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PD-1 Protects against Inflammation and Myocyte Damage in T Cell-Mediated Myocarditis

Margarite L. Tarrio,* Nir Grabie,* De-xiu Bu,* Arlene H. Sharpe,*† and Andrew H. Lichtman*

PD-1, a member of the CD28 family of immune regulatory molecules, is expressed on activated T cells, interacts with its ligands, PD-L1/B7-H1 and PD-L2/B7-DC, on other cells, and delivers inhibitory signals to the T cell. We studied the role of this pathway in modulating autoreactive T cell responses in two models of myocarditis. In a CD8+ T cell-mediated adoptive transfer model, we found that compared with Pd1+/+ CD8+ T cells, Pd1−/− CD8+ T cells cause enhanced disease, with increased inflammatory infiltrate, particularly rich in neutrophils. Additionally, we show enhanced proliferation in vivo and enhanced cytotoxic activity of PD-1−deficient T lymphocytes against myocardial endothelial cells in vitro. In experimental autoimmune myocarditis, a disease model dependent on CD4+ T cells, we show that mice lacking PD-1 develop enhanced disease compared with wild-type mice. PD-1−deficient mice displayed increased inflammation, enhanced serum markers of myocardial damage, and an increased infiltration of inflammatory cells, including CD8+ T cells. Together, these studies show that PD-1 plays an important role in limiting T cell responses in the heart. The Journal of Immunology, 2012, 188: 4876–4884.

Inflammation in the heart, mediated at least in part by T cells, can result from a variety of infections, including viral, bacterial, and fungal, as well as from environmental toxins, drug reactions, and autoimmune disorders. Many cases of myocarditis are idiopathic. Acutely, T cell-mediated myocarditis can cause arrhythmias and heart failure and chronically may lead to dilated cardiomyopathy. Understanding of mechanisms of regulation of T cell responses in the heart is important for development of therapeutic strategies for myocarditis, but these mechanisms are not yet clear.

Various mouse models of T cell-dependent myocarditis exist, and much work has been done in these models to identify important immunologically relevant mediators and regulators of disease. In experimental autoimmune myocarditis (EAM), which is initiated by immunization with cardiac myosin (1), CD4+ T cells are required for disease induction (2), although the relative roles of IL-17-expressing Th17 cells versus IFN-γ-producing Th1 cells is unresolved (3, 4). CD8+ T cells contribute to the inflammatory infiltrate and severity of EAM but are not essential for disease induction (5). In coxsackievirus B3-induced myocarditis in mice, CD4+ and CD8+ αβ TCR T cells both appear to play a role in disease, but the interpretation of the effects of experimental deletion of either subset is complicated by the roles of the T cells in viral clearance (6, 7). γδ T cells also contribute to coxsackievirus B3-induced myocarditis (8). Therefore, viral myocarditis may not be the preferred model for study of T cell subset-specific mechanisms of regulation of myocarditis. In the cMy-mOVA model of myocarditis developed in our laboratory, severe cardiac inflammation and damage are induced exclusively by transferred CD8+ effector T cells specific for a transgenically expressed model Ag in cardiac myocytes (9, 10), providing a unique experimental model in which to assess the contribution of CD8+ T cells to cardiac inflammation, without their confounding role in viral clearance.

PD-1 is a coinhibitory member of the B7/CD28 superfamily of molecules, and together with its binding partners, PD-L1 and PD-L2, represents an important negative regulator of T cell responses to self and microbial Ags. PD-1 expression is induced by physiologic activation of T cells, B cells, and macrophages. PD-1 binds PD-L1, which is broadly expressed on both hematopoietic and non-hematopoietic cells, as well as PD-L2, whose expression is limited to macrophages and dendritic cells (11–13). This pathway has been shown to regulate T cell responses and inflammation in various disease settings, including atherosclerosis (14, 15), allograft vascular disease (16, 17), encephalomyelitis (18), and sepsis (19). PD-1 is described as a mediator of CD8+ T cell exhaustion in the setting of chronic viral infection and cancer (20–22), and a blocking anti–PD-1 mAb treatment is in clinical trials for cancer (23).

Despite a significant body of work devoted to the pathogenesis of myocarditis in various models, relatively little work has been done on the roles of the B7/CD28 family of molecules that likely regulate the activation of pathogenic T cell responses in these models (24, 25). One group has previously studied the effects of blocking PD-L1 and PD-L1 in coxsackievirus-induced myocarditis in mice (24). Additionally, PD-L1 or PD-1 deficiency has been implicated in causing spontaneous myocarditis in MRL mice (26, 27). The PD-1 pathway has also been demonstrated to be important in graft arterial disease in cardiac allografts (28). Finally, it has been reported that PD-1 deficiency in mice on the BALB/c background experience spontaneous dilated cardiomyopathy caused by Abs to cardiac troponin (29, 30). None of these studies specifically addresses if PD-1 on T cells regulates the response of potentially pathogenic T cells. A previous study from our laboratory, using
the cMy-mOVA model of CD8+-mediated myocarditis, found a role for PD-L1 on non-hematopoietic cells in damping T cell responses and secondary neutrophil inflammation (31).

In the current study, we assessed the role of PD-1 in regulating cardiac inflammation and damage in two T cell-dependent models of myocarditis. Using a modification of our CD8+ T cell-dependent model, we transferred naive OVA-specific CD8+ T cells into cMy-mOVA mice, which express a myocyte-restricted membrane form of OVA, and then immunized the recipients with OVA; we found increased severity of disease in the absence of PD-1 on CD8+ T cells. Next, we addressed the role of PD-1 (and PD-L1) in EAM, which is mediated largely by CD4+ T cells independent of CD8+ T cells, and again found an augmented disease severity in the absence of PD-1. Together, our data indicate an important role for PD-1 in protecting the heart from T cell-mediated damage, which is especially relevant given the interest in PD-1 as a clinical target in therapies for cancer and chronic viral infections.

Materials and Methods

Animal studies

The OT-I TCR transgenic mouse strain (32) was provided by W. R. Heath and F. Carbone (Walter and Eliza Hall Institute of Medical Research, Melbourne, VIC, Australia). The OT-I TCR is expressed on CD8+ T cells and is specific for the OVA peptide 257–264 (SIINFEKL) bound to the class I MHC molecule H2-Kb (32). cMy-mOVA transgenic mice on a C57BL/6 background, previously developed in our laboratory (9), express membrane-bound OVA exclusively on cardiac myocytes. Pd1+/− mice on a C57BL/6 background were derived by targeted mutation in C57BL/6 embryonic stem cells, which results in deletion of the IgV domain as described (33), and were crossed with OT-1 TCR transgenic Thy1.1+ mice to generate Pd1+/− OT-1+ Thy1.1+ mice. Wild-type (WT) BALB/c mice were purchased from Charles River. The generation of Pd1+/− and Pd1+/+ mice on the BALB/c background has been previously described (34, 35). All mice were given food and water ad libitum and were maintained on a 12/12 h light/dark cycle under pathogen-free conditions in the Harvard New Research Building animal facility according to institutional and National Institutes of Health guidelines.

Myocarditis induction in cMy-mOVA mice

Male and female C57BL/6 cMy-mOVA transgenic mice between 8 and 20 wk of age were used as recipients. CD8+ T cells were isolated from spleens of Pd1+/− or Pd1+/+ OT-1+ Thy1.1 mice by magnetic bead separation (Miltenyi) and were adoptively transferred i.v. into recipients at 500,000 cells per mouse. In some experiments, naive CD8+ cells were stained with CD90.1–allophycocyanin (BioLegend) to identify and exclude T cells from the analysis. Cells were washed twice more in DPBS before staining with annexin V–PE and 7-aminoactinomycin D (7-AAD) to exclude T cells from the analysis. Cells were then stained with anti-CD8, anti-CD4, anti-CD11b, and anti-Gr-1 Abs. Cells were stained using CD90.1–allophycocyanin (BioLegend) to identify and exclude T cells from the analysis. In some staining, cells were permeabilized using Wash/Perm buffer (BD).

Flow cytometry

Whole hearts were digested in a bicinephato-buffered with 0.895 mg/ml collagenase I (Sigma) and 0.5 mg/ml elastase XIV (Sigma) as described (3). Whole heart digests, spleens, and cardiac draining lymph nodes were made into single-cell suspensions and filtered through 70-μm cell strainers (BD Bioscience). Cells were fixed in 1% paraformaldehyde prior to staining. Abs for CD4, CD8, IFN-γ, IL-17A, Ly-6G, and CD11b were purchased from BioLegend. Abs were diluted 1:100 for staining. In some staining, cells were permeabilized using Wash/Perm buffer (BD).

Quantitative RT-PCR of heart tissue

RNA isolation and quantitative RT-PCR (qRT-PCR) analyses was performed as described (9, 31). Briefly, the apex of the heart was removed after perfusion and snap frozen. RNA was prepared from whole-heart tissue using the RNeasy Kit and DNase I (Qiagen). CD8+ T cells, and again found an augmented disease severity in the absence of PD-1. Together, our data indicate an important role for PD-1 in protecting the heart from T cell-mediated damage, which is especially relevant given the interest in PD-1 as a clinical target in therapies for cancer and chronic viral infections.

Cytokine measurements

ELISAs to detect IFN-γ and granzyme B in culture supernatants were performed using kits from BioLegend and eBioscience. Sera and culture supernatants were analyzed for cytokine concentrations using Luminex bead-based multiplex assays.

Immunohistochemistry

Frozen heart sections were stained with Abs specific for CD4, CD8, F4/80 for macrophages, and GR1 for neutrophils, as described (37).

Statistics

All statistical analyses were performed using Prism software. Differences between two groups of mice were analyzed by Student t test and expressed as mean ± SEM or by the Mann–Whitney U test (for non-parametric data). For experiments with three or more groups, ANOVA with the Bonferroni multiple comparison post test was used. A p value < 0.05 was considered significant.

Results

PD-1 deficiency increases CD8+ T cell-mediated cardiac inflammation and damage

To investigate the role of PD-1 signaling in myocarditis, we first used the cMy-mOVA mouse model of myocarditis, where transgenic OVA is constitutively expressed as membrane-bound protein by cardiac myocytes under the control of the α-myosin H chain promoter (9). Naive CD8+ T cells were isolated from Pd1+/− or
$Pd1^{+/+}$ OT-I mice and transferred to cMy-mOVA mice intravenously. Recipient mice were immunized 24 h later with adjuvant and whole OVA and were sacrificed 7 d later. Histological analysis revealed significantly more myocardial inflammation in recipients receiving PD-1–deficient T cells (Fig. 1A), as well as increased levels of circulating cardiac troponin I (Fig. 1B), a clinical marker of myocyte damage. We have previously established and confirmed in controls in this study that cMy-mOVA mice are tolerant to OVA, and no disease develops spontaneously without OT-1 transfer (9).

Flow cytometric analysis of spleen cells from the cMy-mOVA mice revealed more Thy1.1+ cells in spleens from mice injected with $Pd1^{-/-}$ OT-1 cells compared with that in spleens of recipients of $Pd1^{+/+}$ OT-1 cells (Fig. 1C), indicating that T cells lacking

**FIGURE 1.** PD-1 controls pathologic CD8+ T cell responses in the heart. Naive WT or PD-1–deficient CD8+ OT-1+ T cells were adoptively transferred into cMy-mOVA mice, immunized 24 h later with CFA/OVA, and sacrificed 7 d later. Whole-heart H&E sections were prepared and scored for myocarditis, arrows indicate small inflammatory foci (A), and circulating troponin was measured in the serum (B). Flow cytometric analysis was performed for identification of transferred cells in the spleen (C) and CDLN (D), as well as for identification of dendritic cells (E) and IFN-γ–producing cells (F) in CDLN. Enzymatic digestion of whole hearts was performed, and total cell number (G), infiltrating neutrophils (H), infiltrating monocytes (I), infiltrating transferred OT-1 cells (J), and IFN-γ–producing transferred cells (K) were enumerated by flow cytometry. In (A) and (B), data are pooled from three experiments, each one with its own $Pd1^{+/+}$ and $Pd1^{-/-}$ OT-1 T cell preparations. Each symbol represents the mean of replicate measurements of individual mice, and the horizontal bars are the SEM of all the mice. Data in (C)–(K) represent mean ± SEM (n = 3 mice) of samples from one of the three experiments. **p < 0.01, ***p < 0.001. CDLN, Cardiac draining lymph node.
PD-1 survived longer and/or proliferated more in the immunized hosts. Cardiac draining lymph nodes (9) were harvested and analyzed, revealing an increase in the total cellularity of the node, with a concomitant increase in transferred (Thy1.1+) cells when the OT-1 cells lacked PD-1 (Fig. 1D). We also observed an increase in the total number of CD11c+ dendritic cells, possibly reflecting an increase in tissue-resident dendritic cells migrating into the draining lymph node (Fig. 1E). Additionally, the draining lymph node of mice receiving PD-1 null T cells had more CD8+ IFN-γ+ T cells compared with that of the draining lymph node of the WT group (Fig. 1F). Analysis of enzymatic digests of whole hearts revealed an increase in the total number of infiltrating leukocytes in the mice that received Pd1−/− T cells (Fig. 1G). An increase in both percentage and total number of Ly-6G−CD11b+ as well as Ly-6G−2 CD11b+ cells was observed, indicating an enhanced recruitment of neutrophils and macrophages, respectively, at the site of inflammation (Fig. 1H, 1I). The total number of OT-1 cells in the heart and the percentage of these cells that expressed IFN-γ were greater in mice that received PD-1–deficient OT-1 cells compared with those in mice that received PD-1–expressing OT-1 cells (Fig. 1J, 1K). Together, these data demonstrate an enhanced T cell response, as well as an enhanced infiltration of innate immune cells to the site of inflammation, suggesting that T cell PD-1 is responsible for controlling these responses in vivo.

We performed qRT-PCR analysis for selected genes in cMy-OVA heart tissue at day 7 after immunization, as well as serum cytokine analyses by a cytokine bead assay, to compare further both inflammatory cell and endogenous tissue responses to Pd1−/− and control T cell-mediated myocardial injury (Fig. 2). There were significantly increased levels of IFN-γ (Fig. 2A) and TNF-α (Fig. 2B) mRNA in hearts of mice receiving Pd1−/− OT-1 cells, two signature cytokines of the CD8+ T cell response, as well as an increase in the IFN-inducible chemokine IP-10/CXCL10 (Fig. 2C). Additionally, we found an increase in mRNA expression of MIP1α/CCL3 and RANTES/CCL5 in hearts of mice receiving Pd1−/− OT-1 cells (Fig. 2D, 2E); these chemokines are important in neutrophil and monocyte/macrophage recruitment to inflammatory sites. The PD-1–deficient group also had elevated expression levels of inducible NO synthase compared with those of the control group (Fig. 2F). Analyses of serum cytokines revealed increased levels of TNF-α, IFN-γ, and IL-10 (Fig. 2G–I) and a decrease in the IL-12/IL-23 subunit p40 (Fig. 2J) in the mice receiving Pd1−/− cells, but no difference in the IL-12–specific p70 subunit (Fig. 2K) between groups, consistent with

**FIGURE 2.** PD-1 regulates pathogenic gene expression in the heart and influences circulating pathogenic cytokines. RNA was purified from heart tissue of the mice described for Fig. 1 and used for qRT-PCR analysis of Ifng, Tnfa, Cxcl10, Ccl3, Ccl5, and Nos2 (A–F). Serum samples from the time of sacrifice were analyzed using multiplex Luminex bead-based cytokine assays for TNF-α, IFN-γ, IL-10, IL-12p40, and IL-12p70 (G–K). Data are the mean ± SEM (n = 3 mice). *p < 0.05, **p < 0.01, ***p < 0.001.
a decrease in circulating IL-23. Together, these data indicate an increased inflammatory profile in the hearts of mice given PD-1−/− deficient T cells, with a cytokine profile dominated by IFN-γ and IFN-γ-related chemokines.

**PD-1 deficiency increases OT-I proliferation, intracardiac inflammatory cytokine production, and cytotoxicity**

One possible mechanism by which PD-1 deficiency enhanced myocardial inflammation in our model is deregulated T cell proliferative responses to Ag stimuli. We therefore analyzed in vivo proliferation of the adoptively transferred OT-I cells. Naïve CD8+ OT-I cells were labeled with the fluorescent dye CFSE and injected into cMy-mOVA mice, which were then immunized with OVA plus CFA and sacrificed 72 h later. Lymph nodes draining the immunization site or the heart were collected for analysis. In the lymph nodes draining the immunization site, the majority of Pd1+/+ OT-I cells had undergone minimal CFSE dilution, indicating few cell divisions, whereas a significantly greater percentage of Pd1−/− OT-I cells had undergone multiple cell divisions (Fig. 3A). A similar pattern was seen in the cardiac draining lymph node (Fig. 3B). Together, these data suggest an increased proliferative capacity in T cells lacking PD-1, representing a possible mechanism by which mice receiving PD-1 null T cells experience enhanced disease severity.

To address the possibility that the enhanced myocardial damage in recipients of PD-1−/− OT-I cells was related to enhanced cytotoxic activity of those T cells, we performed in vitro killing assays. We chose MHEC as the target cell as previous data from our laboratory suggest a significant role for nonhematopoietic PD-L1 in the cMy-mOVA model (31). Results from these killing assays showed that coculture of MHEC with Pd1−/− OT-I CTL and OVA peptide resulted in more early apoptotic and dead cells at high T cell/MHEC ratios than that by coculture with Pd1+/+ CTL (Fig. 4A). Supernatants from these assays were evaluated for the presence of cytokines, revealing an increase in IL-2 and a decrease in IL-10 in cultures with Pd1−/− OT-I cells compared with cultures with Pd1+/+ T cells (Fig. 4B). Additionally, supernatants from the cultures with Pd1−/− T cells had more IFN-γ and granzyme B compared with that in supernatants of cultures with Pd1+/+ T cells (Fig. 4D, 4E). Together, these data suggest that PD-1−/− deficient cells are more efficient killers of target cells than WT, providing a mechanism for increased heart damage in the absence of PD-1 on the transferred OT-I cells. Additionally, the elevated levels of IL-10 in the supernatants of the Pd1+/+ group suggest that the Pd1+/+ T cells are exhibiting characteristics of exhausted T cells (i.e., secreting anti-inflammatory cytokines), whereas T cells lacking PD-1 are not. In conclusion, by secreting more IFN-γ and granzyme B and less IL-10, CD8+ T cells lacking PD-1 appear to be capable of inducing more target cell death and inflammation than T cells that express PD-1.

**PD-1 deficiency augments CD4+−dependent EAM**

To investigate if PD-1 also plays a regulatory role in a model of autoimmune CD4+ T cell-dependent myocarditis, we used the EAM model in BALB/c mice. In this model, myocardial inflammation is induced in susceptible strains by immunization with a peptide of the mouse a-myosin H chain (38). WT, Pd1−/−, and Pd1+/+ BALB/c mice were immunized twice, at day 0 and day 7, and sacrificed at day 21. Histological analysis of the heart revealed a significant increase in the pathology of the Pd1−/− hearts compared with the WT and Pd1+/− hearts (Fig. 5A, 5B). Along with increased infiltration into the heart, Pd1−/− mice had significantly elevated circulating troponin levels at day 21 (Fig. 5C), but not at day 14 (data not shown). These data show an increased susceptibility to EAM in the absence of PD-1. Analysis of the cardiac draining lymph node showed an overall increase in the cellularity of the node, but no distinguishable differences in the percentage of T cells or specific subsets (data not shown).

Analysis of mRNA from heart tissue of the EAM mice by qRT-PCR revealed an increase in the expression of IFN-17A, IFN-γ, and the transcription factor RORγt in Pd1−/− animals compared with that in WT (Fig. 6A–C). No significant differences were found when comparing Pd1+/− to either WT or Pd1−/− animals. Notably, no differences were seen in the cytokines IL-6 and IL-23, both of which are considered essential for the development of EAM (4, 39, 40). Additionally, no differences between groups were found in expression of the chemokines RANTES, MIP1α, or MCP1 (data not shown). Circulating cytokine analysis of plasma from these mice revealed significant differences between all three groups in the IL-12/IL-23 subunit p40 at day 14 (Fig. 6D), but not at day 21 (data not shown). These data are consistent with previous studies that report the height of Th17 responses in this model to be at day 14 (3). Together, these data indicate an increase in expression of proinflammatory genes in the hearts of PD-1−/− mice.
deficient mice with EAM compared with that in WT mice with EAM, whereas PD-1–deficient mice showed an intermediate phenotype that was not significantly different from either WT or PD-1–deficient animals.

Immunohistochemical analysis of heart tissue from the mice with EAM revealed an increase in CD4+ T cells in the hearts at day 21 in the Pd1−/− group compared with that in the WT and Pdl1−/− groups. Additionally, we found more CD8+ T cells in the hearts of both Pd1−/− and Pdl1−/− animals compared with that in the hearts of the WT group; the latter group had virtually no CD8+ T cells. We also found an increase in GR1+ cells in the Pd1−/− group, as well as F4/80+ cells, suggesting that the inflammatory infiltrate was a mix of T cells, macrophages, and neutrophils, with neutrophils accounting for the majority of infiltrating cells in the Pdl1−/− group (Fig. 7). Together, these data show an enhanced inflammatory profile in the hearts of mice lacking PD-1 and reveal an important role for the PD-1 pathway in limiting CD8+ T cell responses and secondary neutrophil responses.

Discussion

The data reported in this study provide new insight into the role of PD-1 in regulating pathogenic T cell responses in the heart in vivo. Whereas a significant body of work has been devoted to the study of the PD-1/PD-L1 pathway in many autoimmune diseases, limited work has focused on the role of PD-1 on heart Ag-specific T cells. In this study, we report enhanced cardiac pathology in the absence of PD-1 in two distinct models of myocarditis.

Previous results from our laboratory have demonstrated a role for the PD-1 ligand PD-L1 in endothelial–T cell interactions, both in vitro (41) and in vivo (31). Specifically, in our CD8 adoptive transfer model, recipient mice lacking PD-L1 and PD-L2 on non-hematopoietic cells exhibited enhanced disease pathology and an increase in neutrophilic infiltrate in comparison with recipients that express PD-L1 and PD-L2. Previous studies have also indicated that in addition to PD-L1 interactions with PD-1, PD-L1 can also bind directly to B7-1 on T cells to dampen immune responses (42). Our work addresses an important unresolved question of whether PD-1 is a relevant receptor by which PD-L1 or PD-L2 protects against disease in the heart. Similar to our previous results, in the current study we find that mice receiving PD-1–deficient T cells enhanced pathology, increased clinical markers of myocyte death, and enhanced inflammation, with severe neutrophil infiltration in the heart. This indicates that PD-1 on the T cells is an important receptor for PD-L1 in regulating myocyte damage. It does remain possible that the enhanced myocarditis we previously found in PD-L1–deficient cMy-mOva mice may in part reflect the absence of PD-L1–B7-1 interactions.

FIGURE 4. PD-1–deficient CTLs are more effective killers of cardiac-derived target cells in vitro. Naive OT-1+ CD8+ T cells were isolated from Pd1−/− or WT mice, cultured for 5 d in the presence of IL-2 and IL-12 to generate effector cells, and then rested overnight. Confluent MHEC were pretreated with IFN-γ for 2 h and pulsed with the OVA peptide SIINFEKL. The MHEC were then cocultured with OT-1 effectors at the indicated ratios of effector to target cell ratios for 1 h and were then analyzed by FACS for annexin V and 7-AAD staining (A). Supernatants from these assays were analyzed for the presence of IL-2 (B) and IL-10 (C) using multiplex Lumines bead-based cytokine assays and for IFN-γ (D) and granzyme B (E) by ELISA. Data presented represent the mean ± SEM of three experiments. *p < 0.05, **p < 0.01, ***p < 0.001.

FIGURE 5. PD-1 deficiency exacerbates EAM. WT, Pd1−/−, or Pdl1−/− BALB/c mice were immunized at day 0 and day 7 with CFA/α-myosin H chain peptide and sacrificed at day 21. Representative sections of H&E-stained sections of heart tissue are shown (A). Sections were scored for histopathology (B), and troponin I levels were measured in serum collected at sacrifice (C). Data are mean ± SEM of 7–10 mice per group. *p < 0.05, ***p < 0.001.
Previous studies from our laboratory using our CD8+ T cell-mediated myocarditis model have used effector CD8+ T cells that were derived in vitro and then transferred into the cMy-mOVA mouse. This study, although complementing previous results from our laboratory indicating the importance of PD-L1 in dampening T cell responses in the heart, was performed using naive CD8+ T cells and therefore represents a new adaptation of this model. When we compared myocarditis induced by transfer of in vitro-activated $\text{Pd1}^{-/-}$ versus $\text{Pd1}^{+/+}$ effector OT-1 cells, we observed no significant difference (data not shown). Therefore, our results

**FIGURE 6.** PD-1 regulates cardiac inflammatory gene expression in EAM and the release of circulating cytokines. RNA was isolated from heart tissue of the mice described for Fig. 5, sampled at the time of sacrifice, and analyzed by qRT-PCR for expression of $\text{Iffng}$ (A), $\text{Il17a}$ (B), and $\text{Rorc}$ (C). Serum samples from day 14 were analyzed using multiplex Luminex bead-based cytokine assays for the presence of IL-12p40 (D). Data are the mean ± SEM. *$p < 0.05$, **$p < 0.001$.

**FIGURE 7.** PD-1 deficiency results in increased inflammatory infiltrates in EAM. Immunohistochemical analysis was performed in heart sections of the mice described for Figs. 5 and 6 for CD4, CD8, F480 (macrophages), and Ly6G (neutrophils). Original magnification $\times$10. Total positive staining area was quantified using ImageJ Software. Error bar represent the mean ± SEM. *$p < 0.05$, **$p < 0.01$. 

are consistent with a regulatory role for PD-1 during the initial activation of cardiac Ag-specific T cells, as well as their effector responses.

PD-1 is a known marker of T cell exhaustion, and targeting the PD-1 pathway has been an active area of interest for clinical development. In this study, we provide two mechanisms by which PD-1 controls T cell functionality. First, we report that naive CD8+ T cells lacking PD-1 exhibit an enhanced capacity for proliferation in vivo upon encountering target Ag in the lymph node. Additionally, we show that PD-1 plays a direct role in the effector function of T cells in vitro by demonstrating a heightened killing capacity of mature CD8+ T cells in response to cardiac endothelial cells presenting a target Ag. Additionally, we saw an increase in the CTL effector molecules IFN-γ and granzyme B in these assays, as well as a decrease in the anti-inflammatory cytokine IL-10. We observed less IL-10 production by the CTL effector molecules IFN-γ and granzyme B in these assays.

Our data indicate that there is more cardiac myocyte death and more myocardial inflammation when naive Pd1−/− CD8+ T cells are transferred compared with that after transfer of Pd1+/+ CD8+ T cells. One possible interpretation of these data are that the increased inflammation reflects only a secondary response to increased cytotoxicity of the Pd1−/− T cells with more myocyte death. However, we also saw enhanced proliferation and IFN-γ production by Pd1−/− T cells, and we did not see increased cardiac inflammation when in vitro Pd1−/− CD8+ effector cells were transferred (data not shown). Together, these data argue for a more complex effect of PD-1 in regulating several aspects of the CD8+ T cell response, including proliferation during the in vivo priming phase, the inflammatory functions of the effector CTL that emerge, and their cytotoxic functions.

Because a significant body of in vivo work has previously been focused on PD-1 on CD8+ T cells, we investigated the role of PD-1 on CD4+ T cell-mediated inflammatory disease. For this purpose, we chose the CD4+ T cell-dependent EAM model and also showed that PD-1 exerts a profound regulatory influence on T cell-mediated inflammation in the heart. Our data show enhanced disease severity in the hearts of Pd1−/− mice, with increased cardiac myocyte damage. Again, this model addresses the importance of PD-1 in the initiation of T cell responses and adds significantly to the breadth of knowledge on how PD-1 controls not only CD8+ T cell response but also CD4+ T cell responses. Thus, by using these two complementary models, we are extending the understanding of the PD-1 pathway to a wider range of T cell-mediated heart pathologies. This study represents some of the first work on T cell coinhibition in the EAM model. One previous report showed a role for CTLA-4 in modulating Th17 responses in EAM (25). Additionally, the lack of PD-1 in this model unmasked a role for CD8+ T cells that is not usually seen in EAM. This corresponds to a recent finding from our laboratory that identified a robust CD8+ T cell response in atherosclerotic lesions in mice lacking PD-1 (14).

The basis for the distinct myocardial response in PD-1−/− and PD-1−/−−deficient mice in EAM is not clear. Mice lacking PD-1 showed enhanced disease severity and increased circulating troponins compared with those in WT mice. Notably, PD-L1-deficient mice showed an intermediate phenotype in regard to histology, troponin, and inflammatory gene expression, as well as in immunohistochemical analysis of heart-infiltrating cells. Together, these results leave open the possibility that unlike the CD8-mediated myocarditis model, where PD-L1 is the PD-1 ligand responsible for modulating disease severity, in EAM there may be overlapping roles for PD-L1 and PD-L2 with respect to Ag presentation.

In summary, our studies provide evidence that PD-1 is important for protecting the heart against T cell-mediated injury. Our findings also highlight the mechanisms by which T cell-mediated heart pathology can be enhanced: by increased proliferation and enhanced killing capacity. Additionally, we find that the PD-1 on T cells may contribute to the recruitment of other heart-infiltrating cells, specifically macrophages and neutrophils. Importantly, this work compares two models in distinct genetic backgrounds of mice, with distinct pathologies, broadening the applicability of these observations.

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Disclosures
The authors have no financial conflicts of interest.

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