Deficiency of TNFRI protects myocardium through SOCS3 and IL-6, but not p38 MAPK or IL-1 beta

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ABSTRACT

Tumor Necrosis Factor-α (TNF) plays an important role in the development of heart failure. There is a direct correlation between myocardial function and myocardial TNF levels in humans. TNF may induce local inflammation to exert tissue injury. On the other hand, suppressor of cytokine signaling (SOCS) proteins have been shown to inhibit proinflammatory signaling. However, it is unknown whether TNF mediates myocardial inflammation via STAT3/SOCS3 signaling in the heart, and if so, whether this effect is through the type 1 55-kDa TNF receptor (TNFR1). We hypothesized that TNFR1 deficiency protects myocardial function and decreases myocardial interleukin (IL)-6 production via the STAT3/SOCS3 pathway in response to TNF.

METHODS: Isolated male mouse hearts (n=4/group) from wild type (WT) and TNFR1 knockout (TNFR1KO) were subjected to direct TNF infusion (500 pg/ml/min x 30 min) while LVDP, +dP/dT, -dP/dT were continuously recorded. Heart tissue was analyzed for active forms of STAT3, p38, SOCS3 and SOCS1 (Western blot), as well as IL-1β and IL-6 (ELISA). Coronary effluent was analyzed for LDH activity.

RESULTS: TNFR1KO had significantly better myocardial function, less myocardial LDH release and greater expression of SOCS3 (% of SOCS3/GAPDH: 45+/−4.5% vs. WT 22+/−6.5%) after TNF infusion. TNFR1 deficiency decreased STAT3 activation (% p-STAT3/STAT3: 29+/−6.4% vs. WT 45+/−8.8%). IL-6 was decreased in TNFR1KO (150.2+/−3.65 pg/mg protein) vs. WT (211.4+/−26.08). TNFR1 deficiency did not change expression of p38 and IL-1β following TNF infusion. These results suggest that deficiency of TNFR1 protects myocardium through SOCS3, and IL-6, but not p38 MAPK or IL-1β.
INTRODUCTION

Tumor necrosis factor alpha (TNF) is a proinflammatory cytokine, which plays a role in cellular differentiation, apoptosis and inflammation (23, 28). TNF can be produced locally from different cells including cardiac myocytes, smooth muscle cells and endothelial cells in response to endotoxemia, ischemia, and trauma (23, 24, 31). Locally produced TNF contributes to depression of myocardial contractile function, induction of cardiomyocyte apoptosis, as well as induction of proinflammatory signaling (8, 15, 18). TNF exhibits its biological effect through two distinct surface receptors, TNFR1 (p55) and TNFR2 (p75). Both receptors have been observed in many cell types, including cardiac myocytes (34). Although most animal studies clearly showed that TNF exerts detrimental effects following experimental ischemia and reperfusion (I/R) (6, 20, 22, 25), clinical trials indicated that anti-TNF therapy was associated with increased mortality (7). This led to the important appreciation that the regulatory balance of TNF signaling, likely depends on different TNF receptor-induced pathways with different cellular fates (cell survival versus apoptosis).

TNFR1 initiates the majority of TNF effects in most cell types, including the heart. It has been reported that through TNFR1, TNF exerts negative inotropic effects on the myocardial contractile function (26) and induces cardiac myocyte apoptosis (15). Recent evidence demonstrated significant improvement in myocardial function in TNFR1 knockout mice compared to wild type mice after myocardial infarction (27, 39). While, most studies have focused on TNFR1-induced myocardial dysfunction by calcium dyshomeostasis (45), nitric oxide (NO) production (32), and apoptosis, little information exists in TNFR1-induced myocardial inflammation. Indeed, TNF plays an important role in the execution of an immune response.

Recently, suppressor of cytokine signaling (SOCS) proteins have been reported to inhibit proinflammatory/proapoptotic signaling in the heart (1). They do so by inhibiting intracellular signaling pathways activated by proinflammatory cytokines. However, it is unknown whether
TNF mediates myocardial inflammation via STAT3/SOCS3 signaling in the heart, and if so, whether this effect is through the type 1 55-kDa TNF receptor (TNFR1).

Therefore, we hypothesized that TNFR1 deficiency would protect myocardial function and decrease myocardial inflammation via the STAT3/SOCS3 pathway in response to TNF. The purposes of this study were to determine the effect of TNFR1 on myocardial function and myocardial inflammatory pathways by using mice with a targeted deletion of TNFR1.

MATERIALS AND METHODS

Animals

Wild type (WT) and targeted deletion of TNFR1 (TNFR1KO) mice (Jackson Laboratories, Bar Harbor, ME) were fed a standard diet and acclimated in a quiet quarantine room for two months before the experiments. The animal protocol was reviewed and approved by the Indiana Animal Care and Use Committee of Indiana University. All animals received humane care in compliance with the "Guide for the Care and Use of Laboratory Animals" (NIH publication No. 85-23, revised 1985).

A total of 8 isolated mouse hearts (n=4/group) were subjected to the same protocol with TNF infusion (500 pg/ml/min) for 30 minutes after 15 minutes of equilibration.

Isolated heart preparation (Langendorff) and measurement of cardiac function

Experiments were performed with the use of a Langendorff apparatus as described previously for use in mouse heart. Briefly, mice were anesthetized (sodium pentobarbital, 60 mg/kg i.p.) and heparinized (500 U i.p.), and hearts were rapidly excised via median sternotomy and placed in 4°C Krebs-Henseleit solution. The aorta was cannulated and the heart was perfused with oxygenated (95% O2 / 5% CO2) Krebs-Henseleit solution (37°C). Coronary flow was measured by collecting pulmonary artery effluent. Data was continuously recorded using a PowerLab 8 preamplifier/digitizer (AD Instruments Inc., Milford, MA) and an Apple G4
PowerPC computer (Apple Computer Inc., Cupertino, CA). The maximal positive and negative values of the first derivative of pressure (+ dP/dt and -dP/dt) were calculated using PowerLab software.

**Lactate dehydrogenase assay**

Lactate dehydrogenase (LDH) is released from damaged cells and the measurement of LDH activity indicates the severity of cell death and cell lysis. Coronary effluent was collected and stored at -80°C freezer until enzymatic analysis for LDH activity by using a commercially available kit (Cytotoxicity Detection Kit-LDH, Roche Diagnostics Corporation, Indianapolis, IN).

**Myocardial IL-1β, IL-6**

Myocardial IL-1β and IL-6 were determined by enzyme-linked immunosorbent assay (ELISA) using a commercially available ELISA set (R&D Systems Inc., Minneapolis, MN). ELISA was performed according to the manufacturer’s instructions. All samples and standards were measured in duplicate.

**Western blotting**

Western blot analysis was performed to measure p38 MAPK, STAT3, SOCS3 and SOCS1 proteins. Heart tissue was homogenized in cold buffer containing 20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-Glycerophosphate, 1 mM Na3VO4, 1 µg/ml Leupeptin, 1 mM PMSF, and then centrifuged at 12000 rpm for 5 minutes. The protein extracts (30 µg/lane) were subjected to electrophoresis on a 12% tris-HCl gel from Bio-Rad and transferred to a nitrocellulose membrane, which was stained by Naphthol Blue-Black to confirm equal protein loading. The membranes were incubated in 5% dry milk for 1 hour and then incubated with the following primary antibodies: p38 MAP kinase antibody, phosphor-p38 MAP kinase (Thr180/Tyr182), STAT3 antibody, phosphor-STAT3 antibody (Cell Signaling Technology, Beverly, MA), SOCS-3, and SOCS-1 antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). The membranes were then
incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody and
detection using supersignal west pico stable peroxide solution (Pierce, Rockford, IL). Films were
scanned using an Epson Perfection 3200 Scanner (Epson America, Long Beach, CA) and band
density was analyzed using ImageJ software (NIH).

Presentation of data and statistical analysis

All reported values are mean ± SEM (n=4/group). Data was compared using two-way
analysis of variance (ANOVA) with post-hoc Bonferroni test or Student’s t-test. A two-tailed
probability value of less than 0.05 was considered statistically significant.

RESULTS

TNF-Decreased Myocardial Function Dependent on TNFR1

TNF infusion resulted in a progressive decrease in myocardial function. During the 10
min equilibration period hearts maintained stable LVDP, +dP/dt and −dP/dt, which were
significantly depressed by 30 min of TNF infusion in WT hearts (figure 1). However TNFR1KO
resulted in improved myocardial function compared to WT (LVDP: 37.9+/−4.4 vs. WT 20.6+/−
3.2mmHg; +dP/dt: 1176.4+/−159.1 vs. WT 589.1+/−85.6 mmHg/s; −dP/dt: -877.2+/−115.4 vs. WT
-448.6+/−61.8mmHg/s) with exposure to equivalent amounts of TNF.

Effect of TNFR1 on cardiomyocyte damage in response to TNF

Following TNF infusion, increased LDH release in coronary effluent was observed.
Interestingly, TNFR1 deficiency resulted in less LDH release compared to wild type hearts
(figure 2).

Effect of TNFR1 on Expression of TNF-Induced Myocardial IL-1β and IL-6

TNF infusion resulted in significantly more production of myocardial IL-1β (751 ± 18 vs.
control 362 ± 7 pg/mg protein) and IL-6 (211.4 ± 26 vs. control 74.5 ± 17) (figure 3). TNFR1
deficiency did not change TNF-induced IL-1β expression. However, TNF-induced IL-6
production (150 ± 3.7 vs. WT 211.4 ± 26) was markedly decreased by TNFR1KO.
**TNF Induced Myocardial p38 MAPK Activation**

The myocardial phosphorylated/active p38 and nonphosphorylated/total p38 MAPK were assessed by Western blot (figure 4). More phosphorylated p38 MAPK (% of p-p38/total p38: 29±14.6% vs. control 11.3±0.4%) was observed in the heart exposed to TNF. This result indicated that TNF induced significantly more activation of p38 MAPK. However, there was no significant different in the ratio of phosphor-p38 to p38 MAPK between WT and TNFR1KO following TNF infusion,

**Effect of TNFR1 on TNF-Induced Expression of SOCS-3 and Activation of STAT3**

Following TNF infusion, expression of SOCS-3 protein (% of SOCS3/GAPDH: 45+/4.5% vs. WT 22+/6.5%) was found to be higher in TNFR1KO heart compared to WT (figure 5A). Moreover, TNFR1 deficiency resulted in less activation of STAT3 following TNF infusion (figure 5B). No significant difference in myocardial SOCS-1 expression was noted in response to TNF infusion (figure 5C).

**DISCUSSION**

In the present study, we clearly demonstrated that: 1) TNF induced myocardial contractile dysfunction through TNFR1, which was associated with less LDH release in TNFR1KO hearts; 2) Deficiency of TNFR1 limits acute myocardial inflammation through SOCS3 and IL-6.

Cardiac myocytes themselves produce substantial amounts of TNF in response to endotoxemia, I/R and trauma (23, 24, 31), which then leads to myocardial dysfunction. It is well documented that myocardial TNF is increased in acute myocardial ischemia (12, 24). Our previous studies have shown that I/R-induced myocardial dysfunction was associated with an increase of myocardial TNF production (37, 38). Here, once again, we indicated that TNF infusion into the heart depressed myocardial contractile function. The hemodynamic effects of TNF are characterized by decreased myocardial contractile efficiency and reduced ejection fraction, hypotension, decreased systemic vascular resistance, and biventricular dilatation (23).
TNF has been shown to exert a negative inotropic effect via the disruption of calcium homeostasis (45) and increased production of NO (41, 42, 44). Although both of TNFR1 and TNFR2 are present on the cardiac myocyte membrane, the majority effect of TNF is initiated by TNFR1. It has been reported that TNF disrupted cardiac excitation-contraction coupling through TNFR1-mediated inhibition of L-type Ca\(^{2+}\) channel-induced calcium influx (23). In addition, stress-induced second messenger sphingolipid metabolites have been shown to participate in intracellular signal transduction following TNF binding to the TNFR1. Blockade of sphingosine production abolished TNF-induced contractile dysfunction (26).

TNF has been reported to also induce apoptosis in many cell types, including the myocardium. TNF binding to TNFR1 or Fas resulted in activation of procaspase-8. Caspase-8 then activates downstream caspase-3 and induces the classic extrinsic death pathway (11, 21, 36), which is also responsible for TNF-induced contractile dysfunction in a prolonged myocardial injury (9). However, some scientists concluded that TNF-induced cardiac myocyte apoptosis does not happen in the presence of TNF by itself in vivo (17, 19). Therefore, given that our experimental period (only 30 minutes of TNF infusion) was too brief to observe significant apoptosis, we did not perform apoptotic-related assays in this experimental model. However, the present study showed an increased myocardial LDH release existed following TNF infusion. In addition, TNFR1 ablation resulted in less LDH release. This demonstrates the novel finding that TNFR1 mediates cytotoxicity of TNF.

In addition, TNF is able to induce other inflammatory cytokine synthesis. It has been reported that TNF is an upstream initiator of the reperfusion-dependent cytokine release in experimental canine myocardial I/R (8). Cardiac-specific overexpression of TNF has been shown to elevate myocardial IL-1\(\beta\) and MMP-1 levels in the murine model (16). Using TNF receptor fusion protein has decreased LPS-induced IL-1\(\beta\) in animal experiments (14). Our previous study demonstrated that TNF might mediate production of myocardial IL-\(\beta\) and IL-6 via p38 mitogen-activated protein kinase (MAPK) in response to endotoxemia (38, 40). p38 MAPK
is an important mediator in cellular inflammatory response (29, 30, 33). Indeed, it has been demonstrated that p38 MAPK regulates TNF-induced production of IL-1β and IL-6 in murine embryo fibroblasts (43). Moreover, TNF-induced IL-6 production is mediated by p38 MAPK and this process occurs at the transcriptional level (3, 35). In parallel, the present study demonstrates that direct infusion of TNF into the heart resulted in increased production of IL-1β and IL-6, which is in line with elevated activation of myocardial p38 MAPK in response to TNF. Interestingly though, only TNF-induced IL-6 levels were decreased in TNFR1KO hearts. The mechanism involved in TNFR1-mediated IL-6 production in the heart, therefore, remains unclear.

Recently, suppressor of cytokine signaling (SOCS) proteins have been reported to inhibit proinflammatory/proapoptotic signaling in the heart (1). SOCS proteins may be induced by various stimuli (1) and participate in the important process of controlling proinflammatory signals. They do so by inhibiting intracellular signaling pathways activated by proinflammatory cytokines. It has been demonstrated that TNF induces expression of SOCS3 in the liver (10), rat liver macrophage and mouse macrophage (4). In the present study, we indicated that expression of SOCS3 existed in TNF exposed heart tissue. To date, no data shows the exact mechanisms of TNF mediated SOCS3 expression and what receptor(s) is involved in this process. Herein, we found that a deficiency of TNFR1 increased myocardial SOCS3 protein expression, which is in line with our previous result of an I/R-induced increased expression of SOCS3 increased in TNFR1KO hearts (39). This data suggests that SOCS3 expression may be attributed to TNFR2 signaling. However, the detailed mechanisms need further investigation. In addition, although the trend of higher level in myocardial SOCS1 expression was noted in the TNFR1KO hearts subjected to TNF, there was no significant difference in SOCS1 protein level between wild type and TNFR1KO mice.

SOCS3, in turn, has an inhibitory effect on inflammatory cytokine signaling and myocardial dysfunction (5). It has been demonstrated that recombinant cell penetrating forms of SOCS3 (CP-SOCS3) protect animals from the lethal effects of bacteria and endotoxemia by
suppressing inflammation (13). TNF has been shown to inhibit IL-6 signaling (STAT3 pathway) via SOCS3 in macrophage (4). Therefore, it is not surprising that in this study, increased SOCS3 expression decreased IL-6 production and activation of myocardial STAT3 in TNFR1KO heart exposed to TNF. However, SOCS3 did not affect TNF-induced myocardial IL-1β levels in TNFR1 deficient mice. SOCS3 appears to mediate IL-1β signaling through IL-1 receptor antagonist (IL-1ra) (2). Therefore, a potential explanation for our data is that TNF-induced SOCS3 expression may induce IL-1ra to bind IL-1β, subsequently inhibiting IL-1 signaling without decreasing IL-1β production in TNFR1KO heart.

In summary, these results suggest that TNFR1 plays an important role in myocardial dysfunction. There are complex interactions between signaling proteins such as p38 MAPK and SOCS3 in TNFR1-mediated effects. Further investigation in this area may yield a more complete understanding of TNF signaling in hopes of advancing ischemic heart injury treatments that target specific receptor actions.

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FIGURE LEGENDS

**Figure 1.** Myocardial contractile function following TNF (500 pg/ml/min) infusion in age matched wild type (WT) and TNFR1KO mouse hearts perfused with modified Krebs-Henseleit solution. TNF infusion resulted in a progressive decline in myocardial function in WT, which was improved in TNFR1KO. A. LVDP; B. +dP/dt; C. –dP/dt. Results are mean ± SEM and are represented as % of equilibration (eq). *, (*p<0.05 vs. WT; #p<0.05 vs. equilibration)

**Figure 2.** Lactate dehydrogenase (LDH) release in coronary effluent following TNF infusion. Shown value is equilibration, 10 minutes, 20 minutes and 30 minutes of TNF infusion, respectively. Results are mean ± SEM. *p<0.05 vs. equilibration; #p<0.05 vs. WT.

**Figure 3.** The production of myocardial IL-1β (A) and IL-6 (B) after TNF infusion was assessed by ELISA. TNF induced significantly more IL-1β and IL-6 compared to control. TNFR1 deficiency resulted in less production of myocardial IL-6 but not IL-1β. (Mean ± SEM, n=3-4/group. *p<0.05 vs. control, #p<0.05 vs. WT)

**Figure 4.** The expression of activated p38 MAPK and total p38 MAPK was measured by western blot. TNF infusion resulted in more activation of myocardial p38 MAPK, which was independent of TNFR1. Densitometry data of p-p38 MAPK (% of total p38 MAPK) are shown as bar graph (Mean ± SEM, n=3-4/group). Bottom shown are representative immunoblots (two lanes per group shown).

**Figure 5.** The expression of myocardial SOCS3 (A), activation of STAT3 (B) and SOCS1 protein level (C) after TNF infusion were measured by Western Blot. More expression of myocardial SOCS-3 existed in TNFR1KO compared to WT following TNF infusion. Shown are
densitometry data of SOCS3, SOCS1 (% of GAPDH) and p-STAT3 (% of STAT3) (Mean ± SEM, n=3-4/group, * p<0.05 vs. WT), and representative immunoblots (two lanes per group shown).
Figure 1.
Figure 2.
Figure 3.

A

B
Figure 4.
Figure 5.

A

![Bar graph showing SOCS-3/GAPDH levels in WT and TNFR1KO compared to WT.](image)

B

![Bar graph showing p-STAT3/STAT3 levels in WT and TNFR1KO compared to WT.](image)

C

![Bar graph showing SOCS-1/GAPDH levels in WT and TNFR1KO compared to WT.](image)