SUPPLEMENTAL MATERIAL

Materials and Methods

Animals and treatments

All animal experiments performed in this study adhered to the protocols approved by the Institutional Animal Care and Use Committee of the Northwestern University. Six to ten-week-old wild-type (WT) and IL10-KO (IL10tm1Cgn) mice of C57BL/6J background were procured from Jackson Research Laboratory (Bar Harbor, ME). Before treatments all animals were screened for baseline echocardiography. The cardiac hypertrophy and heart failure were induced by isoproterenol opsmotic pump and transverse aortic constriction surgery (TAC).

Animals and Surgeries:

a) **Isoproterenol pump implantation:** The control group animals (WT and IL10-KO) received saline vehicles and the treatment group animals received isoproterenol (ISO; 45 mg/kg b.wt./day for 14 days) filled into mini-osmotic pumps (model: alzet-20; Durect Corporation, CA. USA). Pumps were dorsally implanted in the mice for 14 days. At the end of the treatment period, pumps were surgically removed. Mice from the IL10 treatment group received subcutaneously injections of mouse recombinant IL10 (50 µg/kg b.wt.) on 0,1,3,5 and 7 (simultaneous with ISO pump to attenuate hypertrophic changes) days or on day 15, 18, 21, 24 and 27 (after removal of ISO pumps for reversal of hypertrophic changes).

b) **Transverse aortic constriction (TAC) surgery:** Caridac hypertrophy was induced in WT mice by constricting to transverse aorta. Briefly, a small midline skin cut was made just above the sternum of the mice and muscles were gently separated until trachea was visible. Partial left side thoracotomy to the second rib was performed with blunt ended spring scissor and the sternum was retracted using a chest retractor. Blunt tip 45° angled forceps were used to gently separate the two lobes of thymus and to clean fat tissue from the aortic arch. A small piece of a 7-0 silk suture (presoaked in sterile saline) were placed between the innominate and left carotid arteries using a 90 degree curved forceps. Two loose knots are tied around the transverse aorta and a small piece of a 27 gauge blunt needle placed parallel to the transverse aorta. The first knot was quickly tied against the needle, followed by the second and the needle promptly removed in order to yield a

constriction of 0.4mm in diameter. Mice from the IL10 treatment group received subcutaneous injections of mouse recombinant IL10 (50 µg/kg b.wt.) on 0,1,3,5, 7,10, 13, 16, 19, 22, 25 and 27 days.

Antibodies and reagents

Antibodies against p-p38, p-STAT-3 (Y704), NFkB, and α-Sarcomeric Actinin (α-SMA) were purchased from Abcam Biotech. (San Francisco, California). Caspase3, cleaved caspase3, Bcl2, Total STAT-3, p38, GAPDH and β-actin were purchased from Cell Signaling Inc. (Boston, MA). Recombinant murine IL10 was obtained from R&D Systems (Minneapolis, MN). Curcurbitacin I (CB 1) was obtained from Calbiochem (San Diego, CA). NFkB activity assay kit were purchased from IMGENEX corporation (San Diego, CA). Isoproterenol was purchased from Sigma (San Diego, CA). STAT3 constitutively active (Stat3-C Flag pRc/CMV) and dominant negative (Stat3 Y705F Flag pRc/CMV) plasmid constructs were purchased from Adgene Inc. (Cambridge, MA). Lipid based transfection reagent, TranFast, was purchased from Promega (Madison, WI).

Cardiac Imaging

Trans-thoracic two-dimensional M-mode echocardiography was performed using Vevo 770 (VisualSonics, Toronto, Canada) equipped with a 30 MHz transducer. Echocardiographic studies were performed before (baseline) and at 7, 14 and 28 days post pump implantation in mice. M-mode tracings were used to measure LV wall thickness, LV dimensions, LV volume and LV mass. The mean value of at least 3-5 cardiac cycles were used to determine the measurements for each animal. Percent fractional shortening (% LVFS) and ejection fraction (% LVEF) were calculated as described previously [1].

Tissue Preparation & Masson`s Trichome Staining

Whole hearts were perfused first with sterile cold PBS followed by 10% formalin for 10-15 min. Atria were removed and ventricles were fixed in 10% phosphate buffered formalin for 24 hours. After fixation hearts were sectioned into two parts, head and apex and paraffin blocks were prepared. Almost 20-25 sections (4 µm each) were prepared from each animal. MT staining was performed by standard methods. Extent of fibrosis was quantified in stained sections with Image-Pro Plus 6.3 software (Media Cybernetics, Bethesda, MD) as described earlier [2]. The sum of 5 fields per mouse was used for statistical analysis to determine significant difference.

Terminal deoxynucleotidyl transferase–mediated dUTP nick end-labeling (TUNEL) staining for cell death study

TUNEL staining was performed as per manufacturers' instructions in paraffin sections (Roche Diagnostic, Mannheim, Germany). Briefly, approximately 4-5, 4 µm thick, paraffin-embedded sections were prepared from mouse hearts. After sequential deparaffinization each section was stained with TUNEL reagents and α-sarcomeric actin (α-SA). DAPI staining was used to count the total number of nuclei. The index of apoptosis was calculated as the percentage of apoptotic nuclei/total number of nuclei in α-SA positive cells.

Quantitative Real-Time PCR

Gene expression levels of ANP, BNP, CD 68, IL-1β, TGF-β and TNF-α were quantified in the left ventricular tissue, NRCM and H9c2 myoblasts as described previously [1, 3]. RNA was collected with RNA STAT-60 (TEL-TEST, Inc., Friendswood, Texas). Total RNA was reverse transcribed with iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA), and amplification was performed using Taqman 7300 (Applied Biosystems, Foster City, CA). Relative mRNA expression of target genes was normalized to the endogenous 18S control gene

(Applied Biosystems, Foster City, CA) and represented as fold change *vs.* respective controls. Specific primers and probes sequences are mentioned in online supplementary Table S1.

Isolation of neonatal rat ventricular myocytes and treatments

NRCM were prepared by enzymatic digestion of hearts obtained from newborn (0–2 day old) Sprague–Dawley rat pups using percoll gradient centrifugation and plated on six-well cell culture grade plates (coated with collagen IV) at a density of 0.85×10^6 cells/well in DMEM/M199 medium and maintained at 37°C in humid air with 5% CO_2 [4]. For *in vitro* experiments NRCM were starved in serum free media (SFM) for 12 hours. NRCM were pre-treated with IL10 (20 ng/ml) along with inhibitors (STAT3 inhibitor curcubitacin I, 500 nM) for one hour followed by isoproterenol (ISO, 1 μM) for various times. After treatments, cells were harvested and used for the mRNA and protein studies.

H9c2 myoblasts culture and transient transfections

H9c2 cells were obtained from American Tissue Type Collection (Manassas, VA; catalog # CRL – 1446) and plated on six-well cell culture grade plates at a density of 0.08×10^6 cells/well in DMEM medium supplemented with 10% FBS and maintained at 37°C in humid air with 5% CO_2. Twelve hours after plating, H9c2 myoblasts (4-5 x 10^6 cells in six well plates) were transiently transfected with DN and CA-STAT3 plasmids constructs using a lipid based transfection reagents TransFast (Promega) according to the manufacturer's instructions and as described in our earlier publication [5]. Transfection efficiency was monitored in protein extracts obtained the cells after 36 hours expression by Western blot analysis of tagged Flag protein and was comparable among transfections. After treatments, cells were harvested and used for the mRNA and protein expression studies.

Preparation of Cell Lysates and Western Blotting

Cells/tissue were lysed in RIPA cell lysis buffer (Cell Signaling, Boston, MA) supplemented with protease inhibitors pellets (Amarsham Biosciences, Piscataway, NJ) [4, 6]. Insoluble debris was removed by centrifugation (25,000 g) for 10 min at 4^{o}C and cell lysates were boiled with Laemmle sample buffer (0.5 mol/L Tris-HCl [pH 6.8], 10% SDS, 10% glycerol, 4% β-merceptoethenol and 0.05% bromophenol blue). Equal amounts of protein (40µg) were separated by SDS-PAGE and assessed by Western blotting in indicated protein(s). β-Actin was used as a loading control.

Electrophoretic Mobility Shift Assay

The following oligonucleotide sequence containing binding sites for NFκB/c-Rel homodimeric and heterodimeric complexes were purchased from Santa Cruz Biotech. (sc 2505) and used for EMSA: 5'-AGT TGA GGG GAC TTT CCC AGG C-3'. Nuclear protein isolation and EMSAs were carried out as described elsewhere [5].

Immunofluorescent Staining

Immunofluorescent staining for p65/RelB (NFkB), Cd68 and α-SA was performed in tissue sections or NRCM plated onto chamber slides (100 viable cells/mm^2) coated with 1 µg/cm^2 collagen IV. NRCM were grown to ~80% confluencey (2 days) in DMEM containing 10% newborn calf serum. The medium was changed to serum-free MEM and immunostaining was performed 24 h later. Prior to immunostaining, cells were washed with PBS and fixed with 4% PFA for 10 min and permeabilized for 10 min using 0.05% Triton® 100. NRCM were incubated for 1 h (22^0 C) with blocking solution (3% bovine serum albumin, 10% Horse serum and 0.2% Triton X-100) to block nonspecific binding. Cells were then incubated at 37^{0}C for 2 h with

primary antibodies and 45 min with the appropriate secondary antibody conjugated to Alexa-488 or Alexa 594 (Invitrogen). DAPI was used to counterstain the nuclei. Samples were covered with mounting media (Invitrogen), overlaid with a coverslips and examined under a fluorescence microscope. For tissue sections all the steps are same after de-parafinization steps.

Statistical Analysis

Differences between data groups were calculated for significance by the use of the unpaired *t* test or 1-way analysis of variance (ANOVA), as appropriate and Tukey`s multiple comparison post hoc test (Graph Pad prism Software Inc., San Diego, CA). The post hoc testing was performed if overall comparison across groups was statistically significant. Two-way repeated-measures ANOVA was used to evaluate the statistical significance of data acquired from the same animal over multiple time points. Survival analysis was performed by the Kaplan-Meier method, and between-group differences in survival were tested by the Log-rank (Mantel-Cox) test using Graph Pad prism Software. Data are expressed as mean ± SEM. For all tests, a probability value of > 0.05 was considered to denote statistical significance

Supplementary Table S1

	Baseline		Day 7				Day 28			
			WT		KO		WT		KO	
LV	WT	KO	ISO	ISO-IL10	ISO	ISO-IL10	ISO	ISO-IL10	ISO	ISO-IL10
PWT;d (mm)	0.77±0.019	0.73±0.05	1.10±0.02	0.96±0.05	1.26±0.04	1.04±0.01	1.05±0.05	1.05±0.05	1.01±0.02	1.05±0.02
AWT;d (mm)	0.73±0.017	0.76±0.028	1.00±0.03	0.99±0.03	1.13±0.07	0.94±0.02	1.14±0.04	1.13±0.031	0.99±0.03	1.03±0.012
Ed;D (mm)	3.77±0.022	3.61±0.039	3.82±0.05	3.74±0.033	4.32±0.19	3.79±0.13	3.91±0.01	3.76±0.17	4.23±0.05	3.84±0.029
vol;d (µl)	54.83±2.42	53.28±1.59	56.71±2.13	55.57±1.42	63.26±2.54	60.66±4.56	69.77±2.90	62.78±3.50	81.27±3.4	60.23±4.46

Supplementary Table S1: Echocardiographic analysis of WT and IL10 KO animals at 7 and 28 days after ISO treatment. LV; left ventricles, PWT;d, diastolic posterior wall thickness; AWT;d diastolic anterior wall thickness, Ed;D; End-diastolic dimension, Vol;d; diastolic volume. N=6-8

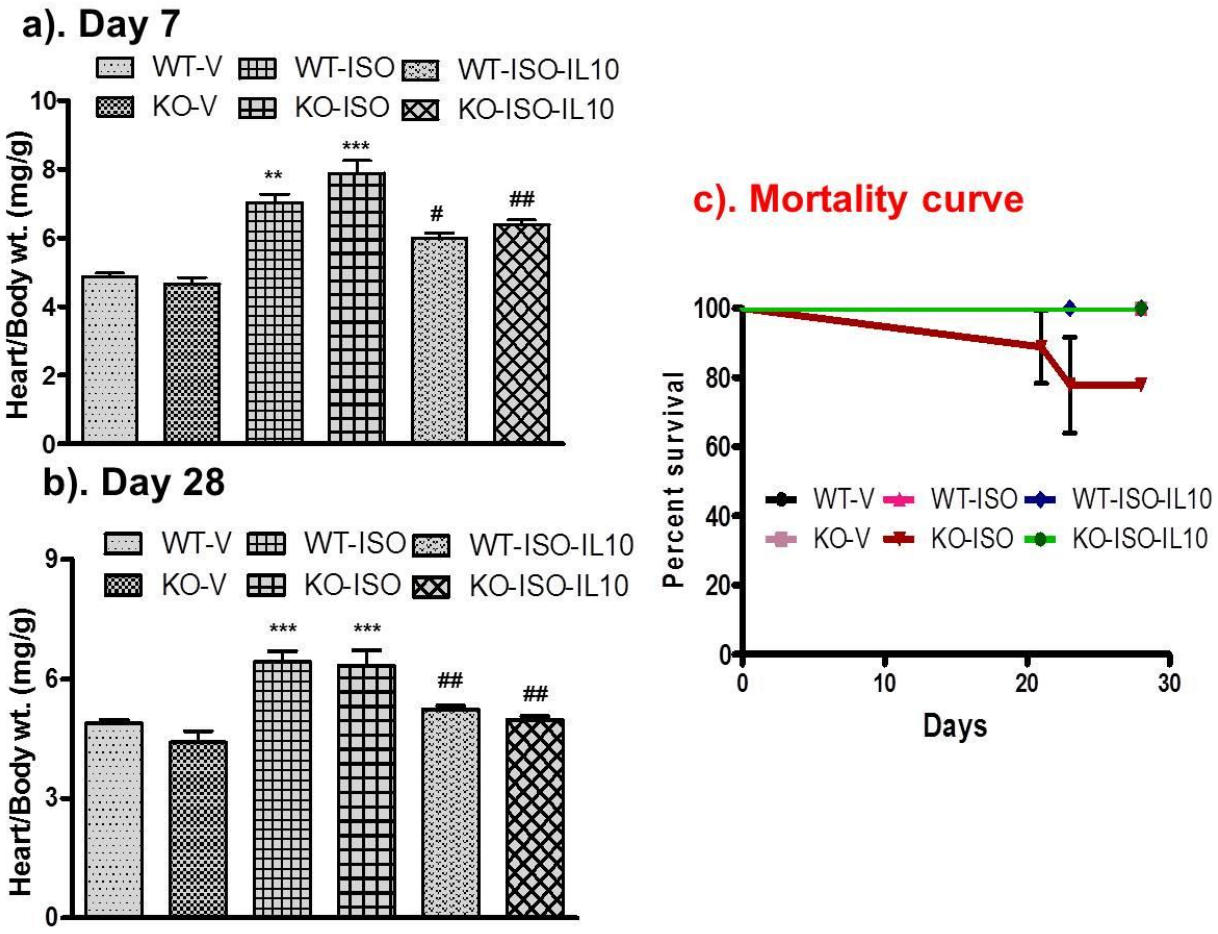
Supplementary Table S2:

A) Rats Primers and Probes

S. No.	Gene	Forward Primer	Reverse primer	Probe
1	ANP	CATGGGCTCCTTC TCCATCA	TGGCCTGGGAGC CAAA	CAAGGGCTTCTTCCT CTTCCTGGCC
2	BNP	CGGGCTGAGGTTG TTTTAGG	GCCGCAGGCAGA GTCAGA	AGACCTCCTGGCTGC AGACTCCGG
3	IL1β	CAGGCTTCGAGAT GAACAACAA	TCCATTGAGGTG GAGAGCTTTC	ATGCCTCGTGCTGTC TGACCCATGT
4	TNFα	CGGGCTCAGAATT TCCAACA	CGCAATCCAGGC CACTACTT	CTACGATGCTCAGAA ACACACGAGACGCT

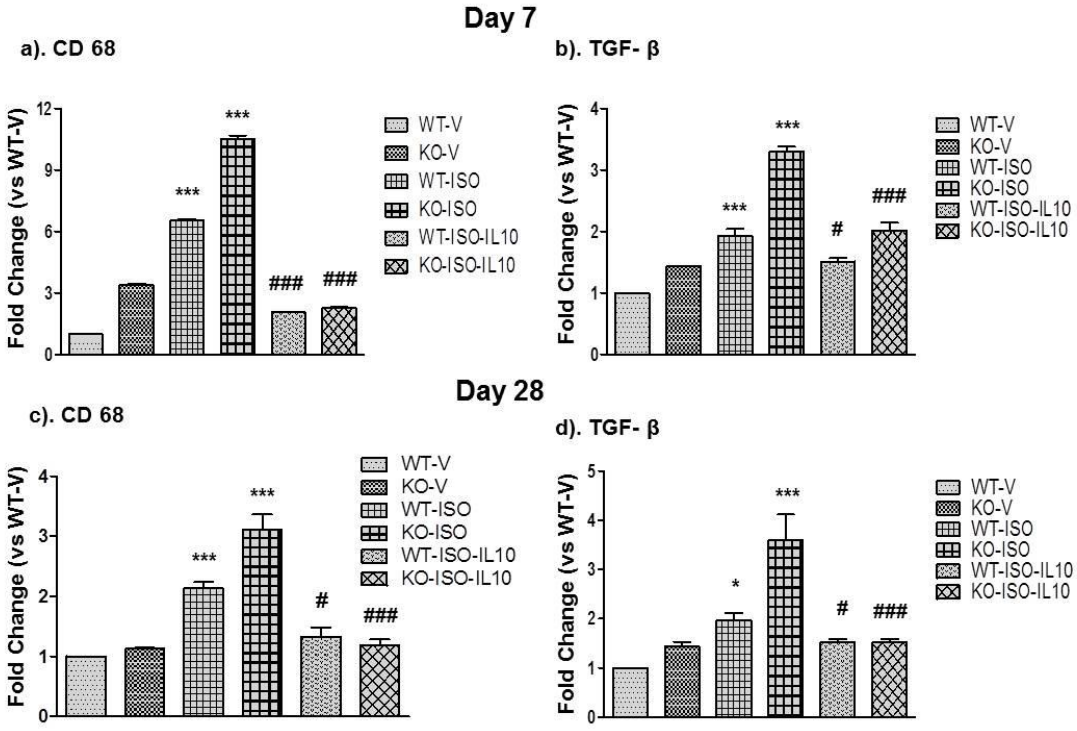
B) Mouse Primers and probes

S. No.	Gene	Forward Primer	Reverse primer	Probe
1	ANP	GGAGGAGAAGATGCC GGTAGA	GCTTCCTCAGTCT GCTCACTCA	AGGTCATGCCCCCGC AGGC
2	BNP	AGCTGCTGGAGCTGAT AAGAGAA	GTGAGGCCTTGGT CCTTCAA	CGGAGGAAATGGCC CAGAGACAGC
3	IL1β	CTACAGGCTCCGAGAT GAACAAC	TCCATTGAGGTGG AGAGCTTTC	AGCCTCGTGCTGTCG GACCCATATG
4	TNFα	GGCTGCCCCGACTACG T	AGGTTGACTTTCT CCTGGTATGAGA	CCTCACCCACACCGT CAGCCG
5	CD68	AGCTGCCTGACAAGG GACACT	TGAGAGGCAGCAA GAGGGA	CCATGTTTCTCTTGC AACCGTGACCA
6	TGFβ	GAGGTCACCCGCGTGC TA	TGTGTGAGATGTC TTTGGTTTTCTC	TGGTGGACCGCAAC AACGCC



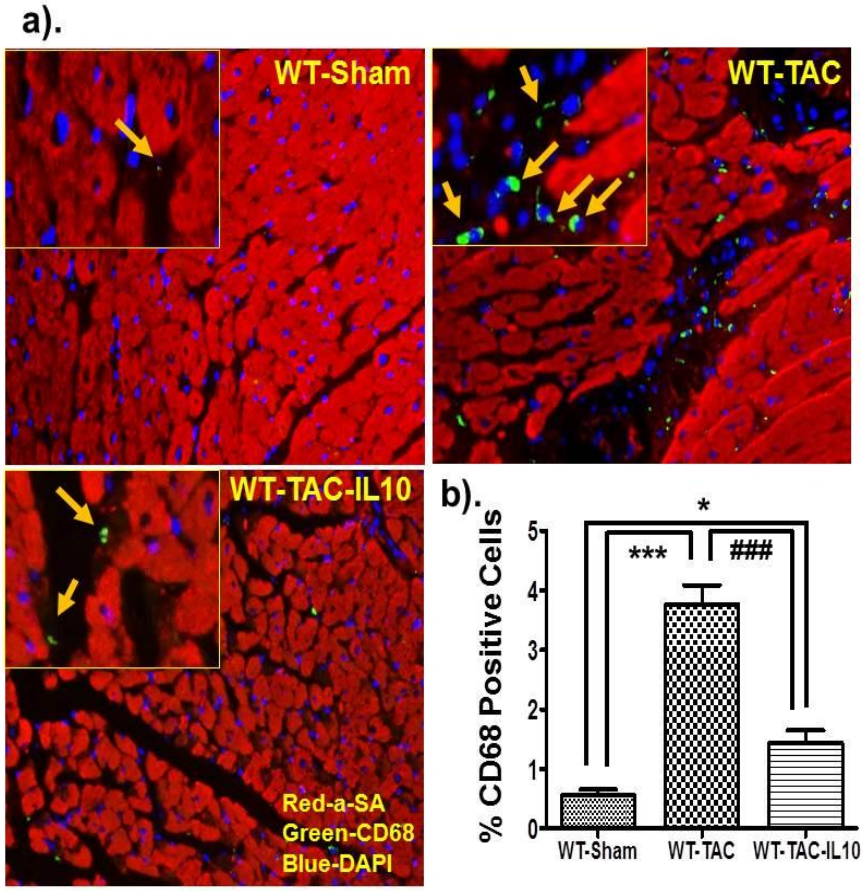
Supplementary Figure S1: IL10 reduced ISO-induced heart weight both wild type and IL10 knockout mice. Changes in heart and body weight ratio were calculated at (**a**) 7 and (**b**) 28 days respectively. ISO treatment significantly increased this ratio, however IL10 treatment significantly abolished the effect of ISO; N=6-8 *** $p<0.001$ vs. WT-V, $^{**}p<0.01$ vs. WT-V, $^{\#\#}p<0.01$ vs. respective ISO treated, $^{\#}p<0.05$ vs. respective ISO treated. N=4-6. (**c)** only IL10 KO mice showed 20% mortality with ISO-infusion which was significantly reversed upon IL10 treatments (N=10).

Supplement Figure S2



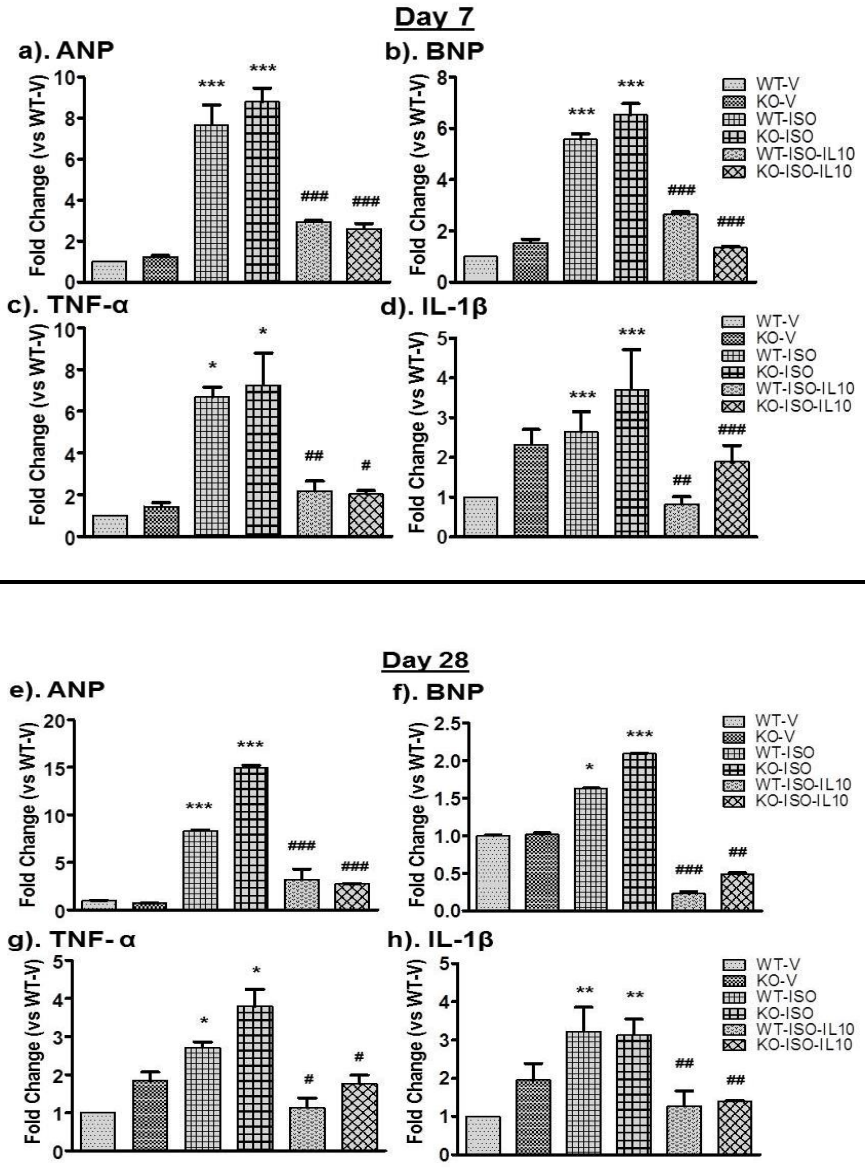
Supplementary Figure S2: Supplementary Figure SII: IL10 markedly inhibited ISO-induced CD 68 and TGF β gene expression. Expression of CD 68 and TGFβ was quantified by Q-PCR in tissues. Relative mRNA expression of target genes was normalized to the endogenous 18S and represented as fold change *vs.* WT-V. ISO-induced increased expression of CD 68 **(a & c) and** TGF-β **(b & d)** was significantly attenuated by IL10 in both WT and IL KO mice. *** $p<0.001$ vs. WT-V, $^{*}p<0.05$ vs. WT-V, $^{###}p<0.001$ vs. respective ISO treated, $^{#}p<0.05$ vs. respective ISO treated. N=4-5.

Supplement Figure S3



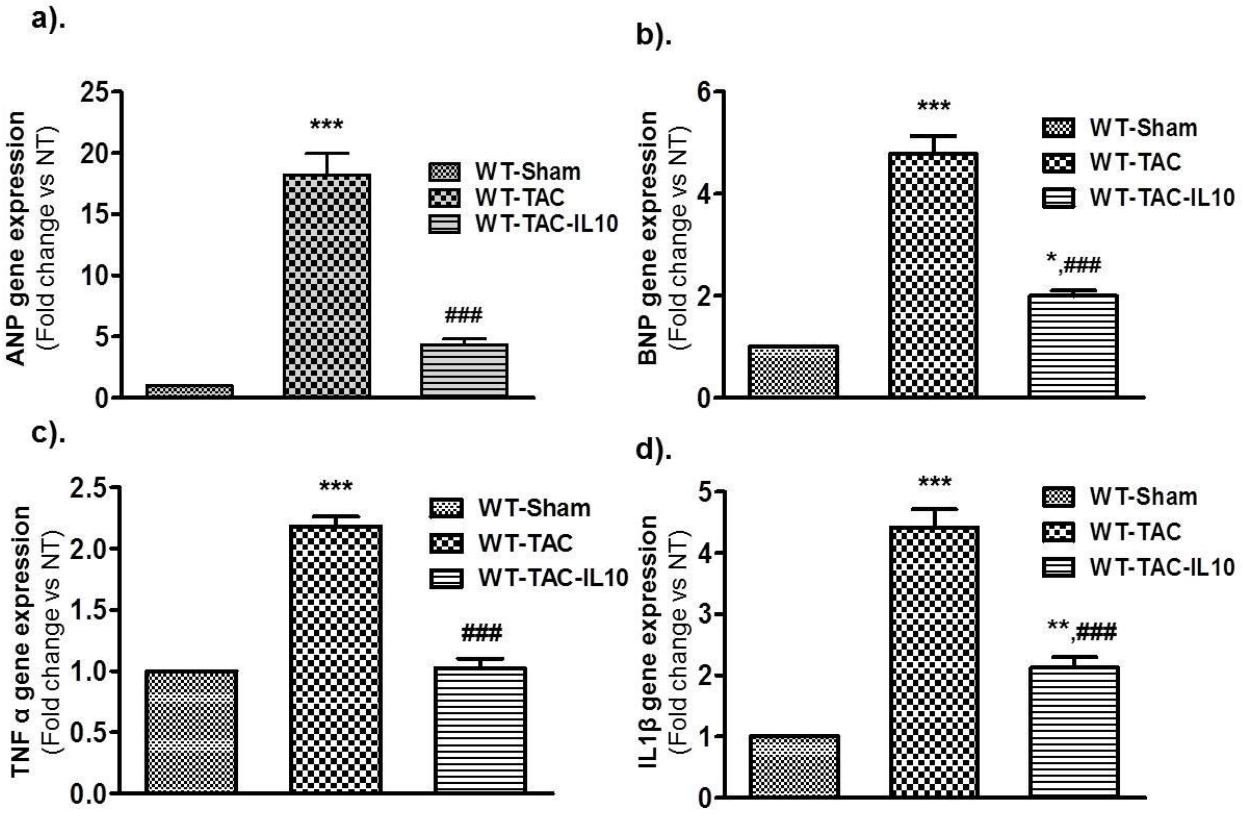
Supplementary Figure S3: IL10 treatment inhibits inflammatory cell infiltration following TAC. (a) Representative images showing CD68+ inflammatory cells (monocyte/macrophages) in myocardial samples from animals treated or not with IL10 after 28 days following TAC surgery. (**b**) IL10 treatment significantly reduced the number of inflammatory cells on day 28 after TAC.

Supplement Figure S4

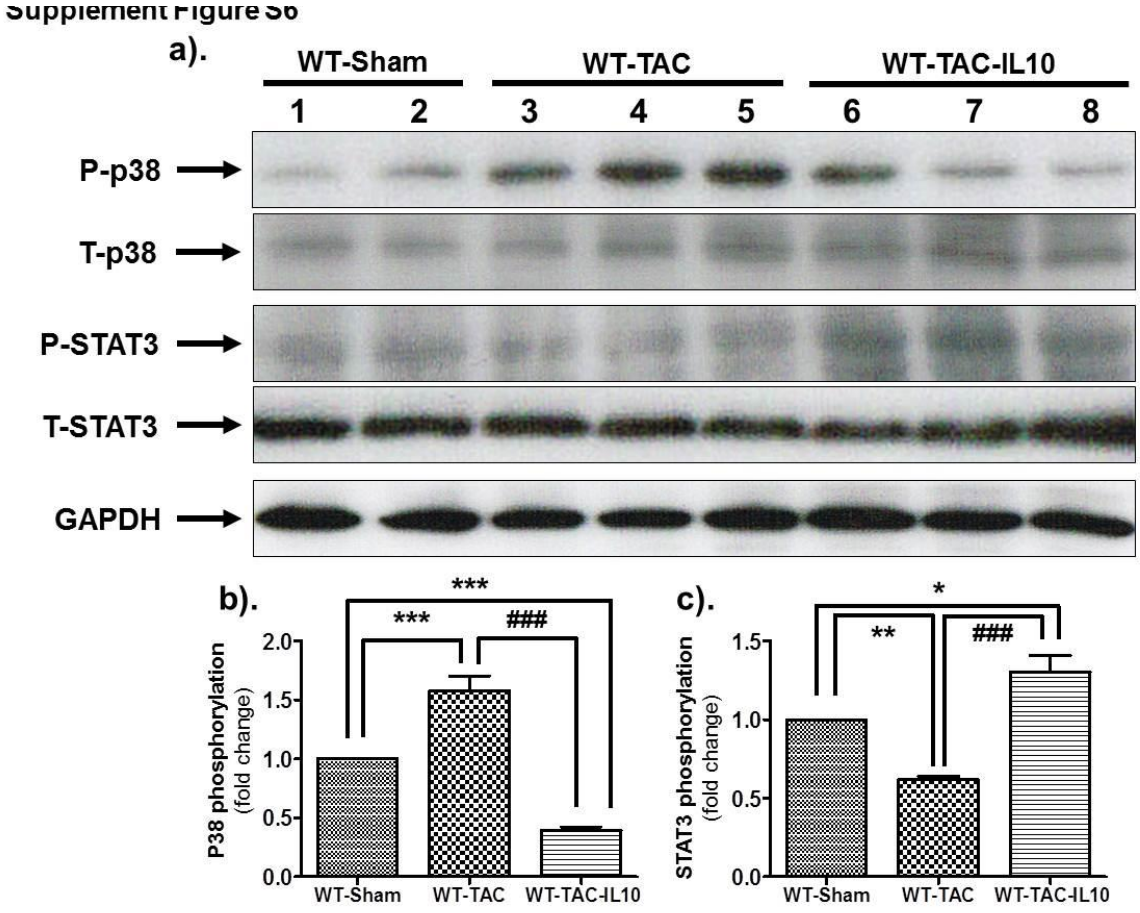


Supplementary Figure S4: IL10 treatment markedly reduces ISO-induced hypertrophic and inflammatory genes expression *in vivo*. ANP, BNP, IL-1β & TNF-α gene expression was quantified in LV tissues by Q-PCR. Relative mRNA expression of target genes was normalized with endogenous 18S and represented as fold change *vs.* WT-V. ISO-induced increase in ANP **(a & e)**, BNP **(b & f)**, TNF-α **(c & g)** and IL-1β **(d & h)** expression was significantly attenuated by IL10. *** $p<0.001$ vs. vehicle. ** $p<0.01$ vs. respective vehicle, * $p<0.05$ vs. respective vehicle, $^{###}$ $p<0.001$ vs. respective ISO treated, $^{##}$ $p<0.01$ vs. respective ISO treated, $^{#}$ $p<0.05$ vs. respective ISO treated. N=4-5

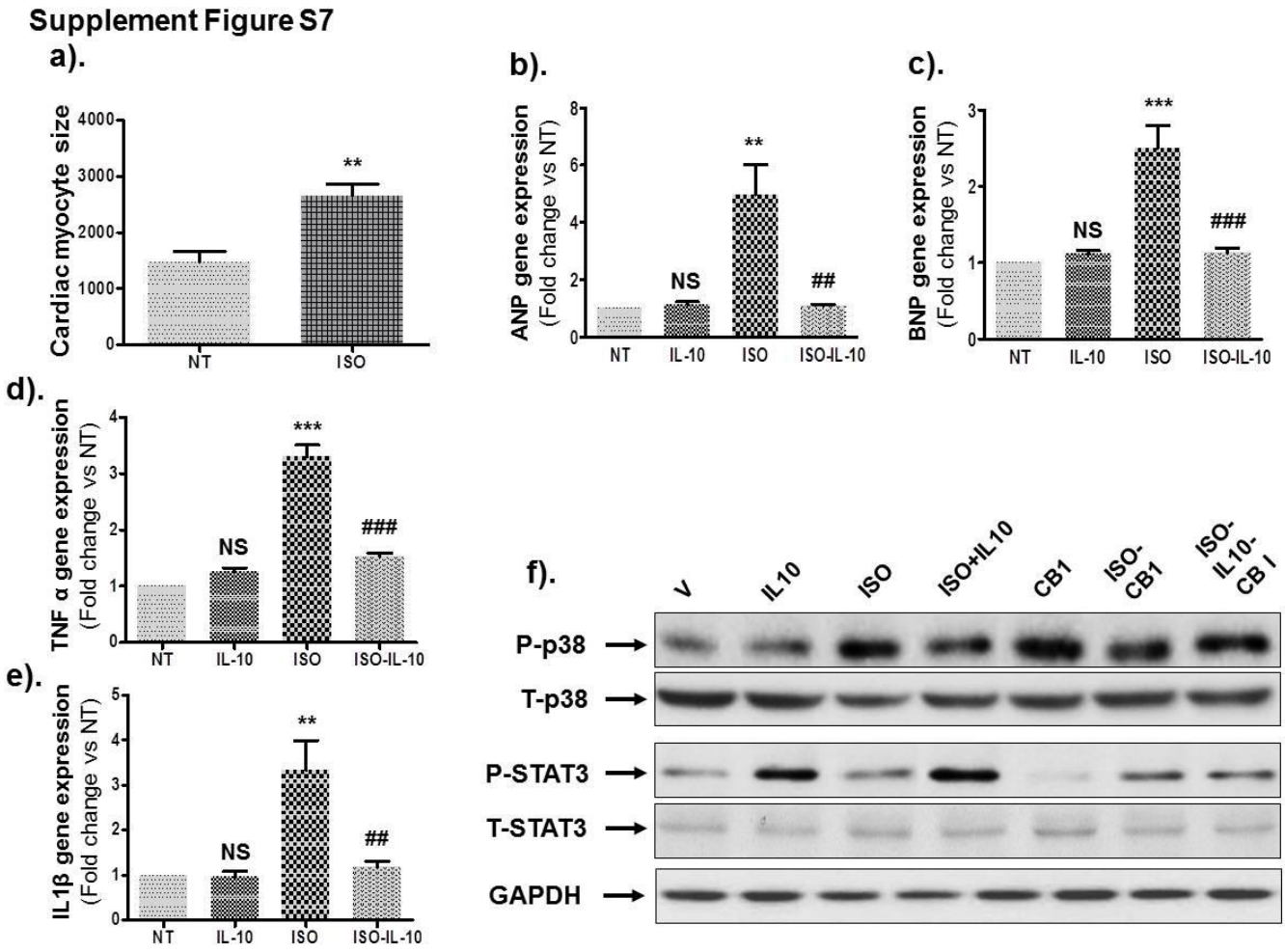
Supplement Figure S5



Supplementary Figure S5: IL10 treatment markedly reduces TAC-induced hypertrophic and inflammatory gene expression ANP, BNP, IL-1β & TNF-α gene expression was quantified in LV tissues by Q-PCR. Relative mRNA expression of target genes was normalized with endogenous 18S and represented as fold change *vs.* WT-sham. TAC-induced increase in ANP (a), BNP (b), TNF-α (c) and IL-1β (d) expression was significantly attenuated by IL10. ***p<0.001 vs. WT-sham. **p<0.01 vs. WT-sham, *p<0.05 vs. WT-sham, $^{###}$p<0.001 vs. WT-TAC. N=4-5

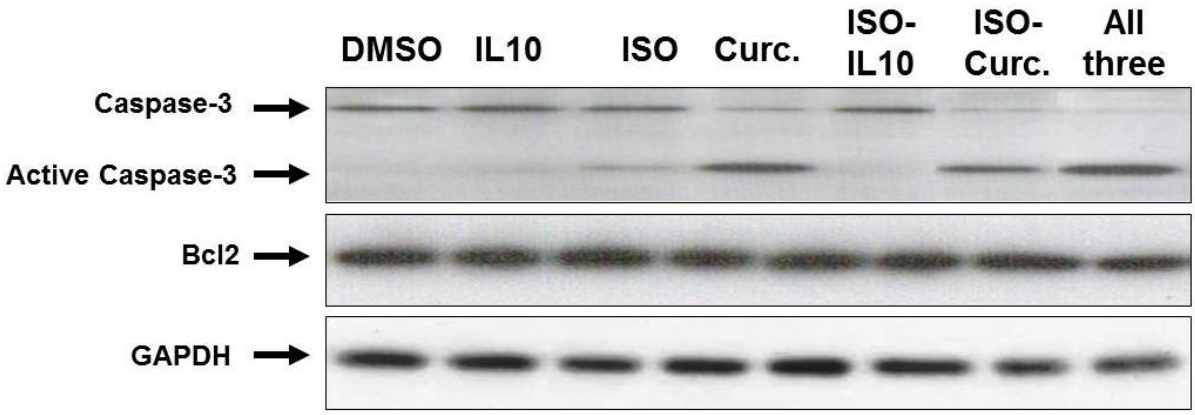


Supplement Figure S6: IL10 treatment markedly reduces TAC-induced hypertrophic signaling pathways. a) Western blots were performed in whole tissue lysates for p38 and STAT3 activation in all treated groups. Respective total proteins were used as normalizing control. IL10 treatment markedly reduced TAC-induced p38 activation (**a & b**). In addition, TAC significantly reduced STAT3 phosphorylation however IL10 treatment considerably attenuated TAC-mediated STAT3 inhibition (**a & c**). β actin was used as loading control ***p<0.001 vs. WT-sham. **p<0.01 vs. WT-sham, *p<0.05 vs. WT-sham, $^{###}$p<0.001 vs. WT-TAC. N=4-5



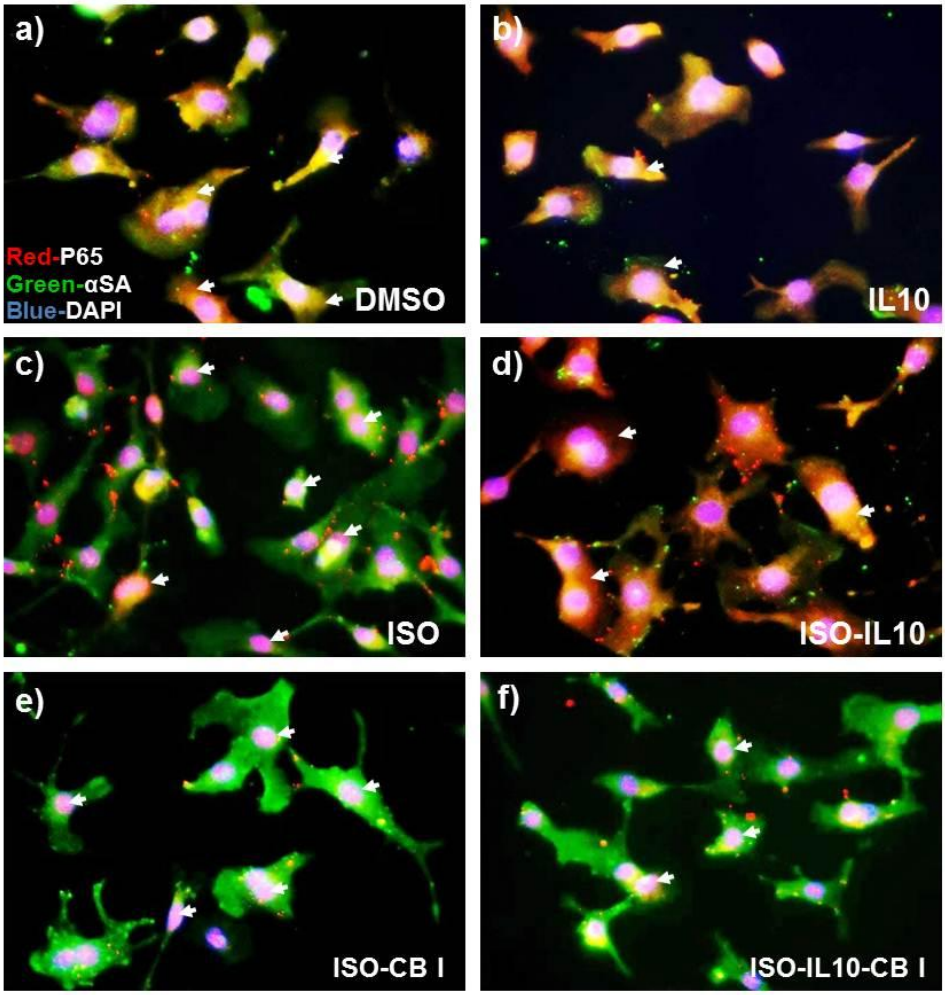
Supplement Figure S7: IL10-reduces ISO-induced hypertropic gene expression and signaling in neonatal rat cardiomyocytes *in vitro* : Neonatal rat ventricular myocytes were isolated and cultured in low glucose DMEM/M199. Almost 12 hours serum starved cells were treated with IL10 for one hour followed by ISO. Myocyte size **(a),** expression of ANP, BNP IL1β & TNF-α was quantified by Q-PCR. Relative mRNA expression of target genes was normalized to the endogenous 18S and represented as fold change *vs.* no treatment group. ISO-induced increased expression of **(b)** ANP, **(c)** BNP, **(d)** TNF-α and (**e**) IL1β was significantly attenuated by IL10. **f)** For Western blot analysis cells were treated with specific STAT3 inhibitor curcurbitacin I (CB I) and/or IL10 for one hour followed by ISO. Western blot analysis was performed in total cell lysates for p38 and STAT3 activation after different treatments. IL10 treatment markedly reduced the ISO-induced p38 activation (phosphorylation) and activated STAT3 phosphorylation. Interestingly, IL10 mediated inhibition of P38 is reasonably dependent on STAT3. β actin was used as control. *** p<0.001 vs. NT, **p<0.01 vs. NT, $^{###}$p<0.001 vs. respective ISO treated, $^{##}$p<0.01 vs. respective ISO treated. N=4-5.

Supplement Figure S8



Supplement Figure S8: IL-10 inhibits ISO-induced caspase 3 activation: To understand the anti-apoptotic mechanism of IL10, caspase-3 and anti-apoptotic Bcl2 levels were measured in the total cell lysate prepared from NRCM treated with different combinations of ISO, IL10 and STAT3 inhibitor for 24 hours. ISO treatment significantly increased active caspase-3. ISO-induced caspase-3 activation was attenuated by IL10 treatment. Interestingly, the anti-apoptotic effect of IL10 was markedly reduced by STAT3 inhibitor. Therefore, the anti-apoptotic action of IL10 is mediated by STAT3. In contrast, there were no significant effects of ISO or IL10 on Bcl2 levels (N=4-5).

Supplement Figure S9



Supplement Figure S9: STAT3 plays critical role for IL10 mediated NFkB inhibition in NRCM. Cells were fixed with 4% PFA for 10 min and permeabilized for 5 min using 0.5% Triton® 100. After blocking, cells were incubated with primary antibodies and appropriate secondary antibody conjugated to Alexa-488 (Green for α-SA) or Alexa 594 (Red for p65) for 45 min. DAPI were used to counter stain the nuclei. **a-f)** Inhibition of STAT3 by STAT3 specific inhibitor curcurbitacin I (CB1) markedly attenuated the IL10 effects on ISO-induced hypertrophy (Original magnification 40X, N=4).

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