Endothelial Programmed Death-1 Ligand 1 (PD-L1) Regulates CD8+ T-Cell–Mediated Injury in the Heart

Nir Grabie, PhD; Israel Gotsman, MD; Rosa DaCosta, BA; Hong Pang, MD; George Stavrakis, MSc; Manish J. Butte, MD, PhD; Mary E. Keir, PhD; Gordon J. Freeman, MD, PhD; Arlene H. Sharpe, MD, PhD; Andrew H. Lichtman, MD, PhD

Background—PD-L1 and PD-L2 are ligands for the inhibitory receptor programmed death-1 (PD-1), which is an important regulator of immune responses. PD-L1 is induced on cardiac endothelial cells under inflammatory conditions, but little is known about its role in regulating immune injury in the heart.

Methods and Results—Cytotoxic T-lymphocyte–mediated myocarditis was induced in mice, and the influence of PD-L1 signaling was studied with PD-L1/L2–deficient mice and blocking antibodies. During cytotoxic T-lymphocyte–induced myocarditis, the upregulation of PD-L1 on cardiac endothelia was dependent on T-cell–derived interferon-γ, and blocking of interferon-γ signaling worsened disease. Genetic deletion of both PD-1 ligands [PD-L1/2−/−], as well as treatment with PD-L1 blocking antibody, transformed transient myocarditis to lethal disease, in association with widespread polymorphonuclear leukocyte–rich microabscesses but without change in cytotoxic T-lymphocyte recruitment. PD-L1/2−/− mice reconstituted with bone marrow from wild-type mice remained susceptible to severe disease, which demonstrates that PD-L1 on non–bone marrow–derived cells confers the protective effect. Finally, depletion of polymorphonuclear leukocytes reversed the enhanced susceptibility to lethal myocarditis attributable to PD-L1 deficiency.

Conclusions—Myocardial PD-L1, mainly localized on endothelium, is critical for control of immune-mediated cardiac injury and polymorphonuclear leukocyte inflammation. (Circulation. 2007;116:2062-2071.)

Key Words: immune system ■ inflammation ■ lymphocytes ■ myocarditis ■ endothelium

The B7 family of costimulatory molecules regulates T-cell functions by modulating responses to T-cell-receptor–mediated antigenic stimulation. In Keeping with these functions, the B7 molecules CD80 and CD86 are mainly expressed by professional antigen-presenting cells. In contrast, other members of the B7 family, including inducible costimulator ligand 1 and programmed death-1 ligand 1 (PD-L1), are expressed by a broader range of cells. For example, costimulator ligand 1 is expressed on human skeletal myocytes, as well as on some endothelia, such as umbilical cord endothelial cells and coronary microvessels, and costimulator ligand 1 is upregulated on various cells under pathological conditions and in response to tumor necrosis factor-α. In addition to its expression on hematopoietically derived antigen-presenting cells, PD-L1 is expressed by renal epithelia, respiratory tract epithelia, pancreatic islet cells, umbilical cord vascular endothelial cells, and cardiac endothelia, where it is largely upregulated in response to interferon (IFN)-γ and lipopolysaccharide. In contrast to PD-L1, PD-L2 (programmed death-1 ligand 2) is restricted to macrophages and dendritic cells. Both PD-1 ligands trigger the programmed death-1 receptor (PD-1) to transduce inhibitory signals through a cytoplasmic immunoreceptor tyrosine-based inhibition motif, but the broad range of body tissues that express PD-L1 suggests that unlike PD-L2, it may regulate more than just lymphoid-tissue–based T-cell activation and cytokine secretion. Previous studies have demonstrated that parenchymal tissue expression of PD-L1 is important for peripheral control of autoimmune T cells that lyse the pancreatic islet β-cells and that the contribution of PD-L1 expressed by hematopoietic lineages is minor in this disease.

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To better understand the functional role of PD-L1 expression in the heart, we used a model of CD8+ T-cell myocarditis developed in our laboratory. In this model, ovalbumin (Ova)-specific T-cell–receptor transgenic CD8+ OT-I cytotoxic T lymphocytes (CTLs) are transferred into CMY-mOva mice,
which express membrane-anchored Ova (mOva) exclusively in cardiac myocytes.9 The severity of disease in this model can be titrated depending on the dose and phenotype of transferred CTLs.9–11 Although the initiation of cardiac disease is dependent on the transferred T cells, secondary inflammatory events involving polymorphonuclear leukocytes (PMNs) play an essential role in the myocardial damage.11 The contribution of molecules expressed by hematopoietic cells, by the transferred CTLs specifically, or by intrinsic cardiac tissue cells can be studied in this model by use of genetically modified bone marrow donors, T-cell donors, or CMy-mOva recipients. We used each of these strategies in the present study to investigate how IFN-γ and PD-L1 regulate CTL-mediated tissue damage in the heart. Our data support the conclusion that IFN-γ-induced PD-L1 on cardiac endothelium plays a major role in limiting inflammatory damage to the myocardium.

### Methods

#### Blocking Antibodies

For specific blocking of PD-L1 in vivo, a Fab preparation was made from purified rat anti-mouse PD-L1 (clone 10F.9G2 rat IgG2b).6,7

#### Mice

Wild-type C57BL/6 mice used in the study were purchased from Jackson Laboratory (Bar Harbor, Me.). C57BL/6 CMy-mOva transgenic mice, which express membrane-bound ovalbumin (mOva) exclusively on cardiac myocytes,9 were maintained on a C57BL/6 Thy1.2 (CD90+) background and are referred to as CMy-mOva. C57BL/6 mice genetically deficient in both PD-1 ligands, PD-L1 and PD-L2 ([PD-L1/PD-L2]−/−), were described recently8 and are referred to as [PD-L1/PD-L2]−/−. CMy-mOva mice genetically deficient in PD-L1 and PD-L2 were created by backcrossing CMy-mOva and [PD-L1/PD-L2]−/− mice and are referred to as [PD-L1/PD-L2]−/− CMy-mOva. CMy-mOva mice deficient in IFN-γ receptor (IFN-γR), referred to as IFN-γR−/− CMy-mOva, were created by backcrossing of CMy-mOva mice with IFN-γR−/− deficient C57BL/6 mice (B6.129S7-Ifngr1tm1Agt/J strain No. 003288, Jackson Laboratory). All CMy-mOva transgenic mice and their derivatives used for experiments were heterozygous for the CMy-mOva transgene. Bone marrow chimERIC CMy-mOva mice were created by conventional methods as described previously.12 The T-cell–receptor transgenic OT-I mouse line,13 which expresses CD8+ T cells specific for Ova peptide 257–264 (SHINEKEL) bound to H-2Kb, was maintained on a C57BL/6 Thy1.1 (CD90.1) background. OT-I mice genetically deficient in IFN-γ (IFN-γ−/−OT-I) were created by breeding OT-I mice with IFN-γ−/− deficient C57BL/6 (B6.129S7-Ifngtm1Ts/J strain No. 002287, Jackson Laboratory). Mice were used for experiments at 8 to 12 weeks of age. Male and female mice were used in separate experiments, with no differences in response to CTL-induced cardiac injury. Mice were housed and bred in the pathogen-free facility at the Institute for Animal Research at the Harvard Medical School in accordance with the National Institutes of Health guidelines for animal research.

#### T-Cell Preparations and Adoptive Transfer

CD8+ CTLs were prepared from naïve OT-I T cells by in vitro antigen stimulation, as described previously.9,10 Activated OT-I CTLs were removed from primary stimulation cultures at day 6, resuspended in PBS, and injected intraperitoneally into CMy-mOva mice, as described previously.9,10 Cell doses transferred in the present study ranged from 25×103 to 1×106 cells, as indicated.

### In Vivo PMN Depletion

Mice were injected intraperitoneally with 300 μg of a purified PMN-depleting antibody (rat anti-mouse Ly6G/Gr-1 monoclonal antibody, clone RB6-8C5)14 in sterile PBS 1 day before and 1 day after adoptive transfer of OT-I CTLs. Additional doses of 200 μg of RB6-8C5 were given 3 and 5 days after CTL transfer, and control animals were injected with equal doses of rat-IgG at the same times. Effective depletion of PMN was verified by Wright-Giemsa–stained tail-blood smears (HEMA-3, Biochemical Sciences, Swedesboro, NJ).

#### Blood Troponin Determination

Blood levels of cardiac troponin-I (cTnl) were measured in tail-vein blood samples,11 by a clinical quantitative immunoassay technique (Advia Centaur cTnl assay, Bayer HealthCare, Tarrytown, NY), which cross-reacts with mouse cTnl as described previously.11

#### Processing and Analysis of Mouse Heart Tissue

Mice were euthanized by CO₂ inhalation. After perfusion, the heart was surgically removed, and transverse sections were prepared for frozen sections, paraffin embedding, and RNA extraction, as described previously.9,10 Isolation of total RNA from heart tissue, CDNA synthesis, and real-time polymerase chain reaction (PCR) analysis were performed. Quantitative real-time reverse-transcription PCR was preformed with SYBR Green PCR mix (Applied Biosystems, Foster City, Calif) and an ABI-5700 Sequence Detection System (Applied Biosystems) as described previously.9 Levels of specific gene expression in tissue samples are presented relative to levels of GAPDH, which were not influenced by the inflammatory conditions or by the treatments applied.

Myocarditis was graded by microscopic examination of hematoxylin-and-eosin–stained sections of formalin-fixed and paraffin-embedded heart tissue, performed in a blinded fashion by a trained pathologist, as described previously.10 In some experiments, the numbers of PMNs and mononuclear leukocytes were counted in 4 high-powered fields per section, 3 sections per heart.

#### Immunohistochemical and Immunofluorescence Staining

Immunohistochemical staining was performed on frozen sections of heart tissue as described previously.9 Specific antibodies used for immunohistochemistry include anti-CD4, anti-CD8, anti-Ly6G, and anti-CD11b (all from BD Pharmingen, San Jose, Calif), as well as anti-mouse PD-L1 (clone 10F.9G2) and anti-mouse PD-L2 (clones No. 19G.12G8 and 19G.7G12) that were developed by Dr Gordon Freeman (Dana-Farber Cancer Institute, Boston, Mass).6 Isotype-matched antibodies were used as controls. For immunofluorescence staining of cardiac PD-L1 and CD31, frozen heart sections were stained sequentially with unconjugated 10F.9G2, biotinylated anti-rat IgGb, Alexa568-conjugated streptavidin (Invitrogen, Burlington, Calif), and Alexa488-conjugated anti-CD31 (BioLegend, San Diego, Calif). Rat IgG2b and Alexa488-conjugated Rat-IgG2a were used as controls, and immunofluorescence was examined by confocal microscopy (Olympus FV1000). Quantification of stains of T cells and PMNs in hearts was evaluated by digital image analysis (online-only Data Supplement).

#### Statistical Analysis

Prism software (GraphPad Software, Inc, San Diego, Calif) was used. Statistical analyses were performed with the Mann–Whitney test for data that were not normally distributed, as determined by the Kruskal-Wallis test, and the Student t test for normally distributed data. A value of P<0.05 was considered to be significant.

The authors had full access to and take full responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.
Results

Low-Dose CTL Transfer Causes Transient Cardiac Inflammation in CMy-mOva Mice

When CMy-mOva mice were injected with $25 \times 10^3$ OT-I CTLs, they developed transient heart inflammation and recovered without manifestations of disease, unlike lethal disease induced by $5 \times 10^5$ transferred cells. CD8+ T cells could be identified in hearts of CMy-mOva recipients as soon as 48 hours after low-dose transfer (data not shown). Numbers of CD8+ cells in the heart typically peaked at 5 to 6 days after adoptive transfer and then declined rapidly, leaving no detectable cells by 10 to 12 days after transfer (Figure 1A). Throughout this time period, only marginal recruitment of endogenous CTLs to the heart occurred, because numbers of CD8+ cells in heart sections closely matched the number of cells that were positive for the allotypic marker CD90.1, exclusively expressed by the transferred OT-I CTLs and not by endogenous CMy-mOva cells that express CD90.2 (data not shown). A relatively small number of endogenous CD4+ T cells were found in hearts at day 5, and scattered clusters of CD4+ T cells persisted at day 11 (Figure 1A). These small CD4+ T-cell infiltrates do not significantly impact disease progression, because transfer of OT-I cells into Rag2-deficient CMy-mOva mice, which lack all endogenous lymphocytes, caused similar myocardial disease as transfer into immunologically normal CMy-mOva mice (data not shown).

Accumulation of mononuclear cells in the heart was associated with myocyte damage, indicated by rising levels of blood cTnl (Figure 1B) and by expression of IFN-γ in heart tissue (Figure 1C). Levels of blood cTnl and of IFN-γ mRNA in heart tissue returned to normal 10 to 12 days after transfer (Figures 1B and 1C) and remained normal for >3 months, with no evidence of recurring inflammation (data not shown).

PD-L1 Expression Is Upregulated in the Heart During CTL-Mediated Cardiac Inflammation

In addition to the increase in IFN-γ mRNA expression in the hearts of CMy-mOva recipients of OT-I cells, an increase in cardiac expression of PD-L1 mRNA was also present, but not of PD-L2 mRNA (Figure 2A). Immunohistochemical staining of heart sections taken from CMy-mOva recipients revealed that in inflamed hearts, PD-L1 protein was detected in a pattern consistent with endothelial expression and, to a lesser extent, on other cells (Figure 2B). Two-color immunofluorescence confocal microscopy showed colocalization of CD31 and PD-L1 staining, consistent with endothelial expression of PD-L1 (Figure 2C). Neither PD-L1 mRNA nor immunohistochemically detectable protein expression was found in hearts of control CMy-mOva mice that did not receive CTL transfers (Figure 2). They were also not found in hearts of T-cell recipients euthanized 10 to 12 days after CTL transfer (data not shown).
Cardiac Endothelial PD-L1 Expressed During CTL-Mediated Myocarditis Is Induced Exclusively by IFN-γ From Pathogenic CTLs

Previous studies have demonstrated that IFN-γ is a powerful inducer of PD-L1 on cultured cardiac endothelia in vitro. To determine whether IFN-γ was responsible for PD-L1 upregulation in the CMy-mOva hearts, we tested PD-L1 expression during inflammation in hearts of CMy-mOva mice deficient in IFN-γ signaling. OT-I CTLs were adoptively transferred into wild-type CMy-mOva or into IFN-γ receptor–null (IFN-γR−/−) CMy-mOva recipient mice. Conversely, we transferred IFN-γ−null (IFN-γ−/−) OT-I CTLs into wild-type (IFN-γ-positive) CMy-mOva recipients. Because of possible diminution of disease severity due to a lack of the proinflammatory effects of IFN-γ, we used a dose of 1×10^6 OT-I cells in these experiments to ensure significant cardiac injury. Five days after transfer, recipient mice were euthanized, and hearts were examined for IFN-γ and PD-L1 gene expression by real-time PCR. GAPDH expression was used as an internal control, and expression of the proinflammatory chemokine IP10 (IFN-γ-inducible 10-kDa protein) was used as a positive control for the endothelial response to IFN-γ (Figure 3A).

Adoptive transfer of wild-type OT-I CTLs induced inflammation in both wild-type and IFN-γR−/− CMy-mOva recipient mice (Figure 3B). We detected significant expression of IFN-γ mRNA in the hearts of both of these recipient groups (Figure 3A, top). No IFN-γ mRNA was detectable in hearts of control CMy-mOva that did not receive T-cell transfers (data not shown). Although wild-type CMy-mOva mice markedly upregulated cardiac PD-L1 after T-cell transfer, the IFN-γR−deficient CMy-mOva did not (Figure 3A, bottom), and expression of IP10 showed similar trends (Figure 3A, middle). Similarly, when IFN-γ−deficient OT-I CTLs were transferred into wild-type CMy-mOva recipients, very little expression of IFN-γ was present in the recipient hearts and PD-L1 induction was lacking compared with a robust induction in recipients that received wild-type OT-I CTLs (Figure 3A, bottom). IP10 mRNA expression in hearts of wild-type CMy-mOva that received IFN-γ−− OT-I CTLs was also significantly reduced compared with recipients that received wild-type OT-I CTLs. Remarkably, when either IFN-γ−deficient OT-I CTLs were transferred or IFN-γ−R−deficient recipients were used, PMN infiltration significantly increased (Figure 3B and 3C).

PD-1 Ligands Control PMN Recruitment to the Heart and Resistance to CTL-Induced Cardiac Injury

To specifically study the significance of PD-1 ligands in cardiac inflammation, apart from other potential effects of IFN-γ, we compared the effects of transferring wild-type OT-I CTLs into wild-type CMy-mOva recipients versus CMy-mOva mice deficient in IFN-γ (IFN-γR−/−) and CMy-mOva mice deficient in IFN-γ and PD-L1 (CMY-μOva mice). We found that PD-L1 upregulation was significantly reduced in IFN-γR−/− CMY-μOva mice compared with wild-type CMY-μOva mice (Figure 2). These findings suggest that PD-L1 expression in CMY-μOva hearts is induced exclusively by IFN-γ from pathogenic OT-I cells.
CMy-mOva mice genetically deficient in both PD-1 ligands [PD-L1/2−−] CMy-mOva]. We found that PD-L1/2−− CMy-mOva developed more severe cardiac inflammation than the control group, which was associated with a several-fold increase in PMNs but no apparent change in CD8+ T-cell numbers (Figure 4A through 4C). Interestingly, we observed that the exacerbated inflammatory response in PD-L1/2−− mice was characterized by multiple necrotic foci with large clusters of PMNs rather than the diffuse distribution of scattered PMNs seen in the wild-type CMy-mOva recipients (Figures 4A through 4C and online-only Data Supplement). Consistent with the intensified cardiac inflammation, PD-L1/2−− recipients had higher levels of blood cTnI and developed lethal disease, whereas their wild-type CMy-mOva controls survived (Figures 4D and 4E). Mice with lethal disease had enlarged hearts and pulmonary and peripheral edema, consistent with cardiac failure. In addition, the wild-type recipients did not show any gross or microscopic evidence of chronic sequelae (data not shown).
Treatment With PD-L1 Blocking Antibody Renders Wild-Type CMy-mOva Mice Susceptible to Necrotic Cardiac Injury

We have not detected significant amounts of PD-L2 expression in murine hearts (Figure 2A) or isolated murine heart endothelium, and it is therefore likely that the effects of PD-1 ligand deficiency we observed are largely due to lack of PD-L1. To confirm this, we used a blocking Fab antibody specific for PD-L1. Wild-type OT-I CTLs were transferred into wild-type CMy-mOva recipients, and the mice were then treated with anti-PD-L1 blocking Fab or control rat Fab. CMy-mOva mice treated with the PD-L1 blocking antibody sustained increased cardiac inflammation and injury compared with mice treated with control Fab (Figure 5). Hearts of anti-PD-L1-treated mice had increased PMN recruitment (data not shown) and elevated blood cTnI levels (Figure 5C) with no apparent change in numbers of cardiac CTLs (data not shown). These data are consistent with a pivotal role for PD-L1, whereas a role for PD-L2 is unlikely.
PD-1 Ligand Expression on Bone Marrow Cells Fails to Rescue PD-L1/2−/− Mice From Cardiac Inflammation and Injury

To assess whether the protective effect of PD-L1 in the model in the present study is mediated by PD-1 ligand expression in heart tissue or by bone marrow–derived cells, we created bone marrow chimera mice that express PD-L1 and PD-L2 in bone marrow–derived lineages but not in nonhematopoietically derived tissue cells, including endothelium. These mice were created by reconstituting lethally irradiated PD-L1/2−/− CMy-mOva mice with wild-type bone marrow. Interestingly, we found the disease induced in these chimeric mice by transfer of OT-I CTLs was as severe as disease induced when mice were reconstituted with PD-L1 and PD-L2–deficient bone marrow, as assessed by histopathological scoring and blood troponin levels (Figure 6A). The tendency to develop large PMN clusters was not abrogated by PD-L1/2 expression on bone marrow–derived cells. Immunohistochemical staining shows clear evidence of PD-L1–positive inflammatory cells within the PD-L1/2–deficient heart on a background of PD-L1–negative endothelium (Figure 6B).

PMN Depletion Reverses Susceptibility to Lethal Myocarditis in the Setting of PD-L1/2 Deficiency

The present data consistently showed increased PMN inflammation and susceptibility to lethal myocarditis in PD-L1/2−/− CMy-mOva compared with control CMy-mOva (wild-type) mice. We previously have shown that when CMy-mOva mice are adoptively transferred with <5×10^5 OT-I CTLs, the severity of ensuing myocardial disease is dependent on PMNs. To determine the relation between increased lethality of PD-L1/2−/− deficient mice and PMN inflammation, we induced disease in PD-L1/2−/− CMy-mOva mice that were treated with PMN-depleting antibodies. The results indicate that PMNs mediate the enhanced disease severity that leads to death in the PD-L1/2−/− deficient mice. As determined by blood cTnI levels, adoptive transfer of 25×10^3 OT-I CTL induced cardiac damage in PD-L1/2−/− deficient mice treated with control rat Ig or with PMN-depleting antibodies (Figure 7A). Nonetheless, PD-L1/2−/− deficient mice treated with control rat Ig suffered 86% mortality, whereas PD-L1/2−/− deficient mice treated with PMN-depleting antibodies survived, as did wild-type CMy-mOva mice treated with control rat Ig (Figure 7B).

Discussion

The present study demonstrates a profound influence of PD-1 ligands in protecting the myocardium from injury mediated by CTL and secondary PMN inflammation and establishes the role of T-cell–derived IFN-γ in this regulatory pathway. Without inflammatory challenges, otherwise genetically normal PD-L1/2−/− mice do not show overt manifestations of dysregulated immunity for many months and do not develop spontaneous myocarditis (N.G. and A.H.L., unpublished data, 2004 to 2006). Although PD-1−deficient BALB/c mice spontaneously develop an unusual chronic dilated cardiomyopathy, that disease appears to be due to an unusual myocardium-specific autoantibody. In the present study, we demonstrate that challenging mice with a dose of myocyte-
At 6 weeks of age, PD-1 blockade indicates that human CTLs are regulated by the PD-1/PD-L1 pathway. The role of this pathway in regulating immune responses in the human heart is unexplored. Limited studies of the role of PD-1 and its ligands in murine cardiac allograft rejection have demonstrated that treatment with blocking anti-PD-1 antibody worsens acute cardiac allograft parenchymal rejection, and agonist reagents that bind to PD-1, when used in combination with immunosuppressive drugs, ameliorate acute cardiac rejection and chronic graft arterial disease. In addition, blocking anti-PD-L1 antibody treatment accelerated graft arterial disease. Those studies could not distinguish between possible influences of the blocking reagents on induction of alloresponses in lymphoid tissues from influences on differentiated effector T cells within the heart or the relative influences on CD4$^+$ or CD8$^+$ T-cell–mediated responses. In the adoptive transfer model we have used, the effects of PD-1 ligand deficiency or blockade are restricted to the effector phase of CD8$^+$ T-cell–mediated myocardial disease.

It is unlikely that our studies of combined PD-L1/2$^{+/−}$ deficiency in the CMy-mOva mouse line have obscured our ability to discern important different influences of PD-L1 versus PD-L2. Our work with cultured mouse endothelial cells and immunohistochemistry of the mouse hearts in this and previous studies indicate that PD-L1 is more abundantly expressed in the heart and likely plays a dominant role in regulating T-cell responses to cardiovascular antigens. Furthermore, our use of blocking PD-L1 antibody recapitulated the effects of combined PD-L1 and PD-L2 genetic deficiency. The marked effects we observed of combined PD-L1 and PD-L2 deficiency on T-cell–mediated myocarditis support the conclusion that these 2 molecules do not represent opposing influences with comparable magnitude.

The influence of IFN-γ in cardiac inflammation has been studied in murine models, and in most cases, IFN-γ has been shown to mediate antiinflammatory effects, contrary to the proinflammatory influence of this cytokine in other tissues. This is true in the noninfectious chronic stages of Coxsackievirus-induced myocarditis and experimental autoimmune myocarditis, and cardiac allograft rejection. This is in contrast to the proinflammatory effects of IFN-γ on the arterial wall in cardiac allograft arteriopathy. A mechanism attributed to the antiinflammatory effects of IFN-γ in myocarditis includes its ability to induce nitric oxide production by inducible nitric oxide synthase. The present data indicate that induction of PD-L1 on cardiac endothelial cells is another significant mechanism by which IFN-γ protects the heart from excessive inflammation.

Recently, interleukin-17–producing CD4$^+$ T cells have been implicated as key contributors to myocarditis, at least in mice. Because interleukin-17 promotes PMN infiltration, it is reasonable to speculate that this cytokine, or others, may mediate the proinflammatory effects of PD-L1 deficiency in the heart. However, in our model, few endogenous CD4$^+$ T cells were recruited into the heart, and the cardiac antigen-specific T cells that initiate disease were purely CD8$^+$ CTLs. Furthermore, we performed quantitative reverse-transcription PCR analyses of myocarditic hearts and found that no elevation of interleukin-17 mRNA expression was present compared with wild-type controls (data not shown).

### Figure 6

PD-1 ligand expression on bone marrow (BM)–derived cells fails to protect PD-L1/2$^{−/−}$ hearts from CTL-mediated injury. At 6 weeks of age, PD-L1/2$^{−/−}$ CMy-mOva recipient mice were lethally irradiated and transplanted with bone marrow taken from PD-L1/2$^{−/−}$ or wild-type CMy-mOva donors. Six weeks later, bone marrow engraftment was confirmed, and hematopoietic lineages were restored as determined by flow cytometry of tail-blood samples (data not shown). Mice were adoptively transferred with OT-I CTLs (25×10$^3$) to induce disease and euthanized 5 days later for pathological evaluation. A, Histological sections were prepared and scored for myocarditis. B, cTnI levels were determined in blood sampled at the time of euthanasia. Data represent 1 of 2 experiments with similar results (n=6 mice/group). Scale bar on top panel indicates 10 μm.

### Figure 6

**A** Recipients: CMy-mOva PD-L1/2$^{−/−}$

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**B** Recipients: CMy-mOva PD-L1/2$^{−/−}$

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The importance of the PD-1/PD-L1 pathway in human diseases is evident from studies of tumor immunity, autoimmunity, and chronic viral infections. The release of suppression of CD8$^+$ T-cell–mediated anti-HIV immunity by PD-1 blockade indicates that human CTLs are regulated by directed CTLs, which normally causes only transient disease, causes robust and lethal inflammatory disease in the absence of PD-L1. Consistent with the view that PD-1 ligands are upregulated in response to inflammation is our finding that PD-L1 expression in the heart is dependent on IFN-γ produced by infiltrating T cells.

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A striking effect of PD-L1 blockade or deficiency in the present study was the enhanced PMN response. The increased numbers of PMNs, and associated necrosis, occurred without detectable increases in T-cell infiltration. This phenotype of PMN-rich inflammation was also seen when IFN-γ or its receptor was genetically ablated. These findings may be due to increased CTL-mediated damage when PD-L1 is missing, which leads to an exaggerated secondary acute inflammatory response. We have shown that blockade of PD-L1 enhances CTL-mediated killing of antigen-pulsed endothelium in vitro, and the same may be true for CTL killing of endothelium or myocardium in vivo. In order for this to be a factor, PD-L1 would have to be expressed on myocytes in the CMY-mOva mice, or the transgenic ovalbumin produced by the myocytes would have to be cross-presented by endothelial cells. We do not have evidence for either of these possibilities. Another explanation is that myocardial endothelial PD-L1 serves as a gatekeeper for PMN infiltration and/or activation, a possibility that will require further investigation.

In summary, the data presented in the present study demonstrate an important role of IFN-γ-inducible PD-L1 in protecting the heart from immune-mediated inflammatory injury. We have previously hypothesized that several mechanisms have evolved to protect the heart from the potentially lethal consequences of cell-mediated immune responses. Our findings suggest that pharmacological manipulation of the PD-1/PD-L pathway, either by cytokine-mediated induction of PD-L1 or by PD-1 agonists, may be therapeutic in the setting of acute immune-mediated inflammation in the heart.

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Disclosures
None.

References


**CLINICAL PERSPECTIVE**

T cells specific for antigens expressed in myocardium contribute to the pathology of infectious and autoimmune myocarditis, as well as cardiac allograft rejection. An understanding of the mechanisms of regulation of T-cell responses in the myocardium can aid in the development of treatments for these diseases. The B7/CD28 families of membrane proteins are involved in both positive and negative regulation of T-cell activation. We used a mouse model of CD8+ T-cell–mediated myocarditis to show that programmed death-1 ligand 1 (PD-L1), a member of the B7 family of immunoregulatory molecules, is essential for protection of the heart from T-cell–mediated injury and associated polymorphonuclear leukocyte inflammation. Of particular interest, the data show that PD-L1 expressed on the endothelium of myocardial microvasculature is the critical site of regulation, not PD-L1 that is widely expressed by cells of hematopoietic origin. Furthermore, interferon-γ acts as an antiinflammatory cytokine in this setting by upregulating cardiac endothelial PD-L1, which in turn limits polymorphonuclear leukocyte infiltration and cardiac injury. These findings highlight the importance of the microvasculature in regulating immune responses in the heart and suggest that therapeutic upregulation of endothelial PD-L1 may be an effective strategy in limiting myocardial inflammation in various diseases.
Endothelial Programmed Death-1 Ligand 1 (PD-L1) Regulates CD8⁺ T-Cell –Mediated Injury in the Heart
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Methods:

Quantification of Immunohistochemical staining by digital image analysis

T cell and PMN infiltration into hearts was evaluated by digital image analysis of frozen sections stained for CD8 or GR1, respectively. Four non-overlapping digital images of the left ventricle of each mouse were captured. The Adobe Photoshop selection tool and the “select similar” function were used to identify all “positive staining” pixels with blue-brown hue, and the number of pixels selected was obtained from the histogram function. Three separate determinations were made for each section to account for variations in hue selections, and the mean was calculated. The sum of positive staining pixels for all four sections was determined. Positive stained pixels were divided by the total number of pixels covering the tissue. The percent staining area was calculated as the quotient of the positive stained pixels over the total pixels.

Online Supplemental Figure

Figure S1: OT-I CTL (25x10^3) were adoptively transferred into wild type or PD-L1/2(-/-) CMy-mOva mice. Five days later recipient mice were sacrificed and immunohistochemistry for neutrophils (Ly6G) was performed on heart tissue. Scale bars indicate 50µm.
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WT CMy-mOva

PD-L1/2(-/-) CMy-mOva