A genetic mouse model recapitulates immune checkpoint inhibitor-associated myocarditis and supports a mechanism-based therapeutic intervention

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Supplemental Material

2 tables

10 figures

Supplemental Tables:

|	Ctla4$^{+/-}$ Pdcd1$^{-/-}$ mice		Ctla4$^{+/+}$ Pdcd1$^{-/-}$ mice	
Heart				
Number of mice evaluated	54		59	
Percentage male	48%		56%	
Percentage female	52%		44%	
Mean age evaluated	154.5 days		164.4 days	
	Male	Female	Male	Female
Percentage of mice with lymphocytic infiltrate	23%	39%	0.09%	19%
Mean lymphocytic infiltrate histology score	2.46	3.71	0.76	1.23
Percentage of mice with T cell infiltrate	34.6%	42.9%	15.2%	19.2%
Mean T cell infiltrate score	3.04	5.73	1.59	2.36
Pancreas				
Number of mice evaluated	44		42	
Percentage male	48%		45%	
Percentage female	52%		55%	
Mean age evaluated	139.6 days		149.4 days	
	Male	Female	Male	Female
Percentage of mice with lymphocytic infiltrate	71%	87%	11%	30%
Mean lymphocytic infiltrate histology score	4.43	5.13	0.68	1.13
Percentage of mice with T cell infiltrate	62%	77%	39%	43%
Mean T cell infiltrate score	3.15	3.27	1.22	1.52
Percentage of mice with exocrine atrophy	43%	57%	5%	0%

Supplemental Table 1. Histological analyses of heart and pancreatic tissues of *Ctla4$^{+/-}$ Pdcd1$^{-/-}$* mice and *Ctla4$^{+/+}$ Pdcd1$^{-/-}$* mice.

Semi-quantitative histologic scores of heart and pancreatic tissues. Mouse characteristics including age, sex, and genotype are denoted. Lymphocytic infiltration was defined by a histologic score of greater or equal to 2 of H&E stained tissue section (see Methods). T cell infiltrate was defined by a histologic score of greater or equal to 2 for CD3 IHC stained tissue sections.

Demog	Cancer Medical History	ICI regimen; number of doses received	Time to onset myocarditis; concurrent irAE	Myocarditis presentation	Immuno-modulators and other support (treatment sequence)	Outcome
66y, F, 50kg	- Treated for metastatic lung cancer (1st line: carboplatin, pemetrexed) - Past history of thymoma (surgery and chest Rx 20 years earlier) complicated paralysis of diaphragm	2nd line: Nivolumab (240 mg/2 weeks); 3 doses	37 days; myositis, hepatitis, arthritis (flare), myasthenia gravis like	Pseudo-ST+ (cMRI +, normal cAngio); Max Trop-T: 6.2$_{ng/mL}$; Max NTpro-BNP: 6.8$_{ng/mL}$; Min LVEF (55%); PVC (10-14,000 /day with triplets); compatible ICI-irAE peripheral muscle biopsy	1/ Corticoresistant (3 days of 500mg IV MP) 2/Plasmapheresis resistant (5 sessions) 3/ Abatacept (500mg IV/2weeks, 5 doses) plus steroids (1mg/kg/day PO prednisone with 5mg/2weeks tapering)	- Progressive normalization of biomarkers of myositis, hepatitis, myocarditis - Complete resolution of PVCs, LVEF remained normal - Discharge home at 1.5 month (stable cancer disease) - Died home at 3.5 months (care refusal, respiratory distress: pulmonary infection and embolism)
71y, M, 70 kg	- Treated for metastatic prostate cancer (previous lines: docetaxel, abitarenone and bone Rx)	Pembrolizumab (200 mg/3 weeks); 2 doses	33 days; myositis; hepatitis; arthritis, myasthenia gravis like	Initial myositis presentation complicated after one week by a complete atrio-ventricular block (cAngio with no significant coronary artery stenosis); Max Trop-I: 1.7$_{ng/mL}$; Max NTproBNP 0.83$_{ng/mL}$; Min LVEF (60%); non-sustained ventricular tachycardia; compatible ICI-irAE peripheral muscle biopsy	1/ Oral prednisone 1mg/kg/day during 1 week for myositis treatment 2/ 3 days of 1000mg IV MP when myocarditis was suspected (complete atrio-ventricular block) 3/ Abatacept 500 mg IV / 2weeks, 3 doses) plus steroids (1 mg/kg/day PO prednisone) with a progressive tapering over 3 months	- Progressive normalization of Trop-I at 3 weeks with a major decreased 24 hours after the first abatacept dose of (0.4$_{ng/mL}$) - complete resolution of ventricular arrhythmia after abatacept - LVEF remained normal - Hospital discharge at day 43 - Alive with stable cancer disease at 4 months, tentative new line (enzalutamide)
68y, F, 63kg	- Treated for thymus carcinoma (previous lines: carboplatine, paclitaxel) -Past history of breast cancer (surgery and radiation 28 and 9 years ago)	Pembrolizumab (200mg/3 weeks); 1 dose	21 days; Myositis; Myasthenia gravis like syndrome	Primarily muscle pain, diplopia, fatique. Then dyspnea (NYHA II-III). Initial TnT: 0.52 ng/ml, max. 3.7 ng/ml. Max. NT-proBNP: 5.6 ng/ml, CK: 6288 U/l. ST-elevations in I,II,III, aVF, V4-V6. Min. LVEF 45%, cMRI+, biopsy +	1000mg prednisone i.v. for 1 day, 500mg for 2 days, 250mg for 3 days; IgG immonoglobulines (2x, day 5-6), plasmapheresis (5x, day 8-12), Abatacept 500mg (5x, every 2 weeks, beginning day 13) with steroid tapering	- Progressive normalization of biomarkers of myositis and myocarditis after Abatacept treatment -LVEF normalization -Tracheotomy with long term ventilation for 2 month - Covered LV rupture, after 3 month - Alive with stable cancer disease 6 month after initial myocarditis

Supplemental Table 2. Case series of patients with ICI-associated myocarditis treated with abatacept

Supplemental Figure 1

Vehicle

Anti-PD-1 + anti-CTLA-4
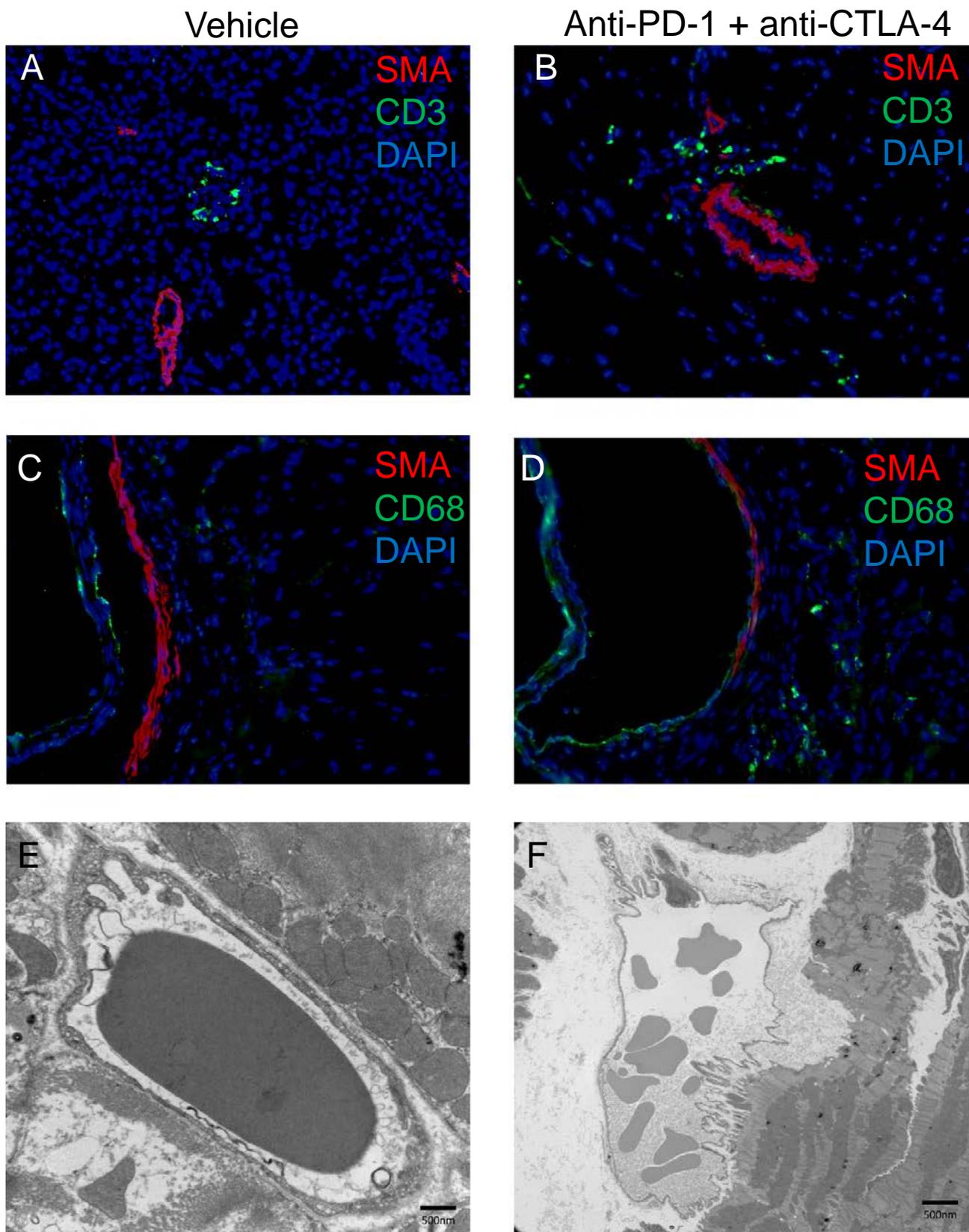
Supplemental Figure 1: Pharmacological inhibition of CTLA-4 and PD-1 leads to increased cardiac immune infiltration in MRL-*Fas^lpr* mice.
A-D) Immunofluorescent staining of cardiac tissue from MRL-*Fas^lpr* mice treated with vehicle or checkpoint blockade therapy.
E-F) Electron microscopy (EM) images of cardiac tissue from MRL-*Fas^lpr* mice treated with vehicle or checkpoint blockade therapy.

Supplemental Figure 2
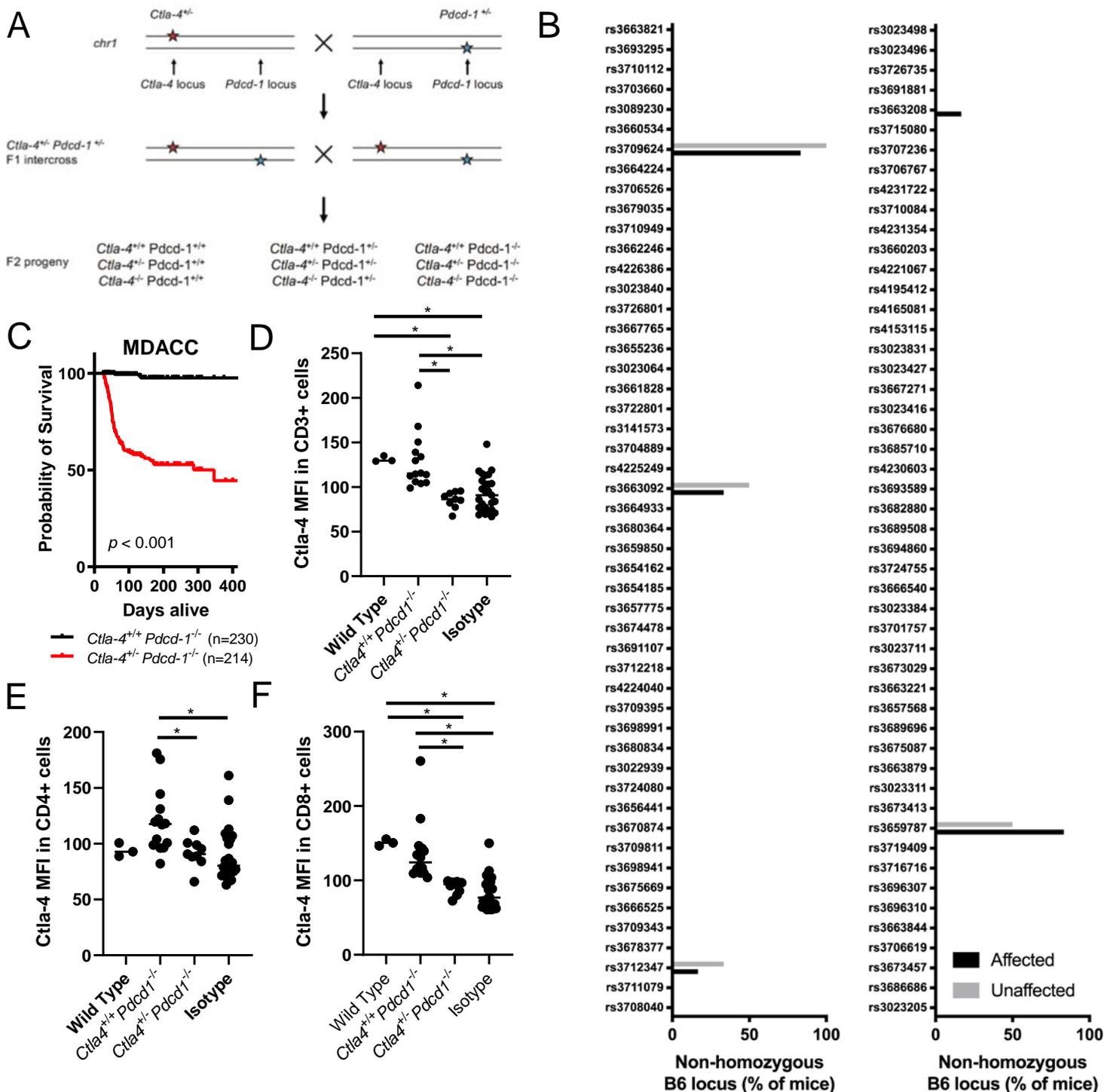
Supplemental Figure 2: Generation and characterization transgenic mice with compound loss of function alleles of *Ctla4* and *Pdcd1*.

A) Schematic of the breeding scheme to generate all potential combinations of *Ctla4* and *Pdcd1* loss of function mutant alleles.
B) 100-SNP panel assessing strain background of *Ctla4+/- Pdcd1-/-* mice displaying clinical signs (affected) and not displaying clinical signs (unaffected). All tested mice harbored 96.5-100% C57BL6/J alleles. Non-homozygous B6 locus is defined as either heterozygous for B6/129 or homozygous for 129 alleles.
C) Kaplan-Meier survival curve of *Ctla4+/- Pdcd1-/-* (n = 350) and littermate *Ctla4+/+ Pdcd1-/-* (n = 400) mice derived from a *Ctla4+/- Pdcd1-/-* by *Ctla4+/+ Pdcd1-/-* breeding cross performed at the MD Anderson Cancer Center (MDACC) vivarium. P-value represents the result of the Mantel-Cox Log-rank test.
D-F) Total CTLA-4 protein levels in in vitro stimulated T cells from *Ctla4+/+ Pdcd1-/-* and *Ctla4+/- Pdcd1-/-* mice lymph nodes. Mean fluorescence intensity (MFI) is displayed on a per mouse basis with mean and standard deviation. Isotype control stained samples for all groups are displayed as well as expression in wild-type C57BL6/J mice. *, p < 0.05 ANOVA with Tukey's multiple testing correction.
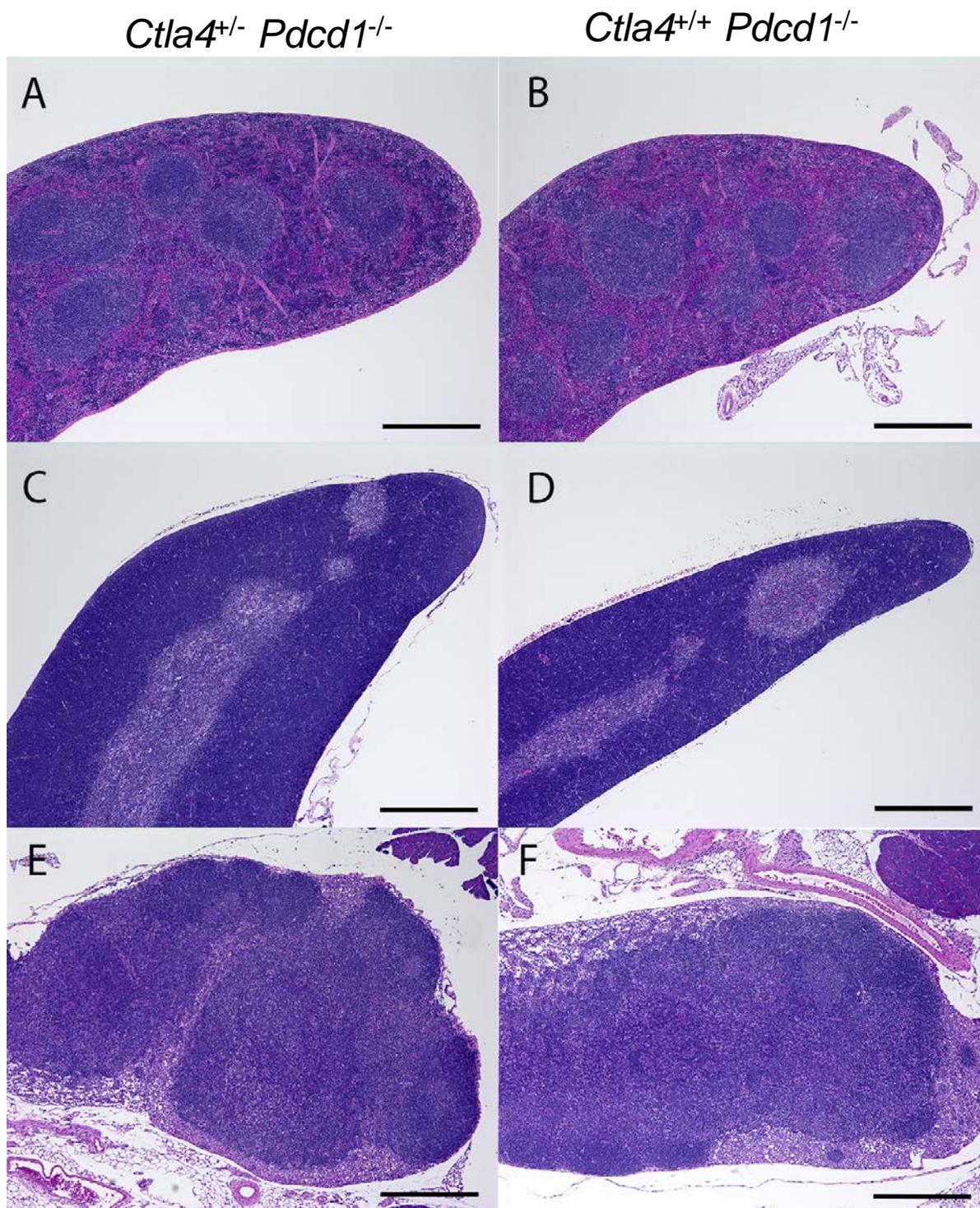
Supplemental Figure 3
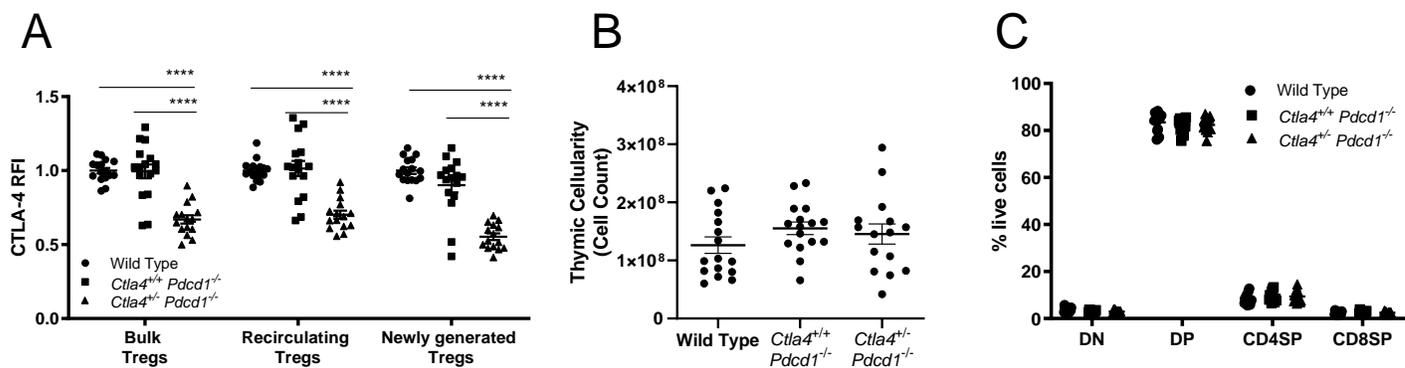
Supplemental Figure 3. Characterization of CTLA-4 expression and thymic development in *Ctla4+/- Pdcd1-/-* mice.

A) Total CTLA-4 protein levels of thymus-derived T regulatory cells from wild-type C57BL6/J, *Ctla4+/+ Pdcd1-/-* and *Ctla4+/- Pdcd1-/-* mice assessed by flow cytometry. Relative fluorescence intensity (RFI) is displayed on a per mouse basis with mean and standard deviation. Treg were identified as CD25+ FoxP3+ cells with newly generated and recirculating Treg cells defined as CD24high Cd44low and CD24low CD44high, respectively. RFI Expression levels are calculated as relative fluorescent intensities, normalized to CTLA-4 expression in C57BL6/J mice. *, p < 0.05 ANOVA with Tukey's multiple testing correction.
B) Thymic cellularity of wild-type C57BL6/J, *Ctla4+/+ Pdcd1-/-* and *Ctla4+/- Pdcd1-/-* mice assessed by flow cytometry.
C) Thymic composition of wild-type C57BL6/J, *Ctla4+/+ Pdcd1-/-* and *Ctla4+/- Pdcd1-/-* mice assessed by flow cytometry. CD4+ CD8+ double negative (DN), CD4- CD8- double positive (DP), CD4+ single positive (CD4SP), and CD8+ single positive (CD8SP)
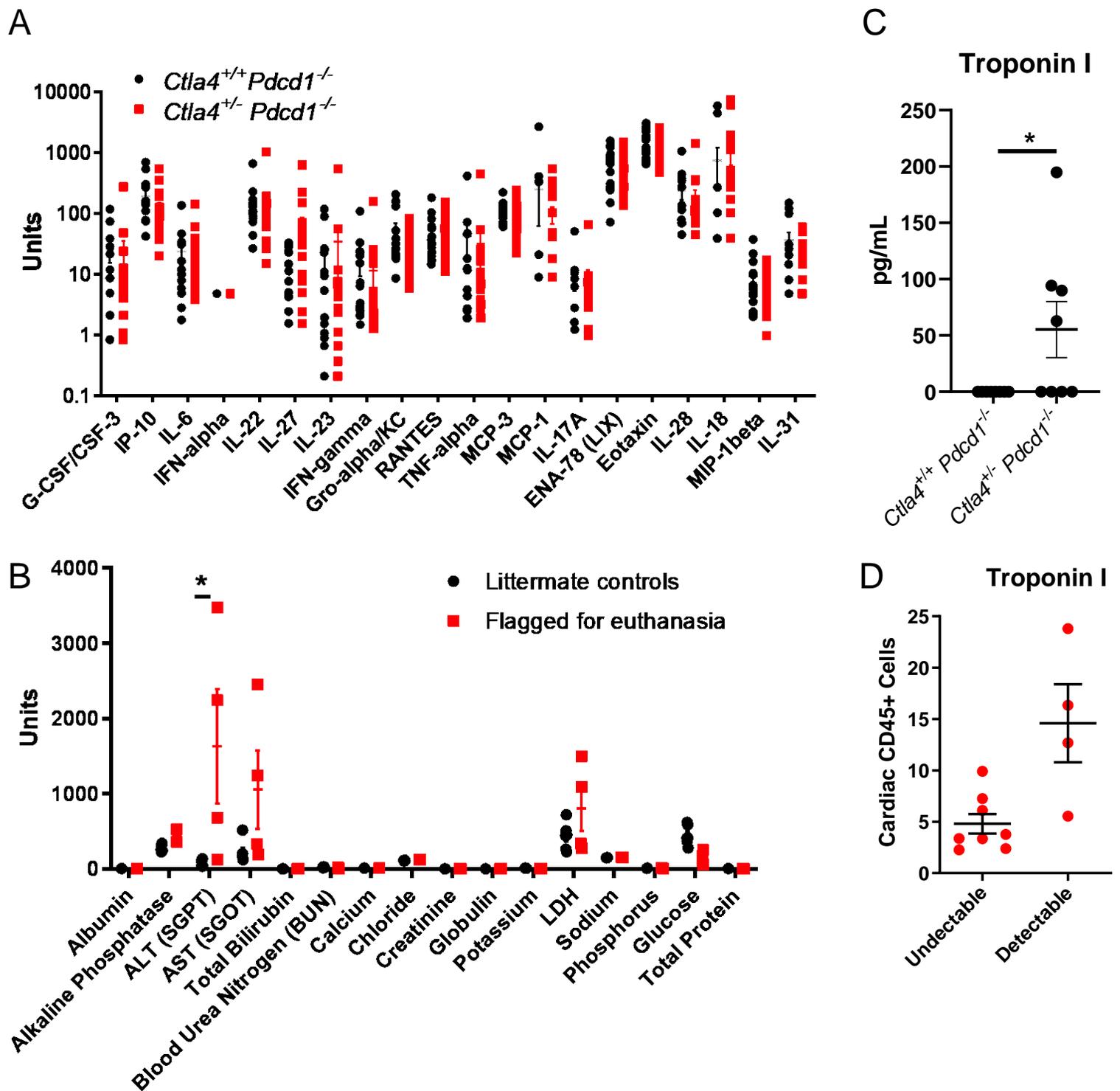
Supplemental Figure 4

Ctla4⁺/⁻ Pdcd1⁻/⁻   Ctla4⁺/⁺ Pdcd1⁻/⁻

Supplemental Figure 4: Absence of lymphoproliferative or abnormal morphology in lymphoid tissues of *Ctla4⁺/⁻ Pdcd1⁻/⁻* mice.

A-B) Representative photomicrograph images of H&E stained spleen tissue from 28-day old *Ctla4⁺/⁻ Pdcd1⁻/⁻* and *Ctla4⁺/⁺ Pdcd1⁻/⁻* mice. Bar = 500 microns.
C-D) Representative photomicrograph images of H&E stained thymus tissue from 28-day old *Ctla4⁺/⁻ Pdcd1⁻/⁻* and *Ctla4⁺/⁺ Pdcd1⁻/⁻* mice. Bar = 500 microns.
E-F) Representative photomicrograph images of H&E stained mesenteric lymph node tissue from 28-day old *Ctla4⁺/⁻ Pdcd1⁻/⁻* and *Ctla4⁺/⁺ Pdcd1⁻/⁻* mice. Bar = 500 microns.

Supplemental Figure 5

Supplemental Figure 5. Characterization of serum properties in *Ctla4⁺/⁻ Pdcd1⁻/⁻* mice.

A) Serum levels of cytokine in *Ctla4⁺/⁻ Pdcd1⁻/⁻* and *Ctla4⁺/⁺ Pdcd1⁻/⁻* mice. Mice analyzed were 29-362 days old. This combined two cohorts, including a young cohort with Ctla4+/- Pdcd1-/- mice that displayed clinical signs as well as aged mice. All comparisons included aged matched littermate controls.
B) Serum chemistry of *Ctla4⁺/⁻ Pdcd1⁻/⁻* mice displaying clinical signs requiring euthanasia and control littermate mice. Mice analyzed were 40-50 days old.
C) Serum troponin levels in *Ctla4⁺/⁻ Pdcd1⁻/⁻* and *Ctla4⁺/⁺ Pdcd1⁻/⁻* mice. Mice analyzed were 40-50 days old. *, P<0.05 T-test with multiple testing correction.
D) CD45+ heart infiltration in *Ctla4⁺/⁻ Pdcd1⁻/⁻* mice where serum troponin levels were detectable via ELISA vs. those where they were not.
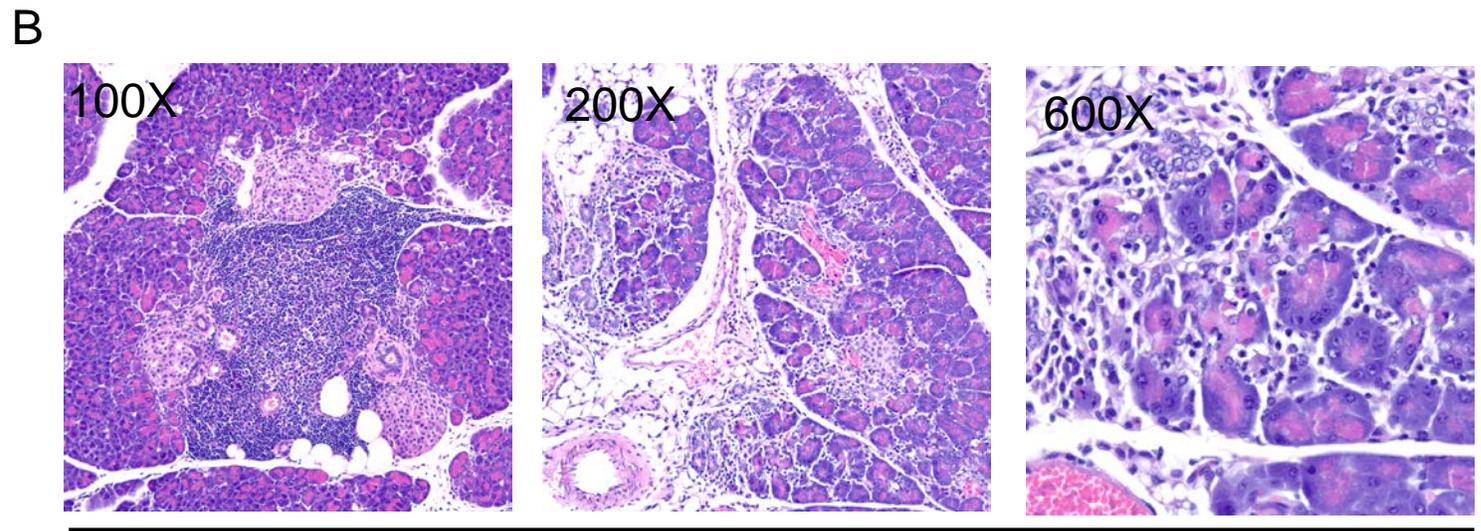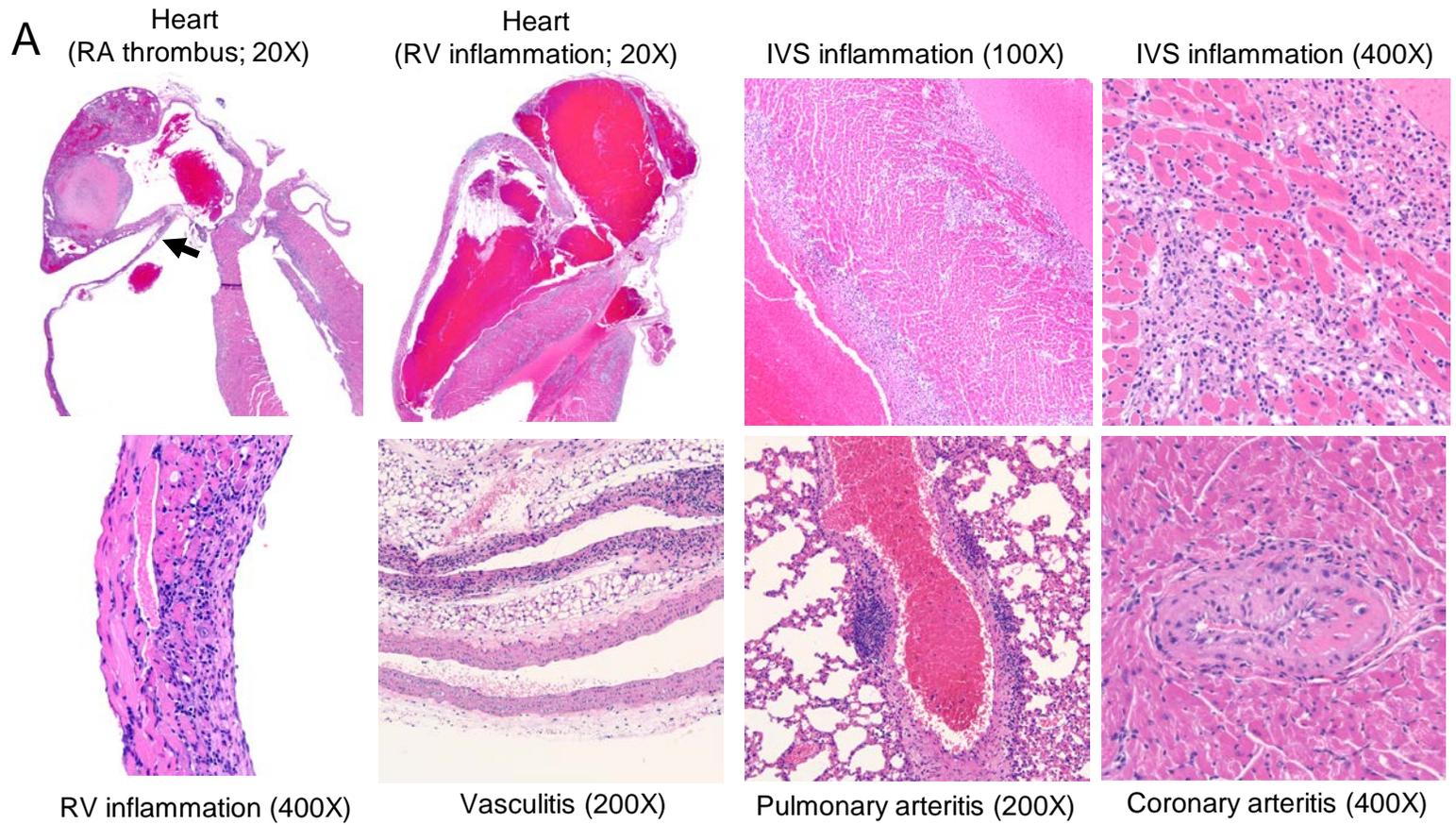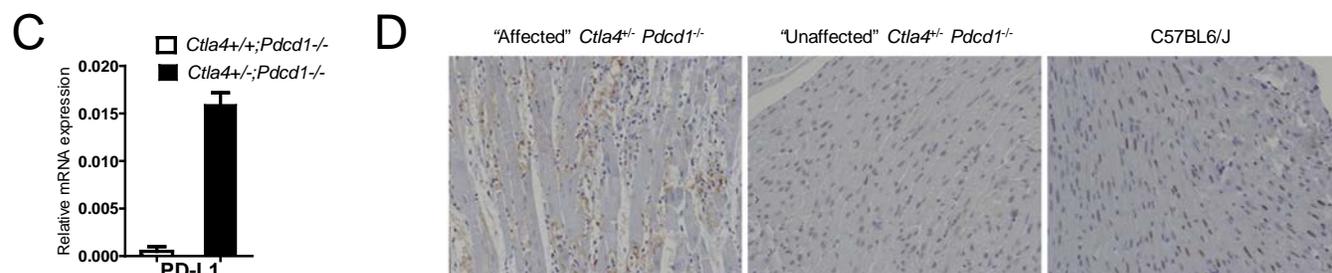
Supplemental Figure 6
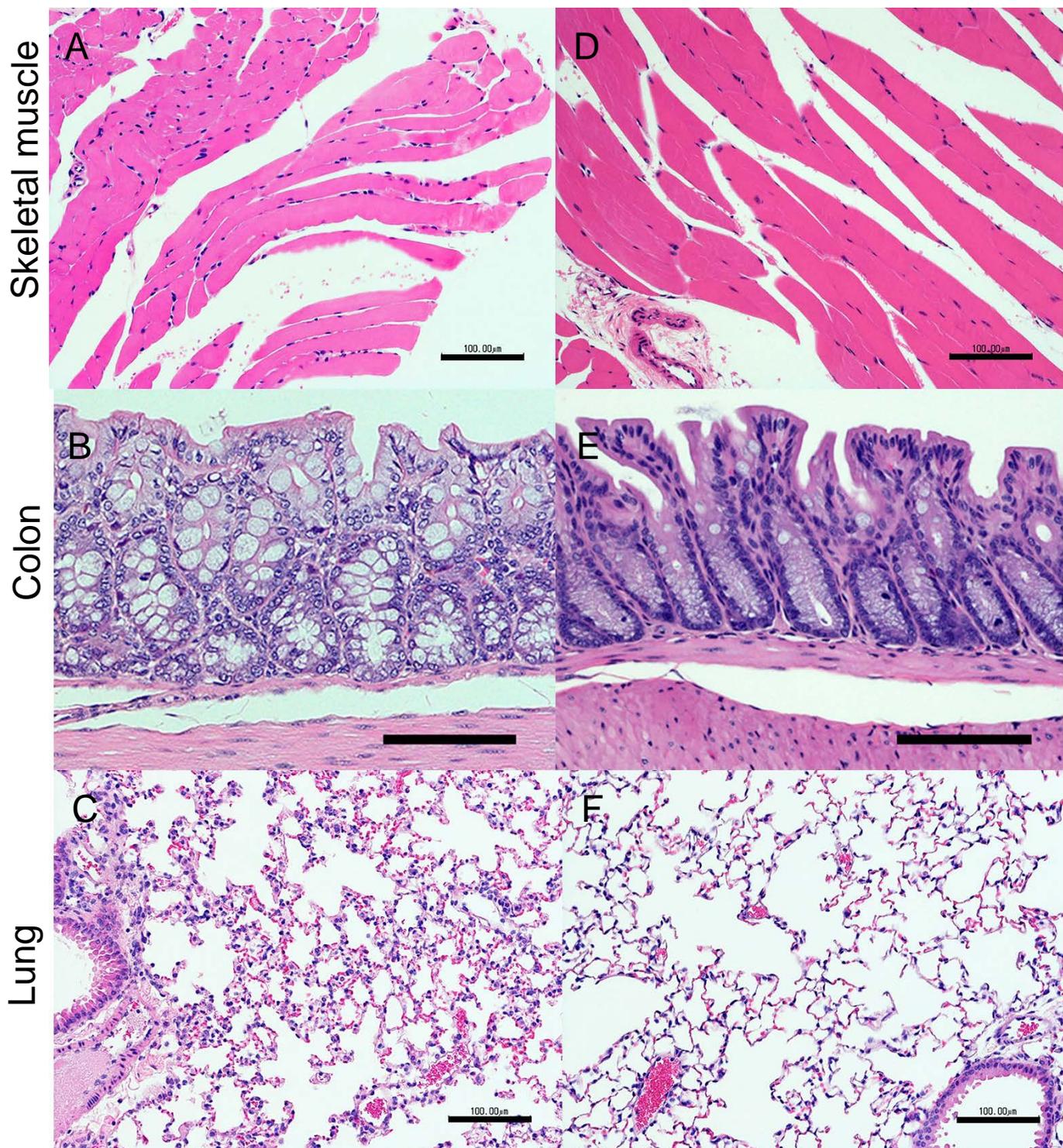
Supplemental Figure 6: Histopathology of skeletal muscle, colon and lung tissue in *Ctla4*⁺/⁻ *Pdcd1*⁻/⁻ mice. Representative photomicrographs of skeletal muscle, colon, and lung from littermate 99-day-old, male heterozygous (A, B, C) and wild-type (D, E, F) *Ctla-4* mice. Note the increased cellularity of the interstitium in the lung of the heterozygous mouse. Cellular infiltrates are not increased in skeletal muscle or colon, compared with the wild-type mouse (difference in degree of mucus distension of colonic goblet cells in the heterozygote is likely due to regional location of sample collection or secondary effects of *Ctla-4* deficiency.) Hematoxylin and eosin. Bar = 100 microns.

Supplemental Figure 7

A

Heart (RA thrombus; 20X) | **Heart (RV inflammation; 20X)** | **IVS inflammation (100X)** | **IVS inflammation (400X)**

RV inflammation (400X) | **Vasculitis (200X)** | **Pulmonary arteritis (200X)** | **Coronary arteritis (400X)**

B

100X | 200X | 600X

Pancreas

C

Relative mRNA expression

□ Ctla4+/+;Pdcd1-/-
■ Ctla4+/-;Pdcd1-/-

PD-L1

0.020
0.015
0.010
0.005
0.000

D

"Affected" *Ctla4+/- Pdcd1-/-* | "Unaffected" *Ctla4+/- Pdcd1-/-* | C57BL6/J

Supplemental Figure 7. Cardiovascular and pancreatic histopathology in *Ctla4+/- Pdcd1-/-* mice.

A) Photomicrograph images of H&E stained heart tissue from *Ctla4+/- Pdcd1-/-* mice.
B) Photomicrograph images of H&E stained pancreatic tissue from *Ctla4+/- Pdcd1-/-* mice
C) PD-L1 mRNA expression in cardiac tissue assessed by qPCR.
D) Example of PD-L1 expression assessed by immunohistochemistry in cardiac tissue in "affected" *Ctla4+/- Pdcd1-/-* mice (displaying clinical signs), "unaffected" *Ctla4+/- Pdcd1-/-* mice, and C57BL6/J wild-type mice for reference.
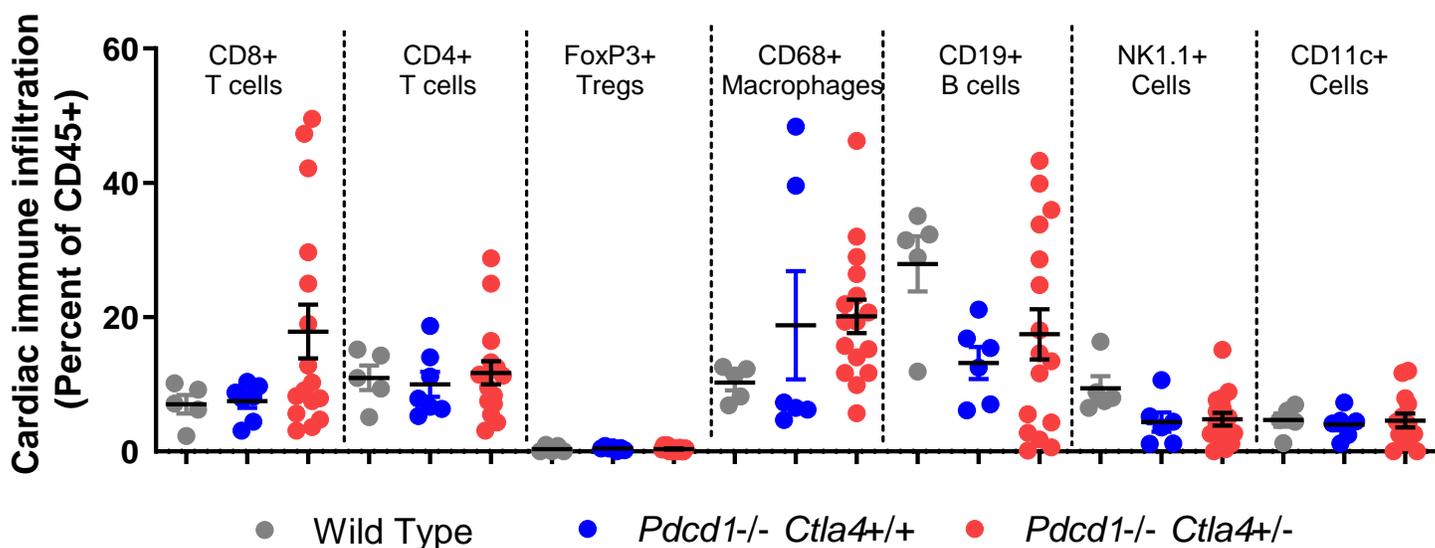
Supplemental Figure 8
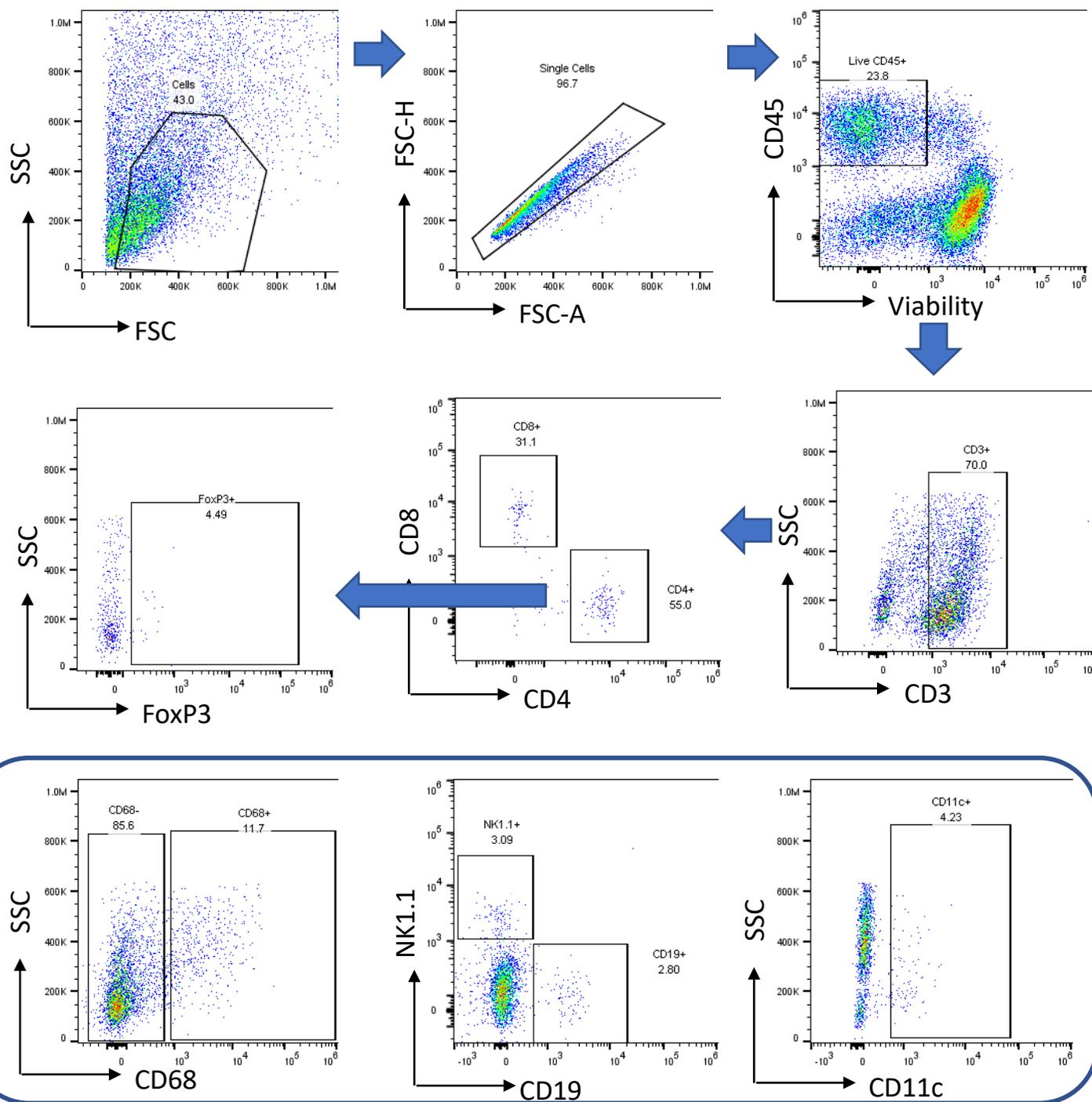
Supplemental Figure 8: Gating strategy/example for characterization of cardiac immune infiltrates *Ctla4+/- Pdcd1-/-* mice.
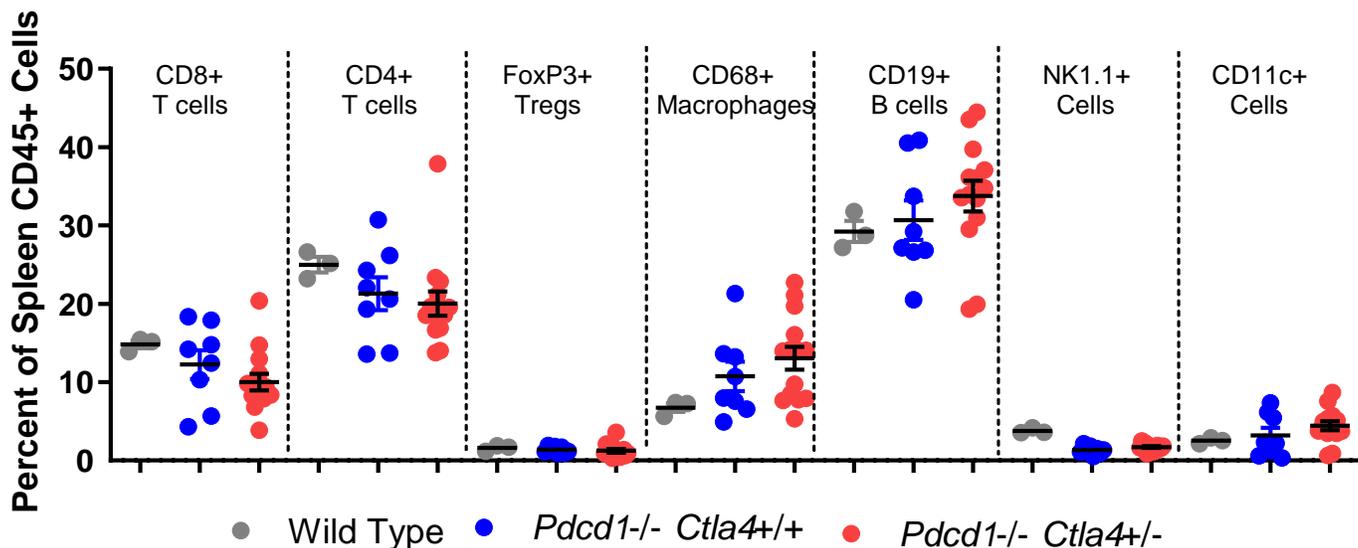
Dissociated cardiac tissue from mice were gated for singlet status, viability, and CD45 positivity, followed by enumeration of the identified populations.
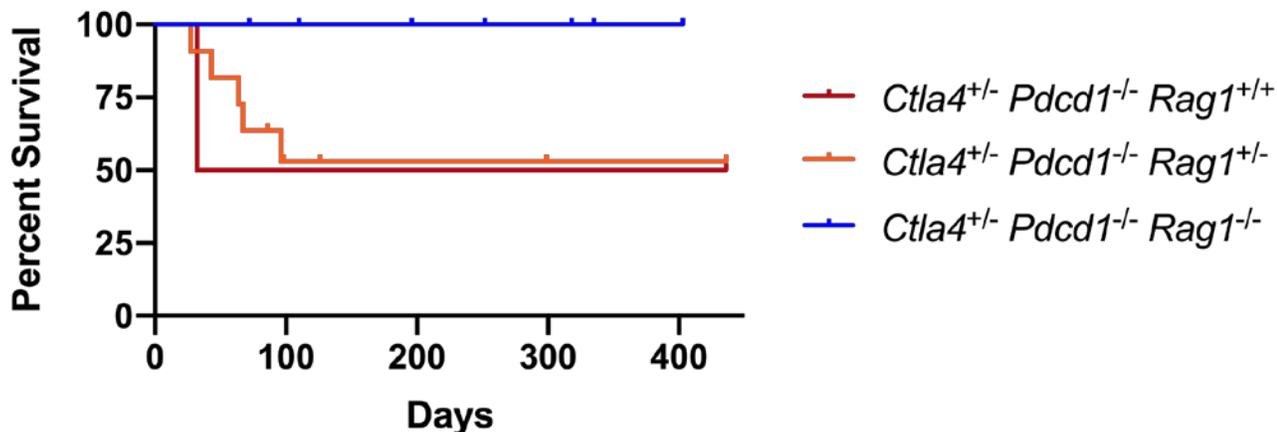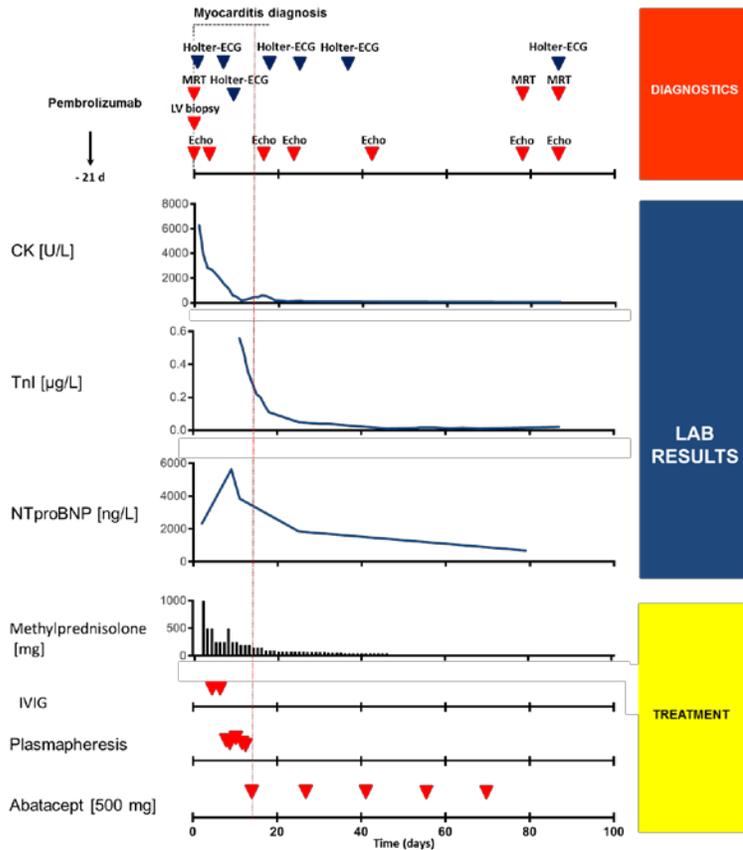
Supplemental Figure 9

A

B

C

Supplemental Figure 9: Characterization of splenic populations among genotypes and survival analysis of female *Ctla4+/- Pdcd1-/- Rag1-/-* mice.
A) Flow cytometry analysis of cardiac immune populations expressed as a percent of total CD45+ cells (No statistically significant different between groups by ANOVA)
B) Flow cytometry analysis of immune populations in murine spleens, matched from Fig. 2E No statistically significant different between groups by ANOVA)
C) Kaplan-Meier survival curve of female *Ctla4+/- Pdcd1-/- Rag1-/-* (n=7) and littermate female *Ctla4+/- Pdcd1-/-* RAG1 competent mice (n=11 and 2 for *Rag1+/-* and *Rag1+/+*, respectively).

Supplemental Figure 10
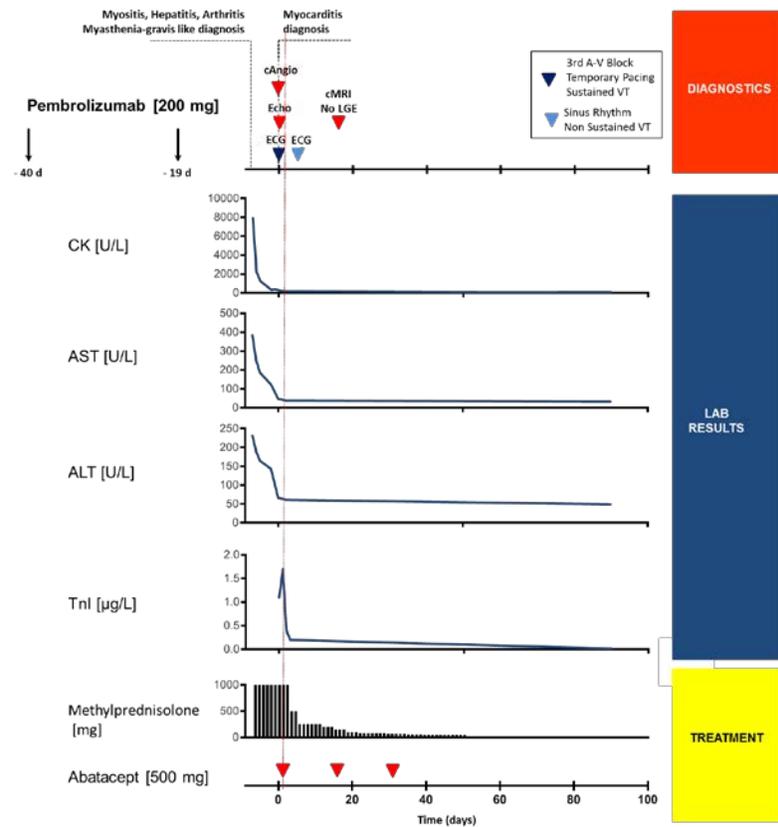
Supplemental Figure 10: Clinical course, diagnostic labs, and therapy in 2 immune-related myocarditis patients treated with abatacept