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Reducing T Cell-Mediated Cardiac Injury With Cpg Oligodeoxynucleotides

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Abstract

Lymphocytic myocarditis is a common condition associated with both infectious diseases and immunological disorders, and is often associated with severe morbidity and mortality, but few effective treatments. In many cases, the pathophysiology involves a failure of central and/or peripheral immune tolerance leading to a cardiac-specific autoimmune T cell response. Previous studies indicate that the PD-1:PD-L1 axis plays an important role in limiting inflammation in the heart. CpG ODN are TLR9 agonists with known immunoregulatory capacity, in part through their potent induction of type I IFN, a known inducer of PD-L1. In this study, we used human tissue to determine the signature of PD-L1 expression in myocarditis. Furthermore, we investigated the *in vitro* activity of CpG ODN as an inducer of PD-L1 in the heart, and tested if this activity is dependent on type I IFN. Lastly, we sought to establish the cardioprotective potential of CpG ODN in a CD8+ T cell-mediated adoptive transfer model of myocarditis in mice. Myocarditic human hearts demonstrate elevated PD-L1 relative to healthy hearts, indicating a possible feedback inhibition on inflammation of translational relevance. CpG ODN robustly upregulates PD-L1 and interferon-related genes in the myocardium, though our data is equivocal as to whether this is a type I IFN-dependent process. Pretreatment of mice with CpG ODN significantly reduced the extent of CD8+ T cell-medaited disease as measured by both histology and serology. Though results did not reach statistical significance, preliminary data suggests that this cardioprotection may not be fully dependent upon PD-1:PD-L1 activity. CpG ODN is known to have other immunoregulatory properties, and our data on gene expression in hearts of treated mice suggest other regulatory mechanisms by which CpG ODN may regulate autoimmunity in the heart. Irrespective of

the mechanism of action, this study provides evidence of the possible therapeutic utility of CpG ODN as a targeted therapy for myocarditis.

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Outside of the Lichtman lab, several other groups have made significant contributions. The Specialized Histopathology Core and Rodent Histopathology Core at Brigham and Women's Hospital (BWH) have both assisted with immunohistochemistry. The James Lederer, PhD, lab has assisted with cytokine bead assays. The Biomarker Research and Clinical Trials laboratory at BWH, led by Dr. Petr Jarolim, completed all troponin bioassays.

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Introduction

The myocardium requires a sterile environment for proper functioning and host survival. Inflammation of cardiac myocytes, or myocarditis, can be caused directly by several "primary" etiologies, including infectious, toxic, or autoimmune. Most commonly, myocarditis is caused by viral infection; in the United States, the dominant infectious agent is the Coxsackie B3 virus (CVB3), while in certain other geographic locations, parasitic infection with *Trypanosoma cruzi* or bacterial infection with *Borrelia burgdorferi* are common causes (1). Certain drugs, such as antiepileptics, penicillin derivatives, and sulfonamides, can precipitate hypersensitivity eosinophilic myocarditis (2). In the setting of ineffective immunosuppression, allograft rejection may result in fulminant alloimmune myocarditis. From a clinical perspective, myocarditis can manifest as a mild disorder, with self-limiting symptoms such as chest pain or dyspnea on exertion, or on the other end of the spectrum, cardiovascular collapse and sudden cardiac death (3). In the case of fulminant myocarditis, inflammation of the left ventricle reduces contractile function and can lead to acute decompensated heart failure. Alternatively, involvement of the conduction system can precipitate acute ventricular arrhythmias and cause sudden cardiac death. In cases where myocarditis does resolve acutely, pathologic remodeling can adversely affect long-term cardiac function and may progress to dilated cardiomyopathy (DCM) (3). Prevalence estimates of myocarditis vary considerably, as definitive diagnosis requires invasive endomyocardial biopsy; however, a Global Burden of Disease working group estimated that myocarditis accounted for 0.5% to 4% of cases of heart failure worldwide (4, 5). Among young adults in particular, myocarditis and DCM represent major causes of heart failure and sudden death worldwide (6).

Unfortunately, even with this significant global morbidity and mortality and an improved understanding of the pathophysiology of myocarditis and DCM, there are relatively few treatment options available (1). For patients with DCM, prognosis remains quite poor, with 40% of patients dying or requiring cardiac transplantation (7). This figure has remained virtually unchanged over the last few decades despite advances in cardiovascular therapeutics for other conditions.

Despite the diversity of upstream precipitants for myocarditis, following these primary insults, many of these entities converge upon a shared disease phenotype that is primarily driven and regulated by the immune system (8). Following myocyte death, the innate immune system mediates the clearance of pathogens and necrotic debris. During this process, the clinical course may take one of two divergent trajectories (1). With rapid neutralization of the inflammatory precipitant and reconstitution of the immunoregulatory environment in the myocardium, patients can experience a self-limited disease course with few long-term sequelae (9). However, the initial inflammatory insult to the heart, even if relatively minor in and of itself, may initiate a "post-viral" immune-driven myocarditis, potentially precipitated by molecular mimicry between myocyte self-antigens and the infectious agent (8). In this setting, even when the offending agent is completely cleared by the immune system, cardiac inflammation may persist wherein an extinguished, exogenous precipitant potentiates a self-propagating, endogenous, autoimmune response. Therefore, the immune system represents a double-edged sword in the pathophysiology of myocarditis, requisite for disease resolution but also capable of exacerbating an otherwise minor insult. Currently, it is not possible to delineate *a priori*

which of these two courses an individual patient's immune system will take (1). Indeed, relatively small differences at the onset of disease may lead to markedly different clinical trajectories (10). An improved understanding of the cellular and molecular mechanisms involved in the immunologic response to inflammation in the heart may provide guidance as to possible immunomodulatory strategies to avoid the initiation of autoimmunity (11).

This Jekyll-and-Hyde nature of the immune system in myocarditis is significantly impacted by the role of tolerance. The immune system has two primary strategies for preventing the development of autoimmunity, central and peripheral tolerance. For T cells, central tolerance refers to the deletion of self-reactive T lymphocytes as they develop in the thymus, whereas peripheral T cell tolerance refers to the varied immunoregulatory mechanisms that suppress mature T cell reactivity towards self-antigen after these cells have left the thymus (12). The pathophysiology of myocarditis involves a breakdown of both types of tolerance (13).

This "two-hit" model with impaired central and peripheral tolerance begins with incomplete clonal deletion of alloreactive lymphocytes. Lv and colleagues have shown that transgenic NOD mice with the human DQ8 major histocompatibility complex (MHC) II allele develop spontaneous myocarditis (14). In this model, the predominant autoantigen is alpha-myosin heavy chain (αMyHC; *Myh6*), an isoform of myosin heavy chain found exclusively in myocytes. Notably, these NOD mice do not express αMyHC on medullary thymic epithelial cells (mTECs), suggesting that there is no central tolerance to αMyHC. Furthermore, transgenic expression of αMyHC in mTECs prevents

the development of myocarditis. Humans with and without myocarditis also both lack αMyHC expression in mTECs and have circulating αMyHC-specific lymphocytes, with myocarditis patients having much higher αMyHC-specific T cell titers. These data suggest that impaired central tolerance allows for at least some heart antigen-autoreactive lymphocytes to escape central tolerance mechanisms, setting up the potential that they will be activated by heart antigens and cause myocarditis (15).

However, peripheral tolerance mechanisms can also prevent the development of autoimmune myocarditis. At baseline, compared to other organs, the heart is relatively immune privileged and is notably devoid of any significant lymphocytic presence. This suggests that evolutionary selective pressures have established higher threshold in the heart than many other tissues for initiating local immune-driven inflammation, which could otherwise disrupt the continuous electrophysiological function of the myocardium that is necessary for survival (13, 16). Alternatively, as discussed below, mice deficient in T cell inhibitory ("checkpoint") molecules are much more susceptible to autoimmune lymphocytic myocarditis compared to mice that express these immunosuppressive molecules (17). These findings point to peripheral mechanisms in place to prevent alloreactive T cells from initiating autoimmunity in the heart.

However, the myocardium exists in an unstable equilibrium with regards to self-tolerance, wherein this tolerance is susceptible to disruption. Binding of pathogen- or damage-associated molecular patterns (PAMPs or DAMPs, respectively) to pattern recognition receptors (PRRs) on antigen-presenting cells (APCs) such as dendritic cells

(DCs) leads to increased APC expression of costimulatory molecules such as CD80 (B7.1) and CD86 (B7.2) (13). Simultaneously, these cells also increase presentation of cardiac self-antigens, such as αMyHC, on MHC II molecules. Autoreactive T cells that have escaped clonal deletion in the thymus are then activated through interactions with both costimulatory proteins and MHC II molecules on APCs. In sum, these changes lead to activation and clonal proliferation of T cells with specificity to heart antigens. As activity of effector T cells leads to further cell death and DAMP-stimulated PRR activation, an initial inflammatory stimulus may ignite a vicious cycle wherein T cell activity potentiates itself through activated DCs. This process may overwhelm baseline mechanisms for maintaining peripheral tolerance in the heart, as described below. Thus, incomplete central tolerance coupled with disrupted peripheral tolerance provides a "two-hit" mechanism whereby, in the context of an insult, a normally immunoprivileged environment is recognized as foreign and runaway inflammation ensues.

Although the development of an autoimmune response involves both the innate and adaptive arms of the immune system, a number of findings point to the centrality of T cells in mediating the pathophysiology in the heart (8). Several mouse models of myocarditis recapitulate many of the cardinal features of myocarditis in humans and are driven by a T cell response. Inoculation of A/J and Balb/C mice with CVB3 precipitates myocarditis in the mice, and T cells from these mice lyse cardiomyocytes *in vitro* (18, 19). These same mouse strains develop CD4+ T cell-dependent myocarditis when inoculated with an emulsion of αMyHC peptide and adjuvant, a widely-used mouse model of inflammatory heart disease known as experimental autoimmune myocarditis

(EAM) (20, 21). Interestingly, both disease models are driven by T cells with TCR that recognize αMyHC epitopes and are capable of inducing myocarditis when αMyHC-specific T cells are injected into susceptible mice (22). Although there is some speculation that antibodies may also play an important role in the pathophysiology of myocarditis, it is not clear that whether autoantibodies against cardiac antigens are causative or merely correlative (8, 9). Given that the significant autoantibodies in myocarditis (against αMyHC or β_1-adrenergic receptors) are of the IgG class, CD4+ helper T cells are still nonetheless involved, due to their integral role in the isotype class switching of these antibodies (9). This abundance of murine data has led to the creation of diagnostic criteria for myocarditis that include the presence of CD3+ and CD4+ T cells on endomyocardial biopsy, as well as the use of anti-T cell therapy for myocarditis in humans (1). Nonetheless, these therapies are not consistently effective and there is a need for more effective myocarditis treatments that directly target T cells (14).

As T cells represent a central mediator in the pathophysiology of myocarditis, it is crucial to understand the molecular mechanisms that govern their activation. While the phenotype assumed by T cells is a determined by a complex and incompletely understood web of interactions, several key mediators determining whether T cells assume a pro-inflammatory or tolerogenic phenotype have been identified. The “two signal” model of T cell activation posits that T cells require both recognition of their cognate antigen on a corresponding APC MHC through their TCR, as well as co-stimulation (23). Many costimulatory molecules are included in the B7/CD28 superfamily. Of note, however, this superfamily contains proteins with both immunostimulatory as well as regulatory effects.

Within the family of CD28-family proteins, the programmed cell death 1 (PD-1): programmed death ligand 1 (PD-L1) axis represents perhaps the most promising new therapeutic target (24). PD-1 is an inhibitory receptor primarily expressed on lymphoid cells, and PD-L1 is the primary ligand of PD-1. Notably, PD-L2 is another characterized ligand of PD-1 that inhibits T cell activation (25). However, evidence suggests that the PD-1:PD-L2 axis has less relevance at least in the context of murine myocarditis (26). PD-L1 is expressed mostly on APCs as well as mesenchymal and endothelial cells (16). Although the exact intracellular signaling mechanisms that lie downstream of PD-1 have yet to be fully established, its cytoplasmic tail includes inhibitory motifs, including the immunoreceptor tyrosine-based shift motif (ITSM), that engage the SHP2 tyrosine phosphatase. This leads to inhibition of PTK-dependent singling events downstream of the T cell receptor (TCR) and the CD28 costimulatory receptor, including activation of PLCγ1, Ras, and PI3K pathways (24). PD-1 activity has been shown to inhibit effector T cell cytotoxicity, survival, and cytokine production (13). PD-L1 expression has been shown to be required for regulatory T cell (Treg) suppression of alloreactive cells (27). Therefore, the PD-1:PD-L1 axis works through multiple mechanisms to inhibit T cell activation and effector function.

Furthermore, there is evidence that the PD-1:PD-L1 axis is specifically important in limiting autoimmunity in the heart. The myocardial endothelium has increased PD-L1 expression relative to other tissue beds, suggesting that PD-L1 is perhaps involved in maintaining the relatively immune-privileged environment under steady-state conditions, possibly through inhibiting extravasation and infiltration of T cells (16). Conversely, the

absence of PD-1:PD-L1 signaling has been shown to either cause or exacerbate T-cell mediated autoimmunity. PD-1-deficient *MRL* mice develop spontaneous and fatal myocarditis (28). PD-1 has been shown to work synergistically with other immune checkpoint molecules to prevent the development of myocarditis in Balb/C mice (29). In an antigen-specific T cell adoptive transfer model of myocarditis (described in *Methods* below), the absence of PD-L1in the recipient mouse converts a self-limited disease into a fatal one (17). Using the same model, PD-1-deficient transferred T cells exhibit enhanced cytotoxicity compared to WT T cells (30).

In addition to these animal studies, there is growing evidence from human cancer immunotherapy trials that PD-1:PD-L1 signaling is involved in preventing the development of autoimmunity. On one hand, monoclonal antibodies targeting immune checkpoint molecules such as PD-1 and PD-L1 have shown marked efficacy in potentiating immune responses against a wide variety of cancers (31). However, the de-inhibition of T cell responses has come at a cost. (32). By preventing PD-1-induced anergy and apoptosis of self-reactive T cells, cancer immunotherapy treatments can potentiate autoimmune responses (33-35). These adverse events, termed “immune related adverse events” (IRAEs), can affect virtually any organ (32). Patients receiving a combination of immune checkpoint agents may be unable to continue treatment in as many as 40% of cases due to severe IRAEs (36). Additionally, combination immune checkpoint blockade treatment has been shown to markedly increase the risk of severe myocarditis, and multiple deaths from fulminant myocarditis have been reported (34, 37). Furthermore, even in cases where patients withstand immune checkpoint blockade

treatment, the long-term sequelae of these medications on the heart is currently unknown (32). Therefore, as suggested by both animal and human experiments, PD-1:PD-L1 signaling may represent an important regulatory mechanism to prevent unchecked T cell infiltration, activation and cytotoxicity in the heart.

Because PD-1:PD-L1 activity has been shown to be a central player in protecting the myocardium, it is crucial to understand how, where, and when this activity happens. Given that PD-1 and PD-L1 are upregulated in response to T cell activation, this suggests that the PD-1/PD-L1 axis may act as a negative feedback mechanism to limit inflammation (13). The molecular mechanisms governing this feedback effect are still being uncovered, but evidence suggests that activation of select Toll-Like Receptors (TLRs), a subset of PRRs, may be involved. TLRs are principally known for their role in initiating innate immune responses against various classes of pathogens, including viruses, bacteria, and parasites (38). However, despite these pro-inflammatory properties, TLRs are also known to have counterregulatory immunosuppressive functions (39). Partially through the release of interferons (IFN), TLRs have been shown to both initiate and then subsequently limit myocardial inflammation (8). On one hand, IFN-γ activity has been shown to be required for disease progression in EAM (40). However, PD-L1 is upregulated in response to both IFN-γ and lipopolysaccharide, a known agonist of TLR4 (41). Furthermore, IFN-γ is required for the PD-L1 upregulation that decreases myocardial necrosis in a CD8+ T cell-dependent murine model of myocarditis (17). Additionally, TLR activation is critical to not only effector T cell maturation, but also differentiation into Tregs (42). In a model of atherosclerosis, deletion of MyD88, a TLR

adaptor important in effector T cell activation, paradoxically had an overall pro-inflammatory effect by primarily decreasing the presence of Tregs (43). These observations suggest that activation of certain TLRs, in part through the upregulation of IFN-γ and subsequent expression of PD-L1, may help regulate the extent of T cell activation (44, 45). At the same time, IFN-γ represents an imperfect immunomodulatory therapy, due to the risk of exacerbating an autoimmune phenotype through T cell activation, in addition to its severe side effect profile (46).

In addition to type II IFNs (i.e. IFN-γ), type I IFNs (e.g. IFN-α and IFN-β) also have a combination of pro- and anti-inflammatory properties (47). This result is not surprising given the remarkable degree of overlap in the downstream signaling between the two IFN families (48). Type I IFNs are known to facilitate viral clearance, increase expression of MHC and costimulatory molecules, and induce expression of chemokines. However, type I IFNs have also been shown to induce expression of IL-10, an anti-inflammatory cytokine, while inhibiting inflammasome maturation and IL-1 production (49). Although evidence suggests that IFN-γ is likely a more potent inducer, type I IFNs have also been shown to upregulate PD-L1 (44, 48, 50). Consequently, type I IFNs also represent a possible immunomodulatory therapy for inflammatory heart disease.

To drive expression of these IFNs, TLR9 ligand are one potentially promising therapeutic approach. TLR9 is an endosomal TLR primarily expressed in plasmacytoid DCs (pDCs) as well as B cells (51), and recognizes unmethylated CpG oligonucleotides (ODN). The response of TLR9 to CpG ODN depends upon the structural characteristics of the

particular CpG ODN, which are typically grouped based upon the chemical composition of their CpG ODN backbone (phosphorothioated or phosophodiester) and whether the DNA is linear or palindromic (51). Binding of type A CpG ODN, composed of a phosphorothioate backbone and a palindromic CpG repeat, to TLR9 stimulates robust production of type I IFN (52). The recently characterized P-class of ODN also demonstrate a robust type I IFN signature (53). Therefore, CpG ODN-TLR9-mediated release of type I IFN, as potentiated by IFN-γ, may be pivotal in inducing peripheral tolerance in the heart through multiple mechanisms, including induction of PD-L1.

CpG ODN has previously demonstrated potential as an immunomodulatory agent in a variety of disease models. Pre-treatment of mice with CpG ODN has been shown to reduce the severity of both cardiac and cerebral ischemic/reperfusion injury, likely in part through immunomodulatory mechanisms (54, 55). Protective roles for TLR9 agonists have also been noted in animal models of asthma, sepsis, viral infection, and heart failure (56-59). Notably, TLR9 agonists are already used in clinical trials for various indications including infections, cancer, autoimmunity, and allergy (60). Furthermore, unlike direct IFN therapies, TLR9 agonists generally have a generally mild toxicity profile (60).

Of note, it has been shown that prophylactic treatment with CpG ODN can attenuate CVB3-induced myocarditis (61). However, this study had several limitations. First, the authors note that the treatment had an extremely limited therapeutic index. At a dose of 10 μg, mice demonstrated reduced mortality and an increased level of virus-neutralizing antibody. However, at a dose of 20 μg, antibody levels were decreased relative to control

and these mouse hearts demonstrated increased viral replication. Furthermore, the authors used a C-class CpG ODN, which, relative to A-class ODN, stimulate a more robust B cell response, and a less pronounced type I IFN signature (51). Therefore, while C-class ODN may be the ideal therapeutic strategy in a pathophysiology with a robust antibody response, for a primarily T cell-mediated phenomenon such an approach is likely suboptimal. Furthermore, the translational potential of such findings is questionable in the light of a narrow therapeutic index. Lastly, as IFNs are known to have potent antiviral activity, the protective mechanism of the CpG ODN treatment in a virally-mediated model may be explained by inhibition of viral replication, rather than a true immunomodulatory therapy. Therefore, the utility of CpG ODN, through a TLR9:type I IFN:PD-L1 pathway, shows theoretical potential as an immunomodulatory therapy for myocarditis, but has yet to be rigorously evaluated.

Statement of Purpose

We hypothesized that CpG ODN would mitigate disease burden in a CD8+ T cell-mediated murine model of myocarditis. Our study evaluates the extent to which pre-treatment of mice with CpG ODN 24 hours prior to disease onset protects mice from fulminant lymphocytic myocarditis. Additionally, if CpG ODN pre-treatment does indeed reduce the severity of myocarditis, we seek to better understand the mechanism by which this occurs. As previous studies suggest that PD-1:PD-L1 interactions may be a main regulator of T cell activity, we aim to establish whether PD-L1 upregulation is the primary mechanism of immunomodulation by CpG ODN, and whether this upregulation requires type I IFN.

Methods

Unless explicitly stated otherwise, all experiments were completed by the thesis author.

Staining and quantification of human tissue

Human heart tissue was accessed through the Brigham and Women's Hospital (BWH) pathology tissue repository with CoolPath software (Tucson, AZ). Control samples were identified by searching for autopsy specimens from deceased patients under the age of 35 with the keyphrase "normal myocardium" in the autopsy report. Any mention of atypical or abnormal findings in the gross or histologic examination of the heart as documented in the final autopsy report disqualified samples from being counted as controls. Myocarditic hearts were identified by searching autopsy and explant hearts for the keyphrase "lymphocytic myocarditis." Only hearts described as having "significant," "severe," "multifocal," or similar descriptors of disease were included in the myocarditis group. Formalin-fixed paraffin-embedded (FFPE) tissue blocks were collected and sent for PD-L1 staining at the Specialized Histopathology Core (SHP) at BWH. PD-L1 staining was quantified with Adobe Photoshop. Staining was quantified by subtracting out non-myocardial background (e.g. white space, lumen, and lung tissue), and then taking the number of positively stained myocardial pixels divided by the total number of myocardial pixels. The average threshold for positive staining was compared between control and disease groups to detect any inconsistency. All case lists and data related to this study were de-identified with respect to patient identification and kept secure. This study was conducted under an approved BWH IRB protocol.

Mice

C57BL/6, IFNAR KO, TNFR2 KO, and OT-I mice were obtained from Jackson Laboratories (Bar Harbor, ME). OT-I mice are T cell-receptor transgenic mice with CD8+ T cells specific for the Ova peptide (SIINFEKL; $Ova_{257-264}$) on the class I MHC H2-K^b. Transgenic Mice with cardiac myocyte restricted membrane-bound ovalbumin (cMy-mOva) mice were previously engineered in the Lichtman laboratory (62). Briefly, DNA segments encoding $Ova_{257-264}$ were isolated and cloned. These residues were inserted into viral vectors and transfected into fibroblasts. This construct was then cloned directly downstream to the mouse cardiac αMyHC promoter. After amplification in competent bacteria, the verified sequence was microinjected into C57BL/6 embryonic stem cells. RT-PCR data confirms that in cMy-mOva mice, ovalbumin peptide is selectively expressed in the myocardium and histologic and ultrasonographic examnination of cMy-mOva mice at 2 years of age are consistent with healthy and normally functional myocardium (62). In short, cMy-mOva mice are genetically identical to C57BL/6 mice except for the presence of ovalbumin peptide in the heart.

All mice used were between 6 and 12 weeks in age. Experiments featured a near-even mixture of male and female mice with no noticeable differences in disease parameters between sexes. Mice were housed in the New Research Building facility as part of the BWH system. All procedures were performed according to protocols approved by the Institutional Animal Care and Use Committee (IUCAC) at Harvard University.

In Vivo *Reagents*

A CpG ODN preparation was created as a combination of two different CpG ODNs. ODN 1585 (InvivoGen, San Diego, CA) is an A-class CpG ODN and ODN 21798 (Miltenyi Biotec, Auburn, CA) is a P-class CpG ODN. Lyophilized CpG ODN was reconstituted in TE buffer and sterile PBS. ODN was administered intraperitoneally (i.p.) in doses of 20 μg/500 μL total injected volume. PD-1 blockade was accomplished with rat anti-mouse PD-1 antibody (29F.1A12, courtesy of Gordon Freeman, Harvard Medical School) and IFN-γ blockade was achieved with rat anti-mouse IFN-γ antibody (XMG1.2; BioLegend, San Diego, CA). Because of the relevance of PD-1 blockade to IRAEs in cancer treatment, we used a similar PD-1 blocking regimen as has been used in basic tumor immunology studies of PD-1 immunotherapy (63). 200 μg of anti-PD-1 were administered on days 0, 3, and 6 (with day 0 indicating the day of adoptive transfer). For IFN-γ blockade use, mice were given 100 μg of XMG1.2 antibody intraperitoneally three hours prior to CpG ODN administration in accordance with dosages used in prior studies (64). Experiments related to IFN-γ blockade were performed by Nafisa Wara.

OT-I CD8+ T Cell Preparation and Disease Induction

OT-I CD8+ T cells were prepared with the assistance of Dr. Nir Grabie. Following OT-I mouse sacrifice, spleens were surgically removed and homogenized through a filter. CD8+ T cells were isolated through CD8a magnetic bead separation (Miltenyi Biotec, Auburn, CA), and activated with soluble anti-CD28 and plate-bound anti-CD3 antibody (BD-Pharmingen, San Diego, CA), as well as IL-2 and IL-12 (R&D Systems, Minneapolis, MN) (62). After 5 days of culture and activation, these cells were

adoptively transferred into cMy-mOva mice through intraperitoneal injection at doses ranging from 40,000 to 250,000 OT-I. PD-1 KO OT-I CD8+ T cells were prepared in an identical manner, and all experiments involving PD-1 KO OT-I CD8+ T cells were completed by Nafisa Wara and Dr. Nir Grabie. Mice were then sacrificed either five or ten days following adoptive transfer, or earlier if dictated by procedure per IUCAC guidelines. For survival analysis, surviving mice were sacrificed 24 days following adoptive transfer.

Processing and Analysis of Mouse Tissue

Mice were euthanized through CO2 asphyxiation and cervical dislocation. Mouse hearts were surgically removed. Blood was collected by heparinizing and cutting the inferior vena cava. Serum was isolated through centrifugation and sent for cardiac troponin I (cTnI) measurement at the Biomarker Research and Clinical Trials laboratory at BWH. Serum cytokine measurement was performed by the laboratory of James Lederer, PhD at BWH through multiplex bead assays. Heart tissue was then prepared for immunohistochemistry (IHC), quantitative real-time RT-PCR (qRT-PCR), or flow cytometry.

For frozen section immunohistochemistry, heart tissue was frozen in optimal cutting temperature compound, cut into six-micrometer-thick sections and fixed with acetone and blocked with hydrogen peroxide. Following primary antibody incubation with rat anti-mouse Gr-1 antibody (BD-Pharmingen), sections were incubated with biotinylated goat anti-rat immunoglobulin (Vector Laboratories, Burlingame, CA). Sections were then

incubated with a horseradish peroxidase-avidin-biotin complex (ThermoFisher, Waltham, MA) and developed with aminoethyl carbazole (ThermoFisher) prior to counterstaining with Gill's number 2 hematoxylin (Polysciences Inc., Warrington, PA). For FFPE IHC, mouse hearts were stored in phosphate buffered formalin and sent to SHP at BWH. H&E staining was performed by the Rodent Histopathology Core at BWH on FFPE tissue.

qRT-PCR was performed by first extracting mRNA from approximately 10 mg of mouse heart biventricular apices with a QIAGEN RNeasy Mini kit (Qiagen, Germany). mRNA was treated with DNAse I prior to elution. Isolated mRNA was confirmed for purity and yield with a NanoDrop spectrophotometer (ThermoFisher). The ThermoScript RT-PCR system was used to generate cDNA (ThermoFisher). We then used the SYBR Green PCR mix (Applied Biosystems, Foster City, CA) to quantify amplification with an Applied Biosystems RT-PCR multiplex machine (ThermoFisher). β-actin was used to normalize gene expression across samples, as done in previous myocarditis studies by the Lichtman laboratory (30). Furthermore, many other studies have used β-actin as a RT-PCR control in assessing the effects of CpG ODN (65-67), and proteomic data shows that beta-actin levels are not significantly changed in response to CpG ODN (68).

Flow cytometry was performed by Dr. Daniel Engelbertsen, PhD. For flow cytometry, whole heart tissue was digested in collagenase mix and then passed through a cell strainer. Following osmotic lysis of erythrocytes, cells were stained with Zombie Aqua (BioLegend) and anti-CD45.2 to identify viable hematopoetic cells. Cells were also stained with anti-Ly6G and anti-CD8 to identify cell populations of interest.

Histologic scoring

Myocarditis was scored on a zero- to four-point scale by examining H&E-stained sections (69). 0 indicates no inflammation; 1 indicates between one and five mononuclear inflammatory foci occupying less than 5% of the cross-sectional area; 2 indicates more than 5 foci with between 5% and 20% of the cross-sectional area involved; 3 indicates greater than 20% involvement without cellular necrosis; 4 indicates diffuse, widespread inflammation with cellular necrosis. H&E slides were examined in a blinded fashion by a trained anatomical pathologist (A.H.L.).

Statistical Analysis

Statistical analyses were performed with Prism software (Graph Pad, La Jolla, CA). Differences between groups were examined with Student's *t* test. Significance was determined by $p < .05$. When samples did not meet assumptions necessary for a *t* test, the non-parametric Mann-Whitney *U* test was used. For Kaplan-Meier survival analysis, a log-rank test was used to determine whether there was a statistically significant difference in the hazard rate between two groups.

Results

PD-L1 is upregulated following lymphocytic infiltration of the human heart

As the protective role of PD-L1 in the human heart is not completely understood, we first sought to establish the possible translational relevance of PD-L1 upregulation in the

human heart. FFPE specimens from healthy (explant $N = 13$; autopsy $N = 2$) and inflamed (autopsy only; $N = 10$) archived human heart tissue as obtained from the BWH pathology core, stained for PD-L1 expression by immunohistochemistry, and quantified (Figure 1a). To ensure that a consistent threshold was used for positive staining across images, the average RGB pixel intensity for pixels identified as staining positively for each image was compared (Figure 1c). No significant difference was noted with Red (189.5 ± 3.839 versus 191.9 ± 2.799; $P = .61$) and Green (178.2 ± 3.771 versus 167.2 ± 3.583; $P = .05$) pixel intensities between control and diseased hearts. Blue color intensities were somewhat higher for the control hearts (164.3 ± 4.642 versus 145 ± 4.694; $P = .011$), corresponding to a lighter (i.e. less stringent) color threshold among control samples. Positive staining was present in some autopsy samples, suggesting that delayed fixation post mortem in autopsies compared to surgical pathology explant specimens does not preclude positive staining. Relative to non-diseased hearts, hearts with documented myocarditis had significantly higher levels of PD-L1 expression (6.71 ± 2.90%) than control hearts (0.516 ± .31%) as per a two-tailed Mann-Whitney *U* Test ($U = 25$; $P = .0041$). Representative images are shown in Figure 1b. Notably, PD-L1 expression was largely localized to inflammatory foci as identified on H&E stains, but included linear staining between myocytes suggestive of endothelial cell staining, as well as dense staining on inflammatory cells.

CpG ODN Upregulates PD-L1 in the Murine Myocardium

As PD-L1 has shown to be an important regulator of inflammation in the murine heart and is upregulated in the human heart following inflammation, we next sought to

understand whether CpG ODN effectively upregulates PD-L1 in the murine heart, and, if so, characterize the spatial dynamics of this upregulation. In addition to quantifying the expression of PD-L1, the expression of other IFN-related transcripts was investigated. MX-1 and IP-10 are both interferon-inducible genes, whereas IRF1 lies downstream of TLR9 activation but upstream of type I IFN production (70, 71). 24 hours following intraperitoneal injection of 30 ug of CpG ODN, both Balb/C ($N = 6$ total) and SCID ($N = 6$ total) mice demonstrated significant upregulation of PD-L1 ($P < .0001$ and $P = .0050$, respectively) and MX-1 ($P = .0482$ and $P < .0001$, respectively) (Figure 2a). PD-L1 upregulation was also found to be significant in C57BL/6 mice ($N = 11$ total; $P = .0058$), though other transcripts were not investigated in this strain. SCID mice were used so as to investigate whether B cell loss would change the transcriptomic signature of CpG ODN treatment, given that TLR9 is expressed on both pDCs and B cells (51). Balb/C mice also showed a significant upregulation of IRF-1 ($P < .0001$), though the difference was not significant in SCID mice ($P = .056$).

Immunohistochemistry demonstrated that the upregulation of PD-L1 was present in a linear, striated pattern suggesting that the protein may be expressed on endothelial cells rather than myocytes (Figure 2b). PD-L1 staining largely corresponded with CD31 staining, an endothelial cell marker.

CpG ODN-mediated upregulation of PD-L1 may occur in the absence of interferon signaling

We then sought to identify the mediators of PD-L1 upregulation in the murine heart following administration of CpG ODN. Because CpG ODN is a known inducer of both type I and type II IFNs (72), we hypothesized that PD-L1 transcript upregulation would be abrogated with blockade of type I or II IFN signaling. However, IFNAR KO mice demonstrated marked upregulation of PDL1 in the context of CpG ODN administration ($N = 5$ ODN versus 4 control; $P = .0098$) (Figure 3a). IFNAR KO mice showed upregulation of IP10 ($P = .0011$) and IRF1 ($P = .0004$). As type I IFN signaling has been shown to be required for PD-L1 expression (44), we next sought to determine if a different receptor may be relaying a type I IFN signal. TNFR2 signaling has been shown to initiate autocrine type I IFN signaling within endothelial cells, and therefore may play a critical role in connecting CpG ODN administration with interferon-related signaling (73). However, in TNFR2 KO mice, CpG ODN administration ($N = 4$) also markedly upregulated PD-L1 relative to controls ($N = 3$; $P < .0001$) as well as for IP10 ($P = .0005$) and IRF1 ($P < .0001$).

To examine the role of type II IFN signaling in CpG ODN-mediated upregulation of PD-L1, we used an IFN-γ blocking antibody (XMG1.2) in C57BL/6 mice (Figure 3b). No difference was detected in PD-L1 upregulation between mice receiving CpG ODN and XMG1.2 ($N = 4$) and just CpG ODN ($N = 4$; $P = .1763$).

CpG ODN Treatment Prior to Initiation of CD8+ T Cell-Mediated Myocarditis Ameliorates Disease Burden

Given that CpG ODN upregulates PD-L1 in the murine heart, we next tested whether CpG ODN pre-treatment can protect against lymphocytic myocarditis in a CD8 T cell-mediated adoptive transfer model of the disease (Figure 4a). At an adoptive transfer dose of 10^5 in vitro activated OT-I cells, mice receiving CpG ODN ($N = 10$) demonstrated a significantly lower ($P = .0001$) serum cTnI compared to mice receiving PBS ($N = 8$) at 5 days after adoptive transfer (263 ± 59 ng/mL versus 776 ± 85.6 ng/mL). Mice without the cMy-mOva transgene demonstrated minimal levels of serum cTnI ($N = 10$; 1.60 ± 1.41 ng/mL). As several troponin readings were above the maximum detectable threshold of 1000 ng/mL with an adoptive transfer dose of 10^5, we next repeated the experiment with a transfer dose of $4 \cdot 10^4$ OT-I cells. Again, mice receiving CpG ODN pretreatment had a significantly lower ($P = .03$) serum cTnI ($N = 12$; 51.2 ± 18.2 ng/mL) compared to PBS controls ($N = 9$; 208.9 ± 79.1 ng/mL). From this, we concluded that myocardial damage by CTLs is significantly abrogated with CpG ODN treatment.

On histologic analysis at day 5, although mice receiving CpG ODN had fewer intramural thrombi, overall histologic scores were not different between the two groups with either adoptive transfer dose (unpublished data). We hypothesized that histologic delineation may occur at a later time point, as smaller differences in initial cardiac injury may magnify into larger differences as inflammation initiates a self-amplifying cycle. Therefore, we histologically evaluated mouse hearts at day 10 after adoptive transfer with $4 \cdot 10^4$ OT-I cells (Figure 4b). Mice receiving CpG ODN ($N = 10$; 1.8 ± .29) had a significantly lower average histologic grade of myocarditis compared to mice receiving PBS ($N = 11$; 3.09 ± .25; $P = .0031$).

As another assay to examine the extent to which CpG ODN ameliorates the burden of lymphocytic myocarditis, we next attempted to measure the degree of neutrophilic inflammation in the heart, as neutrophil recruitment occurs in the context of myocardial cell death, and neutrophils contribute to disease severity in the cMy-mOva model (74, 75). Mice receiving $4{\cdot}10^4$ OT-I cells were sacrificed at day 5 status post adoptive transfer and frozen section immunohistochemistry with anti-Gr-1 (anti-Ly6G) was performed on myocardial cross-sections (Figure 4c). Pre-treatment with CpG ODN ($N = 10$; 7.46% ± 1.86%) led to a significantly less Gr-1 staining compared to PBS-treated controls ($N = 8$; 18.3% ± 2.2%; $P = .0018$). cTnI serology values correlated with percent Gr-1 staining ($r^2 = .689$), with a linear model slope significantly different from 0 ($P < .0001$).

Additionally, we assayed neutrophilic infiltrate via flow cytometry on collagenase-digested hearts (Figure 4d). To increase the number of cells sorted per flow cytometry run, heart tissue was combined within groups according to sex ($N = 4$ hearts per group). Viable cells were identified as $CD45.2^+$ $ZombieAqua^-$. Of this population, neutrophils were defined as $Ly6G^+CD8^-$. Among viable cells in female mice, neutrophils represented 12.8% and of all live cells in the non-ODN treated population versus 4.14% in the ODN-treated population. For male mice, the neutrophils made up 8.1% versus 4.87%. CD8+ T cells were identified as $Ly6G^-CD8^+$. Less significant differences were noted among these cells, with ODN treatment increasing the relative percentage of CD8+ T cells in female mice from 4.27% to 4.76%, and in male mice increasing the percentage from 2.56% to

4.22%. As only one trial was completed and tissue from different mice were combined within the group, statistical testing was not done.

To further characterize the extent to which ODN pretreatment changes the cellular milieu in the context of lymphocytic inflammation in the heart, we next characterized the prevalence of FOXP3+ cells in myocarditic hearts (Figure 4e). FOXP3 is a transcriptional regulator protein constitutively expressed in regulatory T cells (Tregs), a subset of T cells that have essential suppressor activities on effector T cells, mediated by both cell-cell contact with APCs and T cells, and also by the release of immunosuppressive cytokines (76). Of note, CpG ODN treatment has been shown to increase the expression of FOXP3 in other contexts (77). Heart cross-sections were processed as formalin-fixed paraffin-embedded tissue and stained for FOXP3. FOXP3+ cells were manually counted by a blinded technician. To normalize for different heart sizes, the total count of FOXP3 positive cells was divided by the number of myocardial pixels in each specimen. ODN pretreated mice ($N = 10$) had a significantly larger number of area-normalized FOXP3 positive cells than control ($N = 8$) mice at sacrifice 5 days following adoptive transfer ($1.52 \cdot 10^{-4} \pm 6.37 \cdot 10^{-5}$ cells/pixel versus $8.90 \cdot 10^{-5} \pm 3.33 \cdot 10^{-5}$ cells/pixel; $P = .0022$).

Lastly, we examined the RT-PCR signatures of several inflammatory mediators and markers of interest of hearts from cMy-mOva mice 5 days status-post adoptive transfer of $4 \cdot 10^4$ OT-I T cells with ($N = 8$) and without ($N = 6$) pretreatment with ODN (Figure 4f). There was no statistically significant difference in IFN-γ, a known inflammatory marker, but ODN-treated mice did demonstrate increased transcript levels of FOXP3, TGF-β, and

TNF-α. Interestingly, there was no statistically significant difference in PD-L1 transcript at day 5 (unpublished data).

CpG ODN Cardioprotection in OT-I-Mediated Myocarditis May Involve Multiple Mechanisms Beyond PD-L1 Upregulation

CpG ODN is known to upregulate several immunoregulatory factors that could putatively be protective in a T cell model of myocarditis. For example, we have found that CpG ODN administration upregulates 2, 3-indoleamine dioxygenase (IDO) in the murine heart (unpublished data), and in other model systems CpG ODN administration suppresses adaptive T cell responses in a non-interferon dependent manner (78). Therefore, we next sought to establish whether PD-L1 upregulation is required for CpG ODN-mediated cardioprotection.

In an initial experiment, cMy-mOva mice received an adoptive transfer of $4 \cdot 10^4$ OT-I T cells (Figure 5a). In addition, mice were either pretreated prior to adoptive transfer with CpG ODN, a course of three treatments of anti-PD-1 blockade, both, or neither ($N = 6$ for each group). Mice were sacrificed at day 10 after adoptive transfer and FFPE H&E stains were evaluated blindly for histologic scoring. cTnI readings at day 10 after adoptive transfer were indistinguishable from baseline levels (unpublished data).

Though differences did not reach statistical significance, there was a trend toward anti-PD-1 treatment increasing the burden of disease as measured by blind histologic scoring relative to untreated controls (1.67 ± 1.21 versus $2.50 \pm .52$, $P = .26$). In fact, follow up

CpG ODN tended to reduce the extent of myocarditis in both mice receiving anti-PD-1 treatment (2.5 ± 1.22 versus 1.17 ± 1.69; $P = .083$) and mice without anti-PD-1 treatment (1.67 ± 1.21 versus 0.67 ± .52; $P = .092$). Current work is focused on repeating these experiments with greater numbers per group.

To further investigate the role of PD-1 dependency in CpG ODN pretreatment, we next performed several experiments with PD-1 KO OT-I T cells. First, mice were given an adoptive transfer of $4 \cdot 10^4$ PD-1 KO OT-I T lymphocytes with or without CpG ODN pretreatment ($N = 6$ versus 6) and survival was documented to an experiment end date of 24 days, at which point the remaining mice were sacrificed and those data points were censored (Figure 5b). A log rank test failed to detect a statistically significant difference between the two groups ($z = 1.13$, $P = .26$), though CpG ODN pretreatment tended towards increasing survival (median 8.5 days versus 23 days).

In a follow-up study also using PD-1 KO OT-I T cells, we assessed for differences in histologic and serologic markers of myocardial disease five days after an adoptive transfer of $4 \cdot 10^4$ PD-1 KO OT-I T cells ($N = 6$ versus 6; Figure 5c). With respect to histologic scoring, CpG ODN pretreatment tended towards decreased disease, but did not have a statistically significant effect (2.3 ± .44 versus 2.8 ± .38; $P = .37$). Likewise, cTnI readings were on average lower, though not significantly so (98.9 ± 48.5 versus 198 ± 94.3; $P = .37$).

Discussion

There is an unmet need to develop more targeted therapies for myocarditis. As evidence suggests that T cells are centrally important in the pathogenesis of the disease, treatments that act upon T cells represent one potentially useful translational avenue. In this study, we demonstrate that CpG ODN reduces the extent of disease in a CD8+ T cell-mediated mouse model of myocarditis. Furthermore, we establish possible translational relevance of PD-L1 expression in the context of human myocarditis with histologic evidence suggesting a feedback mechanism to limit inflammation in the heart. Lastly, we present some preliminary data to suggest the mechanisms by which this cardioprotection occurs.

Our data indicate that PD-L1 is upregulated in human hearts upon inflammation. This upregulation occurs irrespective of the initial inflammatory stimulus (e.g. viral versus autoimmune), as we received human specimens with a variety of clinical diagnoses and selected them solely based upon histologic characterization. Although this data certainly cannot show causality, this retrospective analysis does suggest that PD-L1 likely plays a protective role, preventing excess cardiac inflammation. Given the known mechanism of PD-L1, a hypothesis positing reverse causality (i.e. that PD-L1 expression predisposes hearts to myocarditis) is unfounded.

Although in some myocarditic human hearts PD-L1 is expressed on cardiomyocytes, the majority of PD-L1 expression was present on endothelial cells. This result corresponds to previous studies done by the Lichtman laboratory of mice with CD8+ T cell-mediated

myocarditis, as demonstrated by confocal dual-antibody immunohistochemistry with PD-L1 and CD31 (17). Furthermore, these results are also in line with the PD-L1 staining patterns demonstrated by our *in vivo* mechanistic experiments with CpG ODN. The similar spatial arrangement of PD-L1 staining in both cases suggests that CpG ODN may be co-opting a similar mechanism to that present in human and mouse hearts with lymphocytic inflammation. The possible importance of endothelial PD-L1 is also suggested by a previous study showing that globally PD-L1-deficienct mice are not rescued with a PD-L1 WT bone marrow transfer, suggesting that deficiency in a non-hematopoetic cell line is responsible for the increased propensity for severe disease in PD-L1-knockout mice (17). Our lab is currently working towards developing mice with an endothelial cell-specific PD-L1 deficiency by backcrossing cMy-mOva mice, mice with VE-Cad-restricted Cre, and mice with PD-L1$^{fl/fl}$ alleles (79). Usage of this strain in an adoptive transfer model would permit the identification of the role of PD-L1 expressed specifically on endothelial cells in limiting myocardial inflammation.

CpG ODN robustly upregulated PD-L1 as well as genes upstream and downstream of type I IFN production. Notably, the upregulation of PD-L1 also occurs in SCID mice. As the major cell types expressing TLR9 are B cells and pDCs, this result suggests that pDCs are the likely regulators of the systemic response to CpG ODN. This result corresponds to other studies suggesting that pDCs are the predominant cell type responsible for the strong IFN upregulation in response to CpG ODN (80). The increased expression of IRF1, which lies upstream of type I IFN production, as well as MX-1, an IFN-inducible gene, suggests that CpG ODN may be acting through a type I IFN pathway

to drive PD-L1 expression. However, we were surprised to see that even in IFNAR KO mice there is a marked upregulation of PD-L1 in response to CpG ODN. Furthermore, we also noted upregulation of genes both upstream and downstream from type I IFN even in this knockout strain. As other work has shown that TNF-α signaling through TNFR2 can initiate an autocrine IRF1-IFN-β loop, we next considered the possibility that TNFR2 signaling may contribute to PD-L1 upregulation, either through an IFN-dependent pathway or otherwise (73). Despite other work suggesting that TNF-α can upregulate PD-L1 on multiple cell lines, we did not observe any effect in TNFR2 KO mice (81-83). Although group sizes were too small to definitively conclude one way or the other, preliminary data from IFN-γ blockade experiments suggests that PD-L1 expression may occur even in the absence of type II IFN signaling. However, it is possible that there was ineffective IFN-γ blockade, and repeat experiments will need to confirm effective blockade by testing for transcriptomic changes expected with IFN-γ blockade (e.g. decreased *CXCL10*, *HLA-DR*) (84). Interestingly, studies have reported mixed results as to whether CpG-mediated immunosuppression is dependent upon type I IFN signaling (78, 85). One possible explanation of our data is that endothelial cells may be able to directly respond to CpG ODN without a systemic pDC-driven upregulation of type I IFN. Recent work has shown that endothelial cells do express TLR 9 and recognize CpG ODN (86). We are currently performing in vitro studies to see if CpG administration directly applied to mouse heart endothelial cells can trigger PD-L1 expression. Other explanations include insufficient blockade through antibody administration (IFN-γ), and redundancy or crosstalk between multiple IFN and TNF signaling pathways leading to no phenotype in single-knockout models (87).

By treating mice with CpG ODN prior to the inflammatory stimulus, our treatment seeks to initiate feedback inhibition prior to the actual inflammatory stimulus, thereby priming the heart in anticipation of a forthcoming insult. Mice receiving CpG ODN prior to disease initiation had less serologic evidence of disease 5 days after adoptive transfer, as well as less histologic evidence of disease 10 days after adoptive transfer. Interestingly, there were no histologic differences at day 5 and serologic differences at day 10. This may reflect the time course of disease, wherein disease intensity peaks, and then either leads to death or begins to resolve. cTnI readings, then, may peak early when cell death is most prevalent. During this time, however, histologic delineation may be challenging. A simple H&E stain may not sufficiently characterize whether cell death is occurring. For example, we have shown that CpG ODN increases the prevalence of FOXP3+ cells in the heart (as well as FOXP3 transcript), though with only an H&E stain both groups would appear to have indistinguishable lymphocytic infiltrates. Furthermore, on simple H&E histology it is not always possible to readily distinguish between luminal, marginated, and infiltrating lymphocytes. As our data suggest that CpG ODN upregulates PD-L1 on endothelium, one mechanism of cardioprotection may be that CpG ODN limits transmigration of T cells into the tissue (41). Alternatively, endothelial PD-L1 may provide immunosuppression through deactivating T cells (88).

The results presented above suggest that CpG ODN represents an effective pre-treatment for lymphocytic myocarditis. Although previous studies have reported upon this immunoregulatory capability of CpG ODN, this result was by no means assured in our

study, as CpG ODN is known to both have stimulatory and inhibitory properties (51). Some have proposed that the overall effect of CpG ODN treatment depends upon the method of delivery, whereby local administration of CpG ODN has a stimulatory effect (e.g. as in serving as a vaccine adjuvant) and systemic administration of CpG ODN has a largely immunosuppressive signature (78). On a molecular level, the difference may be explained by the relative balance of canonical and non-canonical NF-κB signaling (89). Our data show that CpG ODN pretreatment decreases histologic markers of inflammation, reduces serologic markers or myocardial cell death, increases several tolerogenic transcripts, decreases the prevalence of neutrophils while increasing the number of Tregs in the heart. No difference in IFN-γ transcript, a marker of inflammation used in previous experiments in the Lichtman laboratory, was noted with CpG ODN administration (30, 62). However, CpG ODN has been shown to induce IFN-γ in some cases, suggesting that in this instance it may not be a reliable marker of inflammation (90). Likewise, TNF-α transcript, a marker of inflammation specifically used in PD-L1-deficiency experiments in the Lichtman laboratory, was elevated in the group receiving CpG ODN (30). However, CpG ODN has also been shown to upregulate TNF-α, again suggesting that this finding points to the pleiotropic effects of CpG ODN rather than contradictory evidence related to relative levels of inflammation between the groups (91).

In addition to determining whether CpG ODN ameliorates lymphocytic myocarditis, a major aim of this work is to determine the mechanism by which any immunosuppression may occur. Prior studies in our lab indicate that PD-L1 expression plays an important role in limiting inflammation in the heart, and our work has shown that PD-L1 is upregulated

in the human heart upon inflammation and in the murine heart upon exposure to CpG ODN (17, 30). However, our preliminary data is equivocal as to whether PD-L1 upregulation is the predominant mechanism through which CpG ODN provides cardioprotection, suggesting that PD-L1 upregulation may be one of several pertinent mechanisms. Though results did not reach statistical significance in a pilot trail, PD-1 blockade predictably tended towards increasing disease severity among mice not receiving CpG ODN pretreatment. However, both anti-PD-1-treated mice and PD-1-competent mice demonstrated a trend towards less disease when given CpG ODN as measured by histologic grade. This result suggests that other mechanisms of cardioprotection beyond PD-L1 upregulation may be initiated with CpG ODN treatment. To directly investigate the role of PD-L1 in reducing the activity of activated CD8+ T cells, we next eliminated the activity of PD-L1 on these cells through adoptive transfer of PD-1 KO OT-I T cells. These preliminary experiments, though not yet reaching statistical significance, again suggested that CpG ODN pretreatment may have a cardioprotective effect even in the absence of PD-1:PD-L1 signaling. Follow up studies will be focused on repeating these experiments with larger sample sizes.

Several studies have suggested other mechanisms for immunomodulation other than PD-L1 upregulation. Many different models have forwarded the upregulation of IDO as the primary mechanism by which CpG ODN exerts an immunosuppressive phenotype (78, 85, 89). These include other tissue-specific models of autoimmunity such as experimental autoimmune diabetes (a murine model of type I diabetes mellitus) (92), as well as a mouse model of hemophilia A with factor VIII-reactive lymphocytes (93). Indeed, our

own data suggests that there is robust upregulation of IDO mRNA in the myocardium in response to CpG ODN. Classically, IDO has been shown to mediate immunosuppression through enzymatic degradation of tryptophan, an essential amino acid, inducing apoptosis of effector T cells (94). However, IDO has also been shown to have non-enzymatic properties that lead to TGF-β-mediated induction of Tregs (95). Our results do indicate that CpG ODN treatment increases the number of Tregs as well as Treg-associated transcripts. Future experiments will investigate the effect of 1-methyltryptophan (1-MT), an inhibitor of IDO, on the efficacy of CpG ODN treatment. Previous work has shown that 1-MT treatment prevents pDC-mediated, IDO-driven conversion of CD8+ T cells to a Treg phenotype (96).

However, other models have discounted the relevance of IDO in the induction of immunosuppression as mediated by CpG ODN. In an ovalbumin-mediated model of lung inflammation, Mirotti *et al.* report that the protective effect of CpG ODN involved neither type I or II IFN, nor IDO (97). Instead, in this model CpG ODN required functional IL-10 and MyD88 signaling to inhibit inflammation. Additionally, several studies have commented on the capacity of CpG ODN to upregulate inducible nitric oxide synthase (iNOS) and subsequently nitric oxide (NO) (98, 99). NO production has been shown to limit T cell activation and induce regulatory T cells (100). iNOS has been established as a central mediator of disease severity in experimental autoimmune myocarditis, a CD4+ T cell-mediated immunization model of myocarditis (8). Therefore, iNOS represents another putative mechanism of cardioprotection, and will be examined in future efforts to characterize the cardioprotection seen in our model.

Our study has several limitations. First, our CD8+ T cell adoptive transfer model of myocarditis does not fully recapitulate all features of myocarditis in humans, which involves a complex interaction of multiple cell types. Second, several results are preliminary in nature and do not reach statistical significance, requiring follow-up testing. Third, our mechanistic data are largely transcriptomic, and we do not show any corroborative proteomic data currently (e.g. ELISA or Western blots). Fourth, we did not investigate CpG ODN as a reactive, rather than prophylactic treatment. Thus, the translational relevance of this therapy may depend upon an effective screening algorithm to identify patients at risk of developing myocarditis.

In conclusion, we show that CpG ODN is an effective prophylactic therapy in a CD8+ T cell adoptive transfer murine model of myocarditis. Although CpG ODN upregulates PD-L1 in the myocardium and data suggests that PD-L1 is important in maintaining tolerance in both the human and mouse heart, it is currently unclear if the primary immunosuppressive mechanism of CpG ODN is upregulation of PD-L1. This study represents a first step towards addressing the need to find targeted T cell therapies for myocarditis.

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Figures

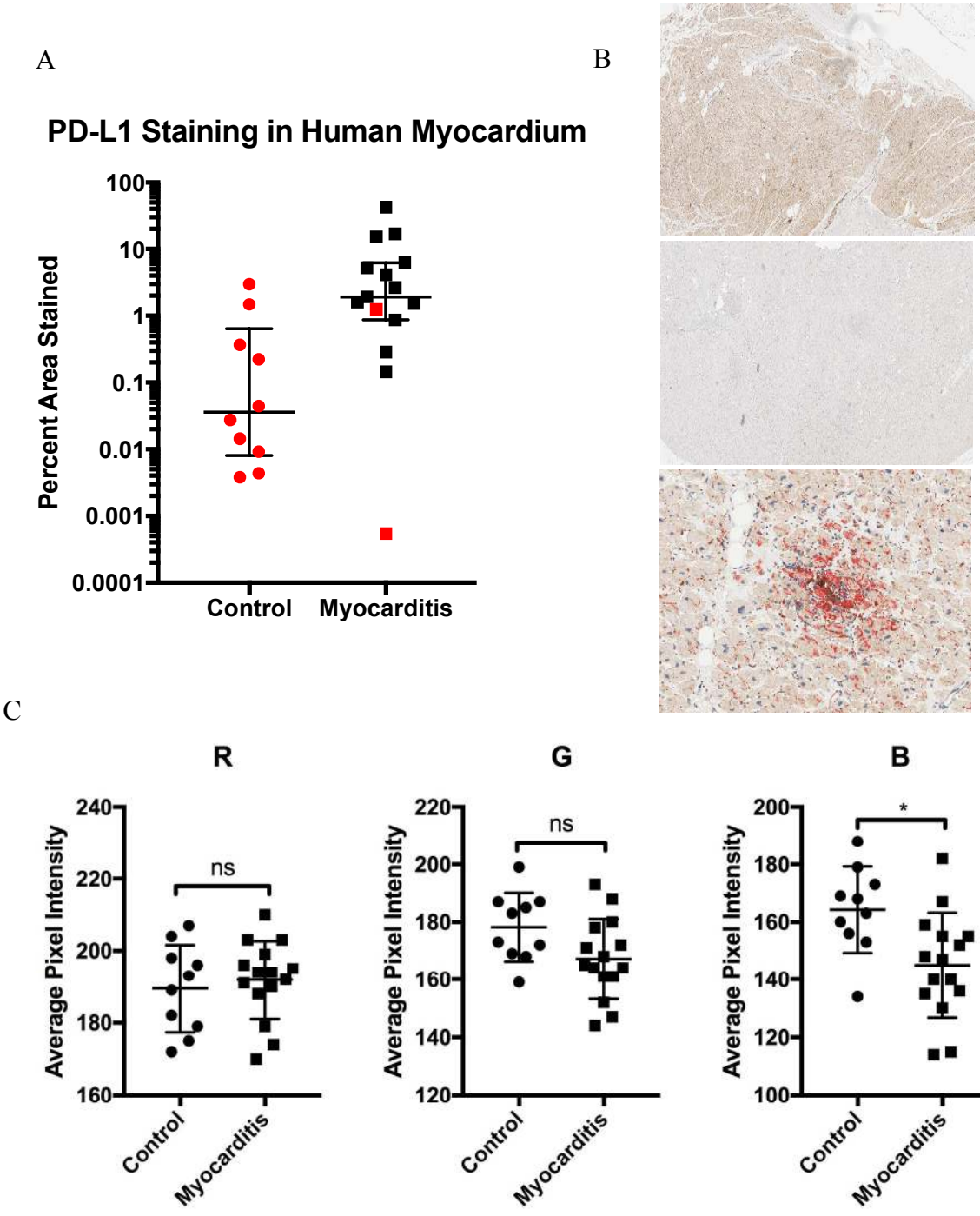
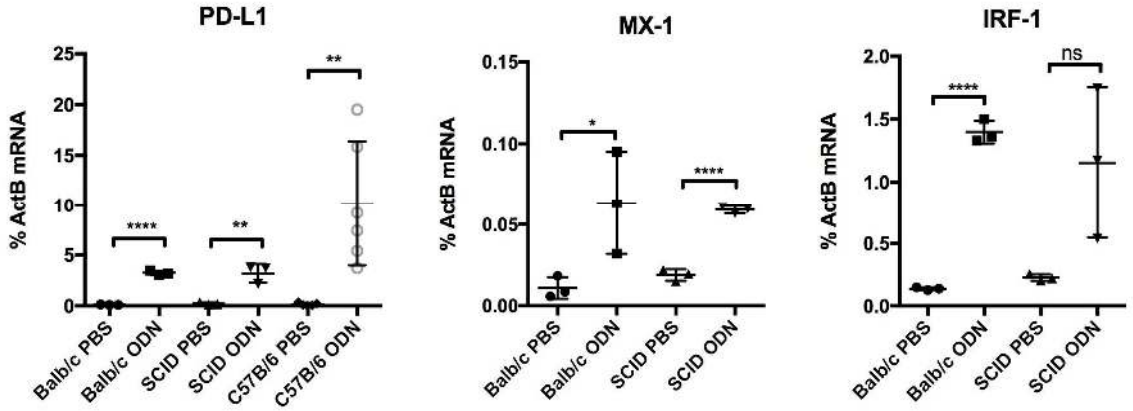


Figure 1. PD-L1 staining in inflamed and healthy human hearts. (A) Percent of pixels staining positive in myocarditic and healthy hearts. Autopsy samples are colored red and explants are colored black. (B) Representative staining images. Top: inflamed heart. Middle: control heart. Bottom: high-magnification image of inflammatory foci. (C) Average RGB values of pixels identified as being positively stained.

A



B

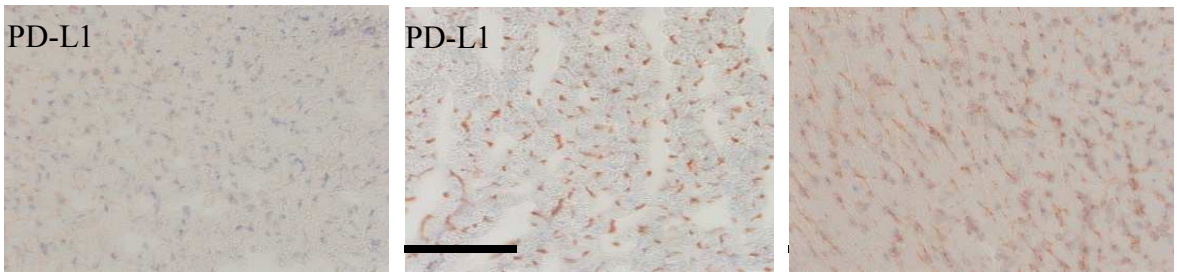


Figure 2. Upregulation of PD-L1 and other interferon-related genes 24 hours status-post intraperitoneal administration of CpG ODN. (A) RT-PCR data from biventricular apices of heats from Balb/C, C57BL/6, or SCID mice after administration of ODN or PBS. Beta-actin (Actβ) has been used to normalize levels of genes between samples. (B) Representative immunohistochemistry from Balb/C mouse hearts. PD-L1 staining in a mouse receiving PBS (left) and CpG ODN (middle). The right shows a CD31 stain of heart from a Cpg ODN -treated mouse. Scale bar represents 50 μm.

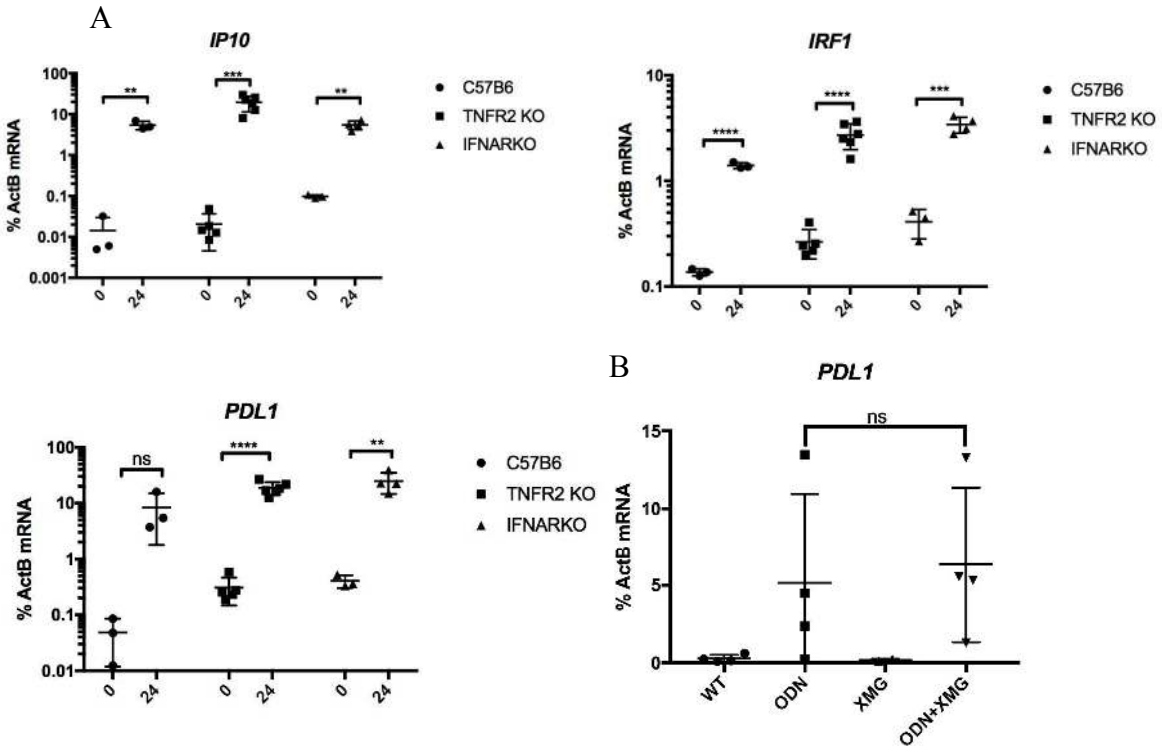
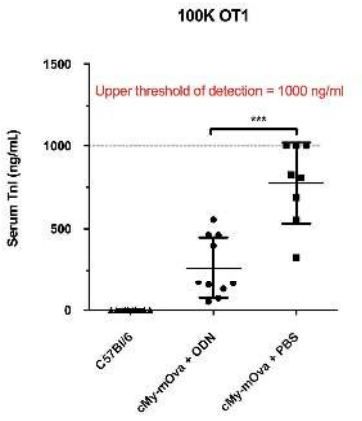
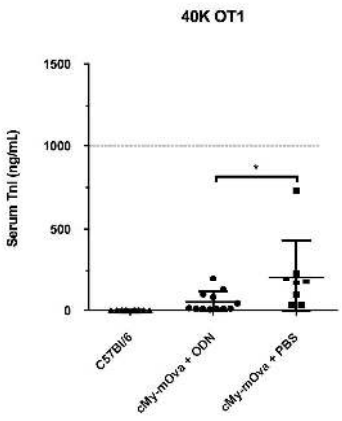


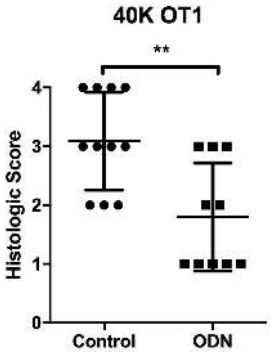
Figure 3. CpG ODN-mediated upregulation of PD-L1 may occur in the absence of IFN signaling. (A) RT-PCR plots of *IP10* (top left), *IRF1* (top right), and *PDL1* (bottom) in murine myocardium 24 hours after administration of CpG ODN administration to C57BL/6, IFNAR KO, or TNFR2 KO mice. (B) RT-PCR plot of *PDL1* expression 24 hours after administration of CpG ODN with or without XMG1.2 antibody, an IFNɣ-blocking antibody.

A

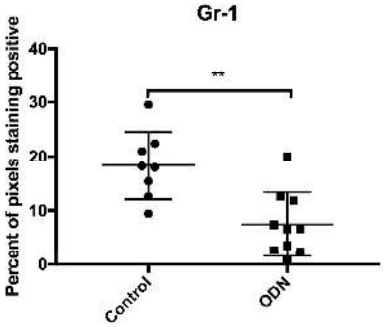

100K OT1
Upper threshold of detection = 1000 ng/ml
***
Serum TnI (ng/mL)
1500
1000
500
0
C57Bl/6
cMy-mOva + ODN
cMy-mOva + PBS

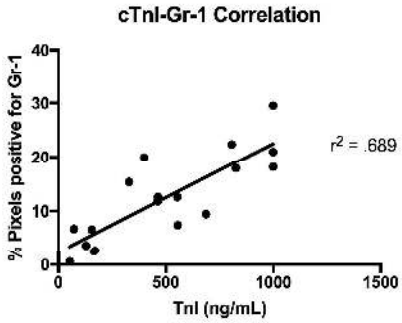

40K OT1
*
Serum TnI (ng/mL)
1500
1000
500
0
C57Bl/6
cMy-mOva + ODN
cMy-mOva + PBS

B

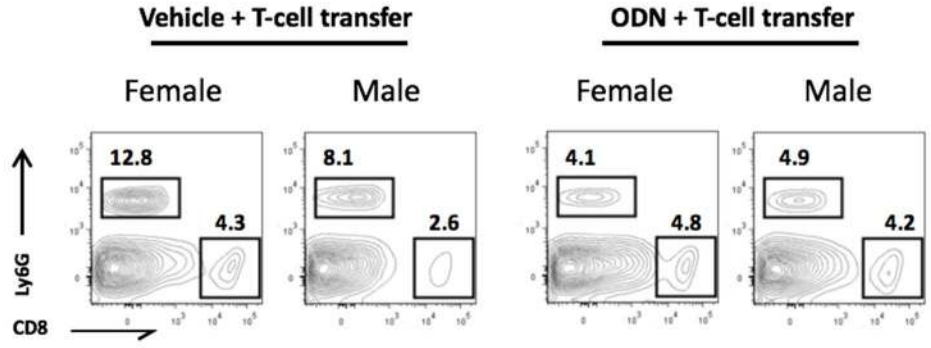

40K OT1
**
Histologic Score
4
3
2
1
0
Control
ODN

C


Gr-1
**
Percent of pixels staining positive
40
30
20
10
0
Control
ODN


cTnI-Gr-1 Correlation
% Pixels positive for Gr-1
40
30
20
10
0
r2 = .689
0
500
1000
1500
TnI (ng/mL)

D


Vehicle + T-cell transfer
ODN + T-cell transfer
Female
Male
Female
Male
12.8
4.3
8.1
2.6
4.1
4.8
4.9
4.2
Ly6G
CD8

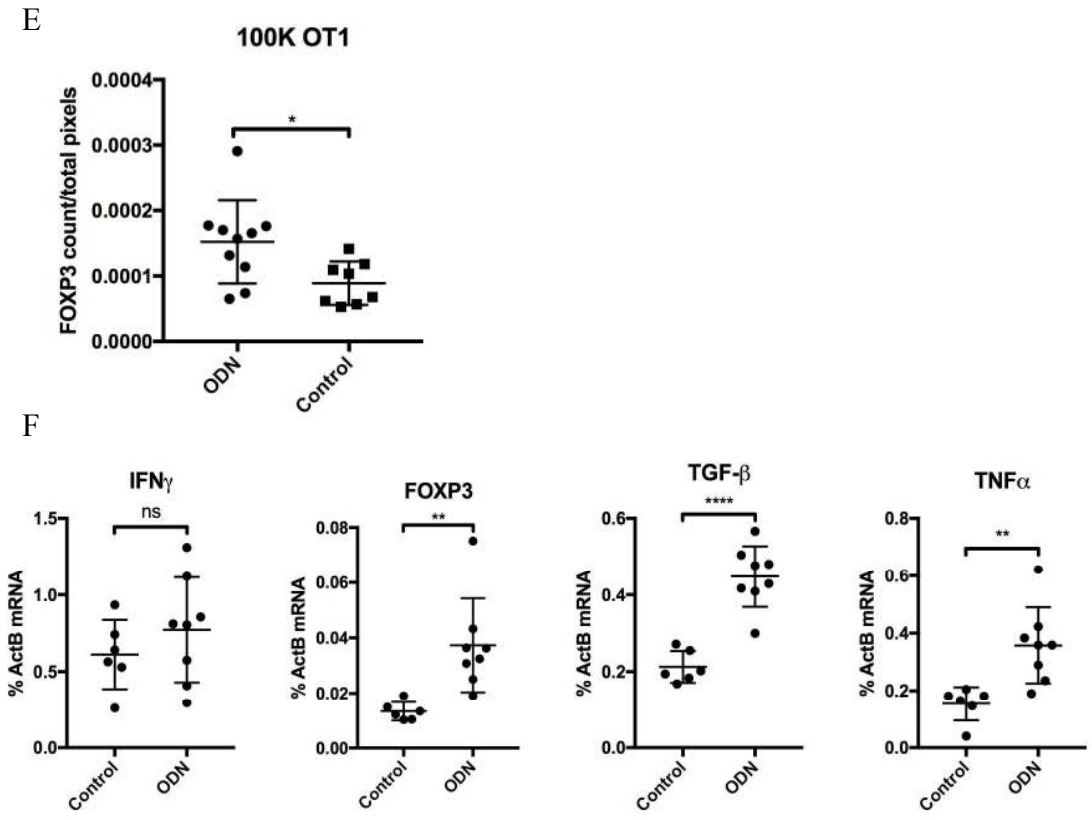


Figure 4. CpG ODN pretreatment ameliorates disease burden in a CD8+ T cell adoptive-transfer model of myocarditis. (A) cTnI histology from C57BL/6 and cMy-mOva mice at 5 days after adoptive transfer of 10^5 (left) or $4{\cdot}10^4$ (right) OT-I T cells with and without pretreatment with CpG ODN. In the adoptive transfer of 10^5 OT-I, several readings were above the maximum detectable threshold of 1000 ng/mL and were counted as 1000 ng/mL for the purposes of statistical testing. (B) Blind histologic scoring at day 10 status post adoptive transfer of $4{\cdot}10^4$ OT-I T cells in cMy-mOva mice with and without pretreatment with ODN. (C) Gr-1 staining analysis of hearts from mice receiving $4{\cdot}10^4$ OT-I T cells at day 5 (left) and correlation between Gr-1 staining and cTnI histology (right). (D) Flow cytometry plot of collagenase-digested hearts from male and female cMy-mOva mice sacrificed at day 5 status post $4{\cdot}10^4$ OT-I T cell adoptive transfer with and without ODN pretreatment. Cells were stained with CD45.2 and Zombie Aqua as viability markers and then CD8 and Ly6G to identify CD8+ T cells and neutrophils. (E) Count of FOXP3+ cells in hearts of cMy-mOva mice 5 days after adoptive transfer of 10^5 OT-I T cells normalized by the size of heart as measured by pixel count. (F) qRT-PCR data from cMy-mOva mice with and without ODN pretreatment 5 days after adoptive transfer of $4{\cdot}10^4$ OT-I T cells.

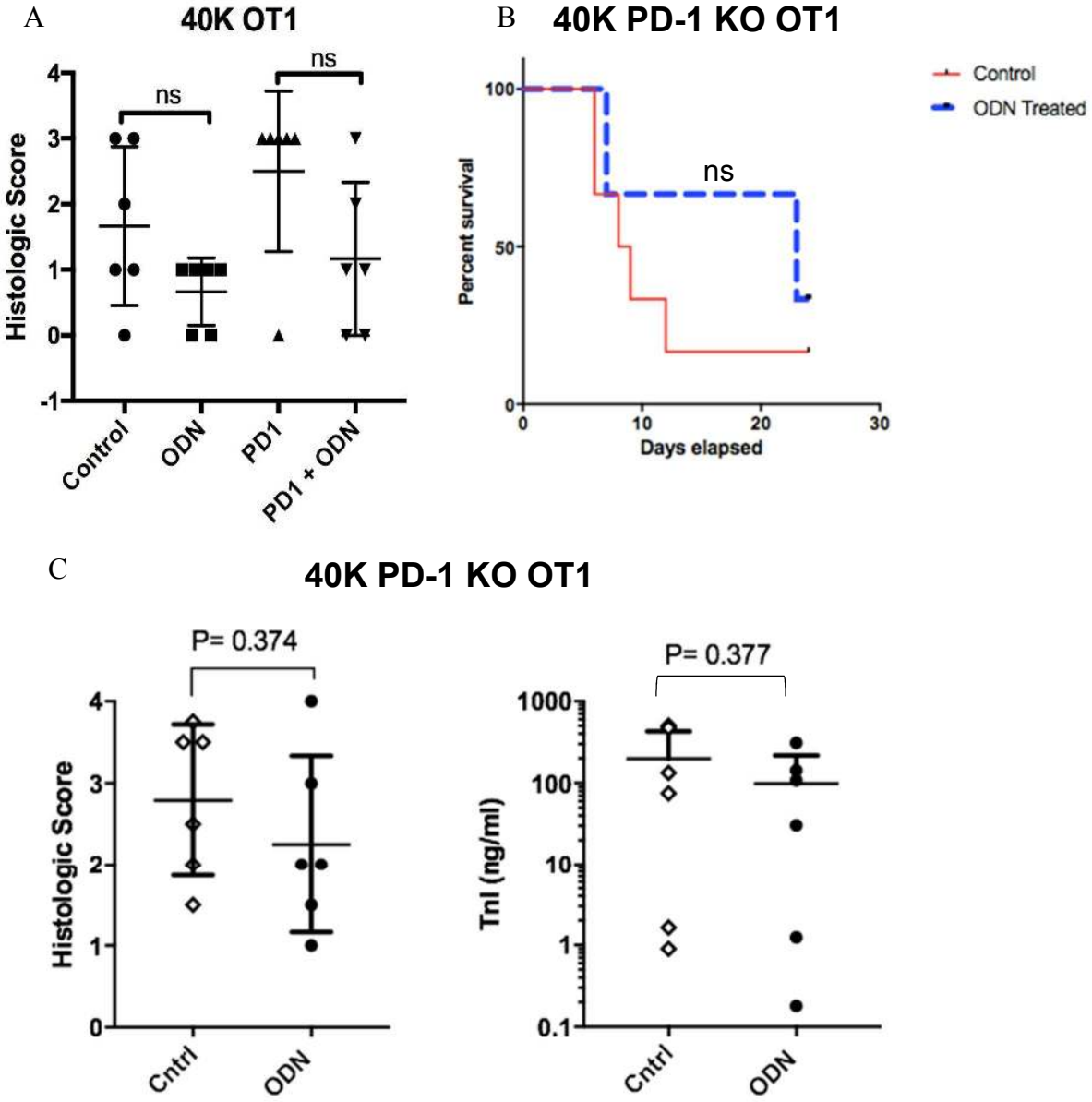


Figure 5. CpG ODN treatment for CD8+ T cell-mediated myocarditis in the absence of PD-1:PD-L1 signaling. (A) Myocarditis histology scores of cMy-mOva mice sacrificed at day 10 following adoptive transfer of $4{\cdot}10^4$ OT-I T cells. Mice received either CpG ODN 24 hours to adoptive transfer ("ODN"), a course of anti-PD1 treatment ("PD1"), both ("PD1 + ODN"), or neither ("Control"). (B) Survival analysis of mice receiving an adoptive transfer dose of $4{\cdot}10^4$ PD-1 KO OT-I T cells with and without CpG ODN pretreatment. (C) Histologic and serologic analysis of mice receiving $4{\cdot}10^4$ PD-1 KO OT-I T cells with and without CpG ODN pretreatment at 5 days status post adoptive transfer.