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## Left Ventricular T Cell Recruitment Contributes to the Pathogenesis of Heart Failure

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### Abstract

**Background**—Despite the emerging association between Heart Failure (HF) and inflammation, the role of T cells, major players in chronic inflammation, has only recently begun to be explored. Whether T cell recruitment to the left ventricle (LV) participates in the development of HF requires further investigation to identify novel mechanisms that may serve for the design of alternative therapeutic interventions.

**Methods and Results**—Real time videomicroscopy of T cells from non- ischemic HF patients or from mice with HF induced by transverse aortic constriction (TAC) revealed enhanced adhesion to activated vascular endothelial cells under flow conditions *in vitro* compared with T cells from healthy subjects or sham mice. T cells in the mediastinal lymph nodes and the intramyocardial endothelium were both activated in response to TAC and the kinetics of LV T cell infiltration was directly associated with the development of systolic dysfunction. In response to TAC, T cell deficient mice (TCR $\alpha^{-/-}$ ) had preserved LV systolic and diastolic function, reduced LV fibrosis, hypertrophy and inflammation, and improved survival compared to WT mice. Furthermore T cell depletion in WT mice after TAC prevented HF.

**Conclusions**—T cells are major contributors to non-ischemic HF. Their activation combined with the activation of the LV endothelium results in LV T cell infiltration negatively contributing to HF progression through mechanisms involving cytokine release and induction of cardiac fibrosis and hypertrophy. Reduction of T cell infiltration is thus identified as a novel translational target in HF.

### Keywords

heart failure; T cells; cell adhesion; left ventricle; inflammation; remodeling

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Heart failure (HF) is still a leading cause of morbidity and mortality, affecting more than 24 million people worldwide.<sup>1,2</sup> It is a very complex syndrome involving the interplay of

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### Disclosures

None.

myocardial factors, inflammation, renal dysfunction, and neurohormonal activation.<sup>3, 4</sup> Based on the observations that patients with HF have increased circulating pro-inflammatory cytokines correlating with disease stage and mortality,<sup>5-9</sup> clinical trials were launched targeting inflammatory mediators such TNF $\alpha$  with infliximab and etanercept. These trials were deemed unsuccessful and were terminated prematurely due to lack of both improved survival and hospitalization rate.<sup>8, 9</sup> The failure of these particular anti-inflammatory agents despite the known activation of the immune system in HF, underscores the importance of better understanding the specific inflammatory mechanisms contributing to the development of left ventricular (LV) remodeling and HF progression.

To date, most of the immune mechanisms studied in HF have focused on the activation of the immune system in response to classical immune triggers such as infectious or autoimmune myocarditis that induce an adaptive immune response mediated by CD4<sup>+</sup> T lymphocytes, or in response to ischemic heart injury provoked by myocardial infarction (MI).<sup>10</sup> In humans, recent studies demonstrate a positive correlation between inflammatory cytokines potentially produced by T cells and LV dysfunction in patients with chronic ischemic HF or with idiopathic dilated cardiomyopathy.<sup>11</sup> Whether this takes place systemically or as a result of T cell infiltration in the heart is still unclear, but, at least in the settings of infection, heart transplantation, myocardial infarction causing heart cell death and when self-tolerance to heart antigens is disrupted in autoimmunity, T cells can infiltrate the heart, which is normally devoid of T cells, and negatively affect cardiac function.<sup>10, 12-14</sup> However, the role T cells play in the development of LV hypertrophy, remodeling, and dysfunction in response to more common pathologies, such as occurs with HF, has only recently begun to be investigated, with recent evidence that CD4<sup>+</sup> T cells promote cardiac remodeling in HF in mice.<sup>15</sup> These recent findings raise additional questions including the mechanisms involved in attracting T cells to the heart, the role they play once in the heart, as well as whether T cells can be a novel translational target for potential therapeutic intervention. In addition, the role of T cells in non-ischemic HF in humans has not been explored. Therefore, better insights into the kinetics of T cell recruitment during the progression of HF, the mechanisms mediating T cell recruitment and the impact they have on cardiac remodeling during HF is warranted. While systemic inflammation and the T cell mediated immune response have recently been associated with the prognosis of HF,<sup>16, 17</sup> whether T cells respond to pressure overload (PO) and develop high affinity for the vascular endothelium as a mechanism that facilitates their infiltration in the heart remains unknown.

In our current investigation, we tested the hypothesis that T cell mediated immune responses and their recruitment into the heart influence cardiac remodeling and contribute to the pathogenesis of HF. Our study demonstrates that human and mouse T cell recruitment into the LV negatively contributes to the pathophysiology of HF. It also highlights the potential of depleting T cells from the circulation over the course of HF to ameliorate cardiac fibrosis associated with HF.

## Methods

### Human Subjects

Blood Samples: from non-ischemic human subjects with NYHA Class III-IV heart failure referred for cardiac catheterization as part of their evaluation for advanced heart failure therapy (n=5); From healthy, non-heart failure volunteers (males 30-45 years of age). Viable LV free wall tissue control (n=3) from the National Disease Research Interchange (NDRI), and from end stage heart failure subjects after LV assisted device (LVAD) support (n=3). The studies were approved by Tufts University Institutional Review Board and all subjects gave informed consent.

### Mice

Bred and maintained under pathogen-free conditions. All protocols were approved by the Tufts Medical Center Institutional Animal Care and Use Committee. Mice were sacrificed at 10-14 wk of age for tissue collection.

### Mouse model of Transverse Aortic Constriction (TAC)

Pressure overload induced HF was produced by constricting the transverse aorta as previously described.<sup>18, 19</sup>

### In vivo transthoracic Echocardiography

Assessed in conscious mice as previously described.<sup>19</sup>. The analysis was performed blinded. M Mode and 2-dimensional images were obtained from the short-axis view, as described previously.<sup>18</sup>

### In vivo Hemodynamics

LV function was assessed by pressure volume (PV) transducing catheter as previously described.<sup>19</sup> Absolute volume was calibrated by the saline injection parallel conductance method as described<sup>19</sup> and data were assessed at steady state. Data were digitized and analyzed with custom software (EMKA version 2.1.10).

**Flow Cytometry** was performed to analyze the immune profile present in heart failure. The data were acquired on a FACSCanto (Becton Dickinson) and analyzed using FlowJo software.

### Histological analysis

Heart samples were excised and LV separated from the Right Ventricle (RV). 1/3 of LV was immediately embedded in OCT and 1/3 fixed in 10% formalin, embedded in paraffin and cut into 5µm sections. Haematoxylin and eosin or picosirius red staining was performed as described.<sup>20</sup> Cardiomyocyte cross sectional area was quantified by tracing the outline of 5-12 myocytes in each section.<sup>21</sup>

### ***In vivo* T cell depletion**

WT C57BL/6 mice were treated i.p. with 300µg/ml of monoclonal αCD3 antibody (BioXcell, West Lebanon, NH) or isotype-matched control mAb starting at 48 hours post surgery and then every 3<sup>rd</sup> day for 4 weeks.

### **Real-time Quantitative Polymerase Chain Reaction (qRT-PCR)**

Total RNA was extracted from mouse heart LV tissues directly using Trizol (Invitrogen). RNA was then reverse-transcribed using the ThermoScript RT-PCR system according the manufacturer's instructions (Invitrogen), and amplified by real-time PCR with SYBR green PCR mix (Applied Biosystems). Samples were quantified in triplicates using 40 cycles performed at 94°C for 30 sec, 60°C for 45 sec, 72°C for 45 sec using an ABI Prism® 7900 Sequence Detection System.

### **Endothelial cell culture**

Human umbilical vein endothelial cells (HUVEC) were isolated and cultured as described.<sup>22</sup> Confluent HUVEC monolayers on fibronectin-coated glass coverslips were stimulated with TNF-α (25ng/ml) for 4 hours before the adhesion assays. Mouse heart endothelial cells (MHEC) were isolated from hearts of newborn C57/BL6 (WT) animals 7-9 days old as described<sup>23</sup>, and also plated on fibronectin-coated glass coverslips and stimulated with TNF-α 4h before the T cell adhesion assay.

### **Videomicroscopy image acquisition and analysis**

T cell interactions with MHEC or HUVECs were observed by videomicroscopy under defined laminar flow conditions in a parallel plate apparatus.<sup>24, 25</sup> T cell interactions with confluent TNF-α activated MHECs or HUVECs grown on glass coverslips observed at 20X magnification. Data was recorded and analyzed using the Nikon Elements Software (NES). Adhesion of T cells on activated endothelial cells was quantified in 6 fields of view per condition.

### **Statistics**

Data are expressed as the mean ± SD unless otherwise indicated. Statistical analyses between two groups were done by student *t* test and Mann Whitney non-parametric test to adjust for non-equal Gaussian distributions among groups. Intergroup comparisons were done by 2-way ANOVA and Bonferroni post-test to adjust for the multiple comparisons. Kaplan Meier analysis with log-rank testing was used for survival analysis. Differences were considered statistically significant at *p*< 0.05 and are indicated with an (\*). Graph Pad Prism software was used in all analysis.

## **Results**

### **T cells from humans and mice with heart failure have high affinity for the activated vascular endothelium and are recruited into the heart's left ventricle**

We used a real time videomicroscopy approach that mimics physiological shear flow conditions in small capillaries and venules to study the ability of CD3<sup>+</sup> T cells from patients

with Class III-IV non-ischemic HF to interact with activated vascular endothelial cells. T cells from HF patients adhered to activated endothelial cells in significantly higher numbers than T cells from non-HF volunteers (Figure 1A and 1B). CD3<sup>+</sup> T cells also infiltrated the LV of patients with non-ischemic end stage HF (Figure 1C), and this was associated with significant cardiac hypertrophy and fibrosis, and the presence of vesiculated pyramids as indicators of pathologically hypertrophied myocytes (Figure 1D-1G). Consistent with the literature, systemic T cells were found to be elevated in patients with HF and were activated based on their ability to differentiate into various T cell subsets compared to control patients (Supplemental Figure 1A-1D). To study this phenomenon further, we evaluated whether T cells responded to PO induced HF using transverse aortic constriction (TAC) induced HF in mice. Consistent with our human data, we observed that CD4<sup>+</sup> T cells isolated from lymph nodes and spleen 4 weeks after TAC adhered to activated mouse heart endothelial cells (MHEC) in significantly higher numbers than those from sham operated controls (Figure 2A and 2B). TAC induced LV recruitment of both CD4<sup>+</sup> and CD8<sup>+</sup> T cells (Figure 2C-2E), whereas Tbet and ROR $\gamma$ T, the signature transcription factors of T helper (Th) type 1 and Th type 17 cells, respectively, were significantly upregulated in the LV, in contrast to Foxp3, the signature transcription factor for Treg cells which remained unchanged between Sham and TAC mice (Figure 2F). Furthermore, the cytokines TNF $\alpha$ , IL1 $\beta$  and IL6, and the Th1 signature cytokine IFN $\gamma$  were significantly upregulated in TAC vs Sham mice (Figure 2G). In addition, TAC resulted in upregulation of RNA of the endothelial cell adhesion molecules, VCAM-1, E-selectin and ICAM-1 (Figure 2H-2J), and significantly enhanced ICAM-1 protein expression in the endothelial cells of the LV intramyocardial vessels (Figure 2K). Taken together these findings demonstrate that PO in mice and humans activates T cells which become pro-adhesive and more prone to interact with the activated vascular endothelium *in vitro*. Moreover, *in vivo*, TAC promotes upregulation of ICAM-1 and other endothelial cell adhesion molecules that can lead to T cell recruitment, and induces pro-inflammatory cytokine release that can further promote T cell survival and subsequent endothelial cell activation.

### **T cell recruitment into the LV is associated with the progression of HF in response to TAC**

We next investigated the time course of LV T cell infiltration after TAC using quantitative flow cytometry to determine whether it was directly associated with the progression of HF. Forty eight hours after TAC, we did not detect significant T cell infiltration in the LV, and at this time point, LV weight and LV systolic function remained similar between TAC and sham controls. Infiltration of T cells to the LV occurred during the development of cardiac remodeling and systolic dysfunction 2 and 4 weeks after TAC (Figure 3A-3C), peaking at 4 weeks. Further analysis revealed that Gr1<sup>+</sup> neutrophils and F4/80<sup>+</sup> macrophages did not follow the same dynamics as T cells (Figure 3D, 3E and 3I). 4 weeks after TAC there was notable increase in infiltrated CD45.2<sup>+</sup> leukocytes (Figure 3F), and these included T cells and CD11b<sup>+</sup> myeloid cells (Figure 3G, 3H and 3I). From our immunohistochemistry studies these T cells included CD4<sup>+</sup> and CD8<sup>+</sup>, possibly IFN $\gamma$  producers given the increased LV levels of IFN $\gamma$  in response to TAC observed in Figure 2. Moreover, we isolated the mediastinal lymph nodes of both Sham and TAC mice and found that it not only was significantly enlarged in TAC mice as compared to Sham (data not shown), but it indeed had more activated T cells that had therefore lost the expression of L-selectin (Figure 3J).

Importantly, we were able to determine the levels of circulating T cells and Th cell subsets and found that these were increased in response to TAC (Supplemental Figure 2), similarly to what we observed in patients with HF (Supplemental Figure 1). Taken together, these data support the idea that T cells respond to cardiac specific damage in the mediastinal lymph nodes, become activated and are recruited to the LV. This recruitment correlates with the development of pathological cardiac remodeling and worsening of systolic function.

### **T cells are major contributors to the pathogenesis of HF**

Based on our findings that T cell recruitment into the LV correlates with adverse remodeling, we next assessed the functional necessity of T cells to promote HF. We specifically tested the response to LV PO in mice deficient in T cells harboring a genetic deletion of the alpha chain of the T cell receptor ( $TCR\alpha^{-/-}$ ). Echocardiography analysis 4 weeks post TAC showed that in contrast to WT mice,  $TCR\alpha^{-/-}$  mice had normal LV dimensions (Figure 4A), preserved systolic function, measured by fractional shortening (FS) (Figure 4B), and preserved LV end diastolic diameter (LVEDD) (Table). This was consistent with invasive hemodynamic measurements revealing that despite similar aortic pressure gradients generated in response to 4 weeks TAC in WT and  $TCR\alpha^{-/-}$  mice (Figure 4C), the stroke volume, end diastolic pressure, and systolic and diastolic functions were all preserved in  $TCR\alpha^{-/-}$  mice (Figure 4D-4G and Table). Intriguingly, of all the mice that underwent TAC surgery, thirty percent of the WT TAC mice died by 4 weeks, whereas no deaths occurred in  $TCR\alpha^{-/-}$  mice (Figure 4H). As expected, the LV of  $TCR\alpha^{-/-}$  mice was free of recruited T cells. Moreover,  $Gr1^{+}$  neutrophils were found recruited in the LV in similar numbers in sham and TAC mice in both WT and  $TCR\alpha^{-/-}$  mice (Figure 4I). In addition, TAC did not induce significant upregulation of endothelial cell adhesion molecules, including ICAM-1, or upregulation of pro-inflammatory cytokines in  $TCR\alpha^{-/-}$  mice (Supplemental Figure 3).  $ROR\gamma T$  and Tbet were also significantly decreased as compared to WT, suggesting that most of the Tbet expression observed in WT mice (Figure 2 and Supplemental Figure 3I, 3J) may be attributed to Th1 cells. Taken together, our results support a role for T cell recruitment to the LV being critical for the observed cardiac dysfunction in response to TAC. The absence of T cells results in a suppressed pro-inflammatory state within the LV, and as such, the deleterious actions of cytokines TNF $\alpha$  and IFN $\gamma$  on cardiac resident cells and endothelial cell activation are prevented. Thus, subsequent immune cell recruitment is avoided and LV function is protected from any negative impact mediated by T cells. This could potentially explain why  $TCR\alpha^{-/-}$  do not develop HF.

### **T cells regulate cardiac hypertrophy and fibrosis in response to pressure overload**

To study the T cell dependent mechanisms leading to improved cardiac function and survival in  $TCR\alpha^{-/-}$  mice, we initially evaluated cardiac hypertrophy in WT and  $TCR\alpha^{-/-}$  mice in response to TAC. As expected, the gross LV weight normalized to tibia length was increased in WT mice in response to 4 weeks TAC. However, the LV weight and size of  $TCR\alpha^{-/-}$  mice was similar between sham and TAC mice, and significantly decreased in  $TCR\alpha^{-/-}$  mice vs WT mice in response to TAC (Figure 5A and 5B). We further analyzed by H&E the cardiomyocyte cross sectional area (CSA) and found that while cardiac myocytes from WT mice have increased CSA in response to TAC, those from  $TCR\alpha^{-/-}$  mice remain

similar in area to the cardiac myocytes from TCR $\alpha^{-/-}$  sham controls (Figure 5C and 5D). Several studies have demonstrated that atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP) are elevated in serum of HF patients; these two molecules are considered serum markers of HF in response to pressure and volume overload, and their increased transcription in LV tissue represents a phenotype of pathologic, rather than physiologic, hypertrophy.<sup>25</sup> Therefore we evaluated the levels of the natriuretic peptides by qRT-PCR using LV tissues and revealed that both BNP and ANP are elevated in WT but not in TCR $\alpha^{-/-}$  in response to TAC (Figure 5E and 5F). We also investigated whether TCR $\alpha^{-/-}$  mice developed cardiac fibrosis, which is intimately associated with LV systolic dysfunction and HF pathogenesis. TAC induced an expected increase in LV fibrosis determined by collagen deposition detected by histology in WT mice. However, LV fibrosis was completely absent in TCR $\alpha^{-/-}$  mice (Figure 6A and 6B). Markers of fibrosis such as TGF- $\beta$ , Collagen type I and alpha smooth muscle actin ( $\alpha$ SMA) were also elevated in WT mice in response to TAC, but not in TCR $\alpha^{-/-}$  mice (Figure 6C-6E). Taken together, these data directly associate the absence of T cells with the absence of cardiac hypertrophy and fibrosis and positions T cells as regulators of these processes.

### T cell depletion after TAC prevents cardiac fibrosis and LV dysfunction

Because of the above observations that genetic depletion of T cells inhibits pathologic remodeling and the HF phenotype, we hypothesized that depletion of T cells in the onset of LV PO prevents pathological cardiac remodeling and may represent a potential novel therapeutic target. We tested whether administration of anti-CD3 antibody could effectively reduce T cells in the setting of LV PO. We therefore depleted WT mice of T cells beginning 48 hours post TAC surgery, when we demonstrate that the mice are responding to TAC but T cells have not yet infiltrated the LV (as shown in Figure 3) (Figure 7A). Approximately 95% of CD3<sup>+</sup> T cells were depleted compared to the isotype control IgG treated animals in both sham and TAC mice after 4 weeks (Figure 7B and 7C). Likewise, CD4<sup>+</sup> T cells were also depleted using the anti-CD3 antibody compared to the control treated mice (Figure 7D and 7E). While IgG antibody control treated mice had CD4<sup>+</sup> T cells infiltrated in the heart in response to TAC, anti-CD3 treatment resulted in absent LV T cell infiltration (Figure 7F). TAC treated mice depleted of CD3<sup>+</sup> T cells also demonstrated absence of cardiac fibrosis, compared to isotype control IgG treated mice (Figure 7G and 7H). Furthermore, TAC anti-CD3 treated mice had increased % fractional shortening in contrast to isotype antibody control TAC treated mice (Figure 7I). While the fibrosis and echocardiography data mimicked the phenotype observed for TCR $\alpha^{-/-}$  mice, WT mice depleted of T cells still developed cardiac hypertrophy (Figure 7J-7L). These data demonstrate the feasibility of reducing T cells in the setting of TAC and suggest a robust anti-fibrotic effect of T cell depletion in the LV that results in improved cardiac function despite the increase in LV hypertrophy.

## Discussion

Our findings in the present study reveal several aspects identifying T cell recruitment into the LV as a negative contributor to pathological cardiac remodeling in HF. Our observations using human T cells and LV tissue from non-ischemic end-stage HF patients indicate

systemic T cell activation, enhanced T cell adhesion to activated vascular endothelial cells under physiological flow conditions in vitro, and T cell LV infiltration, directly correlating with LV myocyte hypertrophy and fibrosis. We obtained similar results using WT mice undergoing PO induced HF, established for the first time the kinetics of LV T cell infiltration, and further characterized T cell activation, LV endothelial cell activation and LV T cell infiltration. Our studies using TCR $\alpha^{-/-}$  mice together with the successful pharmacological depletion of T cells in the setting of LV PO are in support of a role for T cells in PO induced HF as both T cell depletion approaches resulted in significantly attenuated LV fibrosis and improved LV function and HF. Our findings support a model in which LV recruitment of activated T cells in the setting of PO contributes to myocardial dysfunction, remodeling, and ultimately HF. Our findings also suggest that pharmacologic down-regulation of T cell recruitment in the LV may represent a novel strategy for the prevention and potential treatment of HF in humans.

### **Combination of T cell and intramyocardial endothelial cell activation in response to PO lead to LV T cell recruitment and HF**

There is very recent growing awareness that humans with non- ischemic and non- infectious induced HF present chronic systemic inflammation potentially mediated by T cells<sup>26, 27</sup>. T cells are also found infiltrated in the heart in both humans and experimental mouse models in response to classical triggers of the T cell mediated immune response<sup>28</sup>. Intriguingly the mechanisms of action of the T cells and whether they play a role systemically, in the heart, or in both is poorly understood to date. We demonstrate for the first time that human T cells from non-ischemic HF are activated and predisposed to interact with activated vascular endothelial cells in vitro, and infiltrate the human LV in vivo. Our studies in WT mice responding to PO support this concept because naïve non-activated T cells do not have the properly modified adhesion ligands to roll and arrest on the heart vascular endothelium, as we and others have previously described<sup>23, 29<sup>30</sup></sup>. Furthermore, we demonstrate that T cell activation occurs in the mediastinal lymph nodes in response to cardiac antigens, as the lymph node size was significantly increased in response to TAC (data not shown) and a high percent of the T cells present had lost the expression of L-selectin. Moreover, both mice and humans with non ischemic HF had higher numbers of blood circulating T cells and effector T cell subsets, although the nature of the antigens inducing this T cell response remains unknown. The possibility that peptide antigens resulting from cardiac cell death trigger this response as it has been suggested in response to myocardial infarction<sup>31</sup> is unlikely, given that TAC does not induce cardiac apoptosis significantly until 8 weeks post banding<sup>32</sup>, and T cells infiltrate the heart as early as 2 weeks post TAC. We speculate that PO disrupts the immune cell tolerance taking place in the heart allowing self-cardiac antigens to induce a T cell immune response, as it has been described in autoimmune myocarditis.<sup>33</sup> These antigens may include alpha-myosin, which is not expressed in the thymus and therefore can impact immune tolerance two ways: not being negatively selected against by clonal deletion<sup>34</sup> and not being able to positively select alpha- myosin specific natural T regulatory cells. However, this requires further investigation.

Our data indicate that in addition to T cells, CD11b<sup>+</sup> myeloid cells infiltrate the LV after 4 weeks of TAC. This is in line with other models of PO that demonstrate macrophage



infiltration in the heart<sup>35, 36</sup>, however, the similar infiltration of F480<sup>+</sup> macrophages observed in Sham and TAC in our studies suggest these macrophages are not activated. Because endothelial ICAM-1 is significantly upregulated in response to PO, it is possible that T cells and CD11b<sup>+</sup> cells use this pathway to infiltrate the LV. How endothelial ICAM-1 becomes upregulated in response to PO remains under investigation, but one likely possibility is that PO induces cytokine production by cardiac resident cells and these activate the endothelial cells and promote subsequent T cell recruitment<sup>7, 37, 38</sup>. We interestingly identified significant upregulation of the T helper type 1 (Th1) signature cytokine, IFN $\gamma$ , and transcription factor, Tbet. Although these are not exclusively expressed on Th1 cells<sup>39</sup>, the fact that these are not upregulated in T cell deficient mice (TCR $\alpha^{-/-}$ ) suggests that Th1 cells are a major subset of T cells infiltrated in the LV and can alter cardiac myocyte and fibroblast function via IFN $\gamma$ <sup>40</sup>. Further studies will be needed to establish a definitive role for specific T cell subsets in the LV and whether once infiltrated in the LV can further activate heart endothelial cells in vivo, similarly to what we previously described in vitro<sup>41, 42</sup>. Collectively our results support a mechanism in which PO acts two ways: by exposing self-cardiac antigens that result in T cell activation, and by inducing cytokine release in cardiac resident cells that lead to endothelial activation. These are both required for optimal T cell recruitment to the LV, where T cell cytokine secretion of TNF $\alpha$  and IFN $\gamma$  could contribute to cardiac dysfunction.

### Functional necessity of T cells in cardiac remodeling and HF

Whether such a small number of activated T cells infiltrated in the LV can have an impact in cardiac function was evaluated and confirmed in our studies with TCR $\alpha^{-/-}$  mice, which showed preserved adverse cardiac remodeling, including cardiac hypertrophy, cardiac fibrosis and cardiac function in response to TAC. Our data are in agreement with recent data published during the preparation of our manuscript using a different genetic depletion of T cells approach that included the use of recombination activating gene 2 (Rag2 $^{-/-}$ ) mice and the major histocompatibility complex II (MHCII $^{-/-}$ ) mice, both showing blunted adverse cardiac remodeling and preserved ventricular function in response to 6 week TAC. The authors demonstrated that systemic activation of CD4<sup>+</sup> T cells in the draining mediastinal lymph nodes is critical for the T cell immune response observed in response to TAC<sup>15</sup>. Our present study using a different strain of T cell deficient mice, TCR $\alpha^{-/-}$ , and 4 weeks TAC, not only confirms a role for T cells in adverse remodeling and HF, but demonstrate for the first time that T cell infiltration specifically in the left ventricle (LV) of the heart occurs in WT mice as early as 2 weeks post TAC, suggesting a much earlier activation of T cells contributing to the progression of HF. Unlike the previous study, we have also investigated invasive LV hemodynamics in TCR $\alpha^{-/-}$  mice and importantly implicated T cells in diastolic HF, a condition for which there are no current treatments available. Moreover, the protective systolic and diastolic HF phenotype resulted in 100% survival in TCR $\alpha$ . Together with the lack of an LV pro-inflammatory environment observed in these mice, these studies support a mechanism in which cytokine production by infiltrated activated T cells have a negative impact in LV function and survival to TAC.

## Genetic vs pharmacological T cell depletion effects on cardiac fibrosis and hypertrophy

Genetic depletion of T cells using different approaches coincide with the better outcome for HF induced by PO therefore placing T cells as central regulators of pathological cardiac remodeling. It is noteworthy that genetic depletion of T cells can somehow affect other immune subsets during development, as T cells are required by other cells in the immune system to perform their functions effectively<sup>43</sup>. Also, in these genetically modified mice strains, T cell depletion occurs before the onset of PO and resultant ventricular dysfunction, and is thus less directly therapeutically applicable to patients with preexisting heart failure. To circumvent these limitations, we, for the first time, successfully achieved T cell depletion selectively in the setting of TAC. Our data strongly supports a role for T cells in cardiac fibrosis and function induced by TAC that is independent of any whole body genetic modification. Furthermore, it strengthens the idea that targeting T cells after the stimulus for HF has been induced is beneficial in HF prognosis potentially by preventing T cell LV infiltration. While using the genetic and pharmacological depletion approach we recapitulate the finding that T cells can regulate fibrosis in a variety of tissues, more intriguing is the striking observation that the lack of T cells in  $TCR\alpha^{-/-}$  abrogates the hypertrophic response. This phenomenon has been observed in other deficient mice<sup>44-46</sup> including T cell deficient mice<sup>15</sup>, but it is not observed when we pharmacologically deplete T cells in the onset of HF. One could speculate that the remaining T cells in WT mice after depletion can secrete pro-hypertrophic mediators such as Ang II<sup>47</sup> and possibly mediate physiological hypertrophy mechanisms. These mechanisms require further investigation.

## Conclusions and limitations

Our combined observations in humans and mice provide evidence that T cells and their recruitment to the LV are critical regulators of pathological cardiac remodeling in HF in the setting of PO. Our observations are in line with the recently proposed changing paradigm suggesting that certain types of HF occur in novel cell compartments such as the intramyocardial vascular endothelium and the recruited immune cells<sup>48</sup>. One limitation of the human studies is that most non ischemic HF patients take  $\beta$ -adrenergic signal blockers which can affect T cell activation,<sup>49</sup> also affected by hypertension<sup>50</sup>. However, our mouse data corroborates the human data, and the  $TCR\alpha^{-/-}$  mice have similar basal systolic pressure as WT, as assessed by aortic blood pressures in sham mice. Collectively, our data contribute to a broader understanding of the novel role T cells play in cardiac remodeling and highlight that pharmacological T cells depletion prevents HF and may serve as a potential strategy to improve the structural, functional and molecular deficits of the failing heart.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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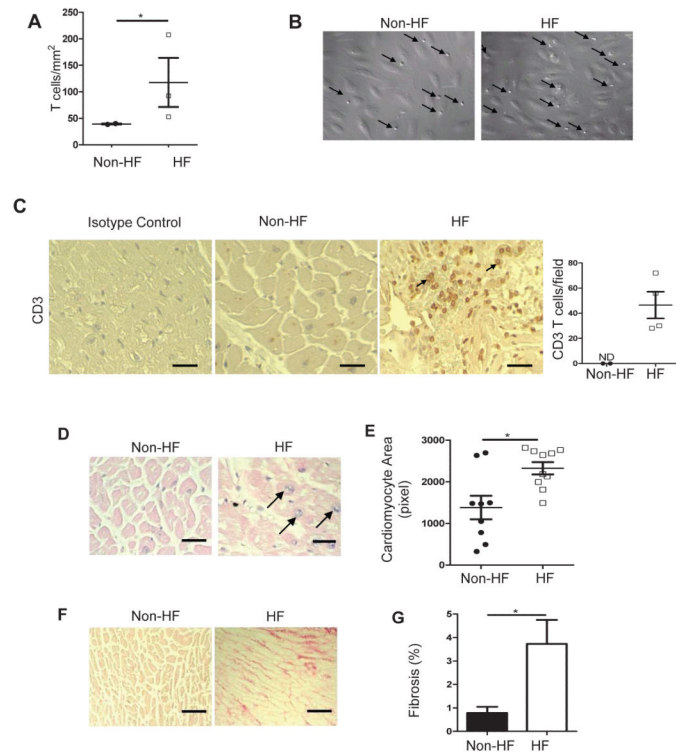
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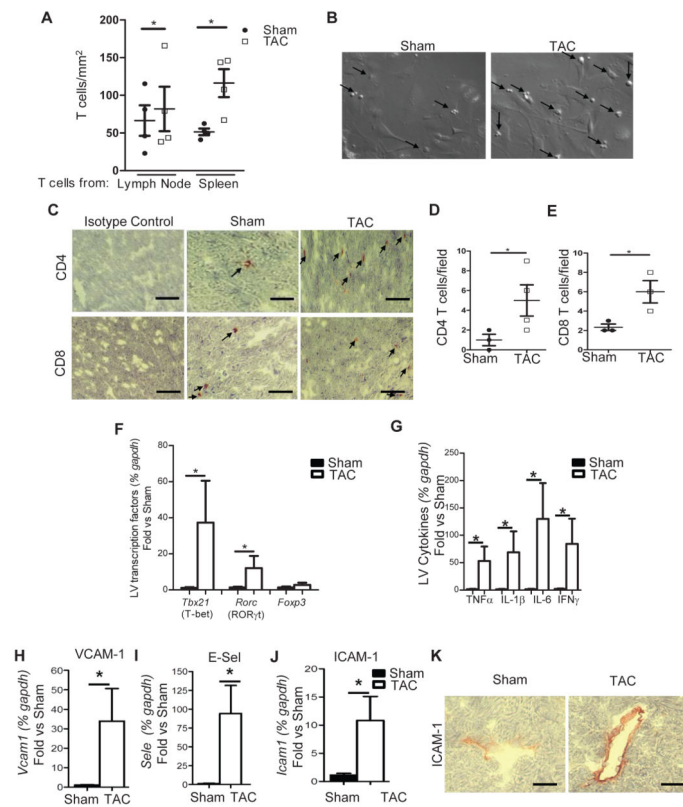
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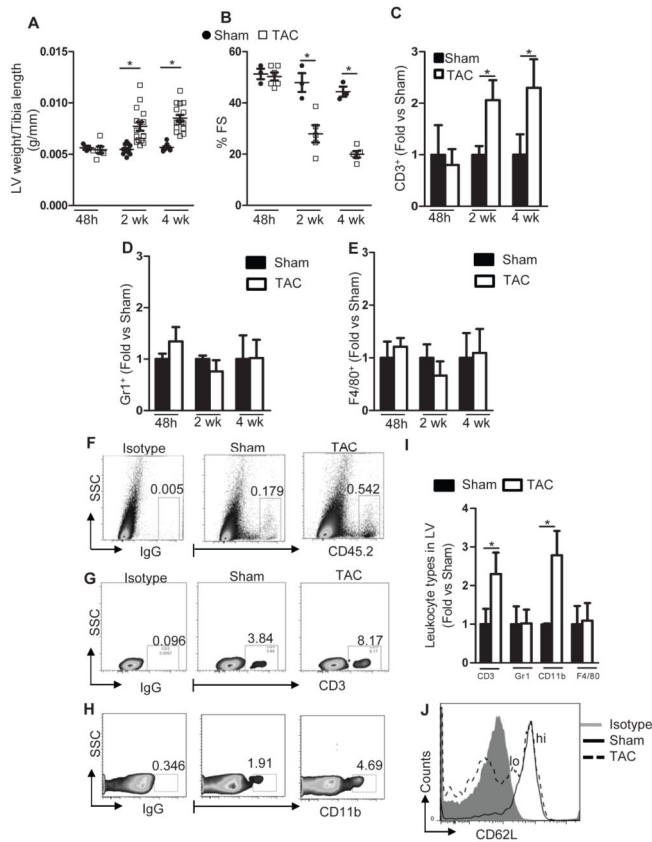
**Figure 1. T cell activation, adhesion to activated vascular endothelial cells and infiltration into the LV in humans**

(A, B) CD3<sup>+</sup> T cells isolated from healthy volunteers or from patients with NYHA-III-IV HF were drawn in a flow chamber across TNF $\alpha$  (25ng/ml) activated HUVEC at a shear stress of 0.8 dynes/cm<sup>2</sup>. Data represents mean  $\pm$  SD of 2 independent experiments using 2 different control and 3 different heart failure samples. Representative images from the videos are shown and arrows point at T cells arrested on the vascular endothelium (B). (C) Non-heart failure (Non-HF) and end stage HF LV tissues were obtained from NDRI and LVAD placements, respectively, and sections were stained for CD3 or isotype control. The arrows point at two representative infiltrated CD3<sup>+</sup> T cells and the quantitative analysis is shown (right). (D, E) Representative H&E staining (D) and cardiac myocyte size quantification (E) of LV sections from non-HF and end stage HF. Arrows point to vesiculated pyramids, standard histological markers of hypertrophic myocytes. (F, G) Representative photomicrographs (F) and quantification (G) of myocardial fibrosis evaluated by picrosirius red staining of LV sections. Data represented as mean  $\pm$  SEM; \*p<0.05. Scale bars, 500 $\mu$ m (C, D and F).



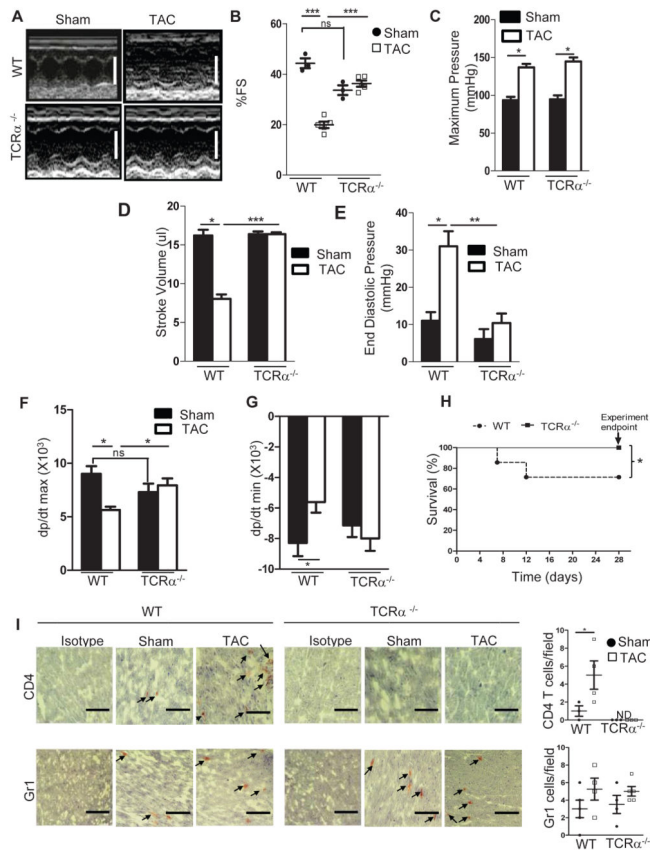
**Figure 2. TAC induces T cell adhesion to mouse heart endothelial cells in vitro and endothelial and T cell activation and recruitment to the heart in vivo**  
**(A, B)** CD4<sup>+</sup> T cells from lymph nodes and spleen isolated from 4 weeks TAC mice or sham surgery were drawn across TNF $\alpha$  (100ng/ml) activated mouse heart endothelial cells at a shear stress of 0.8 dynes/cm<sup>2</sup>. Data represents mean  $\pm$  SD of 4 independent adhesion experiments, each with a pool of T cells from n=3 sham and n=3-5 TAC, and 4 independent MHEC preparations. Data represents the average of 3-6 different fields of view for each condition in the different experiments **(B)**. Representative frames from videos with arrows pointing at T cells arrested on MHEC. **(C-E)** LV tissue sections from 4 week sham or TAC mice were stained for CD4<sup>+</sup> and CD8<sup>+</sup> T cells. **(C)** Representative immunohistochemistry and **(D, E)** quantitative analysis of CD4 and CD8 staining. **(F-J)** RNA expression for T cell subset transcription factors **(F)**, inflammatory cytokines **(G)** and adhesion molecules **(H-J)** in the LV of 4 week TAC WT mice. n=4-6 shams and n=6-10 TAC per group. **(K)** Representative immunohistochemistry staining of ICAM-1 in the LV of 4 weeks sham and TAC WT mice. Data represented as mean  $\pm$ SD; \*p<0.05. Scale bars, 500 $\mu$ m **(C, K)**.



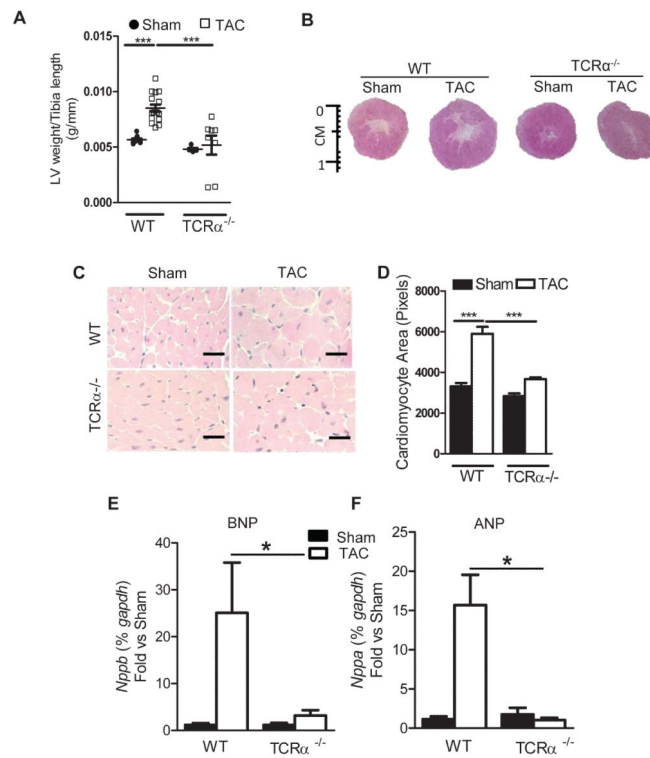


**Figure 3. Time course of T cell recruitment into the LV as cardiac remodeling and systolic function develop in PO induced heart failure**

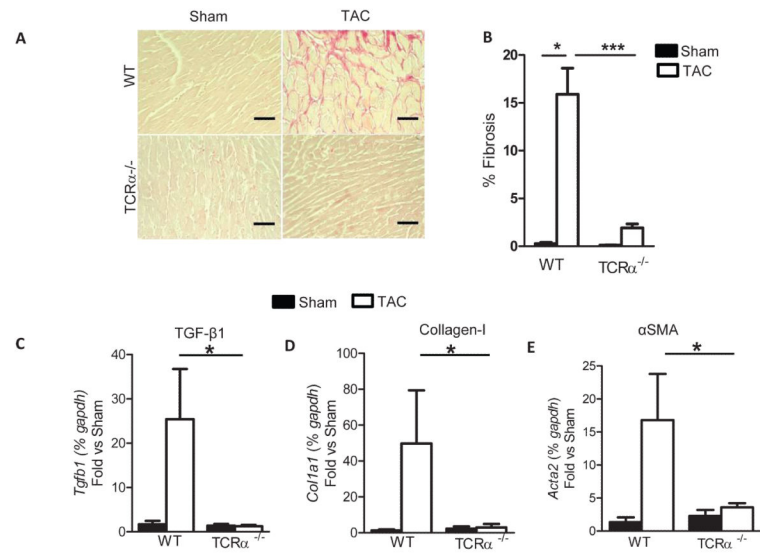
WT mice were subjected to sham (n=3-11) or TAC (n= 5-16) surgery and sacrificed at the indicated times. (A) LV weight normalized to tibia length. (B) Systolic function measured by echocardiography. (C-E) Quantification of LV T cell (C), neutrophil (D) and F4/80<sup>+</sup> macrophage (E) infiltration by FACS during the time course of TAC. (F-H) Representative FACS dot plot for total leukocytes (F) CD3 T cells (G) and CD11b<sup>+</sup> myeloid cells (H) at 4 weeks post TAC. Numbers represent the percent positive in the indicated gate. (I) Leukocyte expression of CD3, Gr1, CD11b and F4/80 positive cells in the LV 4 weeks after sham or TAC mice. (J) Representative FACS histogram of CD62L expression in the mediastinal lymph nodes in sham and TAC mice 4 weeks post TAC surgery. Data represent mean  $\pm$  SD; \*p<0.05.



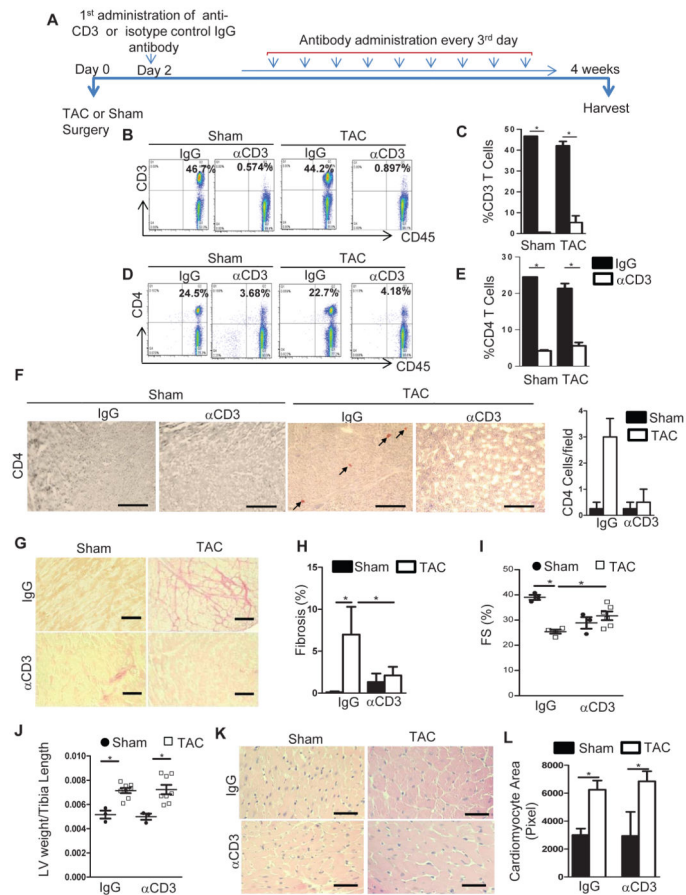
**Figure 4. Preserved cardiac function and survival to PO induced heart failure in TCRα<sup>-/-</sup> mice** (A, B) Representative echocardiograms, white scale bars, 4mm (A) and quantification of fractional shortening (% FS) (B) 4 weeks after sham or TAC surgery in WT and TCRα<sup>-/-</sup> mice (n=3 Sham, and n=5 TAC). (C-G) Invasive hemodynamic measurements in WT and TCRα<sup>-/-</sup> mice measured 4 weeks after sham and TAC surgery (n=3 sham, and n=7 TAC). (H) Kaplan-Meier survival curves in WT (n=19) and TCRα<sup>-/-</sup> (n=10) mice after TAC. (I) Representative immunohistochemical staining of 4 week TAC LV sections for T cells (CD4<sup>+</sup>) and neutrophils (Gr1<sup>+</sup>). Quantification (right) of positive cells/field for n=3-5 mice. Data represent mean ±SD; \*p<0.05; \*\*p<0.005; \*\*\*p<0.0005, ns, not significant Scale bars, 500µm (I).



**Figure 5. Absence of LV hypertrophy in response to PO induced heart failure in TCRα<sup>-/-</sup> mice** (A) LV weight, normalized to tibia length in 4 week sham or TAC mice (WT n=6-11 mice for sham and 11-16 TAC mice; TCRα<sup>-/-</sup> n=5 sham and n=8 TAC). (B) Representative pictures from LV cross sections of WT and TCRα<sup>-/-</sup> mice 4 weeks post sham or TAC surgery. (C) Representative H&E staining of LV sections from mice 4 weeks after sham or TAC surgery (D) Quantification of cardiac myocyte area (WT mice n=4 Sham and n=6 TAC; TCRα<sup>-/-</sup> mice n=4 Sham and n=7 TAC). (E, F) qPCR for hypertrophy markers BNP and ANP. n=5 sham and n=7 TAC mice. Data represented as mean ± SD. \*=p<0.01, \*\*\*=p<0.0001. Scale bars, 500μm (C).



**Figure 6. Absence of LV fibrosis in response to PO induced heart failure in TCR $\alpha^{-/-}$  mice** (A) Representative photomicrographs and (B) quantification of myocardial fibrosis evaluated by picrosirius red staining of LV sections from 4 week sham and TAC mice (WT: n=3-5 sham and 6 mice TAC; TCR $\alpha^{-/-}$  n=4 sham and n=8 TAC). (C-E) qPCR for fibrotic markers TGF $\beta$ , Collagen I, and SMA $\alpha$ . Data represent mean  $\pm$ SD; \*p<0.05; \*\*\*p<0.005. Scale bars, 500 $\mu$ m (A).



**Figure 7. T cell depletion in WT mice post TAC surgery results in preserved cardiac fibrosis and improved systolic function**

(A) Diagram of experimental protocol for T cell depletion with αCD3 Ab in WT sham and TAC mice. IgG Ab was used as control. (B, D) Representative FACS dot plots (C, E) and quantification of CD3<sup>+</sup> T cells (B,C) and CD4<sup>+</sup> T cells (D, E) in lymph nodes of 4 week WT sham or TAC mice treated as in A. (F) Representative immunohistochemical staining of CD4<sup>+</sup> T cells and quantification (right) of LV T cells in the indicated groups 4 week post TAC. (G) Representative photomicrographs and (H) quantification of LV fibrosis evaluated by picrosirius red staining of LV sections in the indicated groups. (I) Echocardiography analysis in Sham and TAC WT mice treated with control IgG Ab or depleted of T cells with αCD3 Ab after sham or TAC surgery. (J) LV weight, normalized to tibia length. (K) Representative H&E staining and (L) quantification of cardiac myocyte area of LV sections among the different groups. Data represent mean ±SD; \*p<0.05. Scale bars, 500µm (n=3-4 IgG and n=4-6 αCD3).

**Table**Characterization of Heart Failure in Wild-type and TCR $\alpha^{-/-}$  mice

Parameters	Sham	TAC (4 weeks)
<b>Total Body Weight, g/Tibia length, mm</b>		
WT (n=Sham 11, TAC 16)	1.457 $\pm$ 0.022	1.437 $\pm$ 0.021
TCR $\alpha^{-/-}$ (n=Sham 5, TAC 8)	1.455 $\pm$ 0.032	1.491 $\pm$ 0.044
<b>Total Lung Weight, mg/Tibia length, mm</b>		
WT (n=Sham 11, TAC 16)	0.009 $\pm$ 0.000	0.01230 $\pm$ 0.001*
TCR $\alpha^{-/-}$ (n=Sham 5, TAC 8)	0.009 $\pm$ 0.000	0.009 $\pm$ 0.000 <sup>†</sup>
<b>Heart Rate, bpm</b>		
WT (n=Sham 3, TAC 7)	535 $\pm$ 45	553 $\pm$ 40*
TCR $\alpha^{-/-}$ (n=Sham 3, TAC 7)	494.7 $\pm$ 49.30	525.2 $\pm$ 42.18
<b>LV end-systolic pressure, mmHg</b>		
WT (n=Sham 3, TAC 7)	114 $\pm$ 12	130 $\pm$ 18*
TCR $\alpha^{-/-}$ (n=Sham 3, TAC 7)	99.95 $\pm$ 8.79	142.3 $\pm$ 9.29*
<b>LV end-diastolic diameter, mm</b>		
WT (n=Sham 3, TAC 5)	3.674 $\pm$ 0.121	4.007 $\pm$ 0.115*
TCR $\alpha^{-/-}$ (n=Sham 3, TAC 5)	3.912 $\pm$ 0.048	3.753 $\pm$ 0.277 <sup>†</sup>
<b>Posterior Wall Thickness, mm</b>		
WT (n=Sham 3, TAC 5)	0.9780 $\pm$ 0.151	1.4 $\pm$ 0.3*
TCR $\alpha^{-/-}$ (n=Sham 3, TAC 5)	1.091 $\pm$ 0.149	0.952 $\pm$ 0.133 <sup>†</sup>
<b>Maximum Pressure, mmHg</b>		
WT (n=Sham 3, TAC 7)	93.46 $\pm$ 7.689	136.912 $\pm$ 8.003*
TCR $\alpha^{-/-}$ (n=Sham 3, TAC 7)	94.90 $\pm$ 8.698	144.8 $\pm$ 9.063*

TAC indicates thoracic aortic constriction; WT, wild-type; and LV, Left ventricle

Values are means  $\pm$  SD,

\* P&lt;0.05, 4 weeks of TAC versus Sham.

<sup>†</sup> P<0.05, TAC TCR $\alpha^{-/-}$  versus TAC WT.