Supplementary Information: Bayer et al Circulation Research 2023

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Supplementary Methods:

In Vivo Echocardiography:

Transthoracic echocardiography was 4 or 10 weeks post surgery, as previously described.[47] Mice were sedated using isoflurane on a heated stage in supine position. Heart rates and respiratory rates were continuously monitored via stage electrodes. Depilatory cream (Nair) was applied to the chest to remove fur, and ultrasonic gel was applied to a 22-55 MHz echocardiography transducer (MS550D Vevo 2100, Fujifilm VisualSonics) to obtain parasternal short axis views of the left ventricle in M-mode with a target heart rate of 400-500 bpm. Cardiac parameters and heart rate were measured by averaging the values from 5 cardiac cycles. All analyses were performed blindly using Vevo 2100 software (see below)

TAC:

Pressure overload was induced by minimally invasive TAC surgery to constrict the transverse aorta of mice as previously described.[13] Sham operated mice underwent the same operation without aortic constriction. 8-10 week old male *Tcra*$^{-/-}$ mice underwent 25G TAC (for comparability to previous adoptive transfer studies[12,48]), then 10 million Th1 cells were injected intraperitoneally 2 days, and 2 weeks after surgery. 8-10 week old male and female *Myd88*$^{fl/fl}$*CD4*^{Cre} mice using Cre^{+} and Cre^{-} littermates underwent 27G TAC (most commonly performed by us and others).[49] Mice were euthanized 4 weeks after surgery and after visual confirmation of asystole tissues were harvested for analysis, or allowed to progress for survival studies up to 12 weeks. Exclusion criteria included WT TAC mice that exhibited no decrease in fractional shortening or LV hypertrophy or inconclusive genotyping. Randomization for each group was performed by a random number generator (https://www.calculator.net/random-

number-generator.html), and all analysis were done blinded using an ID number assigned by a third party. Sample sizes are based on power calculations for the expected decrease in fractional shortening and increase in T-cell infiltration (http://powerandsamplesize.com/Calculators/Compare-2-Means/2-Sample-Equality), showing a minimum of 6 mice per group is needed to detect statistically significant changes with a power of 90%.

Generation and Culture of Th1 cells:

Spleens were excised and crushed through a 40 μM cell strainer, the strainer was rinsed with PBS + 0.5% BSA + 2 mM EDTA, pelleted at 300g, then red blood cell lysis was performed according to manufacturer's instructions (Biolegend 4210301). Naïve $CD4^+$ T-cells were isolated from the splenic cell suspension by positive selection using magnetic beads (Miltenyi 130-117-043). Cells were plated at 2 million cells / mL and differentiated in the presence of anti-CD3 (2.5 μg/mL, Biolegend 100253), anti-CD28 (1 μg/mL, Biolegend 102102), IL-12 (10 ng/mL, Peprotech 210-12), IL-2 (25U/mL, Miltenyi, 130-120-662) and, anti-IL4 (50 ng/mL, Biolegend 504102) for 3 days, expanded for 24 hours in IL-2 alone, then harvested for experiments. T-cells were cultured at 37°C in RPMI medium (Gibco, 11875-093) containing 10% fetal bovine serum (FBS – Atlanta Biologicals S11150), Glutamax (Gibco A12860-01), NaHCO3 (Gibco 25080-094), Penicillin/Streptomycin (Gibco 15140122), 60μM β-mercaptoethanol (Sigma 444203), Sodium pyruvate (Gibco 11360-070). Where indicated T-cells were treated with 1 μM Myd88 inhibitor peptide or scramble peptide (NovusBio NBP2-29328) for 24 hours during activation. Purity and gating strategy for all *in vitro* T-cell use is shown in supplementary Figure 2.

Histologic Analysis and Immunohistochemistry:

LV samples from hearts were fixed in 10% formalin, embedded in paraffin, and cut in 6 μM sections. Slides were deparaffinized using xylenes (Sigma 534056) then hydrated using a decreasing ethanol gradient. Fibrosis was stained in 0.1% Direct Red 80 (Sigma 365548) in saturated picric acid (Sigma P6744) for 1 hour, after 1 min. hematoxylin counter stain (Sigma

GHS2128). Slides were washed with 0.5% acetic acid twice before dehydration and mounting. Immunohistochemistry was performed in frozen LV sections: samples were fixed in ice cold acetone (Sigma 179124), blocked for 10 minutes each with: 10% normal goat serum (Abcam 7481), biotin/avidin blocking kit (Vector labs SP-2001), and with 1% hydrogen peroxide (H1009). Samples were stained with primary antibody against CD4 (Biolegend 100402) for 1 hour (1:200 dilution) followed by incubation with goat anti-rat biotinylated secondary antibody (1:300 dilution, Jackson Immunoresearch 112-065-062). Sections were incubated with Streptavidin HRP (DAKO, K0675) and visualized using AEC Substrate (Sigma A5754), then nuclear counterstained with hematoxylin, and mounted using aqueous fluoromount G (Southern Biotech 0100-01. $CD4^+$ cells were counted manually.

Bulk RNA-Sequencing:

Cell lysis and RNA isolation was performed using an RNEasy kit according to manufacturer's instructions (Qiagen 74004). RNA samples that passed quality checks were used as input to prepare RNA-seq library using Illumina TruSeq stranded mRNA kit per manufacturer instruction. Libraries were sequenced on an Illumina HiSeq 2500 sequencer (Illumina) with a 75 base pair single-end reads format. Raw data in FASTQ format were processed for quality control using FastQC (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/) then mapped to the mouse genome using STAR (http://code.google.com/p/rna-star/), and quantified by featureCounts (http://subread.sourceforge.net/). Using QluCore software, a heat map of differentially expressed genes using hierarchical clustering and a cutoff of $p<0.05$ and log(FC) of >1 for significance. Gene set enrichment analysis was performed against Mouse MSigDB collections (https://www.gsea-msigdb.org/gsea/msigdb/mouse/collections.jsp) using $p<0.05$ for significance.

Analysis of Single-Cell RNA Sequencing Data:

Raw data were downloaded from GEO: GSE122930. 1 and 4-week sham and TAC datasets, which include two replicates, were used for scRNAseq analysis. Well established workflows

based on the Seurat package were utilized for analysis. Briefly, datasets were downloaded, and Seurat objects were merged using the merge function, normalization and dimensional reduction were conducted with the scTransform v2 regularization function, and analyzed using basic Seurat (Version 4) functions.[50] T cells were identified based on *Thy1, Cd3e, Cd4, and Cd8a* expression and created a subset of T cells for analysis using the subset function for downstream analysis. *Cd4, CD8a*, and *Myd88* expression data were visualized using the FeaturePlot function in Seurat.

Analysis of CITE-seq Data:

T-cells were selected from a global object consisting of non-failing donors compared to heart failure of all causes.[25] The T-cells were represented in a UMAP embedding representation using weighted nearest neighbor clustering utilizing RNA and protein information. Differential RNA and protein expression were independently used to identify phenotypically distinct T-cell states within the major CD4, CD8, and NK cell groups. For differential expression analysis FindAllMarkers function from Seurat was used and a Wilcoxon Rank Sum test with a log2FC cut-off of 0.25 and a min.pct cut-off of 0.1 was used. Genes with a log2FC > 0.58 and adjusted p-value < 0.05 were used for annotating cell states.

Immunoblotting:

Cells were lysed in RIPA buffer (150 mM NaCl, 50 mM Tris pH 7.4, 1% NP-40, 0.1% SDS, 5 mM EDTA, 0.1% sodium deoxhycholate, 1 mM DTT) supplemented with protease andphosphatase inhibitors (Roche 4906845001, 4693159001), then cleared by centrifugation at 4°C at 13,000 g. Protein concentrations were determined using DC protein assay (BioRad 5000112) and equal concentrations of protein were subjected to SDS-PAGE in 10% resolving gels unless otherwise indicated, then transferred to PVDF membranes, followed by blocking with Tris buffered saline + 0.1% Tween20 containing 5% non-fat milk or bovine serum albumin (BSA) for 1 hour at room temperature. Immunoblotting was performed overnight at 4°C using

antibodies diluted 1:1000 in 5% non-fat milk or BSA. Antibody sources: IL-1β (CST 12242), IL-33 (R&D AF3626), HMGB1 (Abcam 79823), TRIF (NovusBio NB120-13810SS), GAPDH (CST 2118), MyD88 (R&D AF3109), pERK (CST 9101), ERK (CST 9102), β-actin (Sigma A5441), pP38 (CST 4511), P38 (CST 9212), pP65 (CST 3033), P65 (CST 4764), pAKT (CST 4060), AKT (CST 4691), α-tubulin (CST 3873), α-smooth muscle actin (Sigma A2547), pZap70 (CST 2717), Zap70 (CST 3165), collagen 1 (Invitrogen PA529569) or CXCR3 (Bios 2209R). Blots were then incubated with the appropriate HRP-peroxidase conjugated secondary antibody (1:2000, Rabbit CST 7074, Mouse CST 7076, Goat ThermoFisher A15999) at room temperature for 1 hour, then developed using Pierce ECL (ThermoFisher 32106) and imaged using a ChemiDoc XRS+ System (Biorad). Band intensity was quantified using Image-J.

RT-qPCR:

Cell lysis and RNA isolation was performed using an RNEasy kit according to manufacturer's instructions (Qiagen 74004). For cardiac RNA isolation, LV samples were crushed in Trizol (Qiagen 79306), RNA was chloroform extracted and separated by centrifugation, then the aqueous phase was applied to RNAEasy columns. cDNA synthesis was performed using High-Capacity cDNA Reverse Transcription Kit (ThermoFisher 4368814), and qPCR was performed using SYBR Green (ThermoFisher A25741) on a QuantStudio 6 Flex Real-Time PCR Machine (ThermoFisher), then analyzed for fold change using ΔΔCt method. See Supplemental Table 2 for primer sequences and for amplification conditions.

Flow Cytometry:

Cell suspensions from culture or lymphoid organs were stained using the following antibodies from Biolegend: APC-Cy7 or FITC CD4 (Biolegend 100414, 100406), FITC CD8 (Biolegend 100705), APC IFNγ(Biolegend 505810), PE IL-17 (Biolegend 506904), PerCP Tbx21 (Biolegend 644806), APC-Cy7 TCRβ (Biolegend 109220), BV421 or APC CD45.2 (Biolegend 109832, 109814), PE CD45.1 (Biolegend 110707), APC CD11a (Biolegend 101119), FITC CD49d (Biolegend 103606) PE TLR4 (Biolegend 145404) APC IL-1r (Biolegend 113509) PE-Cy7 IL-33r

(Biolegend 145316), APC CD44 (Biolegend 103012), PE CD62L (Biolegend 104408), BV421 CD25 (Biolegend 102043), FITC CD69 (Biolegend 104506), APC CXCR3 (Biolegend 126512), APC MHCII (Biolegend 107613) APC-Cy7 CD11b (Biolegend 101225), PE CD11c (Biolegend 117307), BV421 Ly6G (Biolegend 127627), FITC CCR2 (Biolegend 150607). Other antibodies included APC Mefsk4 (Miltenyi 130-120-166) and PE Foxp3 (Invitrogen 14-5773-82). Samples were surface stained by incubation with the relevant antibodies diluted 1:50 in phosphate buffered saline (PBS) + 2% FBS for 20 minutes at 4°C. When intracellular cytokine staining was performed, samples were incubated for 4 hours at 37C in complete T-cell media (see above) including 50 ng/mL Phorbol Myristate Acetate (PMA, Sigma Aldrich P8139), 0.1% Ionomycin (Sigma 13909), 0.1% Brefeldin A (Biolegend 420601), and Monensin (Biolegend 420701), followed by surface staining as described. Samples were fixed for 15 minutes at RT with fixation buffer (Biolegend, 420801), then permeabilization by two washes with 1X Perm/Wash buffer (Biolegend, 421002, then stained intracellularly with the appropriate antibodies diluted 1:50 in PBS + 2% FBS for 20 minutes at 4°C. To detect Tbx21 or Foxp3, samples were surface stained as described, fixed using 1X FOXP3 Fix/Perm solution (Biolegend, 421401) for 20 minutes at RT, permeabilized for 15 minutes using 1X FOXP3 Perm solution (Biolegend, 421402), then stained intracellular for 20 minutes with the appropriate antibodies diluted 1:50 in PBS + 2% FBS for 20 minutes at 4°C. Data was acquired an a BD LSRII (BD Biosciences) and analyzed using FlowJo software.

Isolation of Cardiac Fibroblasts and Co-culture:

Adult mice (7-12 week old) were anesthetized, then hearts were excised, perfused with PBS, minced, and digested using 5 mg/mL Liberase TL (Roche, 05401020001) and TrypLE Express (Gibco, 12605036) for 30 minutes with agitation. Digested tissue was pelleted at 300g for 10 minutes, then resuspended in fibroblast growth media – DMEM (Gibco 11995-065) + 10% FBS, Penicillin/Streptomycin (ThermoFisher 15140122), Amphotericin B (Sigma A2411), insulin-

transferrin-selenium (Sigma I3146, and plated on 0.1% gelatin coated plates for 2 hours at 37°C. After this time, non-adherent cells were removed and fresh media was added. Cells were used through passage 3. For co-culture experiments, fibroblasts were plated on 0.1% gelatin coated coverslips in 12 well plates at 200,000 cells / well. Once adherent, cells were serum starved in media containing 1% FBS overnight, then Th1 cells were added at 100,000 cells / well unless otherwise indicated by ratio, and cultured overnight, then washed with PBS to remove non-adherent T-cells. When indicated, Th1 cells were pretreated with 20 μg/mL anti-CD49d (Biolegend clones 9C10 and RI-2) or with 20. Where indicated 5 μg/mL anti-TGFβ (Thermofisher MA5-23795) was added to CFB prior to Th1 addition. Additionally when indicated, cells were washed with 5 mM EDTA to remove adherent T-cells.

T-cell Adhesion and Migration Studies Under Shear Flow Conditions:

T-cell adhesion was determined under defined laminar flow conditions in a parallel plate apparatus using video microscopy (20x objective) and Nikon Elements NIS software as previously described.[51] In brief, Th1 cells were generated as described above, treated with PMA (50 ng/mL, 5 min), and perfused at a concentration of 1 x 10^6 cells / mL at an estimated shear stress of 1 dyne/cm^2 over immobilized ICAM-1 or VCAM-1 (20μg/mL, Biolegend 553006, 553706). Where indicated, Th1 cells were pretreated with 20 μg/mL anti-CD49d (Both Biolegend 103701, 103629) or anti-CD11a (Biolegend 101101) Adherent cells were quantified in 4-5 fields of view. For Transendothelial migration experiments, mouse heart endothelial cells (MHECs) were generated as described previously[51] and cultured on coverslips. MHECs were treated with 125 ng/mL TNFα for 4 hours prior to perfusion of 1 million Th1 cells at an estimated shear stress of 1 dyne/cm^2 with temperature controlled to 37°C. TEM was observed during 10 minutes.

T-cell Chemotaxis:

1 million Th1 cells were loaded on an 8μM transwell insert in complete T-cell media across either media alone of media supplemented with 2 μg/mL CXCL-10 (Peprotech 250-16). After 4 hours, the number of T-cells migrated across the transwell were counted manually.

ELISA:

Th1 cells were cultured in fresh T-cell media at a concentration of 2 million cells/mL for 24 hours, then pelleted at 13,000 rpm for 5', then culture supernatant was snap frozen. Supernatant was run undiluted (IL-2) or diluted 1:5 (TNFα), or 1:100 (IFNγ) Duoset ELISA kits according to manufacturer's instructions (R&D DY410, DY402, DY485).

T-Cell Viability Assays:

Th1 cells were plated on 384 well black optical bottom plates (Nunc 142761) coated with 10 μg/mL αCD43 (Invitrogen, 14-0431-85) at 30,000 cells / well in complete T-cell media or media containing 1% FBS, including 10 μg/mL propidium iodide (Biolegend 421301). The Lionheart imager was used to maintain temperature at 37°C, 5% CO_2, and humidity during the experiment. Kinetic microscopy was performed by the Lionheart imager to quantify propidium iodide uptake every 90 minutes over 72 hours. Wells containing 0.1% Triton X-100 lysed cells were used as a positive control, with propidium iodide uptake baseline subtracted and normalized as % positive control.

T-cell Proliferation:

Naïve $CD4^+$ cells ($CD62L^{hi}CD44^{lo}$) were isolated using a FACS Aria (BD Biosciences). 1.5 million naïve $CD4^+$ cells were stained with 5 μM carboxyfluorescein diacetate succinimidyl ester (CFSE) for 15 minutes at 37°C, then cultured in Th1 polarizing conditions as described above. For proliferation of Th1 cells, 1.5 million pre-activated Th1 cells were stained with 5 μM CFSE for 15 minutes at 37C, then resuspended in T-cell media with fresh IL-2 (25 U/mL). Samples were analyzed by flow cytometry at the indicated time points in culture.

T-cell Activation Assay:

T-cells were generated as described above, and plated onto coverslips coated with anti-CD3 in the polarization conditions described above. At the indicated time points, or after being removed from stimulation for 24 hours, coverslips were removed from media, rinsed with cold PBS, then fixed for 15 minutes in 4% PFA. Immunofluorescence was performed as described in subsequent methods.

Immunofluorescence:

Coverslips or tissues were washed 2 x 10 min. with PBS, then fixed with 4% PFA for 15 minutes. Samples were washed with PBS, then incubated with PBS + 10% normal goat serum and/or 10% donkey serum (depending on species of secondary antibody), and 0.1% triton-X for intracellular staining for 1 hour at RT. Samples were incubated with the following primary antibodies where indicated: αCD4-AF594 (Biolegend 100446), α-alpha smooth muscle actin (αSMA, Sigma A2547), MyD88 (R&D AF3109), TCRβ (Biolegend 109220), CD31 (Biolegend 102520), PDGFrα (R&D AF1062), or Ki67 (Abcam Ab15580) at 1:100 in blocking buffer overnight at 4°C. Samples were washed with PBS then incubated with the appropriate secondary antibody (Thermo A32766, Thermo A11055, Thermo A21432, Thermo A78966, CST 4413) at 1:200 in blocking buffer, then mounted in Vectashield with DAPI (Southern Biotech, 0100-20). For WGA staining, tissues were fixed for 15 minutes in 4% PFA, followed by blocking with PBS + 10% normal goat serum for 1 hour at RT. Tissues were stained with 5μg/mL FITC conjugated WGA (Sigma L4895) in PBS + 10% normal goat serum for 2 hours and mounted in Vectashield with DAPI. For TUNEL staining tissues were deparaffinized as described previously, then stained with *In Situ* cell death detection kit TMR red (Roche 12156792910) according to manufacturers instructions, and mounted in Vectashield with DAPI. Visualization was performed with a Nikon Ti inverted fluorescence microscope, or in Figure S8 using a Leica SP8 confocal microscope. Antibodies were validated based on comparison to unstained and secondary only controls.

Genomic DNA Isolation and PCR:

Tissues were perfused using PBS and then digested using proteinase K (Thermofisher) in Tris buffer at 60°C for 1 hour. DNA was Isopropanol precipitated then used for genotyping using GoTaq Green MasterMix (Promega), using primers listed in Supplemental Table 2.

Group	Fractional Shortening (%)	Heart Rate (bpm)	End Systolic Diameter (mm)	End Diastolic Diameter (mm)	Anterior Wall Thickness (mm)	Posterior Wall Thickness (mm)
TCRα + WT Th1 Sham	37.9 ± 1.9	497 ± 30	2.3 ± 0.2	3.6 ± 0.2	0.73 ± 0.02	0.82 ± 0.09
TCRα + WT Th1 TAC	33.6 ± 2.7	489 ± 20	2.7 ± 0.2	4.0 ± 0.1	1.02 ± 0.09*	0.99 ± 0.05
TCRα + MyD88-/- Th1 Sham	34.6 ± 1.1	460 ± 12	2.4 ± 0.1	3.7 ± 0.2	0.90 ± 0.02	0.79 ± 0.03
TCRα + MyD88-/- Th1 TAC	21.9 ± 1.8*	446 ± 14	3.2 ± 0.1*	4.1 ± 0.1	0.92 ± 0.04	0.91 ± 0.08
T-MyD88$^{+/+}$ Sham 4w	32.9 ± 1.2	494 ± 22	2.3 ± 0.1	3.5 ± 0.1	0.83 ± 0.05	0.74 ± 0.03
T-MyD88$^{-/-}$ TAC 4w	19.4 ± 0.1*	500 ± 5	3.2 ± 0.1*	3.9 ± 0.1*	0.84 ± 0.05	0.89 ± 0.04
T-MyD88$^{+/+}$ Sham 4w	30.5 ± 1.5	473 ± 13	2.4 ± 0.1	3.4 ± 0.1	0.80 ± 0.04	0.76 ± 0.03
T-MyD88$^{-/-}$ TAC 4w	19.3 ± 1.1*	469 ± 13	2.9 ± 0.1*	3.6 ± 0.1&	0.88 ± 0.05	0.88 ± 0.06
T-MyD88$^{+/+}$ Sham 10w	28.9 ± 2.5	524 ± 18	2.8 ± 0.1	3.9 ± 0.1	0.86 ± 0.03	0.77 ± 0.04
T-MyD88$^{-/-}$ TAC 10w	15.6 ± 1.4*	540 ± 6	3.6 ± 0.2*	4.3 ± 0.2	1.08 ± 0.05*	1.04 ± 0.03
T-MyD88$^{+/+}$ Sham 10w	24.2 ± 1.6	482± 14	3.0 ± 0.1	4.0 ± 0.1	0.97 ± 0.02	0.80 ± 0.05
T-MyD88$^{-/-}$ TAC 10w	11.0 ± 1.1*	527 ± 9*	3.8 ± 0.2*	4.3± 0.2	1.08 ± 0.05	1.10 ± 0.05

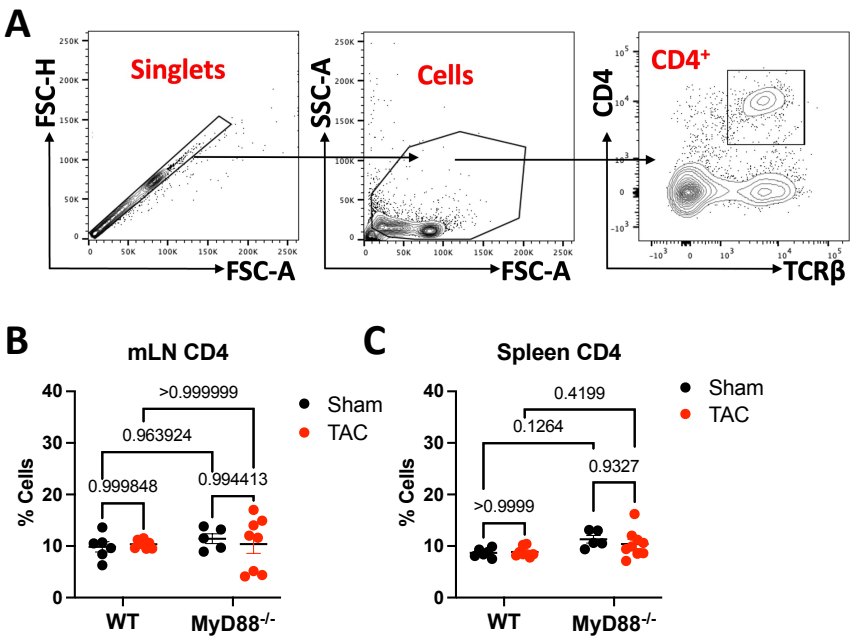
Supplemental Table 1: Cardiac Parameters from TAC

Cardiac parameters as measured by echocardiography or normalized left ventricular weight from TAC experiments. Data are average ± SEM, with * indicating statistical significance using 2-way ANOVA with Sidák's multiple comparison test comparing Sham to TAC ($p<0.05$) and & indicating statistical significance comparing between genotypes.

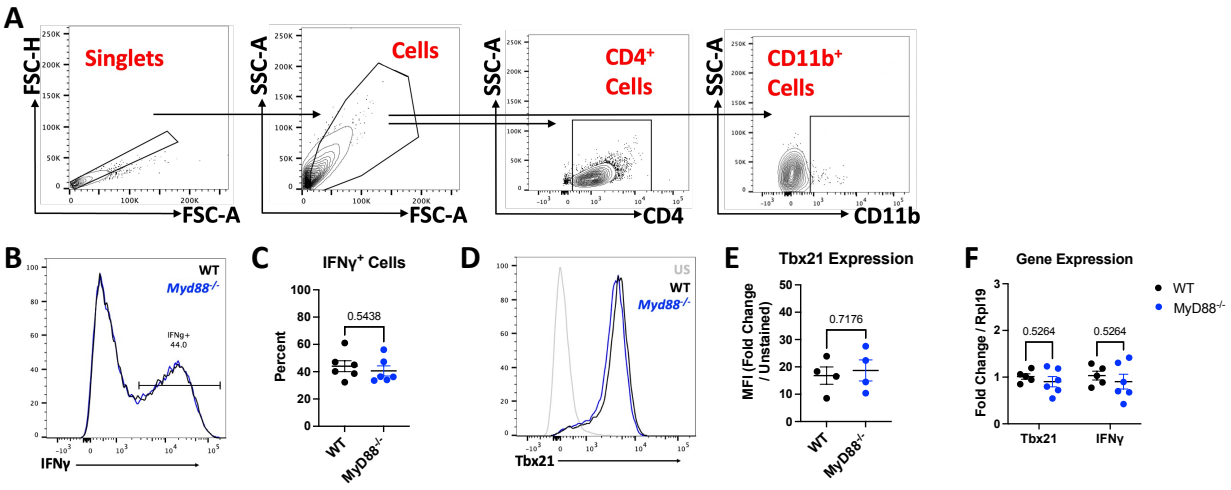
qPCR			
Target	**Forward**	**Reverse**	**Size (bp)**
ANP	GAGAGACGGCAGTGCTTCTAGGC	CGTGACACACCACAAGGGCTTAGG	260
Col1a1	GTATGCTTGATCTGTATCTG	CGATCCTACATCTTCTG	159
Col3a1	CCTGGTCAGTCCTATGAG	CAGGAGCAGGTGTAGAAG	187
Fibronectin	ATGTGGACCCCTCCTGATAGT	GCCCAGTGATTTCAGCAAAGG	124
IFNγ	CGGCACAGTCATTGAAAGCCTA	GTTGCTGATGGCCTGATTGTC	199
IL-6	TGATGGATGCTACCAAACTGG	TTCATGTACTCCAGGTAGCTATGG	96
Rpl19	CCCGTCAGCAGATCAGGAA	GTCACAGGCTTGCGGATGA	58
Tbx21	AACCGCTTATATGTCCACCCA	CTTGTTGTTGGTGAGCTTTAGC	96
Genotyping			
MyD88 Exon 3	ATACCGGAAGCAGATGGATG	AGGCTGAGTGCAAACTTGGT	1800 400

Amplification conditions:
2 min. 50ºC
10 min. 95ºC
40 cycles: 15 sec. 95ºC, 60 sec. 60ºC

Supplemental Table 2: Primers for qPCR and Genotyping, Amplification Conditions

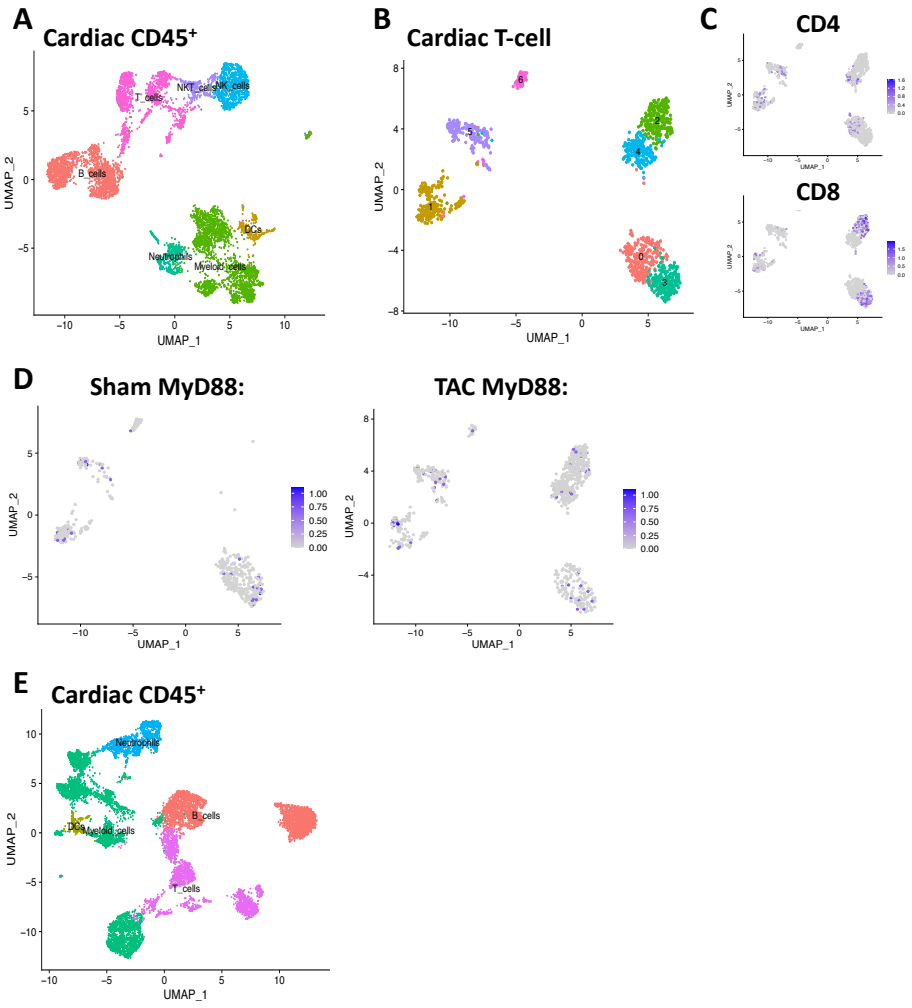


<u>Supplemental Figure 1: Reconstitution of TCRα Mice by Adoptive Transfer:</u>
TCRa$^{-/-}$ Mice were reconstituted with WT or *Myd88*$^{-/-}$ Th1 cells in the onset of 25G TAC or sham surgery, and harvested after 4 weeks. **A.** Spleens or lymph nodes were crushed and stained for analysis by flow cytometry, gating strategy to identify CD4^{+} T-cells in lymphoid organs. **B-C.** Quantification of CD4^{+} T-cells from the mediastinal lymph nodes or spleens. Statistical analysis by 2-way ANOVA with Sidák's multiple comparison test, p values shown.

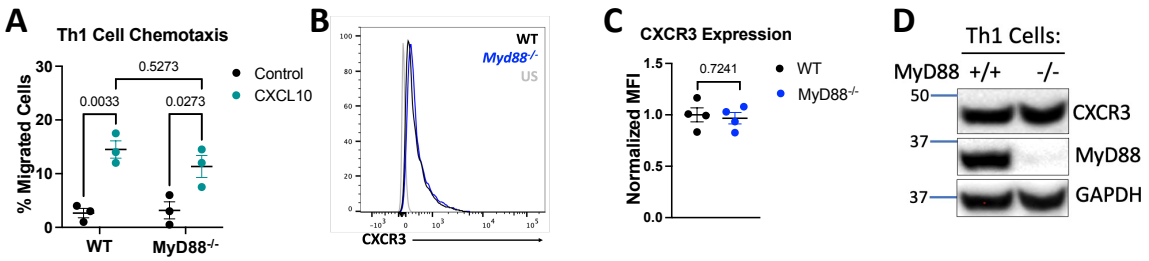


Supplemental Figure 2: Gating Strategy for In vitro T-cell Experiments and Characterization of *Myd88*$^{-/-}$ Th1 cells

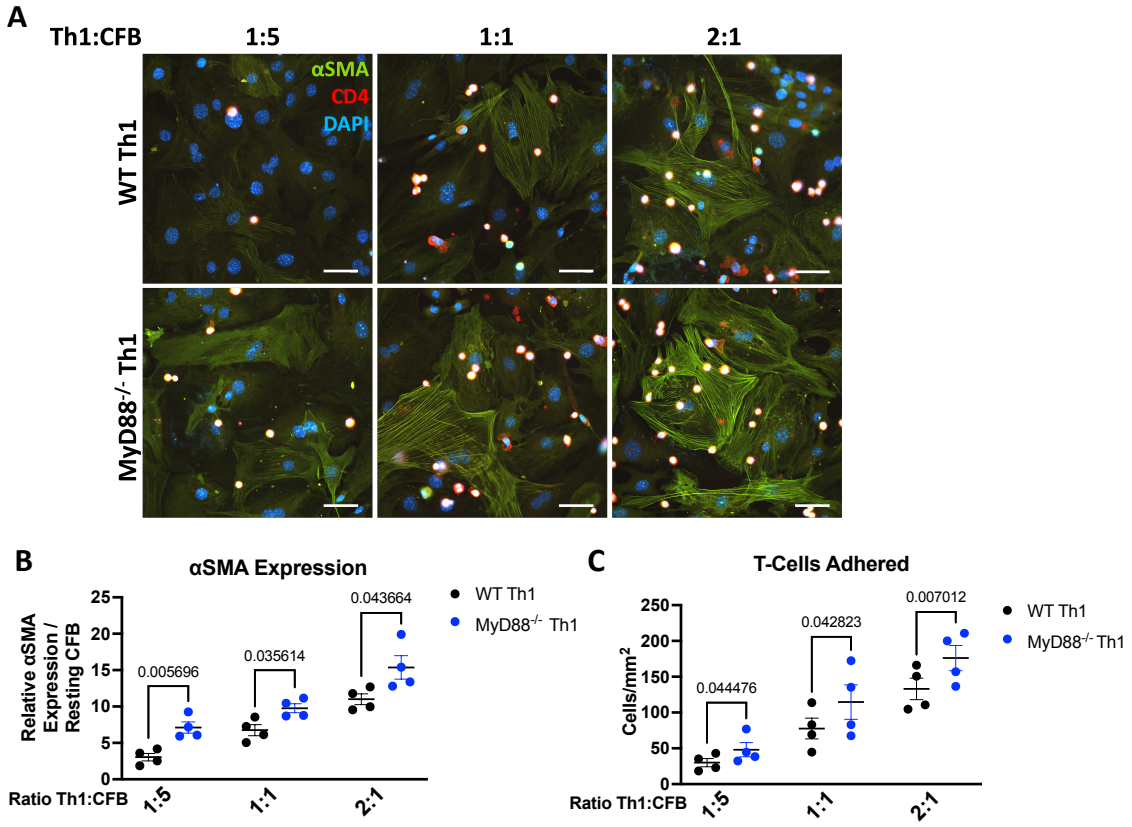
A. Gating strategy used for all *in vitro* T-cell experiments. **B.** WT or *Myd88*$^{-/-}$ Th1 cells were stained for IFN using intracellular cytokine staining, representative flow cytometry plots shown gated on CD4^{+} cells with quantification in **C**. from n=6 independent experiments**. D.** WT or *Myd88*$^{-/-}$ Th1 cells were stained intracellularly for Tbx21, representative flow cytometry plots shown gated on CD4^{+} cells with quantification in **E.** from n=4 independent experiments. **F.** RNA was collected from WT or *Myd88*$^{-/-}$ Th1 cells for qPCR, from n=6 independent experiments. Statistical analyses by T-tests, with p values shown.



Supplemental Figure 3: Cardiac MyD88+ T-cells Are Found at 1 week Post TAC. Pooled 1 week Sham and TAC UMAP of total cardiac CD45+ cells (A) or T-cells (B) with overlay of *Cd4* or *Cd8a* expression (C). D. Relative *Myd88* expression in Sham vs. TAC T-cell clusters. E. Pooled 4 week Sham and TAC UMAP of total cardiac CD45+ cells.

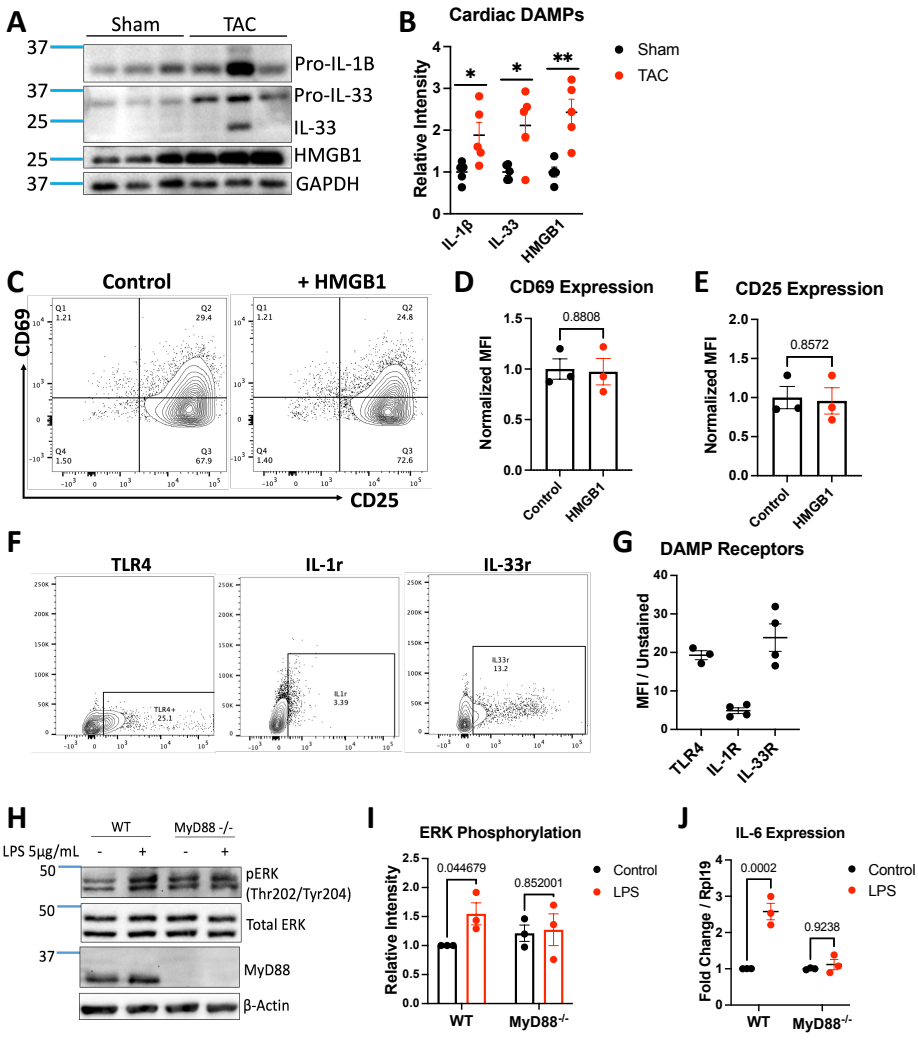


Supplemental Figure 4. *Myd88*$^{-/-}$ Th1 cells Exhibit Similar Chemotaxis to CXCL10.
A. 1 million WT or *Myd88*$^{-/-}$ Th1 cells were loaded onto Transwell inserts with or without a gradient of 2 µg/mL CXCL10, and T-cells migrated were counted manually after 4 hours from n=3 independent experiments. **B-D.** WT or *Myd88*$^{-/-}$ Th1 cells were collected for analysis of CXCR3 expression by flow cytometry, from n=4 independent experiments. Representative plot in **B.** quantified in **C.** or western blot (**D**). Statistical analysis by 2-way ANOVA with Sidák's multiple comparison test for A, or T-test for C. with p values shown.



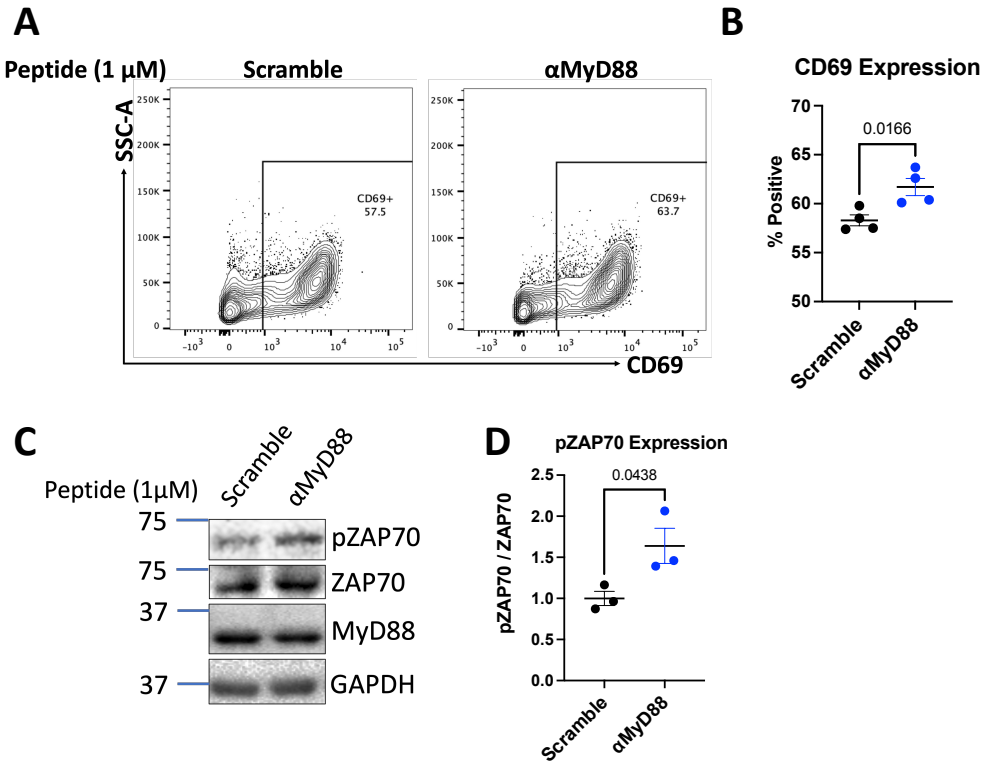
Supplemental Figure 5. *Myd88*$^{-/-}$ and WT Th1 cells Exhibit Dose Dependent CFB Activation.

WT Fibroblasts were cultured with Th1 cells at the indicated ratio (Th1:CFB) for 16 hours, then non-adherent T-cells were washed off with PBS before staining for αSMA and CD4, then imaged by immunofluorescence microscopy. Representative images shown in A. αSMA was quantified using imageJ and normalized to untreated cells (B) and T-cells adhered were counted manually (C). Each data point represents an independent experiment. Scale bars are equal to 50 μm. Statistical analysis by paired T-tests, with p values shown.

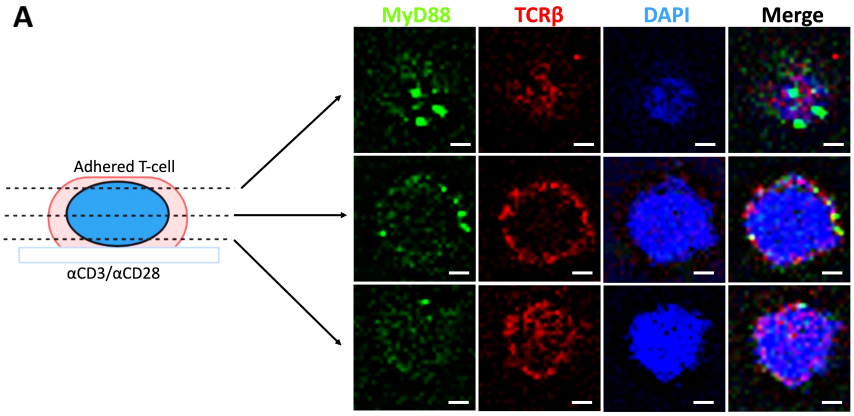


Supplemental Figure 6: Characterization of Cardiac DAMPs and T-cell DAMP sensing

A.WT mice underwent 27 G TAC for 4 weeks, and LV samples were taken for western blot. Representative blot from 3 Sham or TAC mice, quantified in **B.** for n=5 mice total. **C.** Th1 cells were cultured in T-cell media lacking beta-mercapto-ethanol and treated with 10 µg/mL recombinant HMGB1 and 50 µM H_2O_2 for 24 hours, then cells were collected for flow cytometry. Representative plots shown with quantification in **D-E** from n=3 independent experiments**. F.** Representative flow cytometry plots of Th1 cells gated on $CD4^+$ cells, with quantification in **G.** for n=3 or n=4 independent experiments **H.** Th1 cells were treated with 2.5 µg/mL for 1 hour then protein was collected for western blot. Representative blot shown**,** quantified n=3 independent experiments in **I. J.** Th1 cells were treated with 2.5 µg/mL LPS for 16 hours then RNA was collected for analysis of cytokine expression by qPCR, data shown is normalized to control treated cells from n=3 independent experiments. Statistical analyses by T-tests (panels B-E) or 2-way ANOVA with Sidák's multiple comparison test (I-J), with p values shown.

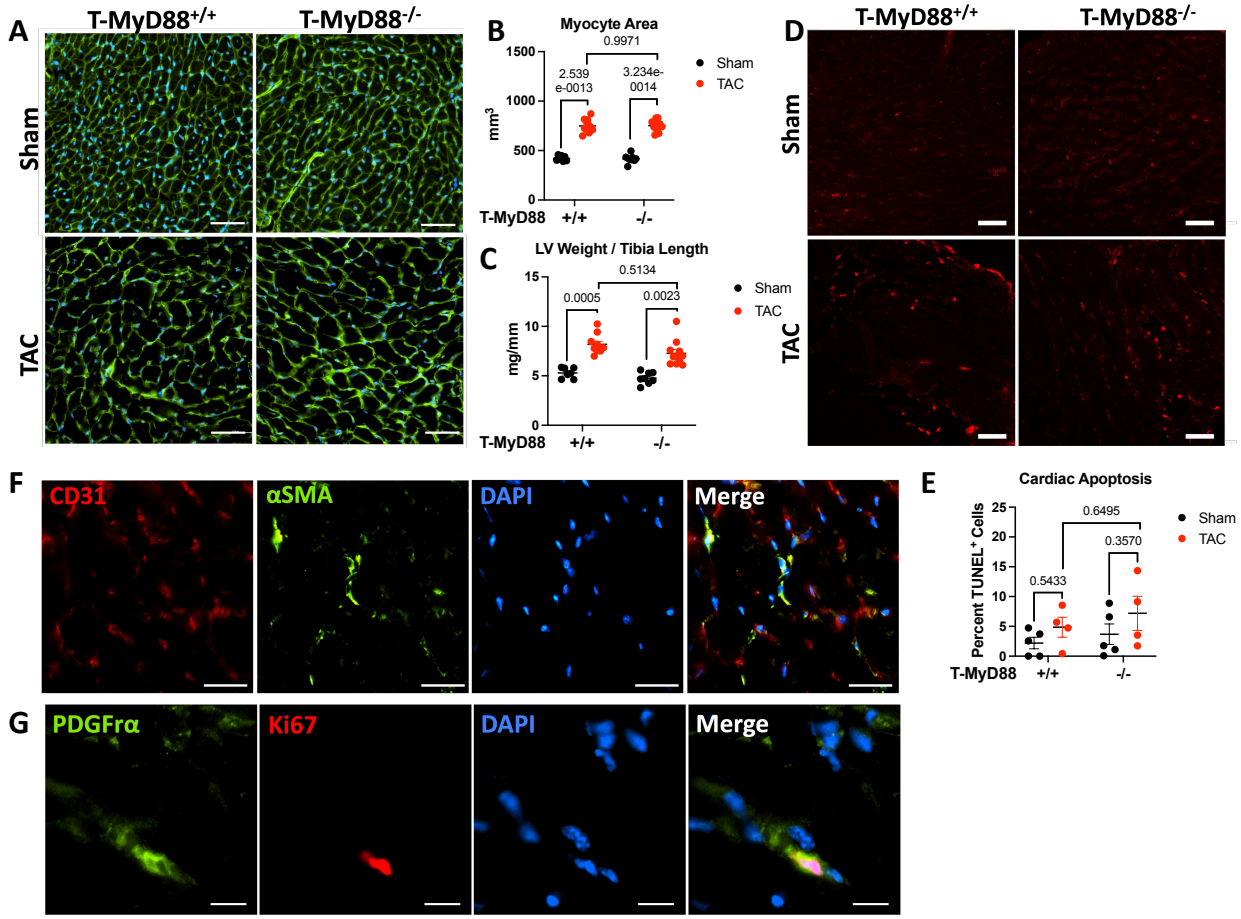


Supplemental Figure 7: MyD88 Inhibition Increases Th1 Activation Status
WT CD4 cells were activated in the presence of 1 µM of MyD88 inhibitor peptide or scrambled peptide for 24 hours. A. Representative flow cytometry plots of CD69 expression with quantification (B). C. Representative western blot of inhibitor treated T-cells with quantification (D). Each data point represents an independent experiment. Statistical analysis by T-test, p values shown.



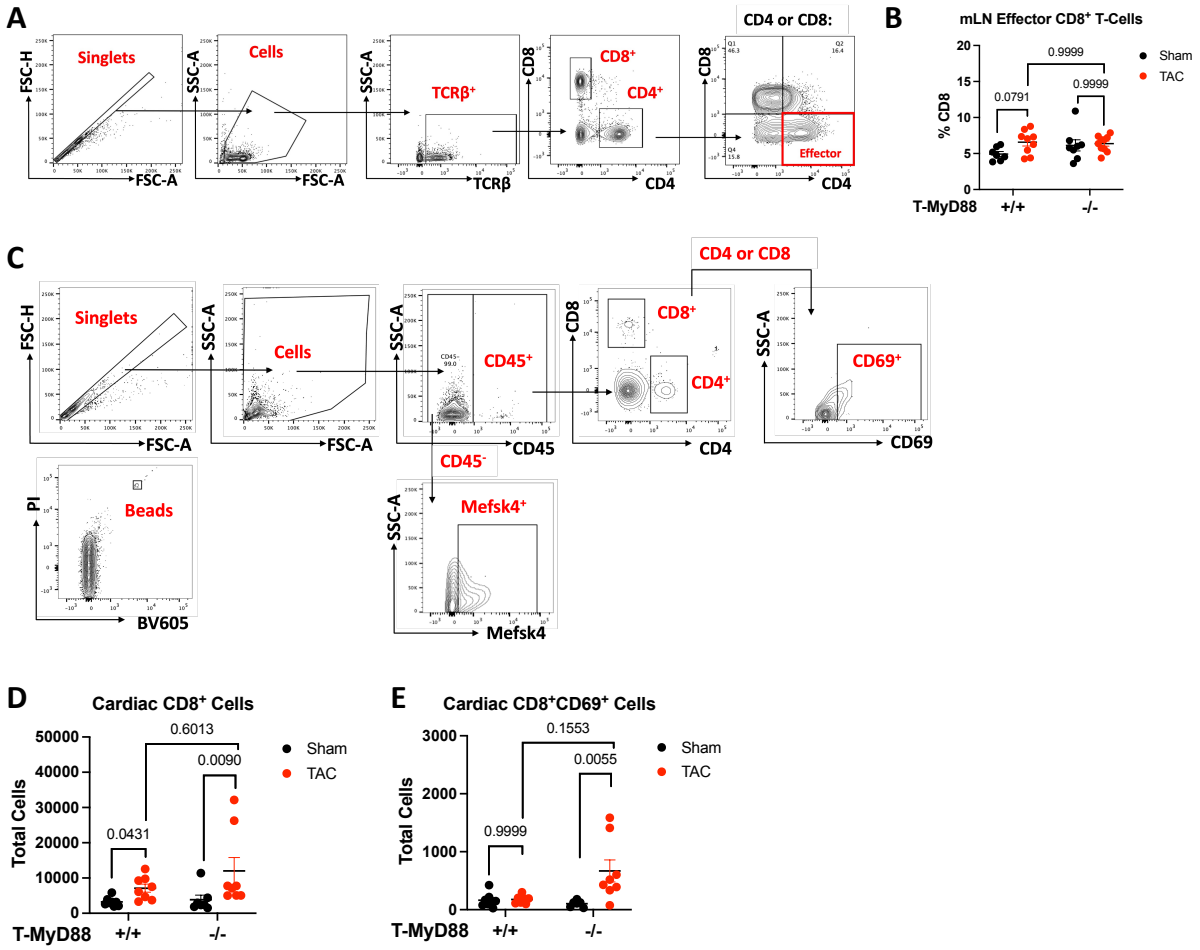
Supplemental Figure 8: Analysis of MyD88 Expression in T-cells by confocal microscopy

A. WT CD4 T-cells were plated on coverslips in the presence of αCD3/CD28 for 24 hours, then stained with MyD88 and TCRβ for analysis by Confocal microscopy with Z-stack imaging. Scale bars are equal to 2 μM.

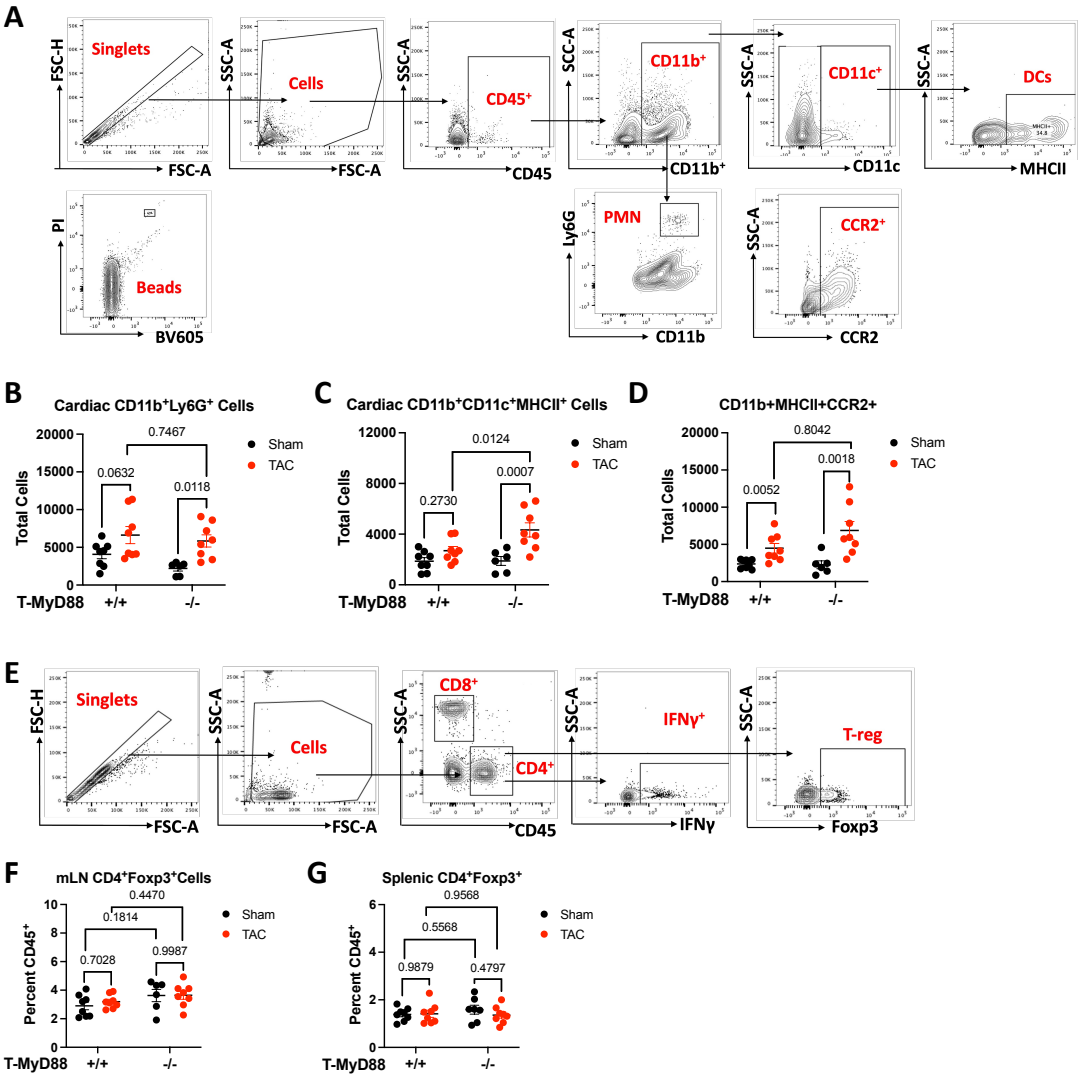


<u>Supplemental Figure 9: T-MyD88$^{-/-}$ Mice Exhibit Similar Hypertrophy and Little Cardiac Cell Death After TAC</u>

8-10 week male and female *Myd88$^{fl/fl}$CD4^{Cre}* mice using Cre^{+} and Cre^{-} littermates underwent 27G TAC or Sham surgery and were harvested after 4 weeks. **A-B**. LV sections were stained with WGA and quantified with ImageJ. **C.** LV weight was measured and normalized to Tibia length. **D-E**. LV sections were TUNEL stained and quantified with ImageJ. **F**. LV sections were stained with CD31 and αSMA, example image from a Cre+ TAC mouse **G.** LV sections were stained with PDGFrα and Ki67, example image from a Cre- Sham mouse. Scale bars shown for are 50 μM (A, D) 25 μM (F), 10 μM (G). Each data point represents an individual mouse. Statistical analysis by 2-way ANOVA with Sidák's multiple comparison test (B,C) or Kruskal-Wallis test with Dunn's multiple comparison tests (C), p values shown.



<u>Supplemental Figure 10: Flow Cytometric Analysis of Cardiac Inflammation</u>
8-10 week male and female *Myd88$^{fl/fl}$CD4^{Cre}* mice using Cre^{+} and Cre^{-} littermates underwent 27G TAC or Sham surgery and were harvested after 4 weeks.. **A.** Lymph nodes or spleen were crushed followed by staining for analysis by flow cytometry, shown is gating strategy for effector CD4 and CD8 T-cells. **B.** Quantification of effector CD8 cells in the mLN. **C.** The LV of each group of mice was enzymatically and mechanically digested for analysis by flow cytometry, shown is gating strategy for cardiac T-cells and fibroblasts. **D-E.** Quantification of cardiac CD8 cells and CD69 expression. Each data point represents an individual mouse. Statistical analysis by Kruskal-Wallis test with Dunn's multiple comparison tests, p values shown



Supplemental Figure 11: Continued Flow Cytometric Analysis of Cardiac Inflammation

8-10 week male and female *Myd88$^{fl/fl}$CD4^{Cre}* mice using Cre^{+} and Cre^{-} littermates underwent 27G TAC or Sham surgery and were harvested after 4 weeks. **A.** The LV of each group of mice was enzymatically and mechanically digested for analysis by flow cytometry, shown is gating strategy for cardiac myeloid populations, quantified in **B-D**. **E.** Lymph nodes or spleen were crushed followed by staining for analysis by flow cytometry, shown is gating strategy for in vivo IFNγ and Foxp3 expression, quantification of T-regs in **F.** mediastinal lymph nodes and **G.** spleen. Each data point represents an individual mouse. Statistical analysis by 2-way ANOVA with Sidák's multiple comparison test (B,C) or Kruskal-Wallis test with Dunn's multiple comparison tests (D), p values shown.