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T-lymphocytes mediate left ventricular fibrillar collagen crosslinking and diastolic dysfunction in mice

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Abstract

Aberrant concentrations of cardiac extracellular matrix (ECM) fibrillar collagen crosslinking have been proposed to be an underlying cause of cardiac diastolic dysfunction however the role of the adaptive immune system in this process has yet to be investigated. Fibrillar collagen crosslinking is a product of the enzymatic activities of lysyl oxidase (LOX and LOXL-3) released by the cardiac fibroblast and possibly cardiac myocytes. Our hypothesis is that stimulation of the TH1 lymphocytes activates lysyl oxidase mediated ECM crosslinking and thereby alter left ventricular function. Three-month old C57BL/J female mice were treated with selective TH1 lymphocyte inducers - T-cell receptor V β peptides (TCR). After 6 weeks, candidate gene expression, tissue enzymatic activity, ECM composition, and left ventricular mechanics were quantified. Lymphocyte gene expression and cytokine assay revealed TH1 immune polarization with TCR administration which was associated with a 2.6-fold and 3.1-fold increase of *LOX* and *LOXL3* gene expression, respectively, and a 55% increase in cardiac LOX enzymatic activity. The ECM cross-linked fibrillar collagen increased by 95% when compared with the control. Concurrently, there was a 33% increased ventricular stiffness, decreased cardiac output, and normal ejection fraction. These data implicate the TH1 lymphocyte in the pathogenesis of diastolic dysfunction which has potential clinical application in the pathogenesis of diastolic heart failure.

Keywords

T-lymphocyte; Fibrosis; Lysyl oxidase; Ventricular remodeling; Diastolic function

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Disclosures

None to disclose from any author

1. Introduction

Lysyl oxidases (EC 1.4.3.13, protein-lysine 6-oxidases) are secreted cuproenzymes that catalyze post-translational modification of the extracellular matrix (ECM) connective tissues. Although widely distributed in a variety of cells, the cardiac fibroblast and possibly the cardiac myocytes are the primary sources of lysyl oxidases in the heart. Of the five members of the lysyl oxidase enzymatic proteins that have been described, the two expressed in cardiac tissue are lysyl oxidase (LOX) and lysyl oxidase-like 3 protein (LOXL3)(Csiszar, 2001). The functional enzymatic activity of lysyl oxidase is to induce oxidative deamination of the ϵ -amino group of selected lysine and hydroxylysine residues of ECM proteins resulting in the formation of allysine and hydroxyallysine. This oxidative deamination is followed by spontaneous condensation to di-valent cross-links which may subsequently form tri- and tetra-valent cross-links(Reiser et al., 1992). Transforming growth factor- β (TGF- β), platelet derived growth factor (PDGF), connective tissue growth factor (CTGF), and angiotensin II induce LOX gene expression via interferon regulatory factor 1 (*IRF-1*) transcriptional factor in numerous tissues (Smith-Mungo and Kagan, 1998; Green et al., 1995; Hong et al., 1999; Tan et al., 1996) however no inducers have yet been described in the cardiac fibroblast. Once expressed, LOX is secreted as pro-enzyme which requires proteolytic conversion to the functional enzyme by pro-collagen C-proteinases encoded by products of the *Bmp-1* gene(Uzel et al., 2001). The natural formation of collagen cross-linkages is to stabilize of the fibrous forms of these ECM proteins in most tissues including the heart, and although initially beneficial, we are suggesting that an over abundance becomes pathologic.

The cardiac ECM composition, including the collagen content and percentage of cross-linking, is a contributing factor of diastole function during the filling phase of the cardiac cycle. The disproportionate formation of the ECM collagen cross-linkages parallels ventricular stiffness (Yu et al., 2006; Yu et al., 2005; Badenhurst et al., 2003; Bradshaw et al., 2009; Norton et al., 1997). Conversely, inhibition of LOX mediated cardiac collagen cross-linking with β -aminopropionitrile markedly reduces ventricular stiffness(Kato et al., 1995). In addition, the collagen cross-linkages appear to protect the fibrillar collagen from matrix metalloproteinase (MMP) mediated degradation(van der Slot-Verhoeven AJ et al., 2005). Therefore, altered ECM biomechanical properties and ventricular diastolic mechanics may result from elevated concentrations of cross-linked ECM collagen.

An enhanced immune response has been coupled with ventricular fibrosis. Hypertensive rodent models have supported a relationship among macrophages, mast cells, and T-lymphocytes and ventricular ECM remodeling(Kagitani et al., 2004; Hinglais et al., 1994; Levick et al., 2009; Yu et al., 2005; Yu et al., 2006). These three cell types are functionally inter-dependent however our focus is the T-lymphocyte as it relates to ECM remodeling. Previously, we observed that altered lymphocyte polarity markedly affects diastolic function and the extent of cardiac collagen cross-linking. We found that mice genetically prone to the lymphocyte T-helper subsets TH1, TH2, or null phenotypes markedly differ in cardiac remodeling in responses to chronic hypertension(Yu et al., 2006). We also demonstrated that induction of TH2 lymphocyte phenotype results in a dilated cardiomyopathy and highly compliant left ventricle which was substantially rescued by selective stimulation of TH1 lymphocyte function(Yu et al., 2005). However, what is not evident is whether a dominant CD4 lymphocyte subtype has a direct effect on the cardiac fibroblast lysyl oxidase gene expression, secretion, and enzymatic function. In the present study, we hypothesized that the induction of TH1 lymphocytic function upregulates lysyl oxidase gene expression and activity and collagen crosslinking; thereby altering cardiac diastolic function.

2. Results

2.1. The effect of TCR V β peptides on the lymphocyte TH1 and TH2 transcriptional factors and cytokine profiles

The T-cell receptor agonists (TCR V β 5.2 and 8.1 peptides) administered at a dose of 200 μ g/mouse every other week for six weeks induced a selective TH1 immune response in comparison to the placebo control group with TH1 responses in the left column and TH2 in the right (Figures 1A–F). Figures 1A–D show that the TH1 genes including: *T-bet* and *IFN- γ* were significantly increased 4.4, and 8.4-fold respectively with no effect on the TH2 genes *GATA-3* and *IL-10*. Likewise, *STAT4* expression was increased by 26-fold ($P=0.02$) without an increase in *STAT6* (data not shown). *IL-12 p35*, *IL-12p40*, and *FoxP3* gene expression did not change, suggesting that the TH1 lymphocyte induction by TCR peptides appeared to be independent of the dendritic and regulatory T-cells (data not shown). The cytokine protein concentrations measured with ELISA from splenic T-lymphocytes harvested from the two groups demonstrated a 14-fold increase in IFN- γ without a change in IL-10 (Figures 1E and 1F). In addition TCR peptides induced the immune factors MCP-1 and IL-6 in splenic lymphocytes by 28-fold and 10-fold respectively ($P<0.001$) but not IL-12p70. Therefore, the TCR peptides selectively induce a TH1 lymphocyte and not dendritic and TH2 cytokines.

2.2. TH1 immune function induces pro-fibrotic cytokines in the heart

Figure 2 demonstrates TCR peptide administration increases the messenger RNA of the recognized LOX inducers, namely; *TGF- β 1*, *CTGF*, *PDGF-A* and the reported transcriptional factor for LOX, *IRF-1*. These data suggest that TH1 stimulation induced cardiac *TGF- β 1* which is understood to induce lysyl oxidase via *IRF-1* in other tissues. Nevertheless, the 7.3-fold increase in *TGF- β 1* with a corresponding 2.9-fold increase in *IRF-1* expression is consistent with the understood induction of the LOX enzyme.

2.3. Induced TH1 lymphocyte function increases lysyl oxidase gene expression and activity

We investigated whether induction of the TH1 lymphocyte phenotype may affect LOX and LOXL3 function since these enzymes are understood to be the rate limiting for fibrillar collagen crosslinking in the cardiac ECM. Six weeks following the initial TCR V β peptide administration, *LOX* and *LOXL3* cardiac gene expressions were increased (Figure 3A). Correspondingly, Figure 3B reveals that cardiac LOX protein levels increased by 54% and Figure 3C shows that the enzymatic activity correspondingly increased by 55% in the TCR peptide group when compared with the control group. Hydroxyproline serves as a means to determine tissue collagen content and the insoluble fraction of the hydroxyproline assay represents the cross-linked collagen. The insoluble fraction increased by 95% in the TCR V β peptide treated mice compared with the controls (Figure 3D). Figures 3E and 3F show that the *in vivo* diastolic function functional parameters demonstrated increased ventricular stiffness (β) by 1.7-fold and decreased rate of ventricular filling over time (dV/dt_{max}) by 31%.

2.4. Increased left ventricular interstitial fibrosis in the TH1 mice

The increased fibrillar collagen is supported by Figures 4A, 4B which demonstrate an increased collagen with picrosirius red staining under polarized light with TCR V β peptides administration. The cardiac fibrosis (Figure 4C), as determined by hydroxyproline assay in these tissues, increased from 1.38 ± 0.07 in the control to 1.86 ± 0.16 μ g/mg dry weight of cardiac hydroxyproline concentrations in TCR V β treated mice ($P<0.05$). Tissues harvested on week 6 did not demonstrate an increase cellular infiltrates with hematoxylin and eosin staining in the TCR peptide group compared with the control group which suggests that systemic induction of the TH1 lymphocyte may induce ECM remodeling that is not secondary to myocardial inflammation (Figures 4D, 4E).

2.5. Induction of TH1 immune function reduces diastolic function in vivo

Ventricular function was measured 6 weeks after the initial administration of TCR V β peptides. In addition to the effect of collagen crosslinking on diastolic function as described above, Table 1 shows that there was decreased cardiac output with preserved ejection fraction. These parameters were supported by a decrease in ventricular end systolic and diastolic volumes. Since the maximum left ventricular pressure (Pmax) decreased by 10%, the decreased cardiac output in the treated group was not due to increased afterload. Since the time constant of isovolumic relaxation (τ) was not significantly altered, these hemodynamic parameters suggest that increased ventricular stiffness accounts for the diastolic dysfunction with the TCR V β treatment.

The transthoracic ECHO confirmed that there was an increased ventricular stiffness in TCR peptide treated mice by a significantly decreased mitral valve flow Doppler E/A ratio as well as lateral wall tissue Doppler Ea/Aa ratio. The area under the E wave (E/E-A VTI) decreased by 22% and the E deceleration time increased by 45%. The parameters related to systolic function did not change including intra-ventricular contraction time (IVCT) and ejection time (ET). The parameter that describes preload (lateral E/EA) (Ommen et al., 2000) did not differ between the two groups suggesting that the decrease in the ventricular filling phase that was not related to preload.

2.6. Direct effect of TH1 induction on lysyl oxidase enzymatic activity in vitro

To determine if the TH1 phenotype lymphocyte can directly induce lysyl oxidase enzymatic activity by cardiac fibroblast, we co-cultured T-lymphocytes with primary mouse cardiac fibroblasts *in vitro* (Figure 5). Most importantly we demonstrate that the TCR V β peptides did not directly affect cardiac fibroblast LOX enzymatic activity. The naive lymphocytes increased LOX activity by 3.7-fold whereas lymphocytes treated with TCR V β peptides increased cardiac fibroblast LOX enzymatic activity by 15-fold compared with control fibroblasts. These data support the concept that TH1 lymphocytes directly induce LOX enzymatic activity of cardiac fibroblasts.

3. DISCUSSION

The present study hypothesized that induction of the CD4 TH1 lymphocyte phenotype alters ventricular diastolic function via induction of ECM remodeling. More specifically, the goal of this study was to examine the effect of systemic TH1 lymphocyte polarization on cardiac ECM collagen cross-linking in a murine model lacking increased ventricular over-load. The TCR V β peptides have been shown to selectively induce a TH1 lymphocyte phenotype and TH1 cytokines in C57BL/6 mice (Yu et al., 2005; Liang et al., 1998; Marchalonis et al., 2001) which is supported by our gene expressions and cytokine determinations.

The most striking finding of this study is the apparent role of immunomodulation on collagen crosslinking. Myocardial stiffness has been attributed to alterations in cross-linked collagen rather than total collagen or collagen (Norton et al., 1997; Woodiwiss et al., 2001). LOX expression are directly correlated with left ventricular stiffness in humans (Lopez et al., 2009) and rodents (Yu et al., 2005; Yu et al., 2006; Lopez et al., 2009; Badenhurst et al., 2003; Bradshaw et al., 2009). This current study demonstrates that heightened TH1 lymphocyte activity stimulates LOX enzymatic activities *in vivo* and *in vitro*. This increased LOX activity relates to the concentrations of cardiac collagen crosslinking and diastolic stiffness. An immune mediated mechanism is supported by our *in vitro* study where the induction of a TH1 lymphocyte cytokine profile directly increased cardiac fibroblast LOX enzymatic activity. The significant value of this work is that this is the first report of direct *LOX* and *LOXL3* gene

induction and the corresponding enzymatic function by the cardiac fibroblast through an increase of TH1 lymphocyte secretory products.

The multifunctional TH1 cytokine, IFN- γ has been shown to induce *IRF1* which is a positive regulatory transcriptional factor of LOX (Yuan et al., 1999; Tan et al., 1996). Song et al have shown that IFN- γ inhibits LOX gene expression through binding to the antagonistic transcriptional factor, *IRF2*, in cultured vascular smooth muscle cells (Song et al., 2000). *IRF1* and *IRF2* appear to be expressed reciprocally and *IRF1* is expressed prior to *IRF2* following stimulation (Horiuchi et al., 1999) suggesting that IFN- γ is operative in the induction of cardiac LOX when *IRF1* expression is greater than *IRF2*. Levick et al demonstrated in the spontaneously hypertensive rat model of myocardial fibrosis that there was a significant increase in myocardial IFN- γ supporting the over-expression of IFN- γ during ECM remodeling (Levick et al., 2009). Recently we have shown that another TH1 cytokine, IL-18, induces *IRF1* and LOX and LOXL3 (Yu et al., 2009). Taken together, these results suggest that the TH1 cytokines, including IFN- γ and IL-18, may directly mediate the increased ECM crosslinking and accumulation of total cardiac collagen through the induction of *IRF1* associated increased LOX enzymatic activity.

The cardiac output of the treatment group was significantly reduced however the ejection fraction was preserved. The ventricular stiffness (β) was significantly increased and correspondingly the dV/dt_{max} was significantly decreased. These hemodynamic data support that the induction of TH1 lymphocyte function directly relates to a condition similar to that of clinical diastolic dysfunction and the mechanism appears to be related to the increased cardiac fibrillar collagen crosslinking.

This study demonstrated that an altered immune background may induce the remodeling processes similar to that observed in response to increased chronic afterload. However in the present study, the afterload in our treated mice was decreased. Previously, we have also demonstrated that a selective stimulation of a dominant TH2 immune condition leads to a markedly dilated heart and decreased ventricular stiffness (Yu et al., 2005; Beischel et al., 2004). Consistent with our findings, Hikoso et al demonstrated that knocking out the TH2 immune function with *STAT6*^{-/-} did not prevent compensatory left ventricular hypertrophy in aortic banded mice and resulted in a 2-fold increase of interstitial fibrosis compared with the banded wild type (Hikoso et al., 2004). Therefore taken together, these studies suggest that TH1 lymphocytes induce an accumulation of fibrillar collagen crosslinking and ventricular stiffness whereas TH2 phenotype may provoke the opposite.

The differentiated TH1 and TH2 lymphocyte cytokine profiles and functions can be biased by T-cell receptor V β variants (Braciak et al., 2003). Twenty-four TCR V β variants have been characterized in mice and humans to date (Rosenberg et al., 1992). Therefore, the T-cell cytokine response depends upon the TCR V β variant epitope agonist ligation and activation. Thus, modulation of specific variants with selective receptor agonists may relate to cytokine synthesis by the T-helper lymphocyte population. We have previously shown that the combination of the two TCR peptides, V β 5.2 and 8.1, induce TH1 cytokines greater than individual peptides (Marchalonis et al., 2005). The lymphocyte gene expression analysis of the study herein supports that TCR V β peptides induce TH1 related genes including: *IFN- γ* , *STAT4*, and *T-bet*, and appear to bypass the dendritic cell since *IL-12p35* and *IL-12p40* gene expression were unchanged. Most importantly, ELISA analysis demonstrated a 14-fold increase in IFN- γ 6 weeks after the initial administration of TCR V β peptides in these C57BL/6 mice.

This report has implications beyond that of the heart. In addition to collagen, LOX also crosslinks elastin (Molnar et al., 2003) which suggests that this paradigm may apply to

vasculature remodeling related to hypertension. LOX has been associated with the genesis and instability of atherosclerotic plaques (Rodriguez et al., 2008); a pathological state that is directly associated with the TH1 lymphocyte subset (Zhou et al., 2000). Moreover, the metastatic potential of tumors is also related to LOX up-regulation and ECM crosslinking (Kirschmann et al., 2002). Therefore, our findings provide compelling data that may also be applicable to hypertension, atherosclerosis, and metastases.

In summary, this study demonstrated that direct immunomodulation of T-lymphocytes affects the cardiac ECM composition, resulting in altered cardiac collagen content and cross-linked collagen, thereby disrupting diastolic cardiac function. A selective means to modulate the T-lymphocyte function in this study was through administration of TCR V β peptides, which shifts T-lymphocyte function toward a TH1 cytokine response; which bypasses the need of co-stimulation by dendritic cells. The *in vivo* polarization to the TH1 cytokine response with the TCR V β peptide treatment induced diastolic dysfunction and associated with the ECM composition. The suggested diastolic dysfunction induction by TH1 lymphocytes has potential clinical application in the pathogenesis of cardiac diastolic dysfunction.

4. Materials and methods

4.1. Mice and peptides

Three-month-old female mice C57BL/6J were obtained from Jackson Laboratories (Bar Harbor, ME, USA). This study was approved by The University of Arizona Animal Care Committee and conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). The mice were randomly divided into a placebo control group (n=14) and TCR V β peptide group (n=14). All mice were studied 6 weeks post-placebo or TCR V β peptide administration. The TCR V β peptides 5.2 and 8.1 (Minotopes Clayton, Australia) were administered on days 0 and 14 at a dose of 200 μ g/mouse i.p. The sequences of these TCR V β peptides were CSPKSGHDTVSWYQQA (V β 5.2) and CKPISGHNSLFWYRQT (V β 8.1). The placebo was phosphate buffered saline used as the TCR V β vehicle.

4.2. RNA Extraction and Real-Time PCR

Cardiac and splenic tissues from 5 mice were harvested then homogenized in TRIzol (Invitrogen Life Technologies, Carlsbad, CA). Diluted cDNA was used for reaction with Quantitect Sybr green PCR kit (Qiagen) 50 μ l, and real-time PCR was performed with SYBR Green using the Rotor-Gene RG-3000 (Corbett Research) in a 72-well rotor. Custom primers were designed using Primer3 and synthesized by Integrated DNA Technologies. The following candidate genes were investigated: *T-bet* (AF241242), *GATA-3* (NM_008091.2), *IFN- γ* (NM_008337), *IL-10* (NM_010548.1), *Pro-LOX* (NM_010728), *Pro-LOXL3* (NM_013586), *IRF-1* (BC003821.1), *TGF- β 1* (NM_011577), *CTGF* (NM_010217.1), *PDGF- α* (BC003817.1), and *PDGF- β* (NM_011057.3). The candidate gene cycle thresholds were normalized by the respective β -actin (BC040513) RNA expression. All data are reported as normalized thresholds and fold change of control compared with treated groups.

4.3. ELISA Cytokine Assay

The purified splenic T-lymphocytes were cultured in 10% FCS RPMI1640 medium at a concentration of 5×10^5 cells/mL on anti-CD3 and anti-CD28 coated plates. After 48 hours, supernatants were collected and stored at -80°C until analysis. The R&D (Minneapolis, MN) cytokine ELISA kits were used for IFN- γ and IL-10 quantification of the supernatants.

4.4. Immunoblot for LOX

Heart tissues were homogenized in a buffer composed of 1 × PBS, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, and 2 M urea, pH 7.4 and freshly added protease inhibitor cocktail (1:200 v/v, Sigma). Samples were centrifuged at 13,000 rpm for 10 min at 4°C. Supernatants were collected and protein concentrations were determined by BCA protein assay reagents (Pierce). Equal amount of protein (20 µg) samples were prepared in a sample buffer and loaded to a 10% precast SDS gel (BioRad). Proteins were electrophoresis with 120V for 90 minutes at 4°C and transferred to PVDF membrane which then was blocked over night in 3% blocking buffer. After four times wash, membrane was probed by 1:5,000 primary antibody (Rabbit anti-mouse LOX antibody, Novus Biologicals) for 1 hour, membrane underwent five times wash and 1:20,000 secondary antibodies (Goat anti-Rabbit antibody conjugated with HRP, Novus Biologicals) were applied. Bands were exposed on film with a horseradish peroxidase substrate system. The LOX molecular weight was confirmed with SDS-PAGE standards (BioRad).

4.5. LOX activity assay

LOX activity was measured by a fluorometric assay based on methods by Trackman et al (Palamakumbura and Trackman, 2002) and performed as described by our previous report (Yu et al., 2006).

4.6. Determination of hydroxyproline and collagen cross-linking

Hydroxyproline (HP) and cross-linking assays of cardiac tissues were conducted as described by Yu et al (Yu et al., 2006). HP levels were quantified by comparison to a standard colorimetric curve of trans-hydroxyproline (Sigma, St. Louis). Collagen cross-linking was determined using cyanogen bromide (CNBr) digestion.

4.7. Quantification of left ventricular mechanics with ECHO and pressure-volume loops

Transthoracic echocardiography was performed on 8 mice per group at 6 weeks. The mice were anesthetized with 2% isoflurane and digital images were recorded at a frame rate of 200 frames/second with a linear 35-MHz probe (Model 707B Scanhead, Visualsonics, Canada). On the day following the echocardiogram analysis, pressure-volume loops were acquired with our previously described Millar CCS methods (Yang B et al., 1999; Yang B et al., 2001). Briefly, the mice were ventilated and the apical portion of the heart and exposed through a substernal-transverse incision and then a 1.4 fr Millar Conductance Catheter (Millar Corporation, Houston, TX) was inserted into the apex of the left ventricle. Pressure-volume loops were acquired and computed as performed by Yang (Yang B et al., 1999; Yang B et al., 2001).

4.8. Cardiac Fibroblast and Lymphocyte Isolation

Cardiac fibroblast isolation was performed as described by Yu et al (Yu et al., 2005). These primary fibroblast cultures were grown to 80% confluence and the fetal bovine serum decreased to 1% prior to co-culturing with lymphocytes.

4.9. Lymphocyte isolation and polarization for co-culture

Splenic lymphocyte isolation was accomplished according to the methods reported by Yu et al (Yu et al., 2005). Subsequent to density separation, the splenic T-lymphocyte population was enriched by panning the monocytes and thereafter removing B-lymphocytes with anti-murine IgG coated plates. TCR Vβ 5.2 and 8.1 peptides were added, at equal concentrations (25 µg/mL) to the enriched T-lymphocytes (10⁶ cells/mL) for 48h or cardiac fibroblast cultures. The treated and untreated lymphocytes were washed three times and added directly to the cardiac

fibroblast cultures at a concentration of $5 \times 10^5/\text{mL}$ for 48h. The lymphocytes were washed off the cardiac fibroblast cultures and the LOX enzymatic activity was determined from fibroblast cell lysate.

4.10. Statistics analysis

Statistical analysis was performed using a two-tailed Student's t test. Data is expressed as Mean $\pm$ SEM

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Transcriptional Factor Gene Expression

Cytokine Gene Expression

Cytokine Protein Secretion

Figure 1.

The effect of TCR Vβ peptides on the lymphocyte TH1 and TH2 transcriptional factors and cytokine profiles. (A) T-bet gene expression, the transcriptional factor for TH1 lymphocytes, (B) GATA-3 gene expression, the transcriptional factor for TH2 lymphocytes, (C) IFN-γ gene expression, TH1 cytokine, (D) IL-10 gene expression, TH2 cytokine, (E) IFN-γ supernatant concentrations, (F) IL-10 supernatant concentrations. This figure supports the finding the TCR Vβ peptide treatment induces TH1 lymphocyte phenotype without altering the TH2 lymphocyte function. (n=4) *P<0.0001 compared with control

Figure 2.

TH1 immune function induces pro-fibrotic cytokines in the heart. The gene expression of the reported lysyl oxidase inducers were analyzed with real-time PCR in left ventricular tissues six weeks after vehicle or TCR V β peptide treatment. These data demonstrate that induction of TH1 lymphocyte phenotype increases the transcriptional factor IRF-1 and the lysyl oxidase inducers TGF- β 1 and PDGF- β . n=4

Lysyl Oxidase Gene Expression and Protein Synthesis

Lysyl Oxidase Enzymatic Activity

Effect on Cardiac Diastolic function

Figure 3.

Induced TH1 lymphocyte function increases lysyl oxidase gene expression and activity. Induction of TH1 lymphocyte function increases cardiac lysyl oxidase gene expression, protein concentration, enzymatic activity, collagen crosslinking, and decreased diastolic function. (A) Gene expression for pro-LOX and pro-LOXL3, (B) immunoblot of cardiac LOX protein (C) cardiac tissue lysate lysyl oxidase enzymatic activity, (D) hydroxyproline assay of fibrillar cardiac collagen crosslinking, (E) the left ventricular stiffness as measured by the slope of the end-diastolic pressure-volume relationship, (F) the derivative of the rate of left ventricular filling over time. (n=4) *P<0.05

Figure 4.

Increased left ventricular interstitial fibrosis in the TH1 mice. Using the collagen stain picrosirius red illuminated with polarized light, these cardiac sections (40×) show that there is an increase in interstitial collagen when comparing control (A) with the peptide treated (B). These sections are at the right ventricular-septal junction in the mid-portion of the ventricles. Moreover, hematoxylin eosin staining panels (C and D) demonstrate that there are no lymphocytic infiltrates in either group and the white bar = 60 microns. Panel E shows that the cardiac hydroxyproline were increased with the TCR Vβ peptide treatment compared with the controls. * $P < 0.05$ compared with control.

Figure 5.

Direct effect of TH1 induction on lysyl oxidase enzymatic activity in vitro. Splenic lymphocytes treated with equal concentrations (25 $\mu\text{g/mL}$) of TCR V β peptides 5.2 and 8.1, or untreated lymphocytes were added to 80% confluent cardiac fibroblast at a concentration of $5 \times 10^5/\text{mL}$. The cardiac fibroblast lysate was collected after 48h for LOX enzymatic activity. The TCR V β peptides demonstrated no direct effect on primary cardiac fibroblasts. The control lymphocytes and the TCR V β peptide treated lymphocytes significantly increased the LOX enzymatic activity *in vitro*. These data strongly suggest that TH1 lymphocyte products directly induce LOX enzymatic activity. (n=3) *P<0.05 compared with control fibroblasts, †P=0.006 compared with control lymphocytes and fibroblasts

Table 1

Induction of TH1 immune function reduces diastolic function in vivo. The weight descriptions include: body weight (BW), and heart weight (HW). The left ventricular functional parameters include: heart rate (HR), cardiac output (CO), ejection fraction (EF), end systolic volume (Ves), end diastolic volume (Ved), maximum systolic pressure (Pmax), and the time of constant of isovolumic relaxation (τ). The ECHO parameters include: ratio of Doppler measured passive and active flow (E/A), ratio of septal tissue Doppler during early diastole to late diastole (Ea/Aa), the ratio of peak E-velocity to the total velocity integral (E/E-A VTI), filling time of mitral valve opening to closure (DT), the time from closure of mitral valve to the opening of aortic valve (IVCT), ejection time from the opening of aortic valve to the closure of aortic valve (ET), and ratio of peak E velocity from Doppler flow across the mitral valve to the tissue Doppler Ea at the mitral annulus during early diastole indicates preload (Lateral E/Ea).

Parameters	Units	Control	Peptide	P value
N		14	14	
BW	g	36.9 $\pm$ 0.8	33.2 $\pm$ 1.9	NS
HW/BW		3.8 $\pm$ 0.1	4.1 $\pm$ 0.1	NS
Hemodynamics measured with conductance catheter				
HR	bpm	565 $\pm$ 9	506 $\pm$ 16	0.004
CO	mL/min	8.4 $\pm$ 0.6	6.4 $\pm$ 0.5	0.03
EF	%	57.8 $\pm$ 2.5	59.9 $\pm$ 3.4	NS
Ves	μ L	11.7 $\pm$ 0.8	9.1 $\pm$ 0.9	0.04
Ved	μ L	25.3 $\pm$ 1.3	20.9 $\pm$ 1.2	0.03
Pmax	mmHg	93 $\pm$ 3	84 $\pm$ 3	0.06
τ	msec	5.7 $\pm$ 0.3	6.5 $\pm$ 0.3	0.08
Hemodynamic measured with ECHO				
N		8	8	
E/A		1.81 $\pm$ 0.09	1.31 $\pm$ 0.02	0.0007
Septal Ea/Aa		1.59 $\pm$ 0.09	1.21 $\pm$ 0.05	0.0001
E/E-A VTI		38.2 $\pm$ 1.9	30.0 $\pm$ 1.8	0.020
DT	ms	51 $\pm$ 3	57 $\pm$ 3	NS
IVCT	ms	9.4 $\pm$ 0.5	10.5 $\pm$ 0.9	NS
ET	ms	43 $\pm$ 3	48 $\pm$ 3	NS
Lateral E/Ea		39.7 $\pm$ 3.8	41.0 $\pm$ 1.5	NS