAC GTC TCC TCC GAATTG-3’ to introduce upstream XhoI and downstream NotI sites and was subcloned into pMKITNeo vector. A mouse PD-L1 cDNA (XhoI/NotI) fragment, a mouse PD-L2 cDNA (EcoRI/NotI) fragment [12], and a PCR fragment of rat GAPDH were gel-purified and labeled with 32P-dCTP by Ready-To-Go™ DNA Labelling Beads (-dCTP) (Amer-sham Biosciences, England; According to the manufacturer’s instructions).

2.10. Northern blot hybridization

Total cytoplasmic RNA from the cultured cardiac myocytes treated with IFN-γ were subjected to Northern blot hybridization with 32P-labeled mouse PD-L1, PD-L2, and rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probes as described elsewhere [1].

![Fig. 3. Northern blot analysis of cultured ventricular myocytes for PD-L1 and PD-L2. Total cytoplasmic RNA (each 30μg) from ventricular myocytes cultured with no addition (control) and with IFN-γ (300 U/ml) for 48 h was hybridized with 32P-labeled mouse PD-L1, PD-L2, and rat GAPDH probes.](image)
2.11. Preparation of RNA and cDNA Synthesis

Mice were killed on day 7 after virus inoculation. The procedures for preparation of total cytoplasmic RNA from the heart tissues and cDNA synthesis were as published elsewhere [21].

2.12. Amplification of cDNA by PCR

To examine the expression of mRNAs of cytokines and other immune mediators in the heart tissues semiquantitatively, we amplified the single-stranded cDNA in a DNA thermal cycler (Perkin-Elmer Cetus, Norwalk, CT) with 1 unit of Ex Taq DNA

Fig. 4. Histological analysis of the effects of blockade of PD-1/PD-1 ligands pathway on the development of myocarditis. Cross-sections of the heart of a mouse (day 7) in rat IgG-treated control group (panel A), anti-PD-1 blocking mAb (RMP1-14)-treated group (panel B), anti-PD-L1 mAb-treated group (panel C), anti-PD-L2 mAb-treated group (panel D), anti-PD-L1 plus anti-PD-L2 mAbs-treated group (panel E), and a mouse (day 28) in anti-PD-1 blocking mAb (RMP1-14)-treated group (panel F) were stained with hematoxylin and eosin. Scale bar=100 μm. Panel G: Mean (±SE) percent area of myocardium undergoing inflammation in rat IgG-treated control group, anti-PD-1 blocking mAb (RMP1-14)-treated group, anti-PD-L1 mAb-treated group, anti-PD-L2 mAb-treated group, and (anti-PD-L1 plus anti-PD-L2) mAbs-treated group.
polymerase (TAKARA BIO Inc., Shiga, Japan) using 5′- and 3′-primers specific for IL-2, IFN-γ, CD40L, FasL, pore-forming protein (PFP), CVB3, and GAPDH, respectively. The primer sequences, annealing temperature, and the number of cycles for IL-2, IFN-γ, CD40L, GAPDH, FasL, PFP, and CVB3 genomes were as published elsewhere [6,22–25]. The PCR was performed denaturation at 94 °C for 1 min, primer annealing for 2 min, and primer extension at 72 °C for 3 min. Expression of these mRNAs was examined using ethidium bromide-stained agarose gel electrophoresis. The band intensity of the PCR products were quantified by analysis performed on a Macintosh computer using the public domain NIH Image program.

2.13. Statistical analysis

One-way ANOVA (using P corrected by Bonferroni/Dunn’s modulus for multiple comparison) was used to evaluate differences between the five groups. Wilcoxon’s two-rank-sum test (Mann–Whitney U test) with p values corrected by the Bonferroni method was used to evaluate differences between the two groups.

3. Results

3.1. Expression of PD-1/PD-1 ligands in Ventricular Tissue

In ventricular tissue of normal mice, PD-L1 was only weakly expressed by vascular endothelial cells and there was almost no expression of PD-L1 on cardiac myocytes (Fig. 1A). There was also only weak or almost no expression of PD-L1 on cardiac myocytes on days 1 to 4 (data not shown). On day 5 after virus inoculation, just after massive cell infiltrations appeared, expression of PD-L1 was clearly induced on the sarcolemma of cardiac myocytes as well as interstitial cells, which were thought to be dendritic cells and fibroblasts, then reached a maximum level on about day 7 and day 14, respectively (Fig. 1B and C, respectively). The expression of PD-L1 on cardiac myocytes continued for more than 4 weeks after virus inoculation with gradual decrease was seen non-uniformly over the myocardium and around the areas of cell infiltration or adjacent to them in serial sections. The expression of PD-L1 on vascular endothelial cells, which reached a maximum level on about day 7, continued for more than 6 weeks after virus inoculation (Fig. 1D). Most of the infiltrating cells, which mainly consisted of NK cells and T-cells, strongly expressed PD-L1 and PD-1 (Fig. 1B and H, respectively). There was almost no expression of PD-L2 in normal ventricular tissue including cardiac myocytes (Fig. 1E). No expression of PD-L2 was induced on cardiac myocytes through the course of acute myocarditis, whereas a part of the infiltrating cells strongly expressed PD-L2 (Fig. 1F and G). The infiltrating cells expressing PD-L2 were T-cells expressing T-cell receptor αβ (data not shown). Time course of the expression of PD-L1 on myocardial cells is summarized in Table 1.

3.2. Induction of PD-L1/PD-L2 on cultured cardiac myocytes by IFN-γ

Next, to confirm the induction of PD-1 ligands on cardiac myocytes, we examined the expression of PD-L1 and PD-L2 on cultured cardiac myocytes treated with IFN-γ in vitro. Fig. 2 shows double-stained cardiac myocytes cultured in a medium with or without IFN-γ for 48 h. Panels (A and B) show the staining pattern specific for PD-L1, and panels (E and F) show that for PD-L2. Panels (C and D), which correspond to panels (A and B), and panels (G and H), which correspond to panels (E and F) respectively, show the staining pattern specific for cardiac myosin heavy chain and indicate that most of the cells were cardiac myocytes. There was very slight or no expression of PD-L1 on cardiac myocytes of the control group (Fig. 2A). After treatment with IFN-γ, most of the cardiac myocytes moderately to strongly expressed PD-L1 (Fig. 2B). There was weak expression of PD-L2 on cardiac myocytes in the control group (Fig. 2E). The expression level of PD-L2 on

---

**Fig. 5.** Effects of anti-PD-1 blocking mAb (RMP1-14) treatment on the expression of proinflammatory cytokine transcripts and CVB3 genomes in the ventricular tissues. Total cytoplasmic RNA was prepared from ventricular tissues of mice from rat IgG-treated control group and anti-PD-1 blocking mAb (RMP1-14)-treated group and analyzed for IL-2, IFN-γ, CD40L, FasL, PFP, CVB3, and GAPDH transcripts by a semiquantitative PCR method. Panel A: Ethidium bromide-stained agarose gel of the PCR. Panel B: Mean (±SE) percent of relative expression levels of PCR products corrected by the levels of GAPDH from ventricular tissues of mice from anti-PD-1 blocking mAb (RMP1-14)-treated group as compared with those from rat IgG-treated control group. Expression of these mRNAs was examined using ethidium bromide-stained agarose gel electrophoresis. The band intensity of the PCR products were quantified by analysis performed on a Macintosh computer using the public domain NIH Image program. *p<0.05, †p<0.02.
cardiac myocytes was not significantly altered by treatment with IFN-γ (Fig. 2F).

3.3. Induction of PD-L1 mRNA in cultured cardiac myocytes by IFN-γ

To confirm the expression of PD-1 ligands at the transcriptional level, we performed Northern blot analysis using a 32P-labeled PD-L1 and PD-L2 probes. Fig. 3 shows the results of Northern blot analysis of the cultured ventricular myocytes by treatment with or without IFN-γ for 48 h. Lower panel shows the levels of GAPDH mRNA for each group as an internal standard. This confirmed that equivalent amounts of RNA were prepared from each group. Upper panel shows the levels of PD-L1 mRNA. Very low levels of PD-L1 mRNA were found in the control group, while marked levels were induced by treatment with IFN-γ. Middle panel shows the levels of PD-L2 mRNA. Almost no significant levels of PD-L2 mRNA were found in the control group as well as in the IFN-γ-treated group.

3.4. Effects of blockade of PD-1/PD-1 ligands pathway on the development of myocardial inflammation (Experiment 1)

The incidence of myocarditis was 100% and no mice were dead in all of the groups. Fig. 4A–E shows the representative sections of the heart of a mouse from Group A (rat IgG-treated control group), B (anti-PD-1 mAb [RMP1-14]-treated group), C (anti-PD-L1 mAb-treated group), D (anti-PD-L2 mAb-treated group), and E ([anti-PD-L1 plus anti-PD-L2 mAb]-treated group), respectively. Extensive cell infiltration and necrosis were seen in the mouse from Group B (Fig. 4B). Whereas both cell infiltration and necrosis were clearly less severe in the mouse from Group A, D, and E (Fig. 4A, D, and E, respectively). The results of the histological study are summarized in Fig. 4G. The (mean±SE) percent area of myocardium undergoing inflammation was significantly increased in Group B (13.61±0.99) as compared with Group A (7.95±1.35; p<0.05), Group D (7.52±1.25; p<0.05), and Group E (8.63±2.70; p<0.05), respectively. The extent of inflammation was also increased in Group C (11.39±1.71) as compared with Group A, but still less than Group B. However, there were no significant differences between Group A and C as well as between Group B and C. This suggests that anti-PD-L1 mAb treatment could not completely inhibit PD-1/PD-L1 pathway in vivo. Thus, blockade of PD-1/PD-1 ligands (especially PD-L1) pathway significantly increased the myocardial inflammation induced by CVB3. To examine the effect of anti-PD-1 mAb treatment on the development of inflammation in more detail, we measured the wet weight and dry weight of the myocardial tissue samples. The (mean±SE, n=8) ratio of (wet weight-dry weight) to dry weight, which

Fig. 6. Panel A: Staining of PD-1 cDNA transfected BHK cells with the anti-PD-1 stimulating mAb (PIM-2). PD-1 cDNA transfected BHK cells and parental BHK cells were stained with the PIM-2 mAb followed by FITC-anti-rat IgG. Fluorescence intensity is plotted on a horizontal logarithmic scale. The vertical scale represents the number of cells. Panel B: Mean (±SE) percent area of myocardium undergoing inflammation in rat IgG-treated control group and anti-PD-1 stimulating mAb (PIM-2)-treated group.
may reflect the interstitial edema induced by inflammation, in anti-PD-1 mAb (RMP1-14)-treated group (2.66±0.02; p<0.01) was significantly increased than that of rat IgG-treated control group (2.26±0.12), indicating the enhancement of inflammation. However, there was no significant difference in the percentage (mean±SE, n=8) of infiltrating NK cells between rat IgG-treated control group (83.74±1.22) and anti-PD-1 mAb (RMP1-14)-treated group (84.18±2.19). There was also no significant difference in the percentage (mean±SE, n=8) of infiltrating of T-cells between rat IgG-treated control group (11.41±0.68) and anti-PD-1 mAb (RMP1-14)-treated group (12.23±0.59). Fig. 4F shows a representative section of the heart of a mouse from anti-PD-1 mAb [RMP1-14]-treated group euthanized on day 28. Myocardial inflammation seemed to be still increased than that of control group on day 7. Myocardial inflammation still existed on day 56, although significantly less severe than that on day 28 (data not shown). This suggested that blockade of PD-1/PD-1 ligands pathway may also prolong the myocardial inflammation.

3.5. Effects of anti-PD-1 blocking mAb treatment on the expression of immune mediator transcripts and CVB3 genomes in the ventricular tissues (Experiment 1)

Next, to investigate the effects of anti-PD-1 blocking mAb (RMP1-14) treatment on the activation of infiltrating cells, we analyzed the expression of IL-2 and IFN-γ, which are mainly expressed by the infiltrating cells and can be good markers for activation [21], and a cytolytic factor PFP, known to be expressed by killer lymphocytes such as NK cells and CTLs. We also examined the expression of CD40L and FasL, which were expressed on the infiltrating cells and were shown to play important roles in myocardial injury [5,6], as well as CVB3 genomes. We amplified the cDNAs from ventricular tissues of mice from rat IgG-treated control group (Group A) and those from anti-PD-1 mAb-treated group (Group B) by a semiquantitative PCR method. The relative expression levels of these immune mediators corrected by the levels of GAPDH were summarized in Fig. 5B. As shown in Fig. 5 (panels A and B), anti-PD-1 blocking mAb (RMP1-14) treatment significantly increased the expression of IFN-γ, FasL, and PFP genomes. Anti-PD-1 blocking mAb treatment also increased the expression of IL-2, CD40L, and CVB3, whereas the differences between the 2 groups were not statistically significant. This strongly suggested that blockade of PD-1/PD-1 ligands pathway enhanced activation of the infiltrating cells. Expression of GAPDH transcripts as internal standard showed that almost equivalent amounts of RNA were prepared from each mouse.

3.6. Staining of PD-1 transfected BHK cells with the PIM-2 mAb

We recently developed another anti-PD-1 mAb (PIM-2), which showed stimulatory effects on PD-1/PD-1 ligands interaction. As shown in Fig. 6A, the mAb PIM-2 was found to react with PD-1 transfected BHK cells but not with parental BHK cells, indicating the specific recognition of PD-1 antigen by this mAb.

3.7. Effects of stimulation of PD-1/PD-1 ligands pathway on the development of myocardial inflammation (Experiment 2)

Next, using this anti-PD-1 stimulating mAb (PIM-2), we analyzed the effects of stimulation of PD-1/PD-1 ligands pathway on the development of myocardial inflammation induced by CVB3 as in Experiment 1. The results of the histological study are summarized in Fig. 6B. The (mean±SE) percent area of myocardium undergoing inflammation was significantly decreased in Group B (4.01±0.74) as compared with Group A (10.04±1.13; p<0.005). Together with the results of Experiment 1, this indicates that modulation of PD-1/PD-1 ligands pathway can significantly improve or worsen the myocardial inflammation induced by CVB3.

4. Discussion

In this study, we demonstrated that PD-L1 (but not PD-L2) was markedly induced on cardiac myocytes and that PD-1 was strongly expressed by most of the infiltrating cells in murine acute viral myocarditis. The induction of PD-L1 on cardiac myocytes was confirmed in vitro by treatment with IFN-γ, which was shown to be mainly synthesized by the infiltrating cells in vivo [1]. This strongly suggested that the expression of PD-L1 on cardiac myocytes with acute myocarditis was induced by the cytokines (such as IFN-γ) mainly released from the infiltrating cells. There have been no previous studies reporting the expression of PD-L1 on cardiac myocytes themselves. Furthermore, in vivo anti-PD-1 blocking mAb (RMP1-14) treatment significantly increased the myocardial inflammation as well as the expression of IFN-γ, FasL, PFP, which can be activation markers for the infiltrating cells. Whereas anti-PD-1 stimulating mAb (PIM-2) treatment significantly decreased the myocardial inflammation. This strongly suggested that PD-1/PD-L1 pathway plays a critical role in suppressing myocardial inflammation through suppressing activation of infiltrating cells and virus replication. Because most of the infiltrating cells, which mainly consisted of NK cells and T-cells, strongly expressed PD-1 and PD-L1, we thought that NK cells as well as T-cells are involved in the mechanism of the in vivo effects of anti-PD-1 mAb treatment. This was supported by the fact that blockade of PD-1/PD-1 ligands pathway did not significantly affect the relative distribution of the infiltrating cell types such as NK cells and T-cells.

For roles of PD-1/PD-1 ligands pathway in virus infection, Iwai et al. [26] reported that effector T-cell proliferation and cell infiltration were increased in the liver of adenovirus-infected PD-1−/− mice as compared with wild-type mice and that remarkably rapid virus clearance was observed in PD-1−/− mice. This contrasts sharply with the present study in which blockade of PD-1 suppressed virus clearance. From the data of our previous studies, it seems that suppression of the effector T-cell activation decreases myocardial inflammation and suppresses
virus replication in our model of viral myocarditis [1-3,6,7]. This is strongly supported by the fact that perforin knockout mice infected with CVB3 developed less severe myocarditis than wild-type did and could control the infection and eradicate the virus [27]. Precise mechanism for this difference is unknown and remains to be clarified. For roles of PD-1/PD-1 ligands pathway in pathological conditions of the heart, Ozkaynak et al. [28] reported that PD-L1.Ig (but not PD-L2.Ig) fusion protein administration in conjunction with subtherapeutic dose of cyclosporin A in fully major histocompatibility complex (MHC)-disparate combinations markedly prolonged cardiac allograft survival. Aramaki et al. [29] reported that pretreatment of intratracheal delivery of alloantigen significantly prolonged cardiac allograft survival and that concurrent administration of anti-PD-1 or anti-PD-L1 (but not anti-PD-L2) mAb abrogated the prolongation. These studies showed that PD-1/PD-L1 interaction plays an essential role in cardiac allograft survival. Although the authors did not demonstrate the expression of PD-L1 on cardiac myocytes of the allografts, this suggested that similar mechanisms through PD-1/PD-L1 interaction may be involved in allograft rejection and acute viral myocarditis revealed in the present study. Okazaki et al. [30] reported that PD-1 deficient mice developed autoimmune dilated cardiomyopathy with production of high-titer autoantibodies against cardiac troponin I, which may have induced heart dysfunction by chronic stimulation of Ca\(^{2+}\) influx in cardiomyocytes. Thus, PD-1/PD-1 ligands pathway seems to suppress humoral autoimmunity as well as cellular autoimmunity against cardiac myocytes revealed in the present study. It is known that direct cytopathic effects of the virus as well as immune-mediated target cell injury play a pivotal role in the myocardial damage in viral myocarditis. Xiong et al. [31] reported that enterovirus infection induced more severe cardiomyopathy with enhanced viral replication in dystrophin-deficient mice than in wild-type mice, supporting that enteroviral infection causes cytopathic effects and viral release by cleaving dystrophin with entero viral protease [32]. This mechanism as well as PD-1/PD-L1 pathway are thought to be important factors in regulating myocardial damage.

For roles of PD-1/PD-1 ligands pathway in autoimmune model, there have been 2 studies reporting that PD-L1 but not PD-L2 was expressed at the site of inflammation such as islets in NOD mice and CNS in experimental autoimmune encephalomyelitis and that PD-1 blockade accelerated the development of inflammation in both diseases [18,33]. The results of these studies are also consistent with our data in the present study. Thus, PD-1/PD-1 ligands pathway plays a critical role in suppressing inflammation induced by viral infection, allograft transplantation, as well as autoimmune mechanism. For roles of PD-1/PD-1 ligands pathway in the regulation of anti-tumor immune response, Dong et al. [34] reported that PD-L1 expression is up-regulated in various tumor cells and that tumor cell-associated PD-L1 promotes antigen-specific T-cell apoptosis, suggesting a mechanism by which tumors may evade immune attack. In the present study, we also analyzed whether blockade of PD-1/PD-L1 pathway affected the apoptosis of the infiltrating cells. However, there was no significant difference in the rate of infiltrating cells underwent apoptosis between rat IgG-treated control group and anti-PD-1 blocking mAb-treated group. The percentage of the cardiac myocytes underwent apoptosis was minimal (less than 1 %) in this model of myocarditis, which was not significantly affected by blockade of PD-1/PD-L1 pathway (data not shown).

By flow cytometric analysis, PD-L1 has been reported to be constitutively expressed on various lymphohematopoietic cell types and essentially expressed by all lymphocytes upon stimulation [14]. Whereas PD-L2 expression is only inducible on macrophages and dendritic cells upon stimulation [14]. In the present study, we found that PD-L1 expression was strongly induced on cardiac myocytes whereas no expression of PD-L2 was induced cardiac myocytes through the course of acute myocarditis. The induction of PD-L1 (but not PD-L2) was also reported on various cell types in various pathological conditions [18,26-28,33,34]. Shin et al. [35] reported that DC-expressed PD-L2 serves a predominantly stimulatory rather than inhibitory function in concert with B7-1 and B7-2 to stimulate T-cells and that this costimulation is mediated via a second receptor other than PD-1. Although the mechanism of the differential expression of PD-L1 and PD-L2 on various cell types is unknown, lack of PD-L2 expression on cardiac myocytes makes PD-1/PD-L1 ligands pathway functions as an exclusively inhibitory regulator for T-cell activation at least in this model of myocarditis.

We reported that blockade of costimulatory signals, which mediate positive signals for T-cell activation, such as ICAM-1/lymphocyte function-associated antigen (LFA)-1, B7-1/CD28, CD40/CD40L, 4-1BB/4-1BBL, and Fas/FasL significantly suppressed the myocardial injury involved in murine viral myocarditis [1,3,5-7]. Our data from the present study suggested that combination of blocking the positive costimulatory signals and potentiating the negative costimulatory signals may be more effective in suppressing myocardial injury without interfering the virus clearance. Studies of rat myocarditis strongly suggested that suppression of myocardial inflammation may improve cardiac function [36].

Acknowledgements

This work was supported by a grant for scientific research from the Ministry of Education, Culture, Sports, Science and Technology, Japan. There is no conflict of interests.

References


